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ANIMAL VIRUS GENETICS

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BERNARD N. FIELDS

RUDOLF JAENISCH

C. FRED FOX

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ANIMAL VIRUS GENETICS

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PREFACE

Animal Virus Genetics was one of the 1980 ICN-UCLA Symposia on topics in molecular and cellular biology sponsored by ICN Pharmaceuticals, Inc., and organized through the Molecular Biology Institute of the University of California, Los Angeles.

The formal presentations at this conference focused on basic genetic model systems, the uses of genetic approaches to study basic problems in molecular biology, and on the increasing application of genetic systems to the study of more complex viral-host interactions such as viral virulence and persistence. The program was constructed to allow close interchange between those working at very basic levels with those more directly involved in applying genetics to models of virus-induced diseases or aspects of viral oncogenesis. We hoped that ultimately this intermingling of individuals with different interests would provide an excellent exchange of ideas and serve to stimulate collaborative research.

The workshops were organized by virus groups rather than research themes. We have placed the summaries of the workshops in Chapter 9. The discussions and often heated debates that emerged were clearly among the highlights of the meeting.

Much of the success of the meeting was due to the session chairpersons: Joseph Sambrook, Peter Vogt, Thomas Graf, Frank Lilly, Purnell Choppin, and David Botstein. In addition, special thanks are due the workshop chairpersons: David Baltimore, Klaus Kister, Alice Huang, Bill Joklik, Arnold J. Levine, Steve Martin, Craig Pringle, Priscilla Schaffer, Sondra Schlesinger, Philip Sharp, Irving Weissman, and Harold zur Hausen, and two keynote speakers: Paul Berg and Hilary Koprowski.

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International Center), which helped to defray the invited speakers' travel expenses. Finally, we are indebted to Fran Stusser and her administrative staff, Diane Slattery and Edward Burgess, for bringing the meeting to fruition.

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IS-ELEMENTS AND TRANSPOSONS

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A. Transposition And Evolution

For a long time, mutation and recombination have been thought to be the driving forces of evolution. Our increased understanding of molecular genetics has let to difficulties with this concept. We have learnt that genes are DNA molecules of 1ooo base pairs or more and that mutations consist in the substitution or addition or deletion of single base pairs. Recombination requires that homologous DNA molecules of considerable length pair. Thus, recombination does not alter the general arrangement of DNA molecules. It is only capable to exchange alleles.

It is doubtful, whether these two mechanisms are capable to explain molecular evolution. How does a protein molecule with a new function evolve? If the gene for the new protein is derived from its ancestor gene by successive point mutations, these point mutations must have been accumulated either by selection or by random genetic drift. If the former is true, we must assume that the two proteins are connected by a continous series of other, functional proteins. This is unlikely. If, however, no such series exists, it is hard to avoid the assumption that the first base substitutions finally leading to the new function do not have a selective value towards this goal. In this case, they must be accumulated by random drift. It is hard to imagine, how this can occur with a protein of 1oo amino acids or more, because the possibilities to be explored by the genetic drift in this case exceed the very large number of $2o^{1oo}$. This problem would be relieved, when the units evolving independently

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were smaller, e.g. in the range of 2o to 3o amino acids. In this case, however, the genes with which we are dealing presently must have been composed from these smaller units by a mechanism capable of combining segments of unrelated DNA. From this point of view, it would be interesting to search for such mechanisms.

The combination of unrelated DNA molecules was not thought to be a major biological mechanism until recently. The two exceptions were the integration of virus DNA molecules into chromosomes of pro- and eucaryotes on the one hand and the in vitro combination of DNA molecules with the help of restriction endonucleases and DNA ligases. Only recently has it become more common knowledge that the new combination of DNA molecules can occur in many organisms from bacteria to insects and higher plants. In those cases, where the study of these phenomena has been possible at the molecular level, a surprising observation was made: the mechanisms responsible for creating new combinations of DNA molecules that have been caracterized up to now do not use random DNA molecules or small recognition sequences. Rather, in all these events, transposable DNA molecules of a length of several hundred to several thousand base pairs are participating in the events that lead to the formation of new DNA combinations.

While the genetic studies of these elements are most detailed presently in maize, the molecular studies have revealed the greatest detail in procaryotes. Here, two kinds of transposable elements have been recognized: the smaller elements, starting at a few hundred base pairs and conspicuously often being of a size around 1.5 Kb, are devoid of any known genes and coding proteins. They are called insertion sequences or, abbreviated, IS-elements. Larger elements, called transposons, are very similar to IS-elements in most properties, but in addition carry genes encoding proteins. These proteins often, but not always, confer resistance to an antibiotic to the bacteria. I will briefly describe some properties of IS-elements and transposons, and will then discuss some of the new studies on the structure of these elements and their implications about the mechanism and specificity of transposition. Detailed reviews have appeared elsewhere (1 - 5).

B. Properties Of IS-Elements And Transposons

IS-elements are normal constituents of the E.coli chromosome. Some of them also occur on E.coli plasmids and bacteriophage. Presently, 6 such elements are known in E.coli K12. Most of them have been observed repeatedly.

Transposition of an IS-element into a gene inactivates this gene and can thus be detected as a mutation (6, 7). If the gene is part of an operon, effects on adjacent genes are observed. Most of these effects are on genes distally in the direction of transcription (6, 7). Most often, the effect is a decrease in the expression of the distal gene, due to transcription stop signals on the IS- element. Enhancement of the expression of the adjacent gene, probably due to the presence of promoter structures on these elements, has also been detected (8).

Deletions occur at an enhanced rate in the vicinity of many IS-elements (9). In some cases, the deletion endpoints could be determined by DNA-sequencing (1o, 11). In these cases, deletions end exactly at the terminus of the IS-element, which itself is not deleted. This suggests the participation of one terminus of the IS-element in deletion formation. Deletion formation may be an abortive transposition event, as will be discussed below.

Adjacent deletions, as well as some other chromosomal aberrations in the vicinity of IS-elements and transposons create a fusion between one IS-terminus and one non-IS-terminus. This mechanism is not yet capable to create new DNA arrangements of non-IS-DNA. Such arrangements could be created, however, when the IS-element would be precisely excised after deletion formation. Precise excision of IS-elements has been demonstrated, but for technical reasons only in those cases, where the precise excision event was not preceded by the formation of an adjacent deletion. For reasons to be discussed below, this latter event may not be possible, and it will be interesting to see a demonstration of it.

Another type of deletion created in the vicinity of an IS-element is the bi-directional deletion created by IS4. Bi-directional deletions remove the IS-elements and sequences adjacent to both of its termini, and thus create new DNA-arrangements (12). The generality of the phenomenon remains to be demonstrated.

In the case of IS2, a new principle for the generation of DNA-sequences has been discovered. Rare mutation events create sequence additions to IS2. Their length is around 5o to 1oo base pairs. The additional sequences are composed of

shorter sequences derived from both preexisting strands of IS2. It is assumed that these sequences arise by replication errors. The newly formed junctions can confer new functions to the IS-elements. They can act as moderate to strong promoters (13).

C. The Structure And Transposition Of IS-Elements

The mechanism of transposition of IS-elements and transposons is known incompletely. In some cases, it could be shown that transposons encode functions, the mutation of which abolishes transposition (14). Function studies with these proteins, however, are still in their early phase. More insight into the transposition mechanism has come from studies of the structure of the elements, because these structures could be analyzed in detail due to the rapid progress in restriction analysis and DNA sequencing. The first observation relevant to the transposition mechanism is the presence of inverted repeats of DNA at the termini of all transposable DNA elements that have been sequenced so far. In the case of IS-elements and some transposons, these inverted repeats are short (up to 4o base pairs) and contain mismatches (1o, 15 - 17). Some of the transposons, however, are flanked by larger elements, which are independent IS-elements themselves (18, 19, 2o). The generality of these findings suggests a functional role of the termini carrying inverted DNA repeats. Possibly, they can be recognized by a protein. This may suggest that in transposition these elements behave topologically as linear rods with two ends rather than as rings, as is well known for bacteriophage lambda (21).

The second relevant observation concerning transposition is the finding that the integration of an IS-element or transposon into a recipient DNA molecule creates a short duplication of recipient sequence at the point of integration (22, 23). Some IS-elements or transposons always create a 5 base pair duplication, others duplicate 9 base pairs, and a few IS-elements do not fall into these two classes. IS4, for instance, creates either an 11 or 12 base pair duplication.(16)

These duplications are different at different integration sites. Thus, they are really formed during integration and are not the result of a recombination event between a preexisting sequence in the recipient DNA and an identical sequence on the IS-element. The integration mechanism is therefore different from that of bacteriophage lambda, where the two common core sequences of the bacterium and the bacterio-

phage recombine (24). Grindley has suggested that a first step in transposition is the introduction of a staggered cut of chracteristic length, followed by the ligation of the two termini of the transposable element with the protruding single stranded ends of the host DNA. The ensuing gap could be filled and would give rise to the duplication flanking the IS-element.

A second set of observations indicates that transposition does not occur by excision from the donor site and integration into the recipient site. The present evidence suggests that transposition is accompanied by replication and that after this event two copies of the transposon are found in the old and a new site respectively (25, 26, 27). This finding can be interpreted in two different ways. Either it means that replication accompanies transposition. Alternatively, it can be assumed that transposition is initiated by an excision event and that the termini of the donor DNA cannot be joined and are therefore lost. In this case, only those chromosomes, (or chromosome arms) which do not participate in transposition, survive.

A more direct demonstration of replication is the observation of replicon fusion. If the transposon Tn3 carries a small deletion in a certain site, transposition is abortive. An attempted transposition between two plasmids leads to a cointegrate structure, which consists of one copy each of the two plasmids joined, at their two junctions, by one copy each of the transposon, which thus must have been replicated during the event (28). While replication is demonstrated unequivocally in this case, it cannot be said with certainty that this event is trapping an intermediate rather than leading to a non-physiological side product. Taken together, however, these observations suggest a transposition by replication rather than a transposition by excision.

Hypotheses have been formulated, according to which the ligation event described above will be linked to a replication of the transposon (29, 3o). These models, in addition to explaining transposition can also explain the formation of adjacent deletions and other aberrations and of replicon fusion. A common feature of these models is the assumption that the primary structure resulting from the replication is not a final product, and has to be converted to this final product by an ordinary recombination event. Since transposition occurs in recA strains, it has been postulated that a site-specific recombination system aids the transposition process. In the case of Tn3, such a site-specific recombination has been demonstrated (28, 31).

The precise excision of IS-elements restoring the wildtype DNA sequence is thus most probably not linked to transposition. It removes not only the IS element, but also one of the duplicated flanking sequences. The mechanism responsible may be the same which gives rise to deletions between short duplicated sequences in the lacI gene (32).

D. The Specificity Of Transposition

IS-elements and transposons can integrate at many sites, but some of them show a certain degree of specificity. In the gal-operon of E.coli, IS1 has a site of preference in the leader sequence, while IS4 is found in one position only in the center of galT. These two IS-elements differ not only by their site of preference. Sequence studies with IS1 have shown that within the leader sequence, several sites can be used for the integration of the IS-element. IS4, however, has been found in its site in three instances always in the same position (33, 16). Tn3, in a certain region, is found at several nucleotides, but some points are strongly preferred (33). The molecular basis for this specificity is not yet clear.

E. Future Questions

In order to get a more detailed understanding of the mechanism of transposition and its specificity, it will be necessary to identify the proteins involved. A first step in this direction will be the study of genes necessary for transposition. In the case of the transposon, the genes necessary may be carried by the transposon itself, as has already been shown for Tn3 (35, 36). Whether the smaller IS-elements encode functions for transposition is presently unknown. Proteins have not yet been identified and may be formed at a low rate, as transposition is a rare event. In the case of IS1, a few peptides, in the range of 7 to 15thousand kilodaltons are predicted from the sequence (1o) and one of them seems to be formed in a cell-free system (Trinks and Ehring, personal communication). In the case of IS4, a large open reading frame, extending over nearly the whole element is present, but it is not transcribed (12). It will be interesting to see, whether this open frame can, in some instances, be translated into protein and whether this protein has a role in transposition.

Elements very similar to the transposons and IS-elements have been found in a number of eucaryotes including maize (37), Drosophila (38), and yeast (39). The ubiquity of these elements suggests that they play an important role in the evolution and/or function of DNA. It will be interesting to study these roles in more detail.

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A MODULAR THEORY OF VIRUS EVOLUTION

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ABSTRACT A modular theory for virus evolution is proposed. The theory envisions viruses as belonging to large interbreeding families, members of which share only a common genome organization consisting of interchangeable genetic elements each of which carries out an essential biological function. Evidence for the theory obtained from the study of temperate bacteriophages is summarized, and the possible application to animal viruses is discussed.

INTRODUCTION

This paper presents an hypothesis concerning the way in which viruses may evolve. The experimental basis for the hypothesis comes primarily from work with what turns out to be a single large family of temperate bacteriophages, whose relationships are not easily explained by classical ideas about evolution along branching trees of linear descents. The modular theory proposed here may also be applicable in accounting for the relationships (many of which are documented elsewhere in this volume) and structural similarities among several groups of animal viruses, notably the adenoviruses and the retroviruses.

The essentials of the modular theory of virus evolution can be stated as follows:

1. The product of evolution is not a given virus but a family of interchangeable genetic elements (modules) each of which carries out a particular biological function. Each virus encountered in nature is a favorable combination of modules (one for each viral function) selected to work optimally individually and together to fill a particular niche. Exchange of a given module for another which has the same biological function (e.g., DNA replication) occurs by recombination among a population of different viruses related only by their similar modular construction. Viruses in the same interbreeding population can differ widely in any characteristic (including morphology and host range) since these

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are aspects of the function of individual modules; more significantly, viruses of the same family containing virtually identical modules can be found as parasites of hosts which themselves are only distantly related.

2. Evolution acts primarily not at the level of an intact virus, but at the level of individual functional units (modules). Selection upon these modules is for:

a) good execution of function

b) retention of flanking homology which ensures proper placement in the virus genome by homologous recombination and thereby guarantees proper regulation

c) functional compatibility with the maximum number of combinations of other functional units.

RESULTS AND DISCUSSION

The Particular Case of the Temperate Bacteriophages P22 and Lambda. The family of bacteriophages which best illustrate this kind of evolution (and which, incidentally, led to the development of the modular hypothesis (1,2)) is one which contains both of the best-studied temperate bacteriophages: bacteriophage lambda (whose normal host is Escherichia coli) and bacteriophage P22 (whose normal host is Salmonella typhimurium). These phages are morphologically completely unlike (Figure 1). Nevertheless, extensive genetic analysis of each led to the observation that their genetic maps were homologous: the order of genes (classified by their functions) is the same (Figure 2) (3,4). The degree of similarity is particularly striking when one examines the arrangement of genetic sites at which transcription begins and regulation is exerted. In fact, the only substantial difference between the two phages in genetic organization concerns the second (immI) immunity region, which P22 has and lambda does not have.

The homology between the maps of phages P22 and lambda, in spite of the gross dissimilarities between these phages in morphology, host range, DNA structure and replication, morphogenesis, and regulation (see ref. 4 for review), stimulated ultimately successful attempts to obtain hybrids between them (2,5). Extensive analysis of such hybrids (2,6,7) showed, surprisingly, that many different possible hybrids are possible, suggesting that homologous crossovers between them at virtually any position has the result of producing viable functional viruses.

Both phages P22 and lambda had already been known to be members of families of bacteriophages within which most recombinants result in viable hybrids. Many viable hybrids of lambda with other members of the "lambdoid" family defined by

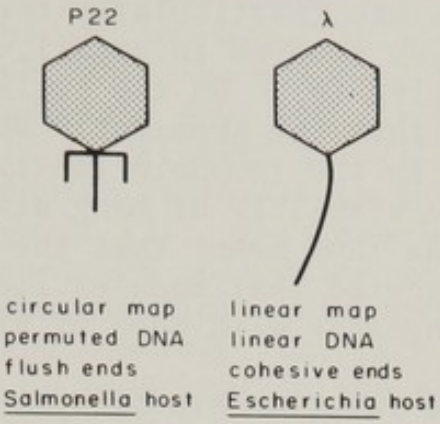

Figure 1. Schematic comparison of the temperate phage P22 and lambda.

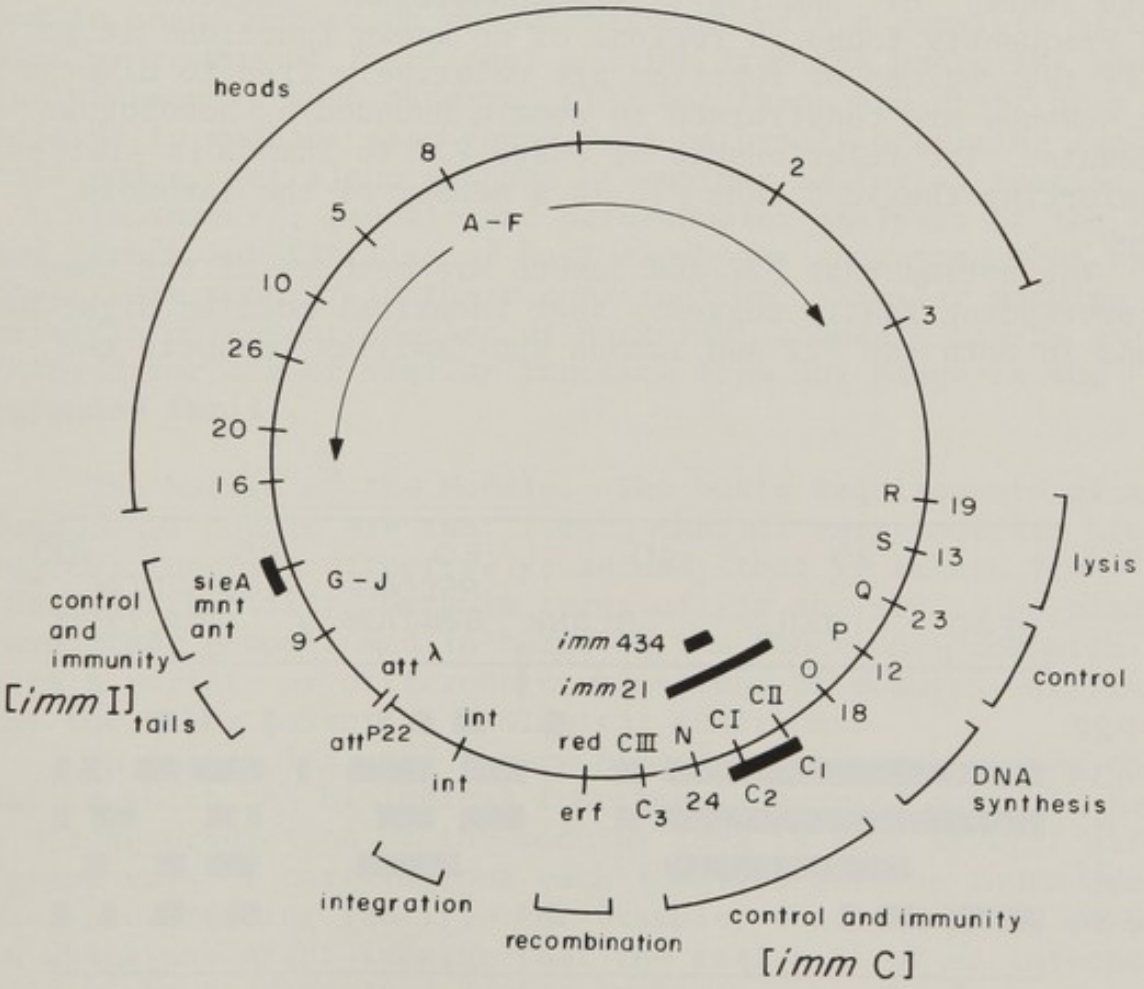

Figure 2. Homology between the order of functions on the genetic maps of P22 and lambda. P22 genes are shown outside the circle, lambda genes are shown inside. The basis for the alignment is given in ref. 3, from which the figure is adopted.

morphology, DNA structure (particularly the cohesive ends), and regulation had been studied in detail, particularly by DNA heteroduplex mapping (8). On the basis of these studies, Hershey (1) proposed that the apparently modular construction of all of the phages in the lambdoid family was the agency of their evolution. The viability of many different hybrids between P22 and lambda thus meant that the two families were in fact one, and suggested that modules from either sub-family were interchangeable with ones of similar function in the other.

This view of the relationship among the lambdoid coliphages (now taken to include the Salmonella phage P22 and its relatives) can best be visualized in the diagram shown in Figure 3 which is an adaptation of Hershey's (1) summary of the DNA heteroduplex analysis of the lamboid bacteriophages. Regions of non-homology are interspersed with regions (often quite short) of homology. The homologous segments are frequently found in regions of no known function: it is as if the regions of function are relatively free to diverge but somehow are constrained to remain bounded by homologous segments. The relationship of phage P22 to lambda is similar, reinforcing the idea that P22 is a member of the lambdoid group.

The notion that P22 and lambda are members of the same interbreeding family suggests that identical modules might be found in both the P22 and lambda sub-families, despite the

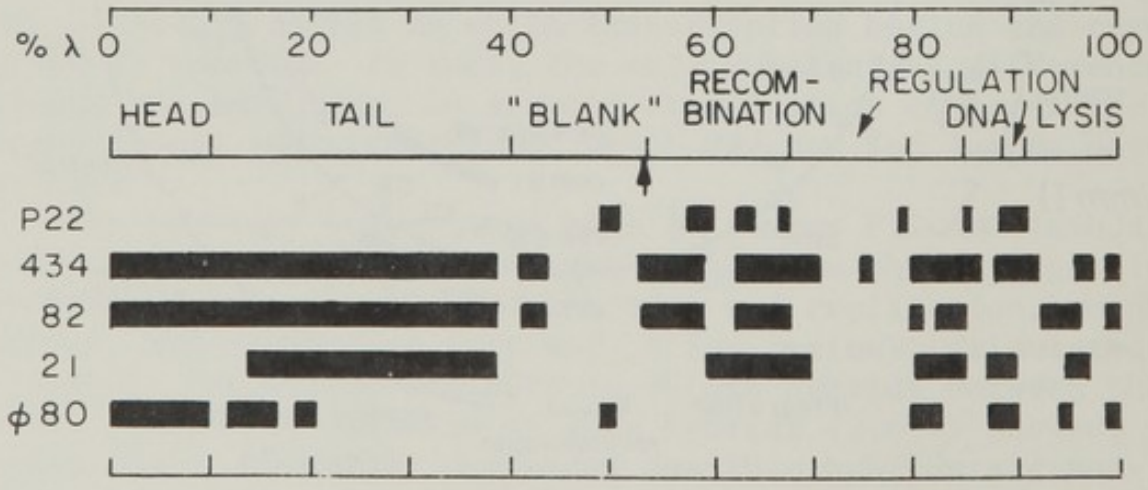

Figure 3. Summary of homology among lambdoid phages is determined by DNA heteroduplex analysis. The bars indicate position and extent of homology between the particular phage and lambda itself. The figure is redrawn from Hershey (1), with P22 data (2,6,9) added. The scale is in % physical length on lambda.

fact that the two sub-families parasitise different species of bacteria. This prediction was fulfilled in the analysis of P22/lambda hybrids, which showed that the P22 immC region (which contains a repressor gene and its operators) was functionally identical in specificity as well as totally homologous (by DNA heteroduplex criteria) to the analogous region of the lambdoid phage 21 (2). This conclusion has since been further confirmed by direct nucleotide sequence analysis (A. Poteete, personal communication). Similarly, by both functional specificity and homology, the late regulator gene 23 of P22 was found to be identical to the late regulator gene Q of phage lambda itself (9).

A more striking example is the recent discovery by Friedman and colleagues (personal communcation) that a segment of DNA which is homologous and functionally indistinguishable from P22's DNA replication module exists as a totally silent genetic element in Escherichia coli K12. In this case, the module seems not to be associated even with a complete virus. Similar rescue of functional modules from the bacterial genome had previously been observed (10) but these had never been related to modules also found in a naturally occurring lambdoid phage, let alone a phage of another bacterial species.

In summary, genetic and heteroduplex analysis of the P22 and lambda sub-families of bacteriophages suggested that these phages are modular in their construction. Similar modules exist in different species of bacteria; a module can be exchanged for one of similar function from any phage in the extended family.

The Nature of the Module. The basic requirements of a functional module are two: first, that it carry out its biological function effectively; second, that it retain its interchangeability, both in terms of its ability to be placed into other genomes and in terms of its functional compatibility with a variety of different combinations of modules carrying out the other essential biological functions.

In principles, the theory places no constraint upon the way in which the biological function is carried out. In fact, a strong prediction of the modular idea is that several different ways of carrying out each function will be found among the interbreeding family. For example, there are many ways in which one might imagine that the cell wall of an infected cell might be dissolved during lytic growth. In fact, the lambdoid phages have two known methods: phages 80 and P22 have a true lysozyme (which breaks a glycosidic bond) whereas phage lambda uses an endopeptidase. The genes specifying these enzymes are completely non-homologous and the proteins are in no way similar except in their biological function.

Yet these genes are found in the same position on the genetic maps of the phages where they first were found and are all interchangeable with each other in hybrids.

Table I lists the modules one can simply define in a temperate bacteriophage of the lambdoid family, along with the number of easily distinguishable mechanisms used by at least one of the modules in the family. However, mechanism is not the only (and probably not the most important) variability in function of modules. Many of the functions of the phage exhibit a specificity (for example for the host surface, or host DNA replication proteins) which can vary among modules. As shown in Table I, the number of distinguishable specificities is very large for some modules (such as the tail, which specifies host range). It may well be that the most important advantage of modular evolution (as opposed to linear descent) is the easy and continual access to a large variety of different specificities.

TABLE I
NUMBERS OF SPECIFICITIES AND MECHANISMS ASSOCIATED WITH LAMBDOID PHAGE MODULES

Function (module)	λ genes	Specificities	Mechanisms
DNA encapsulation	A-F	2	2
Tails	G-J	10+	2
Antigen conversion	b2	10+	2+
Integration	att,int,xis	10+	1
Recombination	red,gam	- - - - - 3 - - - - -	
Early control	N	5	1
Immunity	cI	10+	2
DNA replication	O,P,ori	3	2
Late control	Q	6	1
Lysis	S,R	---	2

The meaning of differences in specificity and function is given in the text. Estimates of the numbers of specificities and mechanisms are based on the literature and upon experiments in the author's laboratory. For example, the large number of tail specificities is based on the observation of very many host ranges; the two mechanisms represent the short tail (P22) and the long tail (lambda) which are obviously mechanistically different.

The Role of Homologous Recombination. The requirement that modules retain their means of interchange must be met by a method which will assure that the whole assembly of modules (i.e. the virus) be properly regulated regardless of which particular modules are present for each biological function. This assurance is intrinsic to the way in which these phages are regulated and by the method of interchange which is homologous recombination. The regulation of the lambdoid bacteriophages (including, of course P22) is at the level of transcription, through a cascade of regulators each of which controls transcription at sites immediately adjacent to itself. Thus the phage repressor regulates transcription in both directions at operators immediately flanking itself. The repressor and its operators are a module which can be interchanged since all the specificity-bearing elements are linked. The transcription leftward includes the positive regulator N (or its many other modular analogues, such as gene 24 of P22) which allows transcription to proceed into the DNA replication, recombination, and late control modules. The late control gene, when expressed, allows all the late modules to be transcribed, as a single operon. Thus a module interchange simply required that homology between (or at the termini of) functional segments be retained. Since recombination will exert specificity for homology, each module (provided it has the homology at its ends) automatically is placed in the right position and orientation to be transcribed in proper sequence. Thus proper regulation is assured no matter which combinations of modules are present, and thus it is easy to account for the observation that virtually all hybrids among the temperate bacteriophages (even using modules that exist as cryptic host genes or which come from distantly related hosts) are viable and normally regulated.

The role of homology also accounts for the observation that there are relatively many instances of homology between phages at positions not known to code for any function (Fig. 3). The conservation of these homologies is a natural consequence of the modular theory: these regions allow correct interchange of modules of like function, and are thus selected since a module which has lost its ability to be placed in the proper sequence has lost a crucial selective advantage.

In summary, the individual module is selected for retention of some common sequences at its ends which ensure ability to be recombined into the proper position in a variety of different phages, as well as for good execution of its biological function. An extension of this idea, of course, is the notion that modules also will be selected for functional compatibility with any other modules where joint function is required. This requirement is to some degree vitiated by the observation that interacting functions are, in these

viruses, usually adjacent genetically and can, under some circumstances, be joined into a single module. This is clearly the case, for example, in the head assembly system: the P22 and lambda systems, which are very different from each other, contain sub-modules (11) with respect to their own subfamilies; but with respect to each other the vast difference in mechanism requires that the entire head region be exchanged as a unit. As might be anticipated, no homology between the two head assembly regions has been observed (Botstein, unpublished observation).

Modular Evolution Accounts for the P22 Antirepressor System. One of the great puzzles in P22 biology has been the observation that the secondary immunity system (immI; 12,13, 14) is entirely deletable with no obvious negative consequences to the normal lytic or lysogenic life cycles of the phage. If one accepts the modular theory, however, the advantage of association with an antirepressor is explicable. Susskind and Botstein (15) showed that P22 antirepressor inactivates the repressors of not only P22, but also of lambda and some of its relatives. It is, in other words, very broad in its specificity for temperate phage repressors. Therefore a phage which carries an antirepressor and infects a cell in which one of its own modules fails to work has a selective advantage, because it will induce the resident prophages and elicit thereby the function of all the modules present in that cell. Induction of the prophages will also frequently result in replication of the prophage DNAs, increasing the probability of a module exchange, and thereby the creation of a new phage containing many of the old modules which is now a fitter host for growth on this particular host.

In fact, the antirepressor advantage nicely illustrates the overall advantage of the modular system in the rapidity and flexibility of response to new environments. Clearly the availability of substitute modules of different specificity and/or mechanism but which have been evolved individually for good function will be an advantage even when a system of antirepressors is not present to increase the frequency of exchange. The module exchanges which have been observed in the laboratory have occurred at frequencies comparable to or higher than the mutation rate. At such frequencies, module exchange seems to offer greater speed and flexibility than evolution by linear descent.

Conclusion. The modular theory of virus evolution has clear experimental support among the temperate bacteriophages of the enteric bacteria. However, there is also similar genetic and DNA heteroduplex evidence for such evolution among other families of bacteriophages: the virulent

bacteriophages of the enterics comprise several families: the T-even group, the T3-T7 group (which has many members among different species of bacteria, including bacteria as widely divergent as E. coli and Caulobacter crescentus (16)). It nicely explains the diffusion of very similar homologous bacteriophages into hosts whose own DNAs have diverged very greatly from each other in nucleotide sequence. It also accounts for the rigorous maintenance of regulatory schemes while units of function (including regions coding for proteins) diverge more rapidly.

It should also be noted that the considerations which make modular evolution seem advantageous for bacteriophages apply equally well to viruses of higher organisms. Many of the signal properties of bacteriophage families which make the modular theory attractive are shared by certain families of animal viruses, notably the adenovirus and retroviruses. These families, as noted elsewhere in this volume, maintain similarity in genome organization, homology and sometimes even interchangeability of functions over a range of widely divergent host species. Quite possibly animal viruses also evolve as a population of interchangeable modules.

ACKNOWLEDGMENTS

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MOLECULAR CLONING OF THE HUMAN CYTOMEGALOVIRUS GENOME (STRAIN AD169)[1]

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ABSTRACT Human Cytomegalovirus (HCMV), one of the herpesviruses, is medically significant both as a cause of birth defects and as a source of problems in immunosuppressed individuals. Because the virus is highly cell-associated, it is difficult to obtain large quantities of pure viral DNA. For this reason we have sought to construct a cloned library of the HCMV genome to aid in further studies of the molecular biology of HCMV infections. The cloning was accomplished as follows: Eco R1 fragments of the AD169 strain of HCMV (0.27 µg of DNA) were inserted into the Eco R1 site of the *E. coli* plasmid PACYC 184. Approximately 9,000 colonies were selected on the basis of tetracycline resistance and chloramphenicol sensitivity. After isolation and Eco R1 cleavage of the recombinant plasmids, the cloned fragments were analyzed for co-migration on agarose gels with authentic Eco R1 digested HCMV DNA. The viral origin of the fragments was determined by hybridization of ^{32}P CMV DNA to Southern blots of the inserts. In those cases where multiple clones containing the same size fragment were obtained, the DNA was cleaved with additional restriction endonucleases to characterize further the insert. To date, we have analyzed 84 clones and the viral inserts obtained represent at least 80% of the genome.

INTRODUCTION

The human cytomegaloviruses (HCMV) comprise a large family of ubiquitous species-specific agents within the herpesvirus family. The virions consist of a large DNA genome (1.5 X 10^8 daltons) enclosed in an icosahedral capsid which is surrounded by an envelope consisting of one or more membranes (1,2).

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These viruses have been associated with a variety of diseases and may have oncogenic potential (see 2,3,4, and 5 for reviews). An infection with HCMV is most often asymptomatic; however, the exceptions are severe enough to make this a medically significant virus. HCMV infection is the most frequent perinatal infection of newborns and involves approximately 1% of all live births. Although only a fraction of these infants exhibit symptoms of generalized cytomegalic inclusion disease, (CMID), several follow-up studies have indicated that a large number of presumably "asymptomatic" patients have suffered some degree of sensorineural hearing loss, mental retardation, and other neurological sequelae (6, 7,8,9). The mechanism of infection and the factors which determine the severity of the disease remain undetermined. HCMV infection also presents a grave danger for individuals undergoing treatment with immunosuppressants. Fatal cases of pneumonia caused by this virus occur in renal and bone marrow transplant patients and in those individuals undergoing treatment for neoplastic disease. HCMV can also cause a form of mononucleosis resembling the infectious mononucleosis caused by Epstein-Barr virus.

A few experiments hint at the possibility that HCMV has oncogenic potential. Infection with HCMV stimulates host cell DNA synthesis and can induce fibroblasts to grow in agarose for several generations (10,11). Furthermore, hamster fibroblasts and Balb 3T3 cells can be transformed in culture after infection with UV-inactivated HCMV (12, and unpublished results of D. Spector). In human cells, although no conclusive data exists, there is one report that human cells can be transformed *in vitro* by a non-inactivated genital HCMV isolate (13).

At the cellular level, a productive HCMV infection is characterized by its cytopathic effect (CPE), a rounding and swelling of the cells accompanied by an increasingly refractile appearance (see 2 for review). Cytoplasmic and nuclear inclusions appear which have been shown to be sites of viral antigens. Although initiation of CPE is rapid, HCMV grows slowly, with an eclipse time of 55 hours. Viral DNA synthesis is initiated in the nucleus between 24 and 48 hours, with maximum synthesis occurring between 48 and 96 hours post infection. Assembly of the virion also occurs in the nucleus, and the virus acquires its envelope as it buds through the nuclear membrane. The events in the infection cycle have not been fully elucidated. It is known that HCMV stimulates host macromolecular synthesis as well as its own viral DNA polymerase. In non-permissive cells, early viral antigens can be detected, but either little or no virus is produced. Non-human cells and human cells of epithelial origin are non-

permissive for HCMV infection. Curiously, the opposite is true *in vivo* with epithelial tissue being the site of active infection.

The realization that HCMV is associated with a variety of human diseases has made it of increasing importance to understand the molecular biology of HCMV infections. In our laboratory, we have developed a number of *in vitro* systems for studying the interaction of HCMV with cells during permissive and nonpermissive infections and the oncogenic transformation of cells by HCMV. A major limiting factor in these studies has been the difficulty of preparing reasonable quantities of pure viral DNA for use as a molecular hybridization reagent. In order to provide large quantities of well-characterized subgenomic fragments of the HCMV genome, we have cloned the DNA from HCMV strain AD169.

RESULTS

Strategy for the cloning of the HCMV genome. The procedure used for cloning HCMV DNA is illustrated in Figure 1. Viral DNA for the cloning was obtained from a low multiplicity infection of human embryonic lung (HEL) cells with a plaque-purified stock of the HCMV strain AD169. We routinely use low multiplicity infections in the propagation of virus to minimize the number of defective particles produced. The extracellular virus was harvested from the medium and isolated by centrifugation through a pre-formed cesium chloride gradient. After treatment with SDS and pronase, the DNA was purified twice by isopycnic cesium chloride centrifugation. The equilibrium density of HCMV DNA (1.716 g/cm^3) differs sufficiently from that of cell DNA (1.699 g/cm^3) to permit efficient separation of the viral DNA from any contaminating cell sequences.

The purity of the isolated HCMV DNA has also been assessed. Because the only contaminant might be human DNA, we have performed the following two experiments. The viral DNA was first labeled to a high specific radioactivity (8 X 10^7 cpm/µg) *in vitro* using α-^{32}P deoxynucleotide triphosphates as substrates and the nick translation activity of *E. coli* DNA polymerase I (14). In the first experiment this labeled HCMV DNA was hybridized in solution with an excess of either uninfected human cell DNA (Cot greater than 10^4) or purified HCMV DNA (Cot greater than 5 X 10^0). The labeled CMV DNA hybridized completely to the purified HCMV DNA and less than 1% to the uninfected human cell DNA. In order to determine if the purified HCMV DNA might contain a low level of reiterated human DNA, a second experiment was performed. High molecular weight DNA from either uninfected human cells or human

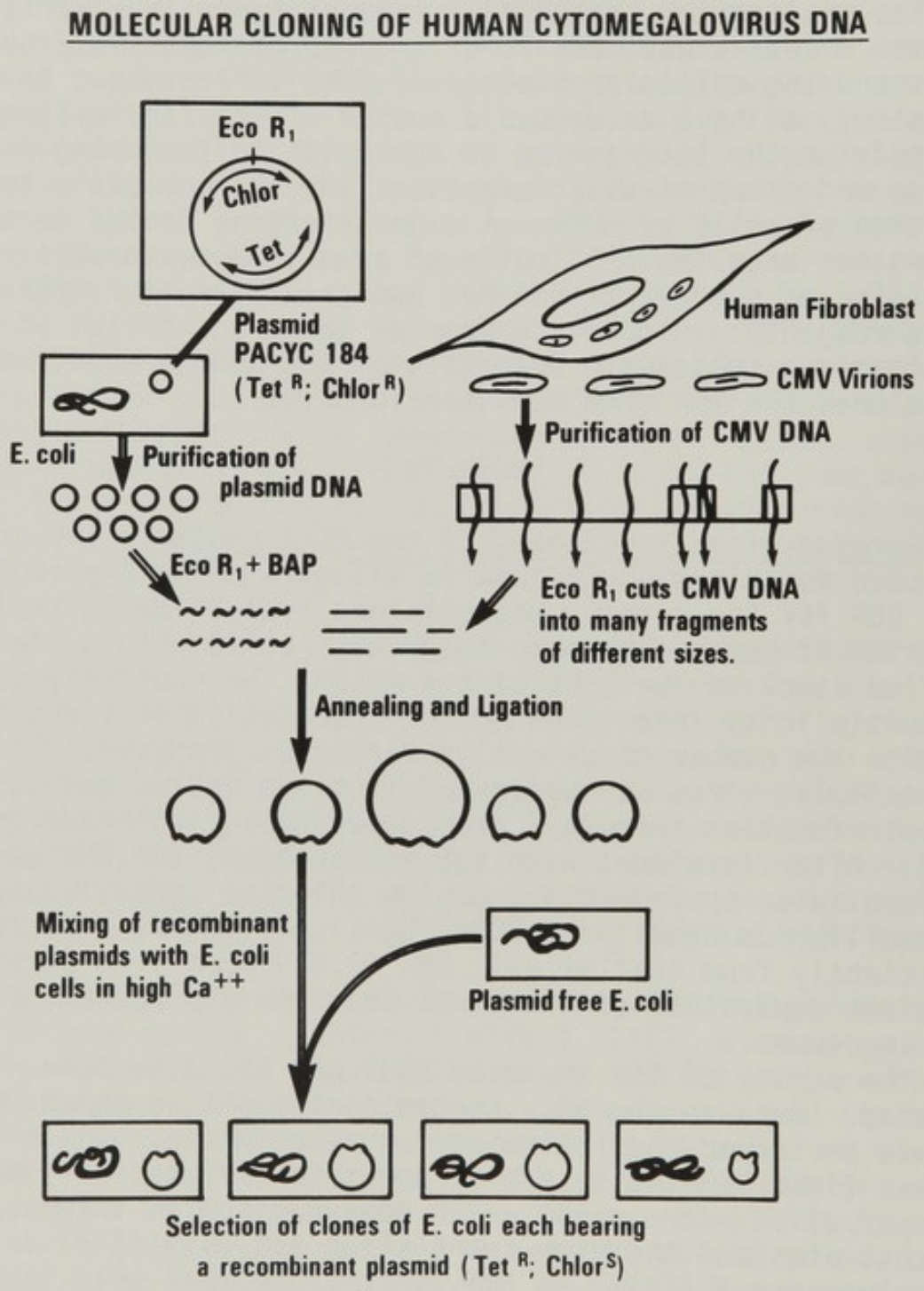

FIGURE 1. Strategy for the cloning of human cytomegalovirus DNA.

cells infected with HCMV was purified, cleaved with Eco R1 restriction endonuclease and the fragments separated by agarose gel electrophoresis. The unlabeled fragments were then transferred to nitrocellulose filters using the method of Southern (15). A large excess of labeled HCMV DNA was then hybridized to the nitrocellulose filters, and the filters subjected to autoradiography. Distinct bands of radioactivity were observed on the filters containing the HCMV infected cell DNA. No bands of radioactivity were observed on the filters containing the uninfected cell DNA even after long periods of exposure to X-ray film. We therefore concluded that the viral DNA was greater than 99% pure.

The cloning vehicle used was the *E. coli* plasmid PACYC 184 (molecular weight of 2.65×10^6 daltons), which carries resistance markers for both tetracycline and chloramphenicol and which contains a single cleavage site for the restriction endonucleases Eco RI, Bam HI and Hind III (16). Purified HCMV DNA was cleaved with Eco R1 restriction endonuclease producing the restriction pattern shown in Figure 2. This cleaved DNA was then ligated to PACYC 184 which previously had been cleaved with Eco RI and treated with bacterial alkaline phosphatase to prevent recircularization of the plasmid alone.

Transfection was accomplished using *E. coli* strain HB101 ($recA^-, R^-, M^-$) and the procedure of Kushner (17) which employs calcium chloride and rubidium chloride to promote uptake of the recombinant plasmid by the bacteria. Transformed colonies carrying recombinant plasmids were selected on the basis of chloramphenicol sensitivity and tetracycline resistance. Since the single Eco RI site in PACYC 184 maps within the chloramphenicol resistance gene, all tetracycline resistant and chloramphenicol sensitive clones should possess plasmid which contains an HCMV insert. In the transfection 0.27 μg of HCMV DNA yielded approximately 10,000 tetracycline-resistant colonies, which corresponds to a transformation frequency of 3.7×10^4 transformants/μg of HCMV DNA. Of these, 90% were chloramphenicol sensitive, indicating the presence of a recombinant plasmid.

Initial Characterization of the Cloned HCMV DNA. For the initial characterization of the clones, plasmid DNA was isolated by means of a rapid purification procedure (18) which involves treatment of the bacteria with lysozyme and detergent. The high molecular weight chromosomal DNA pellets with the cell debris, and the plasmid DNA in the supernatant is purified further by phenol extraction. Although not entirely free of bacterial chromosomal sequences, these preparations are sufficiently pure to allow identification and analysis of the recombinant plasmids. The isolated recombinant plasmids

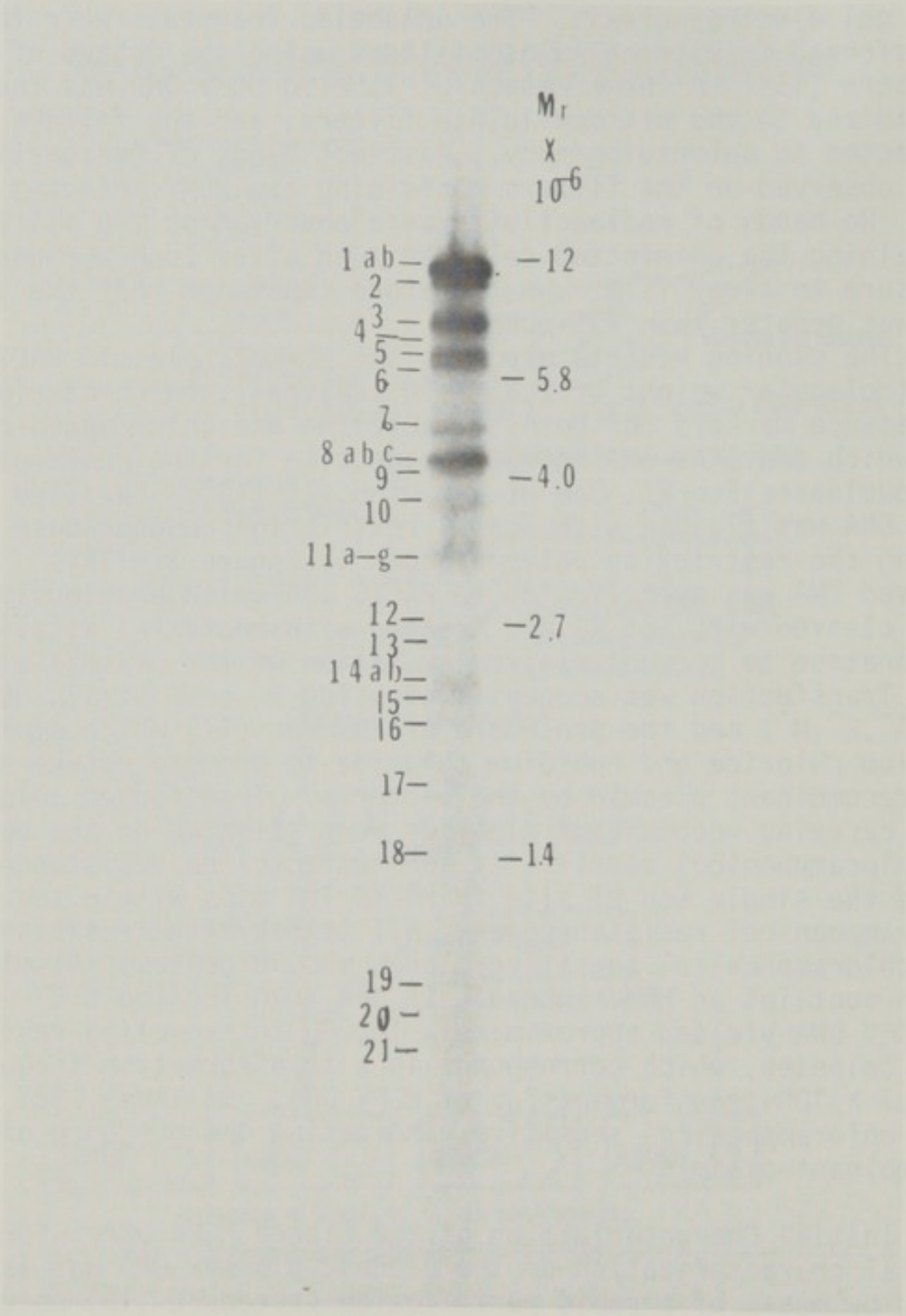

FIGURE 2. Eco RI cleavage of HCMV DNA. ^{32}P-labeled HCMV DNA was cleaved with Eco RI and fractionated on a 0.8% agarose gel. The numbers assigned to the bands of the restriction pattern are shown to the left of the gel.

were then cleaved with Eco RI, and the fragments were separated by electrophoresis through agarose gels to determine the size and identity of the inserted sequences. Assignment of a fragment to a specific HCMV band was made on the basis of co-migration of the cloned fragments with bands from an Eco RI digest of HCMV DNA (Figure 2). The viral origin of the inserted sequences was confirmed by hybridization of nick translated ^{32}P-labeled HCMV DNA to Southern blots of DNA. The gels in Figure 3 (panels A-D) show a representative collection of the clones. Cleavage with Eco RI yields a plasmid band at 2.65 X 10^6 Mr plus the viral insert; lane 1 in panel C of Figure 3 shows the band produced by cleavage of PACYC 184 alone. The length of the insert was not prohibitive in the cloning as evidenced by the large fragments obtained. Clones representing the largest HCMV fragments (1a,1b,2,3,6,7, and 8a) appear in panel A. Panel B shows clones co-migrating with HCMV bands 8b, 8c, 9, 10, 11a and 11b. Bands 11d-g, 12 and 13 are represented by the clones shown in panel C and the smallest cloned inserts, corresponding to bands 14a, 14b, 15, 16, 17, and 18, appear in panel D. Figure 3E shows an example of the hybridization of ^{32}P-labeled HCMV DNA to a Southern blot of the DNA from panel D. The sensitivity of the hybridization method under these conditions is demonstrated by the appearance of a band in lane 5 of panel E, even though there is no trace of DNA by ethidium bromide staining (lane 5 of panel D).

Of the 84 clones analyzed, 48 contained identifiable inserts ranging in size from 1.5 - 11.5 X 10^6 daltons (Table I). We have obtained cloned fragments which co-migrate with 14 of the 21 bands visible in an Eco RI digest of HCMV. Bands 8 and 11 are more highly represented with 5 and 12 clones, respectively, showing co-migrating inserts. Of the bands not represented among our clones, at least two are the termini of the genome and will have to be cloned using Eco RI linkers (see Discussion). The remainder should be obtained upon the screening of more clones. The 36 clones which have not yielded large inserts appear to contain mostly recombinant plasmids carrying small HCMV inserts (Mr of less than 10^6). Experiments aimed at characterizing these small inserts are currently in progress.

Further Analysis of the Cloned HCMV DNA With BAM HI and Hind III restriction endonucleases. Addition of the molecular weights of the visible bands of Eco RI digested HCMV DNA produced a total genome size of 90 X 10^6 daltons which is considerably less than the reported value of 150 X 10^6 daltons (1). The bands of the Eco RI digest also showed variations in intensity, suggesting that some viral sequences may co-migrate on the gel (see Figure 2). In accordance with

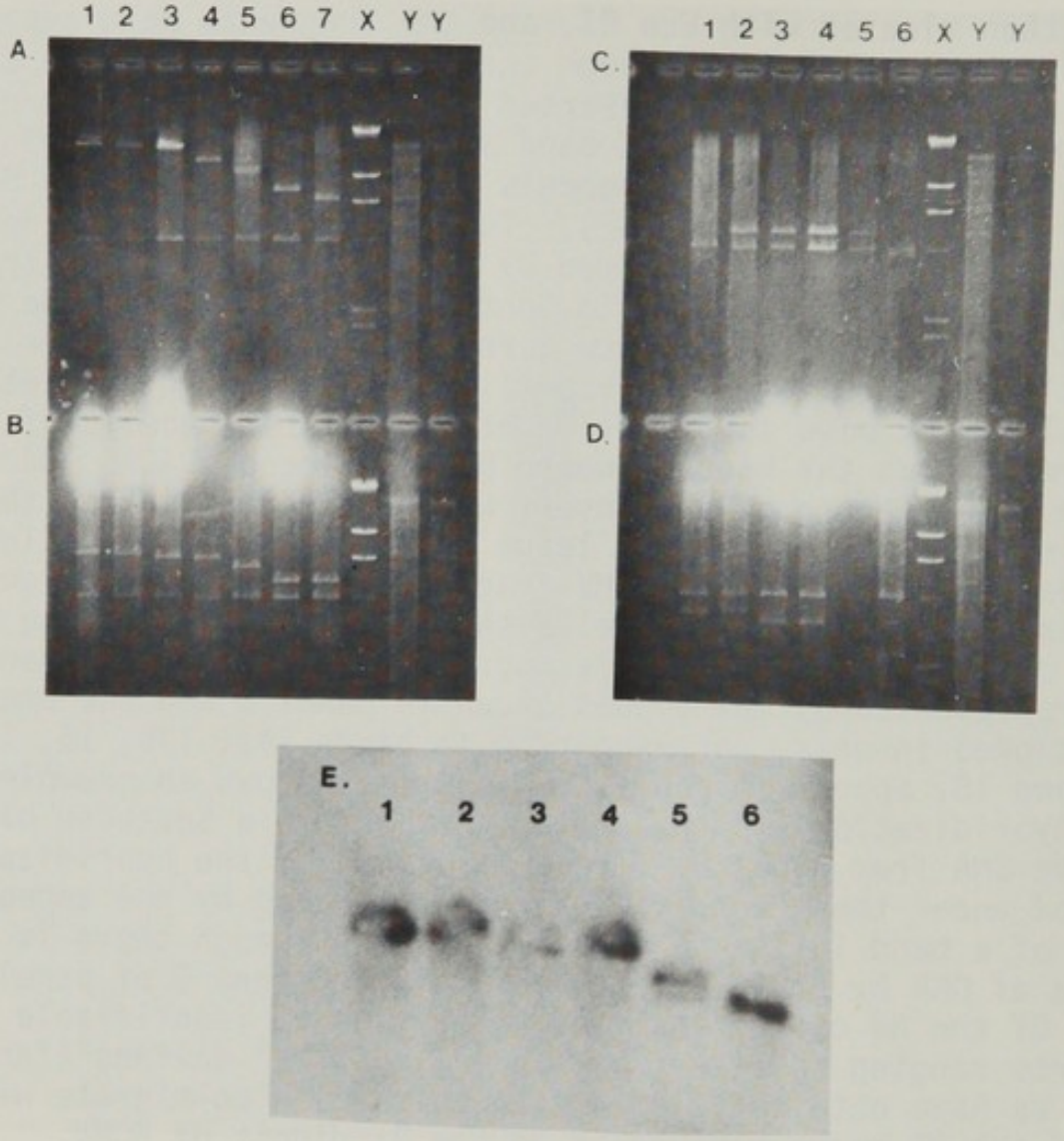

FIGURE 3. A-D. Analysis of a representative collection of the cloned fragments obtained. Recombinant plasmid DNA was purified, cleaved with Eco RI, fractionated on agarose gels, and stained with ethidium bromide. The cloned inserts correspond to HCMV Eco RI restriction bands. Panel A. Lane 1 contains an insert co-migrating with band 1a; 2, band 1b; 3, band 2; 4, band 3; 5, band 6; 6, band 7; 7, band 8a. Panel B. Lane 1, band 8c; 2, band 8b; 3 and 4, band 9; 5, band 10; 6, band 11a; 7, band 11b. Panel C. Lane 1, PACYC 184; 2, band 11d; 3, band 11e; 4, band 11f; 5, band 11g; 6, band 13. Panel D. Lane 1, band 14a; 2, band 14b; 3 and 4, band 16; 5, band 17; 6, band 18. Lanes marked X contain fragments of Hind III cleaved λ DNA (Mr 17.2, 14.6, 5.8, 4.0, 1.4, and 1.2 X 10^6). Lanes marked Y represent Eco RI digested DNA from cells infected with HCMV strain AD 169. Panel E. The DNA fragments from the gel in panel D were transferred to nitrocellulose filters using the procedure of Southern (15) and hybridized with nick-translated ^{32}P-labeled HCMV DNA.

TABLE 1
CLONED ECO RI FRAGMENTS OF HUMAN CMV DNA

Band #	Size of Eco RI Fragment (Mr X 10^{-6})	Number of Clones Analyzed
1a	11.5	2
1b	11.4	1
2	10.5	2
3	8.3	1
4	7.8	-
5	6.9	-
6	6.4	2
7	4.7	1
8a	4.2	3
8b	4.2	1
8c	4.2	1
9	4.0	4
10	3.6	1
11a	3.2	5
11b	3.2	1
11d	3.2	2
11e	3.2	1
11f	3.2	1
11g	3.2	1
12	2.65	-
13	2.55	4
14a	2.4	2
14b	2.4	1
15	2.3	-
16	2.1	3
17	1.8	2
18	1.5	3
19	<1	
20	<1	
21	<1	

this hypothesis, the most intense bands of the Eco RI digest were more highly represented in our collection of clones. To determine whether the prevalence of clones in these particular bands was due to the presence of multiple co-migrating sequences, the uncleaved plasmid DNA was treated with either Hind III or Bam HI. The resulting fragments were fractionated by electrophoresis on agarose gels and examined for differences in Bam HI or Hind III cleavage sites. It was hoped that any differences in the sequences of the inserts would be revealed

by variations in the restriction endonuclease patterns.

One variation in the restriction endonuclease patterns results from the two possible orientations of the insert within the plasmid. When the recombinant molecule is treated with Eco RI, the original components are regenerated; however, treatment with another restriction endonuclease alone (especially with an enzyme which cleaves only once within plasmid DNA) would generate some "hybrid" fragments containing portions of both viral and plasmid sequences. The data in Figure 4 exemplify this point. Panel A shows the fragments which result from Eco RI cleavage of three clones with inserts which co-migrate with HCMV Eco RI band 13. The restriction pattern for the 3 clones consists of a plasmid band of 2.65 X 10^6 Mr and a viral sequence of 2.55 X 10^6 Mr. To determine whether these clones contained identical viral sequences, the recombinant plasmids were digested with Hind III. PACYC 184 has one Hind III site located 1.4 and 2.57 kb (0.95 and 1.7 X 10^6 daltons) from the Eco RI site. The number of Hind III sites

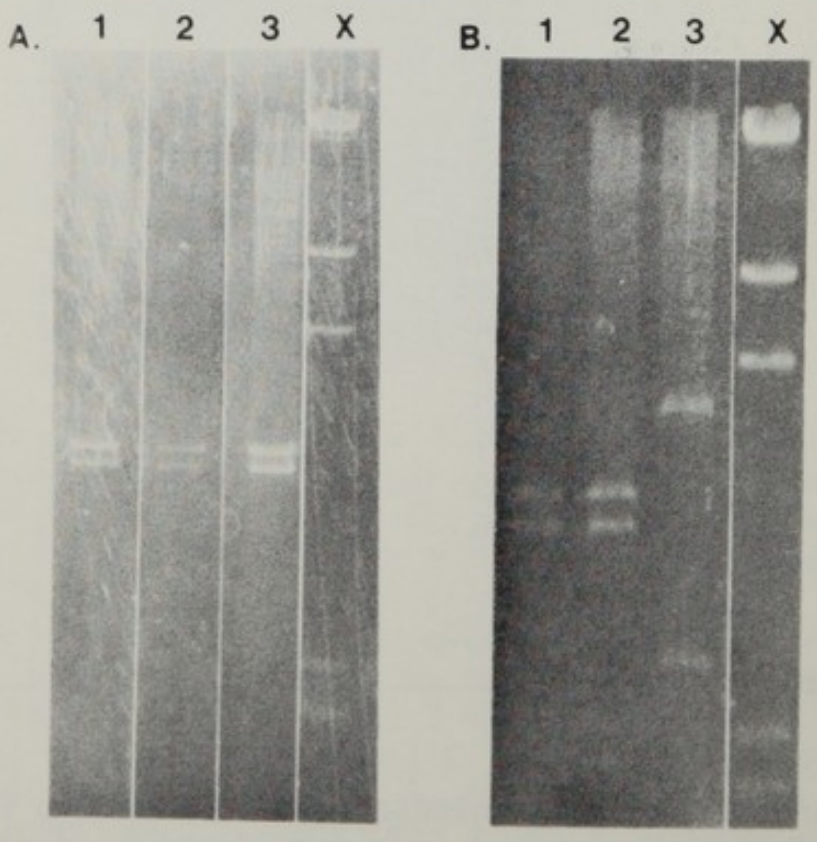

FIGURE 4. Analysis of cloned fragments representing HCMV band 13 with A. Eco RI and B. Hind III restriction endonucleases. The resulting fragments were separated on agarose gels and stained with ethidium bromide. Lane 1, clone 2; 2, clone 19; and 3, clone 20. X, fragments of Hind III cleaved λ DNA (Mr of 17.2, 14.6, 5.8, 4.0, 1.4 and 1.2 X 10^6).

within the viral insert can be determined from the number of fragments produced upon restriction endonuclease digestion of the recombinant plasmid. If no restriction sites exist within the insert, only one band (equal in size to plasmid plus insert) will be produced. If one cleavage site occurs within the insert, two fragments will be produced, each containing a portion of the viral insert linked to one of the plasmid fragments. Because the viral insert can have two orientations within the plasmid, 2 restriction patterns are possible. The simplest interpretation of the data in Figure 4B is that all of the viral inserts co-migrating with HCMV band 13 contain a single Hind III site located 1.2 and 2.6 kb (0.8 and 1.7 X 10^6 daltons) from the ends of the viral fragment. In lanes 1 and 2 of Figure 4B, the smaller of the two viral Hind III segments (Mr of 0.8 X 10^6) is linked to the long (1.7 X 10^6 Mr) plasmid fragment and the larger (1.7 X 10^6 Mr) viral fragment adjoins the small (0.95 X 10^6 Mr) plasmid sequence resulting in bands of 2.5 and 2.7 X 10^6 Mr. The opposite orientation of the viral insert (lane 3) links the short (0.95 X 10^6 Mr) sequence of the plasmid with the small (0.8 X 10^6 Mr) viral segment and vice versa to produce Hind III fragments that migrate at 3.4 and 1.8 X 10^6 Mr. Double digestion of the viral fragment with both Eco RI and Hind III should confirm the inverted orientation of this insert.

We have also analyzed the multiple clones comprising the band 11 group. Panel A of Figure 5 shows the Eco RI restriction pattern from the initial characterization of the clones. All of the clones have a band corresponding to the plasmid at 2.65 X 10^6 Mr and an insert of approximately 3.2 X 10^6 Mr. In panel B, recombinant plasmid from these clones was cleaved with Hind III. Two different restriction patterns were produced: a single band at 5.85 X 10^6 Mr which corresponds to the size of insert plus plasmid (shown in lanes 1-10), and a two band pattern (4.15 and 1.7 X 10^6 Mr) in lane 11.

Since many of the band 11 clones tested had no Hind III site in the insert, we decided to use in our analysis another restriction endonuclease, Bam HI, which also cleaves only once within the plasmid. The results are shown in Figure 5C and Table 2. Digestion with Bam HI produced 6 different restriction patterns (represented by classes 11a-g). The clone (class 11b) which had produced a different restriction pattern with Hind III also generated unique fragments when treated with Bam HI (Table 2). The clones with no Hind III site in the insert fell into the remaining 5 classes (11a, d, e, f, g). In this collection, class 11a was the most prevalent with 4 clones (lanes 1, 2, 3, 10 of Figure 5C) yielding the same Bam HI fragments (Mr of 3.6 X 10^6 and 2.25 X 10^6).

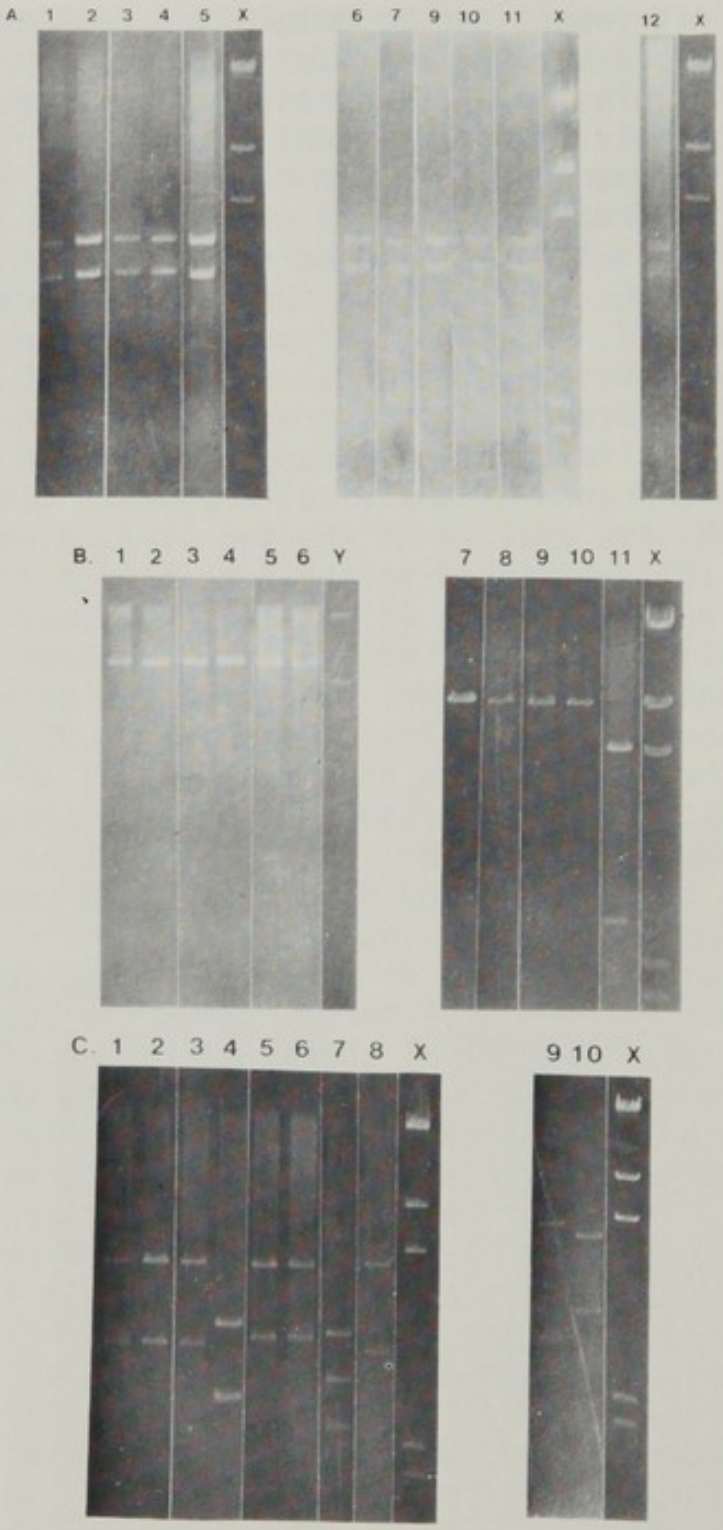

FIGURE 5. Analysis of the cloned fragments representing HCMV band 11 with A. Eco RI, B. Hind III, and C. Bam HI. Lane 1, clone 5, corresponds to band 11a; 2, clone 13, band 11a; 3, clone 52, band 11a; 4, clone 53, band 11e; 5, clone 15, band 11d; 6, clone 24, band 11d; 7, clone 75, band 11f; 8, clone 60, band 11g; 9, clone 80, band 11a; 10, clone 82, band 11a; 11, clone 71, band 11b; X, fragments of Hind III cleaved λ; Y, fragments of Eco RI cleaved λ, (Mr of 15.7, 4.7, 3.6, 3.4, and 3 X 10^6).

TABLE 2
ANALYSIS OF SELECTED CLONES WITH HIND III AND BAM HI RESTRICTION ENDONUCLEASES

Band	Number of Clones Analyzed	Size of Plasmid Plus Insert (Mr X 10^{-6})	Size of Hind III Fragments (Mr X 10^{-6})	Size of Bam HI Fragments (Mr X 10^{-6})
8a	3	~6.9	3.8, 2.8 (3.6, 3.0)[a]	-
8b	1	~6.9	3.1, 2.0, 1.65	-
8c	1	~6.9	6.9	-
11a	5	5.85	5.85	3.6, 2.25 (3.9, 1.95)[a]
11b	1	5.85	4.15, 1.7	3.15, 1.4
11d	2	5.85	5.85	3.6, 2.4
11e	1	5.85	5.85	2.55, 1.7
11f	1	5.85	5.85	2.45, 1.95, 1.5
11g	1	5.85	5.85	3.7, 2.25
14a	2	5.05	5.2	4.8
14b	1	5.05	4.0	5.0

[a]Obtained for the inverted orientation of the insert.

A fifth clone (lane 9) in class 11a probably contains the insert in the opposite orientation. The Bam HI restriction patterns of the clones in classes 11d (lanes 5 and 6) and 11g (lane 8) differed only slightly from those of class 11a and may not deserve placement in a separate class (see Table 2). Class 11f (lane 7) yielded 3 fragments (Mr of 2.45, 1.95 and 1.5 X 10^6) and class 11e (lane 4) produced two fragments (Mr of 2.55 and 1.7 X 10^6) after cleavage with Bam HI. It should be noted that the sum of the molecular weights of the Bam HI fragments for classes 11b and e are less than the expected values. It is possible that some bands contain multiple fragments or that there are small fragments not detectable under the electrophoretic conditions used. These possibilities are being explored.

The results of the Hind III and Bam HI digests for band 11 indicate that multiple co-migrating bands are present. The Hind III digest indicated at least two different co-migrating sequences, and digestion with Bam HI revealed an additional four sequences. We are currently analyzing these clones to determine whether the fragments represent unique sequences or repeats with minor differences between them.

A similar analysis was conducted for other bands with more than one representative clone. In many cases, the clones were identical except for the probable inverted orientation of the viral insert. Bands 8 and 14 were found to have multiple sequences. These data are given in Table 2 along with a summary of the analyses of the band 11 clones. Entries with 2 sets of numbers had clones with inserts in both orientations. For band 8, only a Hind III analysis was performed, which revealed three classes of clones, 8a, b, and c. Hind III and Bam HI treatment of clones representing band 14 also showed that two different sequences were present in this band.

DISCUSSION

Because HCMV is highly cell-associated, it has been difficult to obtain large quantities of pure viral DNA for studies of viral infections at the molecular level. Possession of a cloned library of the HCMV genome would circumvent the problem of low viral DNA yields and would provide homogenous, well-characterized probes for studies on the nature of HCMV infections and the oncogenic potential of the virus. To date we have partially characterized 48 clones which represent 14 of the 21 bands visible in an Eco RI digest of HCMV DNA and approximately 80% of the genome (by weight).

For the cloning, viral DNA was prepared under conditions which minimized the generation of defective virus particles and shearing of the DNA. The purity of the isolated viral DNA was assessed and found to be greater than 99% pure. The cloning vehicle, PACYC 184, was selected on the basis of its ability to incorporate an insert the size of the largest HCMV Eco RI restriction fragment (11.5 X 10^6 daltons). The length of the insert was not a limiting factor in the cloning, as clones containing large viral fragments were obtained as frequently as those incorporating smaller inserts (Table 1).

In cloning, the question of stability, both of the plasmid and insert, is important. At this point, we have no indication of the frequency of recombination of the insert within the plasmid. Some of the co-migrating sequences, such as those obtained for band 11, may be derived from recombination

events, and we are in the process of testing this possibility. We have found that it is essential to maintain high selective pressure on the transformed colonies as transformants either grown or maintained in non-selective media tend to lose the plasmid with relative ease.

Approximately 30% of the clones screened contained extremely small inserts (Mr of less than 1 X 10^6). At this point, we do not know if these inserts are legitimate HCMV Eco RI fragments or products of a recombination event within the plasmid. We have yet to determine the exact size of the inserts and the number of sequences represented in this size class. Experiments are in progress to further characterize these clones.

Of the 7 bands not represented among our clones, at least two must represent the ends of the genome and cannot be cloned using the present method because only one terminus of the viral fragment will have an Eco RI site. The HCMV Eco RI fragments which correspond to the ends of the genome are currently being identified, and will be cloned after gel purification of those bands and the attachment of Eco RI linkers. More than two end fragments may exist if the HCMV genome is organized in a manner similar to that of Herpes Simplex, with long and short regions which occur in four different arrangements (see 19 for review). If this is the case, there could be as many as four different terminal sequences, depending upon the location of the Eco RI sites with respect to any terminal repeats. The other, non-terminal fragments should be readily obtained upon further screening of clones.

The assignment of a cloned fragment to a particular HCMV band has been made solely upon the basis of co-migration of the insert with an HCMV Eco RI fragment. A more definite assignment will need to be made. This will be accomplished by nick translation of the cloned inserts and hybridization to a Southern blot of Eco RI cleaved HCMV DNA. This method should also reveal any extensive regions of homology that may occur in different regions of the genome.

The viral origin of the cloned fragments was demonstrated by hybridization of nick-translated ^{32}P-labeled HCMV to Southern blots of the inserts. This procedure also serves as a measure of the homogeneity of the plasmid preparation, since under our hybridization conditions any minor HCMV fragments are readily revealed.

The addition of the molecular weights of the visible bands of Eco RI digested HCMV DNA results in a genome size of approximately 90 X 10^6 daltons, considerably below the reported value of 150 X 10^6 (1). Similar results were obtained from HCMV restriction patterns produced by Hind III and Bam HI. The estimated genome size is 114 X 10^6 Mr if calculated

from the restriction pattern obtained from digestion with Hind III, and 106 X 10^6 Mr if determined from bands produced by Bam HI (data not shown). We have shown that some of the more intense bands consist of several co-migrating sequences. Addition of the molecular weights of the co-migrating bands into the estimate raises the genome size to 120-130 X 10^6 daltons. It is quite likely that there are one or more additional sequences co-migrating with band 1 due to the intensity of that band.

Since the values obtained for the genome size are below the reported value, it would seem likely that co-migrating sequences are all represented in a single genome; however, in band 11 clones, the close similarities of some of the Bam HI restriction patterns cast some doubt on the uniqueness of these sequences. It is possible that the fragments represented in these clones contain closely related sequences, perhaps from a repeated region of the genome. Other possibilities are that the similar sequences originated from a non-homogeneous population of virus or that they may have arisen through a recombination event in a larger plasmid. These alternatives are currently being tested. We are also in the process of constructing a physical map of the HCMV genome for ordering the cloned fragments.

As a result of the experiments described in this paper, we have available large quantities of purified and well-characterized subgenomic fragments representative of a major portion of the HCMV genome. These cloned fragments should prove to be invaluable hybridization reagents for understanding the molecular biology of HCMV infections.

ACKNOWLEDGMENTS

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STRUCTURAL ORGANIZATION OF THE DNA MOLECULES FROM HUMAN CYTOMEGALOVIRUS

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ABSTRACT From a summation of the sizes of restriction enzyme cleavage fragments we estimate a total genome size of 235 kilobase pairs or 145 x 10^6 daltons for non-defective CMV(Towne) DNA. Electron microscopy of self-annealed single-strands and blot hybridization experiments with isolated restriction enzyme fragments have shown that all CMV(Towne) DNA molecules contain an internal inverted duplication of 7.5% of their sequences creating a "transposable element" -like region occupying almost 30% of the genome. The particular arrangement of these duplicated regions in linear virion DNA resembles that in herpes simplex virus and effectively subdivides the molecule into a large L and a smaller S-segment. The L-segment consists of a unique region of 170 kb surrounded by inverted repeats of 10.5 kb and in the S segment a unique region of 37 kb is encompassed by inverted repeats of 2 to 3 kb. From the complicated patterns of submolar restriction enzyme fragments we conclude that all four possible isomeric forms of the molecules exist in equal proportions i.e. both the L and S-segments can be in either leftward or rightward orientations. However the expected simple ratios of four 0.5 molar terminal fragments and four 0.25 molar internal joint fragments are masked by "stepwise" terminal heterogeneity in which some molecules have one or more additional copies of a 750 bp sequence within both copies of the inverted repeats around the S-segment. Complete cleavage maps for 15 XbaI sites and 23 HindIII sites and a prototype standardized fragment nomenclature

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have been constructed as a basis for subsequent studies on localizing gene functions. Differences between CMV(Towne) and CMV(Davis) DNAs indicate that deletions or substitutions at the junctions between the L-repeats and L-unique region may be a major source of CMV strain variation.

INTRODUCTION

Infections with human cytomegalovirus (HuCMV), a member of the herpesvirus group, are of serious clinical concern especially during pregnancy and in the newborn (cytomegalovirus inclusion disease) and in organ transplant or immunosuppressed patients (CMV pneumonia). The virus remains latent after usually asymtomatic primary infection and inactivated virions have the ability to transform cells in culture (1,2).

We have been constructing physical maps for CMV strain Towne DNA as a prelude to recombinant DNA cloning of individual fragments and attempts to identify specific gene functions. The detailed structure of the genome of human CMV is also of considerable interest from evolutionary considerations because firstly, it lacks any detectable cross-homology with other human herpesvirus DNAs (3; G.S. Hayward, unpublished experiments) and secondly, the size of infectious CMV DNA has recently been reported to be 50% larger than the generally accepted value of 100 x 10^6 daltons for most other herpesviruses (4-7). The only previously published attempt to analyse the structure of human CMV DNA was by Kilpatrick and Huang (4) who interpreted their partial denaturation mapping results to indicate that at least two sequence arrangements of the 150 x 10^6 molecular weight form of CMV(Towne) DNA existed. From these experiments and the observations of "stem-loop" structures in self-annealed strands of CMV strain AD169 DNA (P. Sheldrick, personal communication) it seemed likely that human CMV DNA would have a complex structural organization with inverted tandem repeat features similar to those described earlier for H. saimiri (8,9), herpes simplex (10,11,12) and Epstein-Barr virus DNAs (13,14). We report here the use of electron microscopy, restriction enzyme analysis and blot hybridization experiments to characterize the detailed arrangement of repetitive sequences in CMV(Towne) DNA and to construct the first restriction enzyme cleavage maps of this genome.

RESULTS

Preparation and Passaging of Non-Defective CMV DNA. In earlier studies on human CMV DNA the majority of the molecules in the population appear to have been of the smaller 100 x 10^6 molecular weight form representing non-infectious defective genomes (15,4). These populations give rise to slightly different DNA fragment patterns in agarose gels after cleavage with restriction enzymes in comparison with the fragment patterns from freshly plaque-purified virus passaged at very low multiplicities of infection (16; D. Ciufo and G.S. Hayward, unpublished experiments). In the mapping studies described below, only those CMV DNA preparations that lacked any trace of the "defective-specific" EcoRI 14 kb and BamHI 17 kb bands were used: all virus preparations were grown in diploid human fibroblast cells that were free of mycoplasma (either primary foreskin cells or the MRC-5 line) and passaged at dilutions of 10^{-5} or 10^{-6} essentially as described by Stinski et al. (16). CMV DNA was extracted from either purified cytoplasmic virions or virus particles released into the extracellular medium and separated from remaining traces of host cell DNA by virtue of its relatively high buoyant density (58% G+C-content) in CsCl equilibrium gradients.

Self-Annealed Single Strands of CMV DNA Form Stem-Loop Structures. Denatured CMV(Towne) DNA from purified virions was examined by electron microscopy after reannealing under conditions favoring intrastrand hybridization. Consistent with findings from other herpesvirus DNAs, few if any of the resulting single strands remained intact but a small proportion of the fragmented strands showed evidence for the existence of inverted repetitions. Twelve unambigious stem-loop molecules were photographed and tracings of ten of these are presented in Figure 1. In each case the single-stranded loop portion of the molecules measures between 2.44 and 2.83 times PM2 DNA in length (equivalent to an average of 38 kilobases). In nine of the twelve molecules a relatively short duplex stem can be distinguished which ends with either (1) a spur plus a single-stranded tail, (2) a long single-stranded tail only, or (3) a tiny loop or spur within a longer duplex structure. The distance from the fork of the stem-loop structure to the single-stranded region or spur varies from 0.19 to 0.33 times PM2 lengths (i.e. from 1.85 to 3.16 kilobases). The molecules exhibiting longer duplex structures are of two types: those that fork into two single-stranded tails after an additional 1.00 to 1.05 times PM2 length of duplex DNA (i.e. 10.1 kb;

molecules labeled S1 and S4) and those that do not fork (S3, S8 and S10). All of these longer duplex structures were probably formed by interstrand annealing between two complementary fragments. The two molecules consisting of an S-loop and short duplex stem, the spur, an adjacent long duplex stem

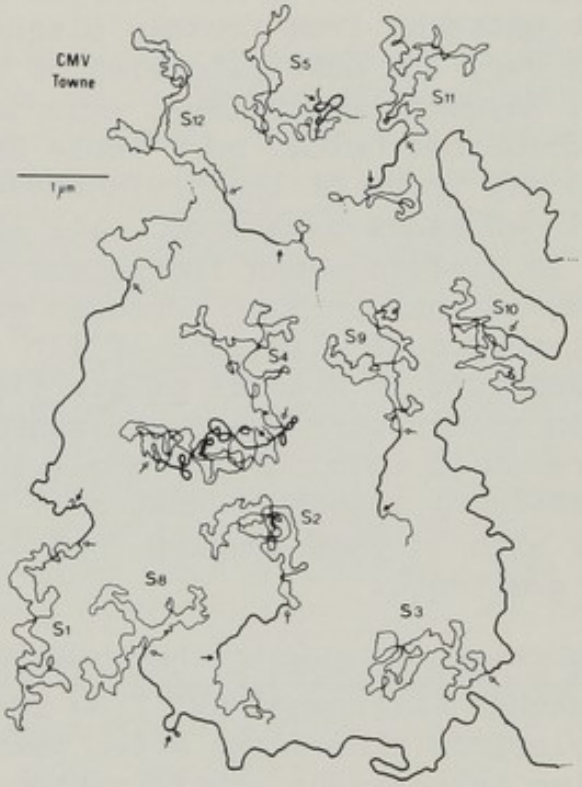

Figure 1: Tracings from electron micrographs of "stem-loop" structures formed by self-annealing single strands of human CMV(Towne) DNA. The DNA at low concentration was denatured with alkali, neutralized and incubated at 68° for 2 hr with slow cooling. Samples were diluted into 40% formamide and spread onto a hypophase containing 10% formamide for electron microscopy. Admixed circles of phage PM_2 DNA were used as the references for length determination. The self-annealed molecules are labeled S1, S2, etc., for identification purposes. Open arrows denote the forks between the single-stranded loops and adjacent duplex stems whilst solid arrows indicate the terminus of the short stem whenever clearly demonstrated by a spur or single-stranded tail.

and finally a single stranded fork (S1 and S4) closely resemble the "dumb-bell" shaped structures observed with fully intact HSV DNA strands after self-annealing (10,11), except that the bulk of the longer L loop is missing here. We conclude that at least one end of CMV(Towne) DNA contains an S-segment with a unique sequence of 38 kb surrounded by an inverted repetition of approximately 2 to 3 kb. These studies also provide tentative evidence for the existence of a second longer inverted repeat of 10-11 kb in a similar location to that around the L-segment in HSV DNA.

Restriction Enzyme Digests of CMV DNA Contain Complex Patterns of Submolar Fragments. In addition to the existence of inverted repeats, both the L- and S-segments in HSV DNA occur in either leftward or rightward orientations relative to one another resulting in four equimolar subpopulations of DNA molecules that are termed "inverted permutations" or "structural isomers" of each other (12,17). This fact was readily deduced from the clear and unambigious observation of four 0.5 molar and four 0.25 molar fragments in certain restriction enzyme digests of HSV DNA, especially those of the HSV-1(MP) strain with HindIII and BglII (12,18). In CMV DNA however the situation is much more complicated: all enzymes tested yielded submolar fragments (Figure 2) but none fit into the simple 0.5M and 0.25M pattern. For example, BamHI yields at least five visible submolar fragments ranging from approximately 0.15M to 0.3M relative to the majority 1.0M species. An examination of the HindIII cleavage pattern reveals at least eight submolar species with varying molarities. XbaI digests in constrast show only two submolar species both of approximately 0.5M. For convenience, during the initial stages of the mapping analysis, these submolar species are referred to as m1, m2, m3, etc. in each digest and the molar fragments are referred to by letters A, B, C, etc. in order of decreasing size.

As shown below, the principal explanation for the complex submolar fragment patterns proved to be stepwise length heterogeneity within fragments containing portions of the inverted repeats. A similar phenomenon was observed previously in the so-called "split-band" effect with fragments containing the internal and extermal termini of the L-segment in HSV DNA (19) and subsequently in EBV DNA also (20), but the effect is far more pronounced here in CMV(Towne) DNA.

Terminal Fragments Include Both Molar and Submolar Species. A key point in the proof of the inversions of the L and S segments in HSV DNA was the demonstration of four

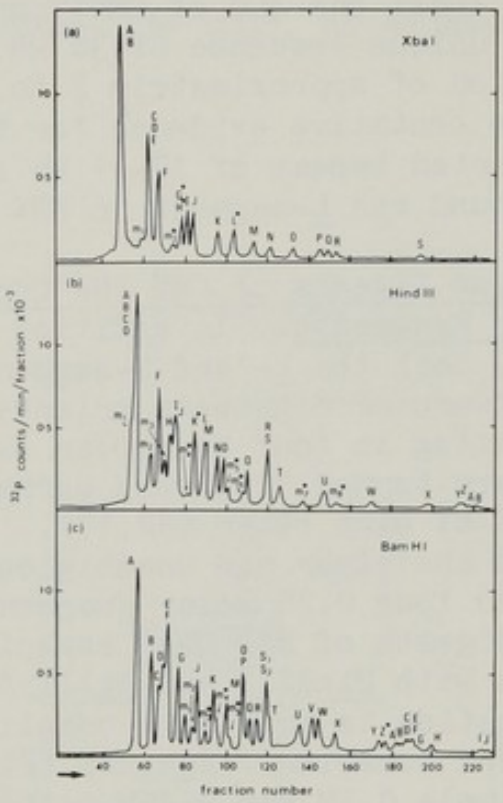

Figure 2: Gel profiles of ^{32}P-CMV(Towne) DNA fragments resulting from limit digests with the restriction enzymes XbaI, HindIII or BamHI. Electrophoresis was carried out in 30 cm long cylinders of 0.6% agarose gel which were then cut into 1 mm fractions for radioactivity determinations. The standard A, B, C fragment nomenclature is used to conform with that given in the cleavage map in Figure 6, except that obvious submolar species are given the preliminary labels m_1, m_2, m_3, etc., to correspond with the description in the text. Solid circles indicate terminal fragments (see Figure 3).

separate species of terminal 0.5M fragments. All four of these fragments disappeared from the gel profiles when the intact linear viral DNA was first digested back from the 5' ends by brief incubation with lambda exonuclease (12). The results of similar experiments with CMV(Towne) DNA are presented in Figure 3. The arrows indicate those bands that are absent from the profiles after lambda 5' exonuclease

treatments: e.g. the XbaI bands labeled m2, (G,H) and I (but not m1) and the HindIII m4, K, m5, m6, m7 and m8 species. In BamHI digests the m3, m4, m5 and Z species but not m1 or m2 proved to be terminal fragments by this criterion. The observations that the smaller submolar

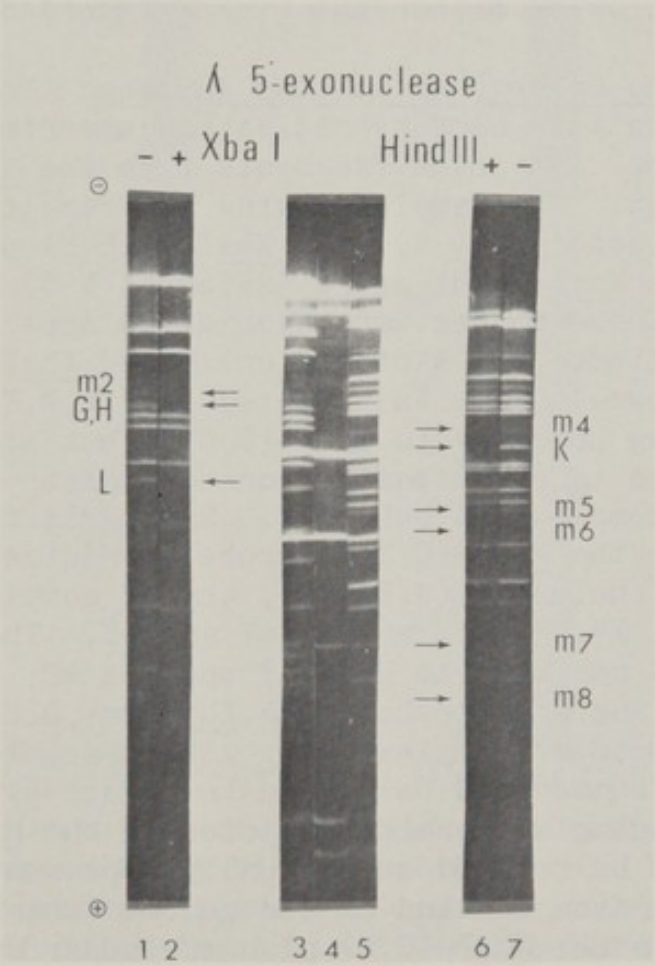

Figure 3: Identification of terminal fragments in CMV(Towne) DNA by the lambda exonuclease procedure (12). Gels 1 & 2 and 6 & 7 show a comparison of ethidium bromide stained gel patterns of DNA incubated briefly with (gels 2 & 6) and without (gels 1 & 7) lambda exonuclease prior to digestion with HindIII or XbaI. All fragment species which disappear from the profile (i.e. end fragments) are identified by arrows. The centre panel shows comigration of the CMV(Towne) XbaI digests (gel 3) and HindIII digests (gel 5) with reference HindIII fragments from phage lambda DNA (gel 4) for use in size determinations.

species come from the ends of the molecule but that larger ones do not is consistent with the notion that they are all associated with an "inversion" phenomenon similar to that found in HSV DNA and that the larger species represent internal "joint" fragments containing the same DNA sequences that are present in the smaller submolar fragments. In general, the 1.0M bands that contain terminal fragments are presumably explained as representing pairs of identical 0.5M species created by symmetrically placed cleavage sites within both copies of either the L or S inverted repeats.

At Least One Segment of CMV DNA Inverts. To confirm the above interpretations blot hybridization experiments with isolated terminal and joint fragments from the XbaI digest were carried out. An example showing the results with total CMV DNA probe (slices 1 & 4); the XbaI-(C,D,E) probe (slices 2 & 5); and the XbaI-(G,H) probe (slices 3 & 6) is presented in Figure 4. These probes were hybridized separately to strips from cylinder gel blots of unlabeled XbaI and HindIII digested CMV(Towne) DNA. As expected the (C,D,E) probe exhibited strong homology to XbaI-(C,D,E) but also to m_1, m_2, (G,H), L and to additional minor components in the (m2,G,H,I,J,) complex and behind K. Similarly in the HindIII profile the XbaI-(C,D,E) probe hybridized to at least 15 bands including (A,B,C,D), the m2 complex, F, the m3 complex, m4, K, m5, m6, T, m7 and m8. The XbaI-(G,H) probe exhibited homology to HindIII species m2, F, m3, m5, m6, m7, and m8 and also to both the XbaI-(m_1,D,E) complex and the XbaI-(m_2,G,H) complex. Our interpretation of these data are summarized below and in Figure 4. The m5, m6, m7 and m8 submolar terminal species in the HindIII digest must all be related and actually represent two sets of 0.5M species from one end of the genome (the same end as all the bands in the XbaI-(G,H) plus m2 ladder complex). Each 0.5M fragment apparently splits into at least 3 subspecies differing in size by 750 bp with the intermediate sized subspecies being the most abundant. Thus the V, V*, V** set and the P, P*, P** set each represents a heterogeneous terminal fragment from the leftward or rightward orientation of one segment of the genome and the G, G*, G** and H, H*, H** sets are the equivalent species in the XbaI digests. The difference in size between the 11-14 kb Xba-G and H species and the 5-8 kb P amd V complexes accounts for the homology of the XbaI-(G,H) probe to the internal HindIII-T and -F fragments. Other experiments revealed that the opposite end of CMV DNA gives rise to the XbaI-L and HindIII-K terminal fragments which cross-hybridize. Although about 10% of the molecules also appear to contain

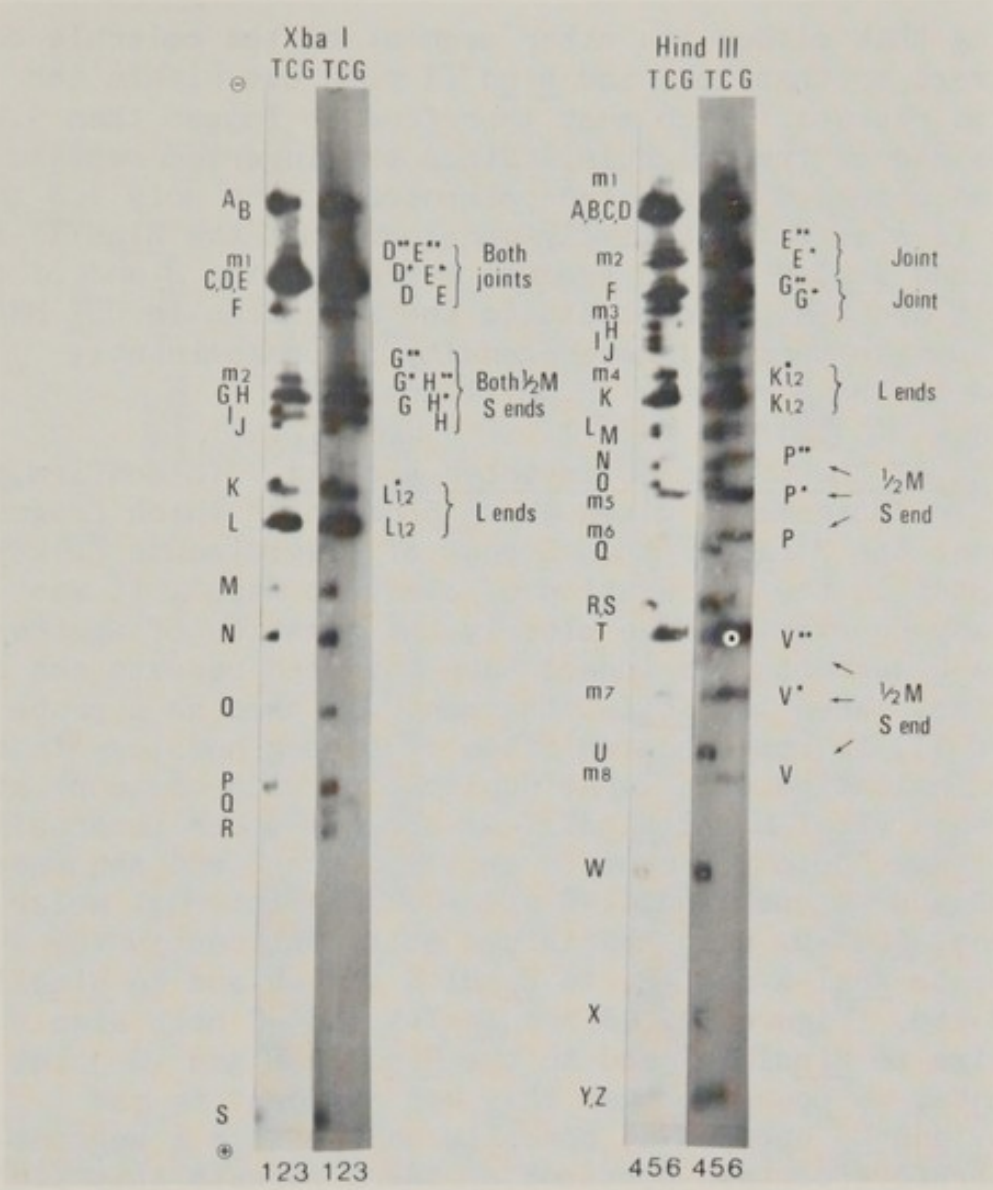

Figure 4: Detection of S segment duplications and terminal heterogeneity by blot hybridization. The figure shows autoradiographs (at two different exposures) after hybridization of "nick-translated" ^{32}P-labeled DNA probes to "Southern" millipore blots of cylinder gels containing fractionated XbaI or HindIII digests of CMV(Towne) DNA. The blot of each gel was cut into three slices each of which was hybridized separately to a probe of total CMV DNA (slices 1 & 4), the isolated XbaI-(C,D,E) band (slices 2 & 5) or the isolated XbaI-(G,H) fragment band (slices 3 & 6). Our interpretation of the location of terminal and joint fragments is given alongside the figure (see text). Bands labeled G, G*, G** etc., represent the stepwise heterogeneous versions of related subspecies.

an extra 900 bp added to XbaI-L (=L*) or HindIII-K (=K* or m4), these two fragments are essentially 1.0M species

implying that either the other segment of the molecule does not invert or that XbaI and HindIII both cut within the inverted repeats, which must therefore be longer than 9.7 kb at this end of the molecule. Since the inverted repeats observed around S by electron microscopy were only 1.8 to 3.2 kb in size we tentatively conclude that the HindIII-P and -V and XbaI-G and -H species represent the S end of the molecule and that in contrast to the situation in HSV DNA the step-wise terminal heterogeneity is predominantly located in the S repeats in CMV(Towne) DNA.

Size of the L Segment Inverted Repeats. To confirm the tentative assignments given above concerning which fragments represent the S rather than L ends of the molecule (a key first step in the preparation of cleavage maps), it was necessary to examine more closely the question of whether or not the L segment does indeed have inverted repeats and of what size. When the XbaI-L fragment was used as a probe (Figure 5), it revealed two sites of strong homology in the genome indicative of a large duplication with one copy at the L ends (XbaI-L and HindIII-K) and the other internally in the same "joint" fragments that contain S end sequences (XbaI-D and -E and HindIII-E and -G). An internal molar fragment, XbaI-N, also hybridized at two places in the genome, to XbaI-N and -P, to BamHI-G and -U and to HindIII-O and -W etc. (Figure 5). Since XbaI-N and -P both also hybridize to HindIII-K and to the HindIII-E and -G joint fragments, we conclude that they map adjacent to the XbaI-L_1 and L_2 species at opposite ends of the L segment and therefore contain portions of the L repeats that lie more than 9.8 kb from the termini. In other words XbaI-N and -P consist of both unique and repeated sequences and span the junctions between the L unique region and L repeats. In addition, their map locations provide direct evidence that these repeats are inverted. In the BamHI digest BamHI-Z (2.5 kb) represents the two identical 0.5M L segment termini and the 2.0M band BamHI-(S_1, S_2) must contain two identical 1.0M species that each lie entirely within the inverted repeat regions and adjacent to Z_1 and Z_2.

The size of the L inverted repeats deduced from these results is close to 10.5 kb exactly the same as that implied by our tentative electron microscopy evidence. Because every restriction enzyme we have tested cleaves at least once within the L repeats of this strain of CMV DNA our biochemical and hybridization analysis does not supply any evidence for or against inversions of the entire L segment. However, it seems likely that the three intermolecular

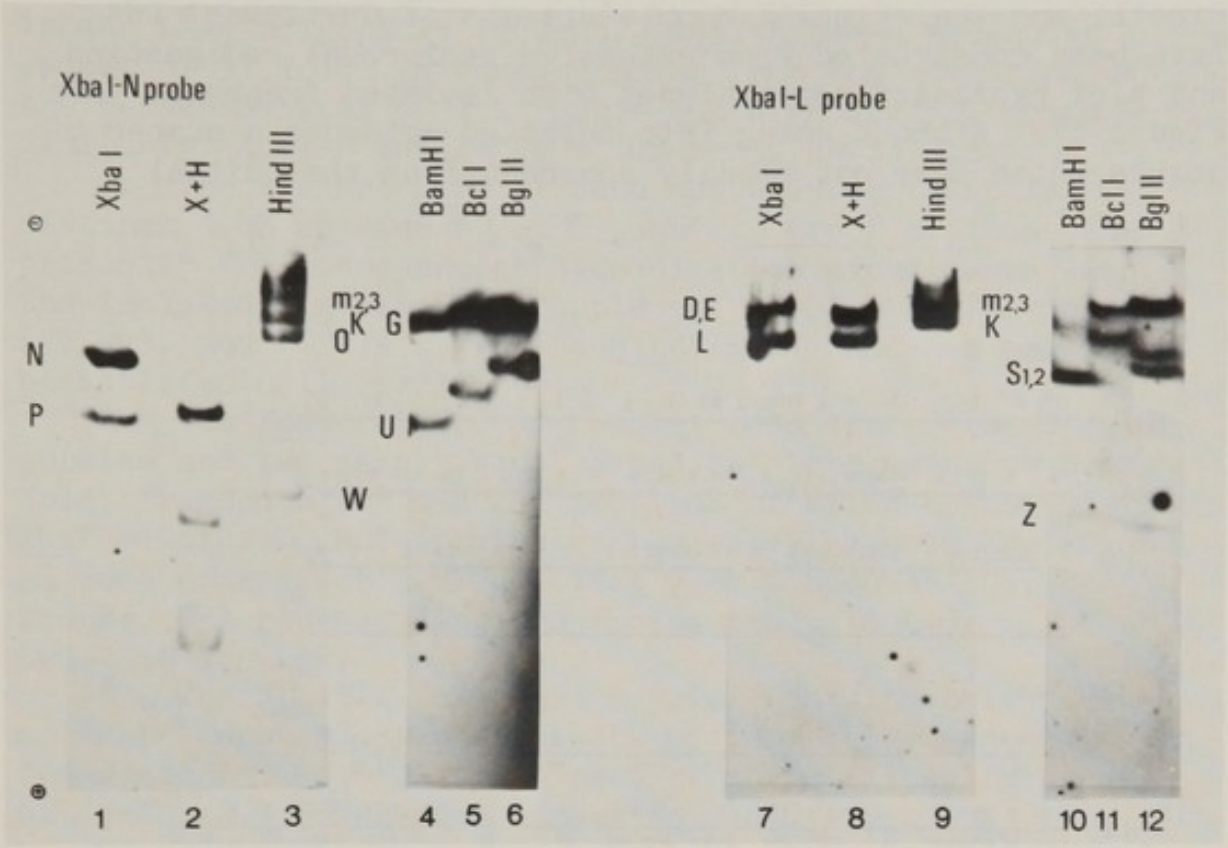

Figure 5: Detection of inverted duplications around the L segment by blot hybridization. The autoradiographs were obtained from experiments similar to that described in Figure 4 except that the "Southern" blots were prepared from 1.0% agarose slab gels in which individual slots contained various total digests of unlabeled CMV(Towne) DNA. The two nick-translated ^{32}P-hybridization probes used were isolated DNA bands containing the L segment terminal fragment XbaI-L (slots 7-12) or an adjacent internal fragment from one side of the L-segment XbaI-N (slots 1-6). Each probe hybridized to at least two fragments from different positions in the DNA molecule.

reannealed structures (with S stem-loops) that do not fork after 10-11 kb of duplex DNA on the L side (molecules S3, S8 and S10 in Figure 1), could only have been formed from complementary strands derived from an internal L end and an external L end of molecules with opposing orientations of their L unique regions.

XbaI and HindIII Cleavage Maps of CMV(Towne) DNA. Complete maps for virtually all fragments of the XbaI, HindIII and XbaI/HindIII double digests of CMV(Towne) DNA have been constructed from extensive reciprocal redigestion and blot hybridization analyses with isolated fragments (see Figure 6). Without going into detailed evidence a number of points which were not readily apparent from the initial

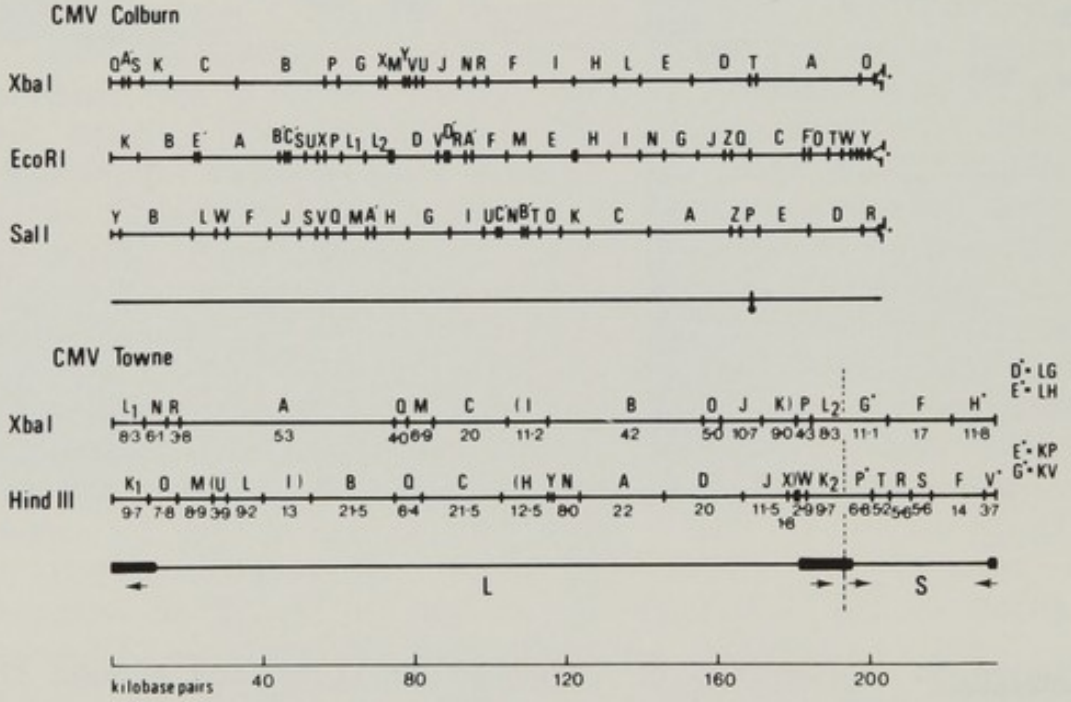

Figure 6: Physical maps of the CMV(Towne) and CMV(Colburn) genomes. Only one selected prototype orientation of CMV(Towne) DNA is given: the positions, sizes and letter designation of all fragments in each of the other three structural isomers can be inferred directly from the standardized fragment nomenclature information given in he single-line map. Numbers below the individual fragments in the CMV(Towne) cleavage maps indicate sizes in kilobase pairs. Solid areas and arrows show the position, dimensions and relative orientations of the inverted repeats. Heterogeneous terminal fragments from the S segment and joint regions are identified with asterisks. The maps of CMV(Colburn) DNA, a human isolate but clearly of simian origin, are shown for comparison and drawn to the same scale. The solid circle shows the location of cross-homology with mouse and human cell DNA.

fragment profiles and preliminary nomenclature assignments are listed below.

1. The XbaI-A and -B fragments appear to be considerably larger than originally thought. XbaI-A has a molecular weight of 35 x 10^6 daltons or 52 kb from the summation of the measured sizes of its double cut HindIII/XbaI components. Similarly, XbaI-B totals 26 x 10^6 or 39 kb in size.

2. The largest HindIII band actually contains four distinct 1.0M species A, B, C, and D. Proof of this came primarily from independent hybridization experiments with the isolated HindIII/XbaI double cut species AB (22 kb), BA (20 kb), and CC (19 kb). The HindIII-m_1 band has yet to be satisfactorily explained.

3. The XbaI-(m1,C,D,E) complex consists of the C molar species and two nearly equal sized but heterogeneous 0.5M joint fragments (D* and E*, portions of which constitute the XbaI-m1 band). Note that strictly speaking both XbaI-D and -E each consist of two identical 0.25 M species (D_1, D_2 and E_1, E_2) representing the fusion products L_1G, L_2G, L_1H and L_2H.

4. The HindIII-(m2,F,m3) complex contains only one molar fragment (F) plus two sets of 0.5M heterogeneous joint fragments E* and G*. Again each of these is really a pair of idential 0.25M fragments E_1, E_2 and G_1, G_2.

5. The XbaI (m2,G,H,I,J) complex actually consists of two internal 1.0M fragments labeled I and J plus two sets of three or more heterogeneous 0.5M fragments from the S ends (G* and H*). The latter two sets of submolar fragments although similar in size have only the 2-3 kb S repeat in common and are otherwise distinct sequences. The corresponding submolar species in HindIII digests are P* (P, P*, P**, etc.) and V* (V, V*, V** etc.), portions of which were referred to earlier as m5, m6, m7 and m8.

A Standard Fragment Nomenclature. After establishing the number, molarity and location of all of these fragments, we applied the same standardized rules for revised nomenclature of herpesvirus DNA fragments that were established for HSV DNA at the 1976 Cold Spring Harbor Herpesvirus Workshop (18). This simplified nomenclature for the CMV(Towne) DNA cleavage map is presented in Figure 6. The selection of prototype orientations for both the L and S segments to permit an unambigious single line map was quite arbitrary. Note that the relative order of HindIII-(U,L,I) in this tentative map has not been finalized and that the evidence for placing the section XbaI-(I,B,O,J,K)/HindIII-(H,Y,N,A,D,J,X) in the particular orientation given here is relatively weak. Several smaller fragments, XbaI-Q and

HindIII-Z,A',B' etc., have yet to be mapped.

DISCUSSION

Several lines of evidence over recent years have suggested that human CMV DNAs are closer to 150 x 10^6 molecular weight than the commonly regarded figure of 100 x 10^6 daltons for a typical herpesvirus genome. Indeed the 100 x 10^6 molecular weight molecules observed earlier in CMV DNA preparations proved to be noninfectious and therefore represent an unusual form of defective genomes whose structure has yet to be fully elucidated (16). Summation of the sizes of the fragments in our CMV(Towne) cleavage maps gives a total length of 234 kb or 145 x 10^6 daltons for XbaI (235 kb for HindIII) directly confirming the results from electron microscopic and sedimentation measurements on nondefective CMV DNA populations (5,6,7). Therefore, the human CMV(Towne) genome is larger than that of any other herpesvirus known and may even be 20% larger than that of the poxviruses (21).

Earlier work by Kilpatrick, Huang and Pagano (22) and Huang et al. (23) has indicated considerable variation amongst the cleavage patterns of different human isolates of CMV and the existence of at least one isolate, CMV(Colburn), which more closely resembles an African green monkey CMV isolate (GR2757) than other human CMVs. Our laboratory has constructed restriction enzyme cleavage maps of CMV(Colburn) DNA and found no evidence for an invertible L and S structure or terminal 0.5M fragments in this genome (D. Ciufo and G.S. Hayward, manuscript in preparation). The CMV(Colburn) DNA molecule is approximately 132 x 10^6 daltons in size but has no detectable sequence homology with CMV(Towne) and another related human isolate, CMV(Davis), by blot hybridization tests (Figures 6 & 7). In addition, the simian CMVs (Colburn and GR2757) both show localized cross-homology with highly repetitive sequences found in many different mammalian cell DNAs, whereas human CMV(Towne) and (Davis) DNAs do not.

As shown in Figure 7, although CMV(Towne) and CMV(Davis) have considerably different cleavage patterns, they show extensive cross-homology within almost all restriction fragments except those such as XbaI-N and -P and HindIII-O and -W of CMV(Towne) which map at the junction of the L-unique and L-repeat sequences. Since another human isolate, CMV(AD169) apparently has much shorter L repeats (8 kb) than does CMV(Towne) (P. Sheldrick, personal communication) and also lacks homology with CMV(Towne) DNA at these

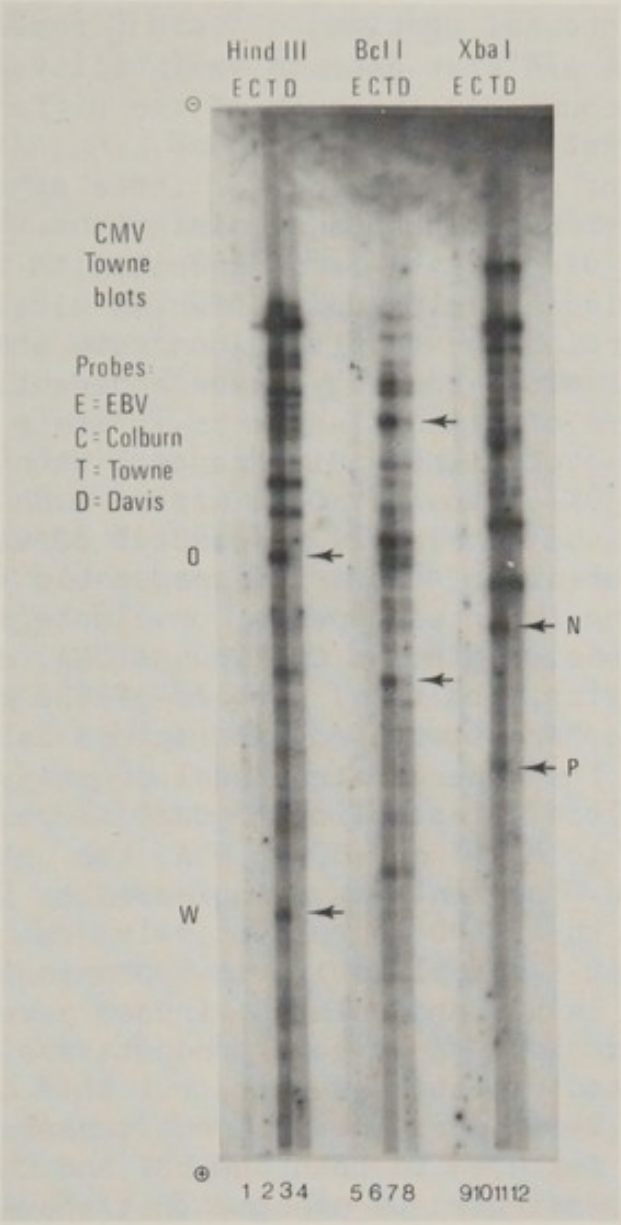

Figure 7: Comparison of CMV(Towne), CMV(Davis), EBV and CMV(Colburn) DNA by blot hybridization. The figure shows an autoradiograph from an experiment similar to that described in Figure 4. The blots of each of three long cylinder gels containing HindIII, BclI or XbaI cleaved CMV(Towne) DNA were cut into four strips and hybridized separately with total ^{32}P-"nick-translated" probes of either EBV(B95-8) DNA, CMV(Colburn) DNA, CMV(Towne) DNA or CMV(Davis) DNA. Arrows indicate fragments of CMV(Towne) DNA that are significantly lacking in cross-homology with CMV(Davis) DNA. EBV and CMV(Colburn) exhibit little or no detectable cross-homology with the other human CMV isolates.

sites (R. Pritchett, personal communication), we suggest that both CMV(Davis) and (AD169) are deleted by 2000-4000 bp

at the extreme internal portions of both L repeats relative to CMV(Towne) DNA and that genetic variability at these locations may account for the bulk of the differences encountered amongst human CMV isolates.

The genomes of representatives of three other groups of mammalian herpesviruses (herpes simplex virus, H. saimini, and Epstein-Barr virus) have been described in some detail (8-14). These viruses, although differing widely in biological properties and despite a complete absence of residual sequence cross-homology, have apparently retained the common feature of L and S segments in their genomes throughout their evolutionary divergence. When viewed in a circular format 30% of the physical map of each of these viral genomes can be considered to consist of various different arrangements of either "transposable element"-like regions containing inverted repeats, or clusters of multiple tandem duplications. In human CMV(Towne) DNA, despite the 50% increase in size, the total portion of the genome composed of these "accessory" repeat regions is once again almost 30%, with the general structural organization of the whole molecule closely resembling that of herpes simplex virus. Relative to HSV-1 and HSV-2 DNA, the unique portions of both the L and S segments have increased by 50% to greater than 190 kb and 40 kb respectively, but the amount of DNA involved in the duplication is approximately constant at 13 to 15 kb. In HSV and related viruses several of the most abundant immediate-early gene products are encoded within the inverted repeat sequences, but this is not known as yet for CMV. Because of the apparent conservation of these structural features in both the HSV and CMV subgroups of mammalian herpesviruses we presume that there must be strong selective pressures to retain the inverted repeats either as structurally necessary features involved in the mechanism of DNA replication or to provide genetically important functions in which gene dosage or orientation effects play some essential role.

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THE NUCLEOTIDE SEQUENCE OF THE HEPATITIS B VIRAL GENOME AND THE IDENTIFICATION OF THE MAJOR VIRAL GENES[1]

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ABSTRACT. The complete sequence (3221 nucleotides) of the hepatitis B viral DNA (adw2 serotype) is reported. The long strand has four major polypeptide coding regions with an aggregate translational capacity of 1613 amino acids (4839 nucleotides). Two genes coding for the major viral proteins have been identified; the previously described surface antigen gene coding for a protein of 25,398 daltons and the core antigen gene, which codes for a basic polypeptide (21,335 daltons) with a striking protamine-like sequence at its C-terminus. There are two other putative peptide coding regions: A, which overlaps the surface antigen gene and may code for a protein up to about 95,000 daltons and B, which partially overlaps the core gene and may code for a peptide of about 16,000 daltons. The short strand of the virus is largely devoid of possible peptide coding regions. A single segment capable of coding a peptide of 94 amino acids is identified.

INTRODUCTION

The hepatitis B virus is the etiological agent for a major form of hepatitis particularly prevalent in Asia and Africa. This form of hepatitis may also be causally related to the development of hepatic tumors (1).

The HBV virion (also called the Dane particle) is a 42 nm particle comprised of a lipid-containing surface envelope as well as a core or nucleocapsid containing the viral DNA. HBV also produces 22 nm particles and 22 nm filaments of various lengths that contain viral proteins but not DNA (2).

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The HBV derived particles obtained from infected humans have been characterized immunologically and at the molecular level. There are apparently several strains of the virus that can be recognized by differences in the hepatitis B surface antigen (HBsAg). All contain a group specific determinant a and usually one of each pair of subtype determinants (d/y and w/r) (3,4). In addition, HBV contains a "core" antigen (HBcAg) (5); and the e antigen (HBeAg) (6,7).

HBsAg is comprised largely of two major polypeptides (∿25,000 and ∿30,000 daltons) found in the surface coat of Dane particles as well as in the 22 nm particles (8,9). These molecules are antigenically indistinguishable (5) and contain identical amino acids at the N-terminus and probably at the carboxy-terminus (10). It has been proposed that the protein moieties are identical and that the larger is a glycosylated derivative (5). Five to seven other polypeptides (36,000-97,000 daltons) (11) have been detected in HBsAg preparations, some of these polypeptides also cross react with HBsAg antibodies. Core particles produced by removal of the surface coat contain a major polypeptide (the core protein, HBcAg, estimated to be about 19,000 daltons), and several additional polypeptides (25,000-80,000 daltons), including an endogenous DNA polymerase activity (12,13).

Hepatitis B viral DNA is a partially double stranded circular molecule (14). The long strand is approximately 3200 nucleotides while the short strand varies from 1700 to 2800 nucleotides in different molecules (15). The 5' end of the short strand appears to begin at a single site but the 3' end extends variably along the long strand. On addition of appropriate nucleoside triphosphates the endogenous DNA polymerase activity of the virion makes double stranded full length circular DNA with cohesive ends. The nicks are approximately 310 bases apart (16).

In order to help identify the HBV genes, to study the pathology associated with HBV infection, we and others have cloned in bacteria (9,17-19) and have now sequenced the virus and viral fragments (9,20,21,22). The surface antigen gene has been identified from the known partial amino acid sequence (9,20,22). From the complete nucleotide sequence of the virus, reported here, we can identify five possible coding regions for polypeptides of significant length (>70 amino acids). Four are on the long strand. Besides the surface antigen, we identify the gene coding for the core protein and two other regions 'A' which can code for a protein of <93,000 daltons, 'B' coding for a protein <16,000 daltons. The short strand contains a single region 'C' that could code for a protein of <9,000 daltons.

CLONING AND SEQUENCE ANALYSIS OF THE HEPATITIS B VIRUS

DNA from Dane particles (prepared from pooled sera received from Merck Sharpe & Dohme Research Laboratories) was cloned as an EcoRI fragment and two BamHI fragments ligated respectively into the plasmids pBR325 and pBR322 (9). The physical map containing the cleavage sites of restriction endonucleases was prepared for the entire EcoRI fragment (pHBV-3200) and for the two BamHI fragments (pHBV-1900 and pHBV-1300) (9). Sequence analysis of the independently isolated BamHI constructed clone (pHBV-1900) ensures the existence of a single EcoRI site therefore the EcoRI constructed clone contains the complete genome. The resulting sequence is reported in Figure 1 including translation of each strand in the three possible phases.

IDENTIFICATION OF POLYPEPTIDE CODING REGIONS

Currently it is not possible to identify genes solely from the DNA sequence because of the possibility of intervening non-coding regions (introns). Nevertheless, putative coding regions can be identified from the positions of start and stop codons. Sites near the initiation and termination of transcription can also be located, although with less certainty. These sequences include the Hogness TATAAAG-like sequence (24,25), the ribosomal binding site which is complementary to the 3' end of 18S rRNA (26,27), and the AAUAAA-like sequence found in the 3' untranslated region near the polyA addition site of most eucaryotic mRNAs (28). The very few TATAAA, and AAUAAA-like sites in the HBV genome restrict the number of possible transcripts. In contrast there are many putative ribosomal binding sites, thus the sites of initiation of translation cannot usually be determined.

Inspection of the sequence of each strand in the three possible translation frames shows that the coding capacity is severely limited by the plethora of stop codons as well as the available AUG (methionine) start codons. (We neglect here the possibility of GUG initiation.) In particular the majority of the short strand is unavailable for coding. This may be of little consequence since much of this strand is not present in the isolated virus and hence may be unavailable for transcription. On the short strand there is only one "open" region sufficient to code for peptides larger than 80 amino acids (D, base 3018 to base 2731; 94 amino acids). However this coding region may be bisected by a break in the nucleotide sequence since it is in the vicinity of the nick in the DNA (16). The nearest TATAAA-like sequence (TATAAG) is 310 bases upstream (base 107). The nearest AAUAAA-like sequence (AATAAC) at

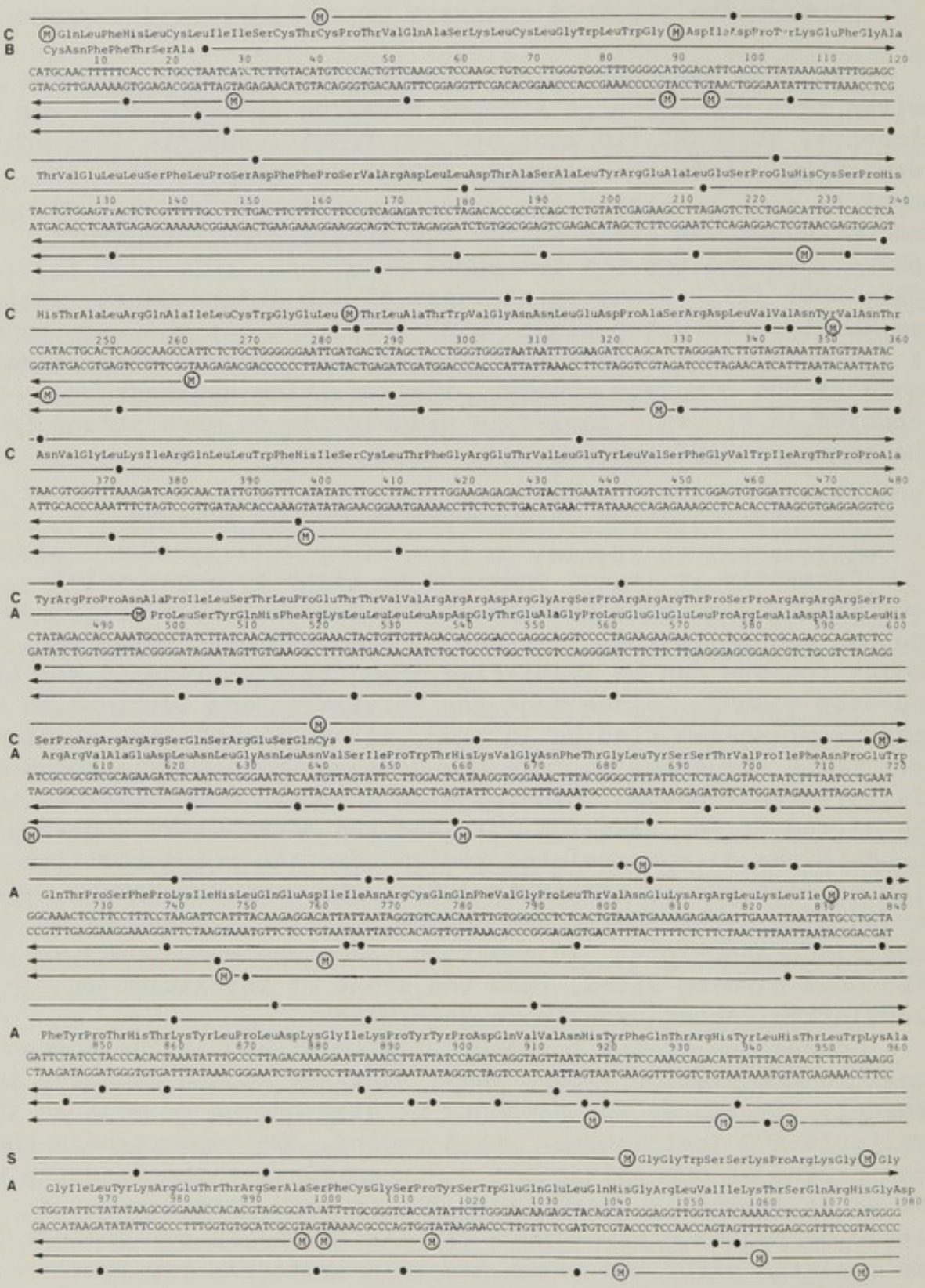
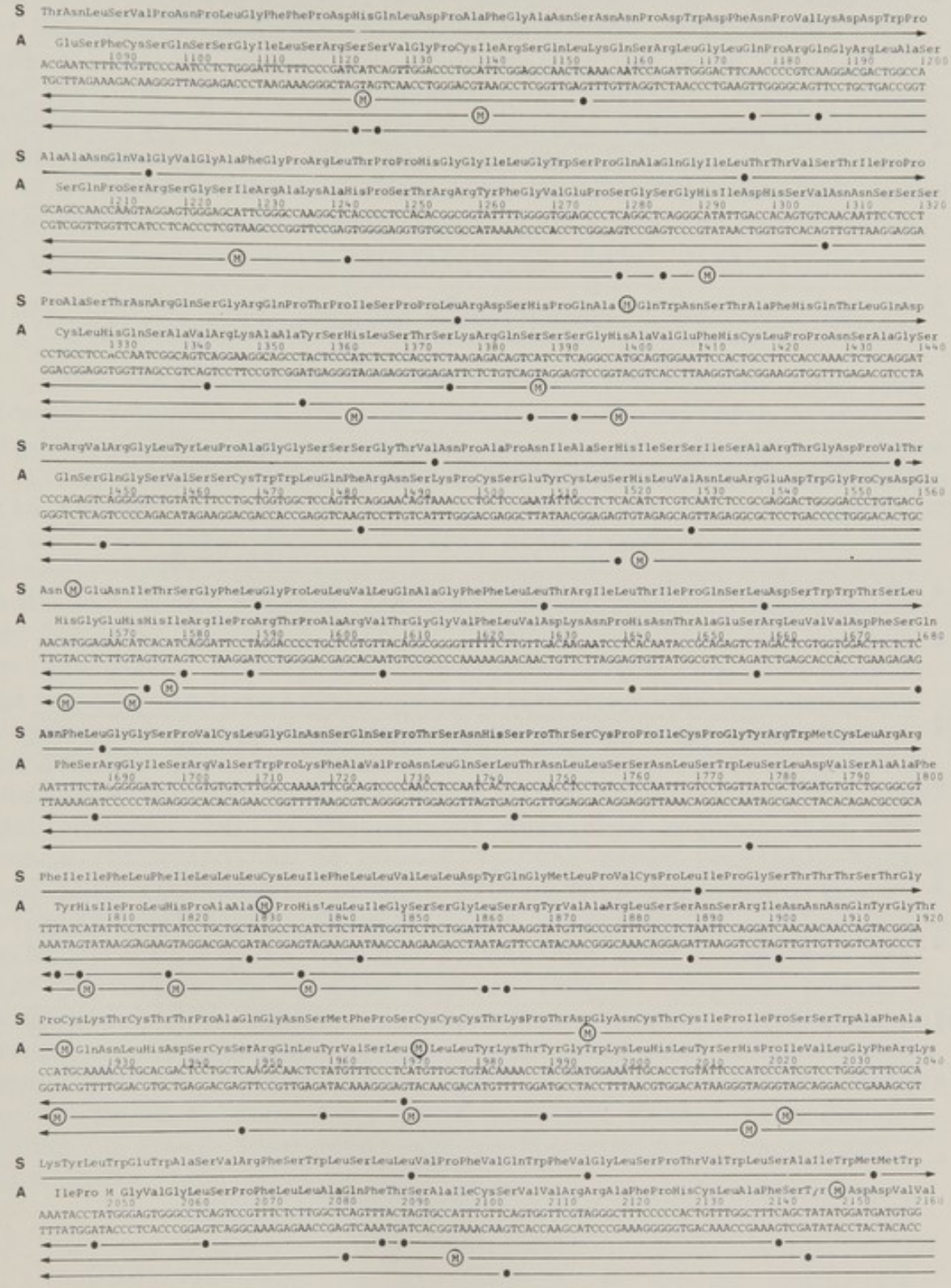


FIGURE 1. The DNA sequence of the HBV genome and translation of the major open coding regions in the six possible reading frames. For convenience the translation is depicted next to the strand corresponding to the mRNA, the transcription occurs from the opposite strand, thus the top nucleotide sequence corresponds to the short viral strand. The possible



methionine start signals are emphasized by an encircled M, where the stop codons are labeled by a closed circle. Regions of less than 80 amino acids or devoid of methionine starts are shown by lines. The letters at the left of the figure identify the coding regions: S: surface antigen gene; C: core antigen gene; A: putative A gene; B: putative B gene; and D: puta-

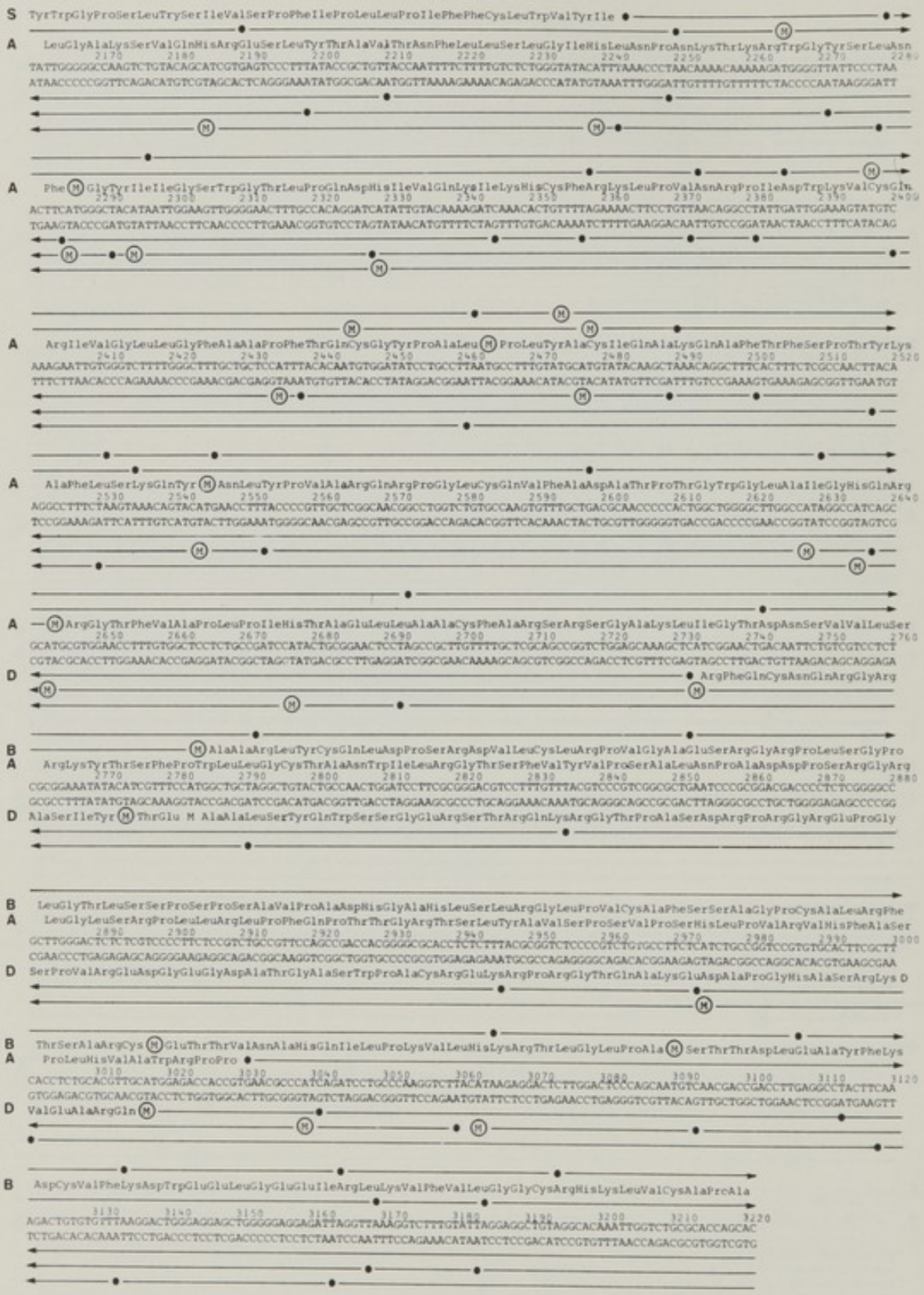


FIGURE 1 (continued)

tive D gene. DNA sequencing was carried out by the method of Maxam and Gilbert (23).

base 2273, is 458 nucleotides downstream. Thus the putative transcript (neglecting the nick) would be 1053 bases. A number of other possible coding regions for even smaller peptides are evident; for example, D overlaps a region (base 2973 to base 2790) which could code for a basic protein of 61 amino acids).

In contrast to the short strand, all three reading frames of the long strand contain large open areas for translation. In these areas we have identified the major viral genes.

SURFACE ANTIGEN GENE

The coding region for the surface antigen has been previously reported (9,20). The amino acid composition of the adw HBsAg (5,8) and determined sequence of this gene product (10), as well as the congruence of our restriction enzyme map with that of an adw2 HB virus (29) suggests the cloned HBV DNA is the adw2 serotype. The amino acid sequence contains two strongly hydrophobic regions surrounded by more hydrophilic regions rich in proline and cysteine residues. The complete HBV sequence provides further information about the surface antigen gene. The lack of homologous sequences elsewhere in the genome eliminates the possibility that the two dominant proteins of the surface coat of the virus were coded by similar but distinct genes. However, there is an open area available for coding a precursor (<400 amino acids from bases 1041 through 2241). The nearest available Hogness-like sequence (TATATAAG) is at base 970. The first available AAUAAA-like site (AATATA) is at base 2767. This predicts a transcript of 1797 bases. We have found a transcript of about 1800 bases hybridizing to HBV DNA in the RNA from a hepatoma cell line producing the surface antigen (30; P. Gray, J. Edman, P. Valenzuela, W.J. Rutter, accompanying article). Although this transcript likely defines the gene, its location cannot be considered definitive, since another Hogness sequence (TATAT) can be found at base 400, and a possible AAUAAA-like site, AACAAA is found just after the coding region at base 2250. This transcript would be 1850 bases.

THE CORE GENE

Because of the paucity of structural information concerning the core protein, the localization of the gene is more tenuous. We conclude the region from base 2 to 643 is the core gene sequence on the following grounds.

1) If translation begins with the ATG at base 89, a polypeptide of the desired size (185 amino acids, 21,335 daltons) would be formed. We believe that translation does

indeed start at this codon because it is preceded by a distinctive palindromic structure (7-35 bases prior to the ATG) containing a ribosomal binding sequence CCTT; the previous ATG at base 2 has no proximal ribosomal binding site. More decisively, there may be a nick in the viral DNA strand at base 9 (22) perhaps eliminating the ATG from the core transcript.

2) Studies on the core protein by Shih and Gerin (personal communication) indicate that it contains a single methionine residue; CNBr cleavage results in fragments similar to the size predicted from the proposed sequence (0.65 and 0.35 of the original mass). No other area (without splicing) fits the constraint imposed by this datum. In particular the only other possible region (in 'A'); has 3-4 methionine residues.

3) The core protein interacts with DNA. The proposed molecule is basic (31 basic vs 19 acidic residues) with a strong bias for arginine (24) vs. lysine (2) residues. The most striking feature is a protamine-like (31,32) region at the carboxy terminus which contains 17 arginine out of 35 residues, with the remainder being largely composed of proline and serine residues. The amino terminal portion of the molecule (to residue 80) is acidic (13 acidic vs 7 basic residues).

Recently, Pasek et al. (22) have obtained independent evidence showing that a portion of this region when expressed as a fused protein with ampicillin in E. coli yields a molecule reacting with core antibodies.

The localization of the transcript of the core gene is in doubt because of the presumptive presence of a nick at base 8 (22). Transcription could begin at this nick, or alternatively, at a TATAAA-like sequence (TACATAAG) at residue 3060. An AAUAAA-like sequence (AAATTAA) is found at residue 822. The core gene, like the surface antigen gene, gives no evidence of an intervening sequence. If an intervening sequence were present, it would have to be small and should not change the reading frame (other frames have early stop signals).

POSSIBLE CODING REGIONS A AND B

The longest continuous nucleotide sequence devoid of a stop codon (>80% of the long strand) begins at ATG (base 495) and ends at a stop codon (base 3030); it overlaps the surface antigen gene. Depending on which ATG is used for initiation of translation, the polypeptide could vary from about 131 to 845 amino acids. The proximal Hogness sequences are TATAAAG (base 104) or TATAT (base 400). There are no obvious AAUAAA-like sequences until AATAAT at base 308; termination of transcription could also occur at the nick (base 8). This sequence predicts a basic protein rich in histidine with a large proportion of paired basic amino acids.

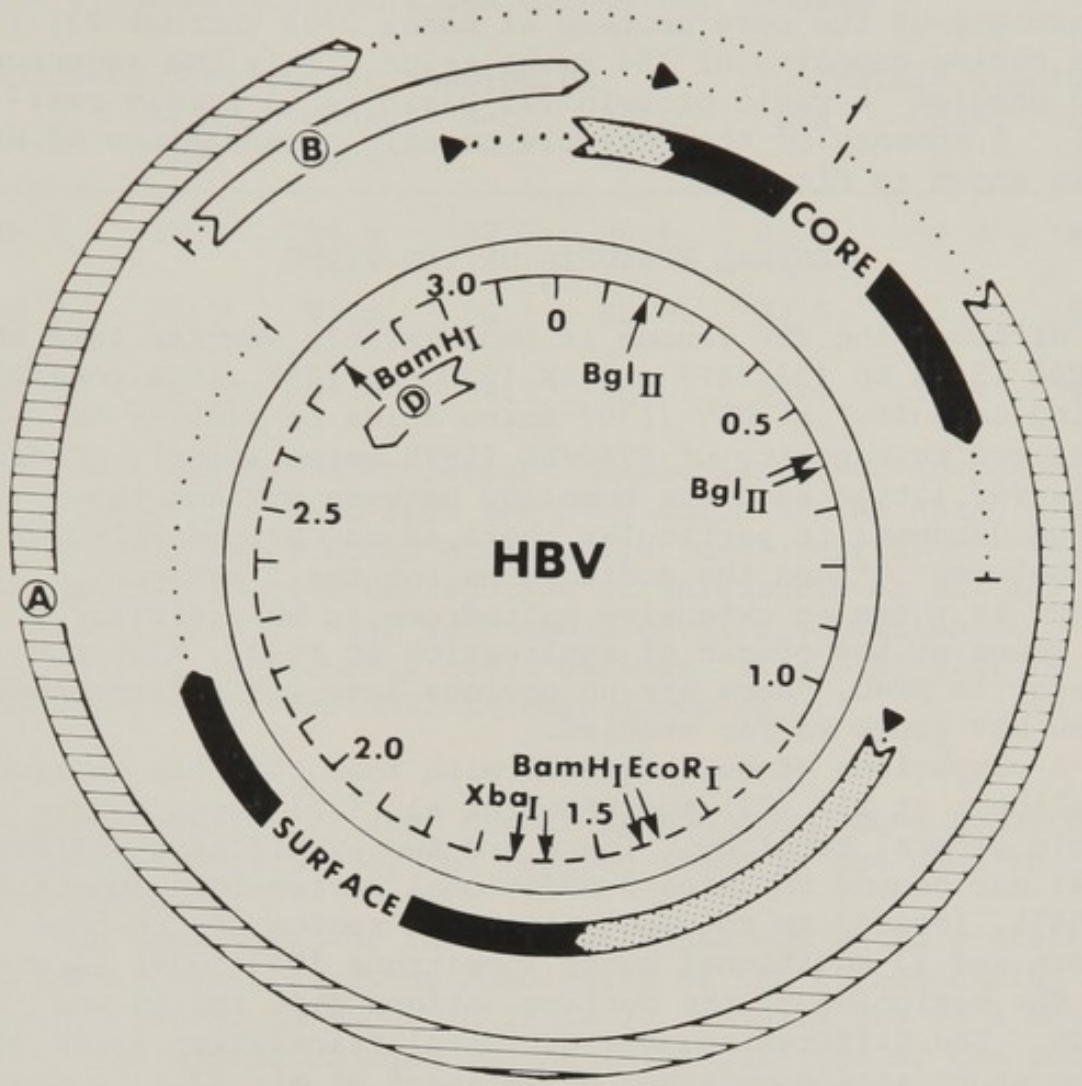


FIGURE 2. Possible genetic organization of HBV DNA. The 3221 base pair genome of HBV is presented as a partially double stranded circle. The major gene loci are indicated by boxes representing protein coding regions. The filled areas represent the known polypeptides. The non-solid areas correspond to possible precursors (surface or core antigens) or as yet unknown gene products (A,B,D). The interrupted lines flanking the coding regions represent putative transcripts. In these transcripts the closed triangles indicate probable start regions (deduced from Hogness TATAAAG-like sequences) and the vertical lines indicate possible regions at the 3' end of the message (the nearest AAUAA-like sequence). The restriction enzyme cleavage sites are shown as physical references.

The small coding region B is immediately adjacent to the 5' terminus of the core protein at bases 2783 through 23; it has a coding capacity of 154 amino acids. This DNA sequence would predict a basic protein (22 basic vs 13 acidic residues). A summary of the possible genetic organization of HBV DNA is shown in Figure 2.

GENERAL FEATURES OF THE VIRUS

Although the HBV genome is considerably smaller than that of SV40 (5200 bp (33,34)) and BK (5000 bp (35)), the possible "coding capacity" of HBV (1707 amino acids is similar to the actual coding capacity of SV40/BK (1876 amino acids). There is however little sequence homology between HBV and the SV40/BK genomes; in particular there is no obvious relationship between 'A' and the coding area for the T antigen. Neither is there an extensive palindrome in HBV, similar to that found at the origin of replication in SV40. Also in contrast to SV40, there are no obvious intervening sequences in the HBV genes so far studied.

A comparison of our sequence with that recently obtained by Galibert et al. (21) and Pasek et al. (22) shows considerable general homology. There are two significant differences: our genome contains 6 additional nucleotides (positions 542-547), located in the protamine-like region of the core protein and 33 additional bases (positions 1047-1079) located near the beginning of the surface antigen gene and in the 'A' region. The different number of 'open' translation areas (4) in the short strand obtained by Galibert et al. (21) is due to an altered distribution of stop codons and our requirement of an ATG codon for initiation.

Table I compares our DNA sequence data (adw2) with that of Galibert et al. (ayw) (21) and that of Pasek et al. (adyw) (22). Each of the sequenced HBV sequences is derived from viruses exhibiting different HBsAg antigenic subtypes. Thus the amino acid differences can be analyzed from the DNA sequence. The adw2 subtype HBsAg contains 17 different residues when compared with ayw subtype and 19 different residues when compared with adw. The HBsAg of ayw and adw are more similar, with only 7 different residues. Similarly, the nucleotide sequences of these subtypes are more homologous to each other than to adw2. A notable difference between the viruses is the length of the genomes. The ayw and adw genomes are 3182 bp long while our HBV adw2 DNA is 3221 bp, 39 bp longer than the others. These length differences are due to changes at two positions; there are six additional nucleotides near the carboxy terminus of the HBcAg gene and 33 additional bases near the start of the HBsAg gene. The significance of these

TABLE I: Comparison of Three Cloned HBV Genomes

Comparison	Nucleotide Homologies					Protein Homologies	
	Total DNA	HBsAg Gene	s-c	HBcAg Gene	c-s	HBsAg	HbcAg
A vs B	89.2	94.8	92.6	90.6	83.1	92.5	94.6
A vs C	88.4	95.1	90.1	90.6	83.5	91.6	94.6
C vs B	96.1	97.3	94.2	96.0	95.8	96.9	95.6

Values are expressed as percent homologies. "s-c" represents the intergenic region from the stop codon of the HBsAg gene to the start codon of HBcAg gene, while "c-s" represents the opposite region. The HBV subtype of Valenzuela et al. (A) (data from Fig. 1, also Valenzuela et al., submitted for publication) is probably adw2, Galibert et al (B) (21) is ayw, and Pasek et al. (C) (22) is adyw (complex).

"inserts" within our adw2 genome is not clear, but since both are multiples of three, the reading frame is the same in all three variants.

The homologies of the various HBsAg and HBcAg proteins are more similar than the homologies of the corresponding DNA coding regions, as shown in Table 1. This is due to a majority of nucleotide changes in the third position of the codon, resulting in no amino acid substitution. The amino acid changes which do occur are conservative, such as one hydrophobic residue for another hydrophobic (e.g. valine to alanine). The overall homologies for various portions of the HBV genomes are relatively consistent; there appears to be no mutational "hotspots" or major genetic drift between the HBsAg and HBcAg genes.

The sequence for the virus contains four well delineated coding areas, (nelecting intervening sequences), two of these are now identified. The predicted amino acid sequence gives insight into the properties of the proteins themselves and perhaps into the structure of the virus. The unique structural aspects of the surface antigen are the presence of two strong hydrophobic regions flanked by more hydrophilic regions rich in proline (therefore little α-helix), and cysteine. By interactions at one hydrophobic region, this polypeptide may form aggregates that can be covalently linked by intermolecular S-S bridges. The other hydrophobic region may interact with the lipid of the surface coat.

The "core" protein with its protamine-like domain at the

carboxy-terminus must be a DNA binding protein; its more acidic amino terminal domain is available for other purposes (binding to the surface antigen?). Both SV40 and BK viruses code for presumed DNA binding proteins; VP2 and VP3 have a strong basic domain at the carboxyl terminus. This region however contains lysine residues and is not as strongly basic as the protamine-like domain of the core protein.

The predicted properties of the core protein suggest a mechanism of HBV pathogenesis. The production of the core protein in quantity could lead to the replacement of histones and other chromosomal proteins, much as these proteins are replaced by protamines during the course of spermatogenesis (36). This may alter the expression of host genes, with possible pathological consequences. If this hypothesis were correct, core proteins but not surface proteins should be found in the chromatin of the infected liver cells. Furthermore production of core proteins in quantity should be deleterious to any host cell.

Our understanding of the genetic complement of the virus remains incomplete. If the endogenous DNA polymerase is coded by the virus, it is likely that coding region A is the gene, since DNA polymerases are large molecules. The HBe antigen is a component of the core, but has not been well identified. The possibility of transcription of non-coding RNA molecules also should not be overlooked.

The availability of cloned, sequenced hepatitis viral DNA allows a direct approach to control of the virus via the production of a surface antigen vaccine. Toward this end, we have linked the surface antigen coding sequence to a bacterial expressing plasmid in order to obtain the synthesis of this protein in bacteria.

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CORRELATING GENETIC MUTATIONS OF A BACULOVIRUS WITH THE PHYSICAL MAP OF THE DNA GENOME[1]

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The ability to map temperature-sensitive mutations of a baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), has been demonstrated. A marker rescue technique, based on the ability of a restriction endonuclease fragment of wild-type AcNPV L-1 DNA to recombine with a ts mutant DNA genome was employed to determine approximate locations of several ts mutations of AcNPV with respect to the physical restriction endonuclease fragment map of AcNPV L-1 DNA. Two mutants, ts N332 and ts B837, are defective in occlusion but non-defective in extracellular non-occluded virus formation. Thus, scoring for rescue of these mutants by wild-type fragments was based on visualizing occlusion bodies within plaques produced at the restrictive temperature following transfection. In addition, five plaque morphology mutants producing few polyhedra per cell, FPs, were isolated after serial passage of AcNPV in TN-368 cells. Analysis of the DNA genomes of these mutants with restriction endonucleases revealed that one mutant, FP-D, contains an additional sequence of DNA located at 66 md units (75%) on the AcNPV L-1 physical map.

INTRODUCTION

Compelling reasons for studying the mechanism of baculovirus infections derive from the current use of these viruses as biological pesticides (1, 2) and their potential use as

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transducing vectors for recombinant DNA research in invertebrates (3). Of fundamental interest, some baculoviruses undergo a fascinating and complex development process involving at least two distinct forms of the virus (4, 5). Most of the approximately 300 baculoviruses described in the literature are highly pathogenic but a persistent baculovirus infection in cell culture has been described (6). In addition, baculovirus-like particles apparently have an intimate role in certain invertebrate host-parasite relationships and are believed to be vertically transmitted (7).

Members of the family Baculoviridae have enveloped, rod-shaped capsids containing circular, double-stranded DNA genomes (4, 5, 8). Progeny nucleocapsids, produced in the nuclei of infected invertebrate host cells, may acquire an envelope by budding through cellular membranes resulting in the formation of extracellular nonoccluded virus (NOV) (9, 10, 11). Some baculoviruses have an additional option; the nucleocapsids may acquire an envelope within the nucleus and then be embedded in a crystalline protein matrix by a process known as occlusion (12, 13). Occlusion is temporally controlled, occurring late in infection as the rate of extracellular NOV formation declines (14). Occlusion is host-dependent and possibly tissue-dependent (13, 15).

Both extracellular NOV and occluded forms have important roles in virus transmission. Occluded viruses effect horizontal transmission from organism to organism. The occlusion body protects the enveloped nucleocapsids in the environment and in passage through the alimentary canal to the midgut where alkaline pH and enzymes apparently facilitate disruption of the occlusion body thereby releasing infectious virions at the primary infection site, the midgut epithelial cells (4, 9). Extracellular NOV effects transmission of virus from cell to cell via the invertebrate hemolymph or via the cell culture media thus permitting virus spread throughout the organism or culture (9, 16, 17).

Two types of baculoviruses undergo occlusion: nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs). The two viruses are distinguished on the basis of the occlusion body morphology; an occlusion body of GVs contains only one enveloped nucleocapsid whereas an occlusion body of NPVs contain many enveloped nucleocapsids per occlusion body. Occlusion bodies of NPVs are known as polyhedra and appear as large (approximately 5 microns in diameter) refractive, multi-faceted, crystals under the light microscope.

Interest in the molecular biology of baculoviruses is now focusing on *Autographa californica* nuclear polyhedrosis virus (AcNPV) as a model baculovirus system. Several established lepidopteran cell lines (such as *Spodoptera frugiperda*, IPLB-SF-21 and *Trichoplusia ni*, TN-368) serve as hosts for the pro-

duction of both extracellular and occluded virus forms of AcNPV. Excellent yet simple methods for plaque assay permit cloning (18, 19). Information concerning AcNPV replication in cell culture (14, 20), structural proteins (21, 22, 23), induced proteins (24), and genotypic variants (18, 25, 26, 27, 28) is rapidly accumulating. Physical maps of restriction endonuclease cleavage sites of the circular, covalently closed DNA genome of AcNPV have been constructed (26, 27). The AcNPV genome is approximately 85 million daltons and is predominantly unique sequence DNA (26).

Temperature-sensitive (ts) mutants of AcNPV were isolated recently (29, 30) and a variety of interesting phenotypes were observed. Several mutants were defective in both NOV and occluded virus formation. One set of mutants produced occlusion bodies but was defective in formation of infectious extracellular NOV at the restrictive temperature (29). Another set of mutants produced normal levels of extracellular NOV but was defective in occlusion body formation. Although there is only one major protein (polyhedrin) composing the matrix of the AcNPV polyhedra, complementation analysis revealed that at least five genes are involved in polyhedra formation. The proportion of mutations affecting polyhedra formation suggests that perhaps as much as one-half of the DNA genome is involved in the control of occlusion.

Serial passage in cell culture of AcNPV or its closely related genotypic variant *T. ni* NPV results in the progressive selection of virus strains with decreased ability to produce polyhedra in TN-368 cells (31, 32). Plaques produced by these viruses contain few polyhedra thus giving rise to the term FP to distinguish these viruses from normal (MP) viruses (31, 32). The FPs produce more NOV than MPs and thus have a growth advantage. The FP characteristic is genetically stable and may be involved in systemic infections (33).

Construction of a genetic map of the AcNPV genome based on correlating ts mutants and FP mutants with the physical map of restriction endonuclease sites would provide greater depth in understanding the strategy of the baculovirus infection process as well as provide a basis for future manipulation of the genome. Location of the genes controlling occlusion would, for instance, define a potential region for inserting passenger DNA. This region is most likely dispensable for viral replication in cell culture and deletion of the region would result in a virus defective in transmission in nature. A marker rescue procedure for mapping ts mutations of AcNPV on the physical restriction endonuclease fragment map is described in this manuscript. In addition, restriction endonuclease analyses of several FP mutants have revealed the presence of an addition mutation.

RESULTS

Transfection of *S. frugiperda* Cells With AcNPV DNA. A method for transfecting *S. frugiperda* cells, IPLB-SF-21, was developed based on the $CaCl_2$ technique described by Graham and Van der Eb (35). AcNPV L-1 DNA (18) was diluted in a solution of RHeBS buffer (8.0 g/l NaCl, 0.2 g/l KCl, 0.1 g/l $NaH_2PO_4 \cdot H_2O$ and 5 g/l HEPES, pH 7.0). Calf thymus DNA was added to a final DNA concentration of 20 µg/ml. A 2.0 M solution of $CaCl_2$ was added to a final concentration of 0.125 M. Following a 10 minute incubation at room temperature to allow for precipitate formation, the DNA-calcium suspension was applied to 60 mm tissue culture dishes containing $3x10^6$ attached cells. After 20 minutes at room temperature, 4 ml of complete TC-100 media (18) was added and the plates incubated at 27°C. For a "glycerol boost" (36) the cells were washed once with TC-100 media at 7 hours post infection, treated for one minute with 20% glycerol in RHeBS, washed once with TC-100 media and finally overlaid with TC-100 media containing 0.5% Seakem agarose. A linear dose response curve is observed with an average of $3.5x10^4$ pfu/µg DNA (Fig. 1).

For routine work, the "glycerol boost" was eliminated and TC-100 media containing 0.5% Seakem agarose was placed on the cells one hour after incubation in liquid TC-100 media at 27°C. The linear dose-response was maintained with this procedure but the pfu/µg DNA ratio was decreased approximately 50%.

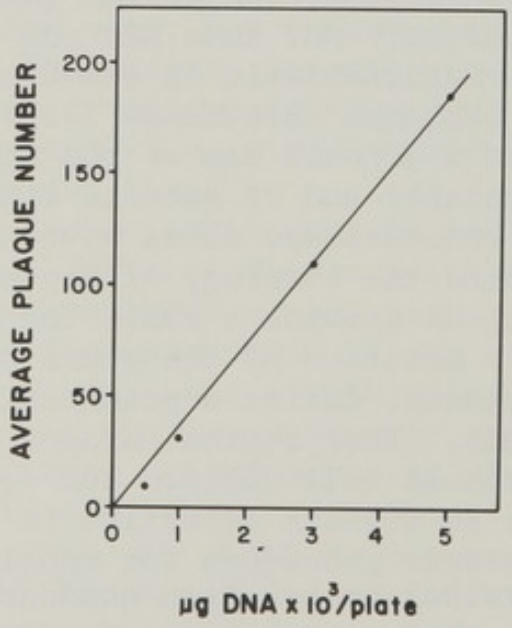

FIGURE 1. Linear dose-response with AcNPV L-1 DNA using a $CaCl_2$ transfection procedure.

Marker Rescue of ts Mutants of AcNPV L-1. A marker rescue procedure, based on recombination of a ts mutant DNA with a restriction endonuclease fragment of wild-type AcNPV L-1 DNA (36, 37) was developed to map ts mutants on the AcNPV physical map. Restriction endonuclease fragments of wild-type AcNPV L-1 DNA were isolated following gel electrophoretic separation (26). Each fragment was mixed with mutant DNA in a ratio of 10 molecules of fragment to one mutant DNA molecule. The mixtures of DNAs were used to transfect *S. frugiperda* cells by the $CaCl_2$ technique. Cells were maintained at the restrictive temperature, 32.5°C, during incubation. After six days, cells were stained with neutral red and examined for plaque formation (18). For those mutants nondefective in extracellular NOV formation, it was necessary to screen each plaque under the light microscope for occlusion body formation. Table I presents data obtained in rescue of ts mutants B1074, B837 and N332 with BamHI fragments (29).

The mutant ts B1074 is defective in infectious NOV production and nondefective in occlusion body formation at the restrictive temperature (29). Rescue of this mutant is thus reflected in the increase in full-sized plaques formed. Table I indicates that Bam-A preferentailly rescues B1074. To a lesser extent, Bam-B also rescues B1074 probably owing to contamination of the Bam-B fragment with Bam-A since these two fragments are difficult to resolve electrophoretically.

The mutants B837 and N332 are defective in polyhedra production but nondefective in NOV production. The increased quality of plaques achieved when *S. frugiperda* cells are maintained at 32.5°C on a long-term basis allows observation of plaques of N332 as well as B837 at the restrictive temperature

TABLE I
MARKER RESCUE OF ts MUTANTS OF AcNPV WITH BamHI FRAGMENTS[a]

	BamHI Fragment						
DNA	A	B	C	D+E	F	G	No
No DNA	0	0	0	0	0	0	0
B1074	150(100)	50(100)	1(100)	5(100)	2(100)	1(100)	4(100)
B837	30(45)	20(20)	29(0)	18(0)	26(0)	19(0)	25(0)
N332	38(83)	25(47)	23(0)	25(0)	22(0)	26(0)	38(0)

[a]Data are reported as the number of pfu produced at the restrictive temperature (32.5°C). The second number in parentheses indicates the percent of plaques with polyhedral occlusion bodies.

although these plaques lack polyhedra. Rescue of B837 and N332 is based on observing polyhedra production by light microscopy in plaques produced at the restrictive temperature. Table I indicates that Bam-A rescues both B837 and N332 with Bam-B again showing rescue to a lesser extent probably due to contamination with Bam-A. The percent of plaques producing polyhedra is remarkably high. In scoring for polyhedra production, approximately half of the "polyhedra-containing" N332 and B837 plaques had only a few cells containing polyhedra suggesting that the plaque was of mixed genetic type. This suggests the possibility of recombination during secondary virus infection in plaque development.

Rescue of N332 with the Xma fragments of AcNPV L-1 indicate that Xma-A rescues this mutant. These results, in conjunction with the Bam-A rescue results, indicate that N332 maps between 0-29.3% of the AcNPV physical map (Fig. 2).

Restriction endonuclease analysis of FP strains of AcNPV. Serial propagation of AcNPV in TN-368 cells resulted in the selection of virus strains which produced few polyhedra per cell in TN-368 cell cultures. Doubly plaque-purified FP isolates maintained their FP characteristic upon continued passage as previously reported for TnNPV (33). Although the FP characteristic was not apparent when tested in the IPLB-SF-21 cell line, the strains exhibited the FP characteristic when tested in TN-368 cells after passage through the *Spodoptera* cell line. The DNAs of five FP isolates were analyzed by restriction endonuclease analysis. Two of the five isolates had restriction endonuclease fragment patterns which varied from the patterns of the parent strain (an MP strain with the restriction pattern of AcNPV L-1) DNA. One of these FP iso-

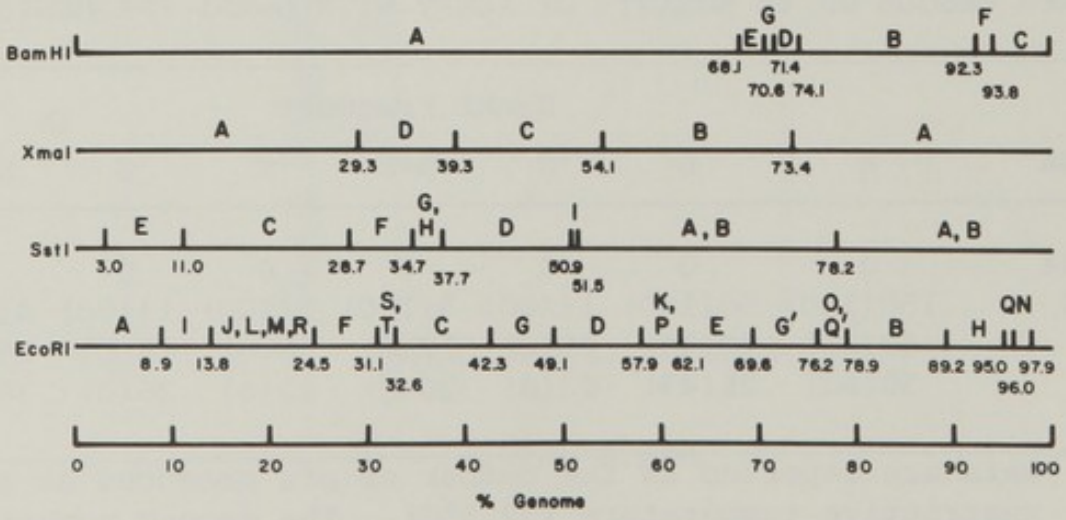

FIGURE 2. The Physical Map of AcNPV L-1 DNA.

lates, FP-D, was analyzed in detail. A comparison of the HinDIII, EcoRI, SstI, XhoI and BamHI fragment patterns of FP-D (D) and the MP parent (P) is presented in Fig. 3. In addition, an XmaI digest and a double-digest of BamHI and SstI were performed. The results of the single digests are summarized in Table II.

The alterations in the FP-D fragment pattern indicate that FP-D contains an additional DNA sequence located in a position of the AcNPV L-1 physical map between 74.1 and 76.2% of the genome (see Fig. 2). In each pattern examined, at least one new fragment, unique to FP-D, was observed. Concomitant disappearance of one fragment present in the parent strain was difficult to prove conclusively in some cases. To demonstrate that SstI-AB (see 27 for Sst nomenclature) and BamHI-B fragments were altered, it was necessary to do a double SstI-BamHI digestion. The fragment produced from cleavage at 74.1 and 78.2 (Fig. 2) was missing in FP-D and three new fragments (4.25, 3.80 and 0.55 md) unique to the FP-D double SstI-BamHI digest were observed. The alteration of HinD-L (26) is also consistent with an addition mutation between 74.1 and 76.2%. Alteration of Xho-J suggests that the placement of this fragment by other research workers (27) may not be precise.

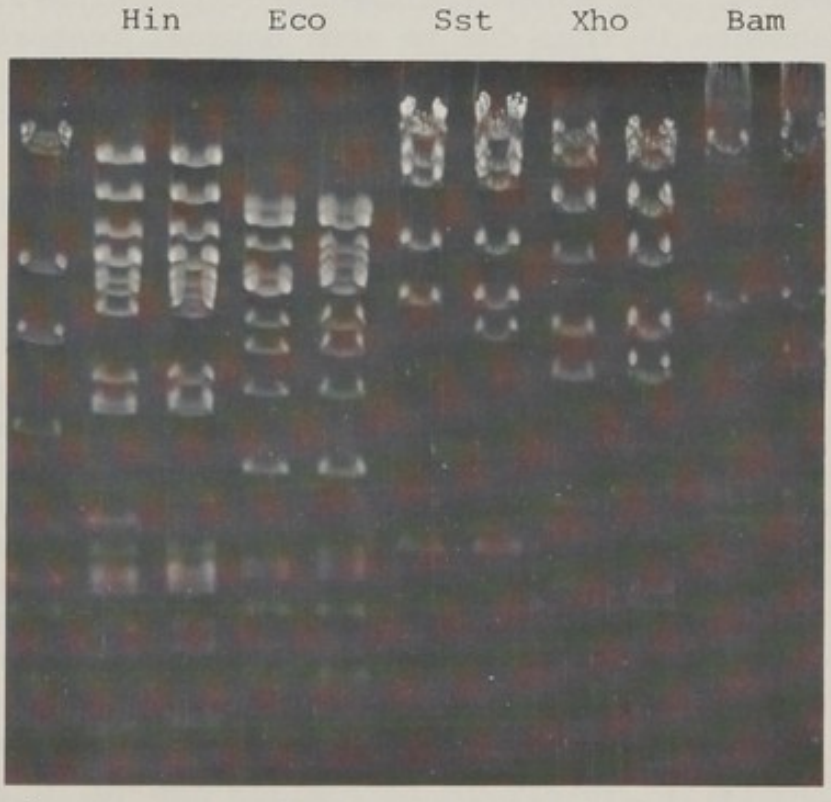

FIGURE 3. Restriction endonuclease analysis of FP-D.

TABLE II
ALTERATIONS IN FP-D DNA FRAGMENT PATTERNS

Restriction Endonuclease	L-1 Fragments Altered in FP-D	New Fragments[c] Observed in FP-D
HinDIII	L	5.3, 2.4[b], 1.65
EcoRI	G'[a]	6.5, 1.6, 1.0
SstI	AB[a]	4.6
XhoI	J	6.8, 2.3[b]
BamHI	B[a]	4.3
XmaI	A[a]	2.7

[a]Difficult to observe fragment alteration due to large size or comigration with other fragments.
[b]Appears in sub-molar quantities.
[c]Units are daltons x 10^{-6}

An unusual feature of at least two of the FP-D patterns, HinDIII and XhoI, is the appearance of fragments in apparently sub-molar (nonstoichiometric) quantities (see Fig. 3 and Table II). Since the Hin-L and Xho-J fragments are totally absent, all FP-D DNAs must contain an additional DNA sequence within these fragments. The nonstoichiometry of the HinD-2.4 and Xho-2.3 fragments is thus due to some unusual feature of the inserted DNA itself. The nature of this feature and the precise size of the additional DNA sequence remain to be determined.

DISCUSSION

A variety of temperature-sensitive mutations of AcNPV may be mapped in relationship to the physical map by a marker rescue procedure based on recombination between the mutant DNA genome and a wild-type fragment. Mutants, defective in occlusion but nondefective in NOV synthesis, produced some plaques of apparently mixed genetic type upon rescue. Such mixed plaques may be due to mutant NOV, produced in the first (or first few) infected cell(s), encountering another cell containing a wild-type fragment during plaque development. Certain mutants, only partially defective in polyhedra production such as FP mutants or the ts-IVb mutants (29), may be more difficult to map by the marker rescue technique.

One FP mutant, analyzed by restriction endonuclease analysis only, contained an additional DNA sequence which may be of host origin since more than two restriction nuclease

fragments of unique size are produced by some enzymes (eg. EcoRI produces three fragments unique to FP-D). Although integration of viral DNA into the host chromosome is believed to lead to many oncogenic DNA virus addition mutations, essentially nothing is known concerning possible baculovirus interaction with the host chromosome despite suggestive information in this regard (7).

AcNPV is clearly proving to be an excellent as well as fascinating model baculovirus system. Exploring the gene organization of the virus using the marker rescue technique described herein will provide valuable insight into the mechanism of infection and permit future sophisticated manipulation of the DNA genome.

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ANALYSIS OF VSV GLYCOPROTEIN STRUCTURE AND GENOME STRUCTURE USING CLONED DNA[1]

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ABSTRACT We have determined the COOH-terminal and NH_2-terminal sequences of the vesicular stomatitis virus (VSV) glycoprotein (G). The COOH-terminal sequence was deduced from the DNA sequence of a cloned DNA insert derived from the 3'-end of the G mRNA. The NH_2-terminal sequence was deduced from the sequence of a DNA primer extended on the VSV genome from the M gene into the adjacent G gene. We have shown that an uninterrupted hydrophobic domain near the COOH-terminus of G spans the lipid bilayer and that the highly basic COOH-terminus resides inside the virion. The functional significance of both the COOH-terminal and NH_2-terminal sequences is discussed.

The sequences in the VSV genome at the junctions of the NS-M, M-G, and G-L genes were determined using DNA primers derived from cDNA clones. The junctions have the common sequence

(3')AUACUUUUUUUNAUUGUCNNUAG(5')

in which the underlined dinucleotide is the intergenic region. The sequence preceding this dinucleotide is complementary to each mRNA at the site of polyadenylation, and the sequence following the dinucleotide is complementary to the capped 5'-terminal sequence of each mRNA. Polyadenylation by repetitive copying of the 7 U residues and other possible transcription events at these junctions are discussed.

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INTRODUCTION

We have previously described the isolation and characterization of cDNA plasmids containing sequences derived from vesicular stomatitis virus (VSV) mRNAs (1). DNA from these plasmids has been useful for obtaining structural information from important regions of the VSV glycoprotein and from the intergenic regions in the VSV genome (2,3). These studies are summarized here.

VSV Glycoprotein Structure and Function. Enveloped viruses such as VSV serve as useful model systems for the study of the structure, biosynthesis, and function of membrane proteins. VSV virions contain a single glycoprotein, G, which forms spikes on the surface of the virion. This 70,000 dalton glycoprotein contains two asparagine-linked complex oligosaccharides (4,5) and is positioned in the virion such that almost 90% of the polypeptide chain is external to the lipid bilayer (6,7). G protein is responsible for both the binding of virus to host cells and for inducing uptake of virus by the cell (8,9). Also, during virus maturation the interaction between the internal components of the virion and the portion of G exposed on the cytoplasmic face of the plasma membrane probably directs virus budding.

The G protein is inserted into the rough endoplasmic reticulum (RER) as a nascent polypeptide chain (10,11). Both ends of the molecule play important roles. Like the majority of secreted and membrane proteins, the nascent G polypeptide has a short hydrophobic NH_2-terminal signal or leader peptide (12 -15) which appears to initiate association of the nascent polypeptide-mRNA ribosome complex with the membrane of the endoplasmic reticulum and which is removed prior to chain termination (16). Unlike secreted proteins, however, membrane proteins like G are not discharged completely across the microsomal membrane and instead become anchored stably in the membrane. Preliminary studies have suggested that G is bound to microsomal membranes at a site very near its COOH-terminus (15,17). Hence, this region of G must differ from the COOH-termini of secreted proteins in some fundamental but not as yet not understood way so as to halt extrusion into the lumen of the RER.

A knowledge of the complete primary amino acid sequence of both ends of this polypeptide is clearly critical to a molecular understanding of how G interacts with cellular membranes. Because only partial amino acid sequences had been determined for the NH_2-termini of both the leader peptide and of the mature protein (13,14) and no sequence had been determined for the COOH-terminus of the protein, we used two different strategies to obtain the G mRNA sequences encoding these domains. Furthermore, we have been able to demonstrate

that G interacts with the membrane of the mature virion near its COOH-terminus. We discuss features of this COOH-terminal sequence which anchor G in the membrane and which may be critical in formation of functional virions.

VSV Intergenic Structure and Function. The genome of VSV is a single-stranded RNA molecule containing approximately 10,000 nucleotides (18). This virion RNA (vRNA) is copied by a virion-associated RNA polymerase (19) which transcribes the five structural genes sequentially in the order 3'-N-NS-M-G-L-5' into the five monocistronic mRNAs (20,21). Synthesis of a short leader RNA (47-48 nucleotides) encoded at the 3'-end of the genome precedes the synthesis of N mRNA (22). The five mRNAs are modified and have capped and methylated 5'-termini and 3'-termini poly(A) (23).

The discrete mRNAs and leader RNA could be generated either by cleavage of a precursor RNA or by separate initiations and sequential synthesis along the genome. The vRNA sequences at the junctions of the five structural genes must therefore contain specific signals triggering either the termination of transcription, polyadenylation, and initiation or cleavage and polyadenylation. We have determined the sequences in VSV vRNA at the junctions of the NS-M, M-G, and G-L genes using primers derived from cDNA clones (3). Together with the sequence reported by McGeoch (24) for the N-NS junction these results provide the complete intergenic sequences from the VSV genome. The nature of the transcription and polyadenylation signals at these junctions are discussed.

RESULTS AND DISCUSSION

Determination of the COOH-terminal G Protein Sequence. Katz and Lodish (17) and Chatis and Morrison (15) observed that newly synthesized G protein was oriented in vesicles derived from the RER such that proteolysis degraded only about 3,000 daltons of the protein. The tryptic peptides of G which were degraded appeared to map to the COOH-terminus of the protein. Assuming that the orientation of G in the mature virion was similar to that in vesicles derived from the RER, we expected the COOH-terminal portion of G to contain both that part of the polypeptide which spans the lipid bilayer and that part which is inside of the lipid bilayer, presumably interacting with internal virion components.

To obtain the 3'-terminal G mRNA sequence and the corresponding COOH-terminal protein sequence, we have sequenced an insert from a cDNA clone which contains 470 nucleotides derived from the 3'-end of the G mRNA. Figure 1 illustrates the region of G mRNA sequence included in this cDNA clone and

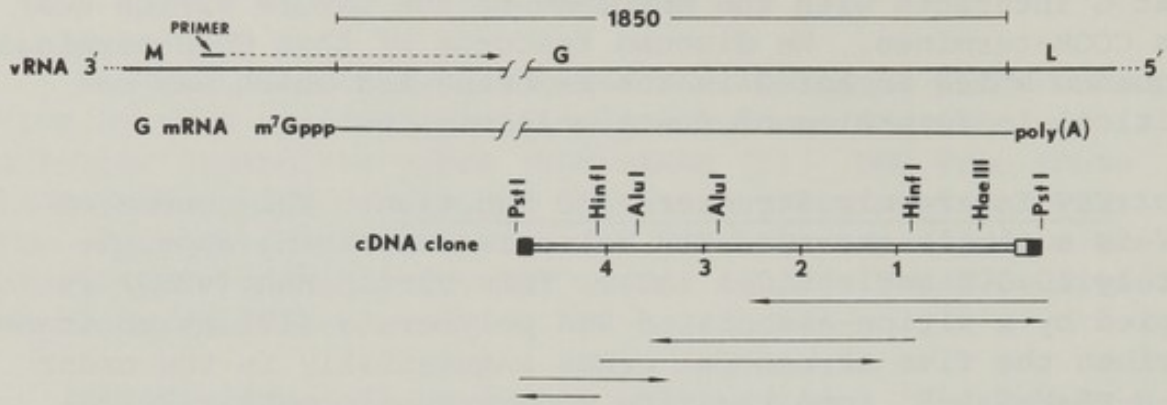

FIGURE 1. Diagram showing the methods used to obtain the 5'-terminal and 3'-terminal G mRNA sequences. The region of the VSV genome containing the G gene and the flanking M and L genes is shown. A single-stranded DNA primer derived from a cDNA clone of the VSV M mRNA was extended from the indicated position in the M gene into the G gene and then sequenced to obtain the 5'-terminal G mRNA and protein sequences. The G mRNA and the region of the mRNA sequence containing the pG65 cDNA clone (double lines) are indicated. Arrows represent the number of nucleotides sequenced from each ^{32}P-labeled 5'-end. Restriction sites used in sequencing are indicated. Lengths are in hundreds of nucleotides.

the restriction sites used to generate fragments for complete sequencing of both DNA strands by the Maxam-Gilbert procedure. The mRNA sequence derived from the sequence of the cDNA clone is shown in Figure 2b.

The reading frame for the G protein was deduced as follows. The length of G mRNA is about 1,850 nucleotides without poly(A) and it encodes a polypeptide of 62,500 daltons (25) or about 570 amino acids. In the mRNA there are 30 noncoding nucleotides at the 5'-end (3), leaving the capacity to encode 606 amino acids. Thus we would expect no more than 108 noncoding nucleotides at the 3'-end of the mRNA. The mRNA sequence has an uninterrupted reading frame extending toward the 5'-end from the UAA terminator located 100 nucleotides from the poly(A). This must be the proper reading frame because the two other frames are blocked many times including termination codons at positions 449 and 468. The amino acid sequence predicted for this open reading frame is shown in Figure 2b. Note that the predicted sequence contains an uninterrupted hydrophobic domain (boxed) near the COOH-terminus.

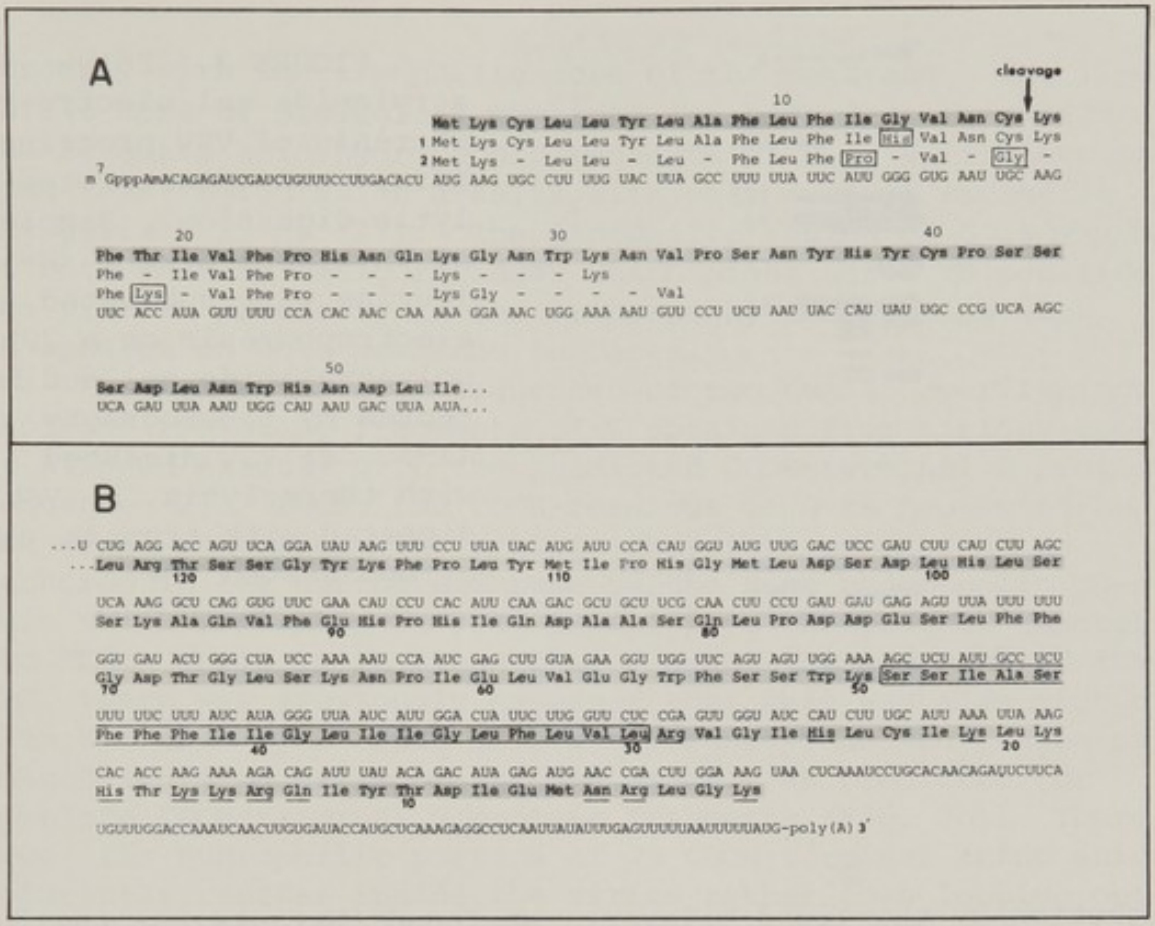

FIGURE 2. G mRNA and protein sequences. A) 5'-terminal G mRNA and protein sequences. The shaded protein sequence was predicted from the cDNA (mRNA) sequence beginning from the single initiator AUG codon in the ribosome binding site sequences determined previously from ribosome protected G mRNA (12). Partial amino acid sequences numbered 1 and 2 were determined by Lingappa et al. (13) and Irving et al. (14), respectively. These sequences were determined by direct sequencing of cleaved and uncleaved forms of G proteins. Boxed residues do not agree with those predicted from the cDNA sequences. B) 3-terminal G mRNA and protein sequences as determined from cloned DNA. The amino acid sequence shown was predicted from the mRNA sequence.

The previous data on the orientation of G in microsomal membranes (15,17) led us to suspect that the amino acid sequence deduced above contained the domain of G associated with the membrane of the virion. However, to test this directly we digested VSV virions with proteases and determined partial amino acid sequences of those fragments of G that were protected from protease by the lipid bilayer. As shown by Mudd (6) and Schloemer and Wagner (7), digestion of VSV with protease results in complete disappearance of the intact G protein and the appearance of a small fragment (c[illegible] 7,000 daltons) [illegible] was presumably protected from

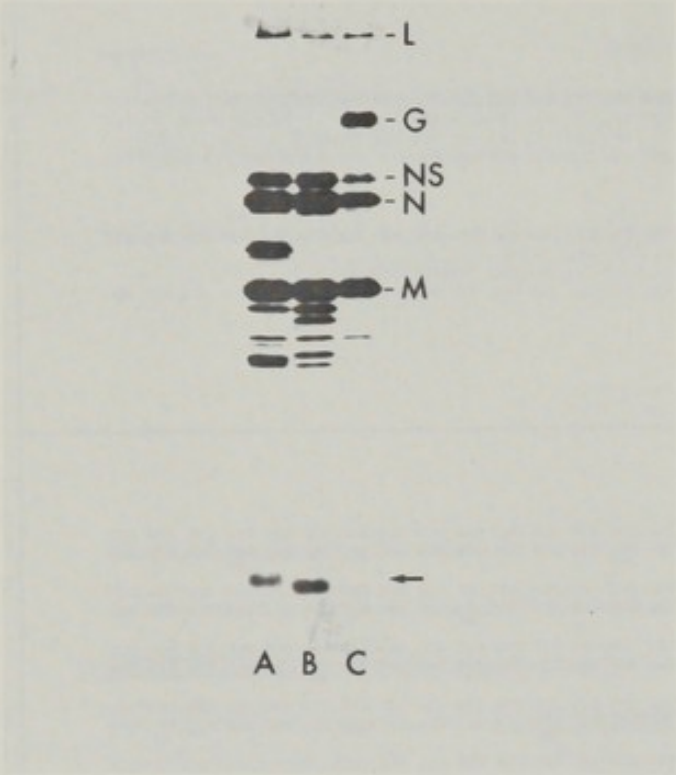

FIGURE 3. Polyacrylamide gel electrophoresis of VSV proteins before and after proteolytic digestion. Samples of VSV labeled with ^{3}H-leucine were subjected to electrophoresis on a 20% polyacrylamide gel and detected by fluorography (19). a) VSV digested with thermolysis, b) VSV digested with trypsin, and c) undigested VSV.

digestion by the lipid bilayer. We found that virions purified after proteolysis contained peptides of ca. 6,700 daltons (trypsin digestion) or ca. 7,000 daltons (thermolysin digestion) which were not present prior to digestion (Fig. 3). We isolated both of these fragments and determined the partial NH_2-terminal amino acid sequences of each (2). These sequences could be aligned exactly within the predicted COOH-terminal sequence of (Fig. 2b). The new NH_2-terminus generated by trypsin resulted from cleavage after the lysine located 64 amino acids from the COOH-terminus of the G protein. Similarly, the slightly larger fragment generated by thermolysin resulted from cleavage on the NH_2-terminal side of the leucine located 66 residues from the COOH-terminus.

Features of the COOH-terminal Domain. Previous work has shown that insertion of the VSV G protein into the RER occurs when the protein is a nascent chain (10,11). Insertion stops when the majority of the protein is inside the lumen of the RER. A region near the COOH-terminus spans the membrane and a small portion is exposed on the cytoplasmic face (15,17). The COOH-terminal G protein sequence predicted here from the nucleotide sequence of a cDNA clone shows an uninterrupted hydrophobic domain beginning 49 amino acids from the COOH-terminus and ending 30 amino acids from the COOH-terminus. Presumably this hydrophobic region and the flanking charged amino acids act as a signal to stop the transfer of the protein across the membrane. This region might disrupt a hydrophlic channel (16) through the membrane and then interact

strongly with the lipophilic core of the membrane. The lipophilic core of biological membrane is about 3 nm thick (26). The 20 amino acid hydrophobic sequence of G is sufficient to span this region as an α-helix with each residue advancing the helix 0.15 nm (27). The lipophilic region of G is bounded at both ends by basic residues which might serve to position the protein precisely in the membrane by interacting with phosphates on both membrane surfaces.

Partial amino acid sequences of two small, overlapping, protease resistant fragments of G obtained from virions could be aligned exactly with the predicted COOH-terminal G protein sequence (2). Thus, the COOH-terminus of G is protected by the viral membrane. The protein therefore appears to be anchored in the virion membrane by the same hydrophobic domain which initially stopped transfer of the protein through the RER. While it is clear that G spans the membrane of the RER, there was previously no proof that this was also true of G in virions. The sizes of the membrane protected fragments calculated from gel mobility are exactly as predicted for complete COOH-terminal G protein fragments (Fig. 2b). Therefore, the hydrophilic portion of 29 COOH-terminal amino acids apparently resides inside the virion rather than looping outside the viral membrane. Figure 4 illustrates the orientation of G that is suggested by our data. Of the 29 "internal" residues, 15 are basic and 2 are acidic suggesting ionic interactions with internal virion components.

The complete sequence (131 amino acids) of the human erythrocyte membrane protein glycophorin has been determined (28). This protein contains an uninterrupted hydrophobic domain of 23 amino acids which spans the lipid bilayer leaving 36 COOH-terminal residues inside and 72 NH_2-terminal residues outside the plasma membrane. Thus the location relative to the COOH-terminus and the sizes of the hydrophobic portions in VSV and glycophorin are very similar, although there is no exact sequence homology. The inter-

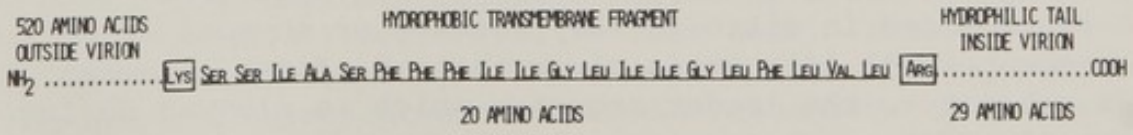

FIGURE 4. Orientation of G in the VSV membrane.

nal COOH-terminal portion of glycophorin is highly charged, but does not show the strongly basic character of the internal portion of VSV G. This basic character may be specific for viral structure. The complete sequence of fowl plaque virus hemagglutinin (a glycoprotein) has been deduced recently from the sequence of a cDNA clone (29). The sequence predicts a hydrophobic region near the COOH-terminus as in VSV G and glycophorin. Thus the orientation of G in the viral membrane that we have demonstrated here may be a characteristic of most viral glycoproteins.

Determination of the NH_2-terminal G Protein Sequence. To obtain the 5' terminal sequence of the G protein mRNA and the corresponding NH_2-terminal protein sequence, we used the approach illunstrated in Figure 1. A 52 nucleotide DNA primer isolated from a cDNA clone of the VSV M protein mRNA was labeled at its 5'-end using [γ-^{32}P]-ATP and polynucleotide kinase. After hybridization to the single negative strand of VSV genome RNA, the primer was extended into the adjacent G gene using reverse transcriptase and unlabeled deoxynucleoside triphosphates. Primer extended more than 350 nucleotides was purified by gel electrophoresis and sequenced using the Maxam-Gilbert procedure (30). This methodology has been described previously (3). This sequence is shown in Figure 2a with a predicted G protein sequence of 53 amino acids beginning at the single AUG codon in the ribosome binding site. Two partial sequences of the NH_2-terminus of G protein synthesized *in vitro* (13,14) are largely in agreement with, but differ in a few positions, from the sequence predicted from the cDNA (Fig. 2a). The single difference with the sequence of Lingappa et al.(13) is in a region of protein sequence that was considered tentative. Because three base changes would be required to convert the glycine codon (GGG, position 13) to specify the histidine reported (13), we believe that this assignment was probably incorrect. The three differences with the sequence of Irving et al. (14) might be due to VSV strain differences. However, this possibility seems unlikely at positions 12 and 19 because at least two base changes would be required to convert each codon to specify the amino acids suggested. From the previous protein sequencing data on G synthesized in vitro in the presence or absence of cellular membranes (13,14), it is clear that the first 16 amino acids constitute the leader sequence which is cleaved during or shortly after membrane insertion.

Features of the NH_2-terminal Domain. We have deduced an NH_2-terminal sequence of 53 amino acids for the VSV G protein including the complete sequence of the leader peptide, from the sequence of a DNA primer extended on the VSV genome from the adjacent M gene into the G gene. The structure of the 16 amino acid leader peptide is typical o[illegible]

other secreted and membrane proteins (31) in that it has a central core of hydrophobic or non-polar residues (positions 4-14) and hydrophilic residues at both ends (positions 2,3, 15 and 16). Most leader sequences also contain at least one proline or glycine (31) and the sequence predicted from the DNA has a glycine at position 13 (Fig. 2).

Determination of Intergenic Sequences. The basic methodology for determining sequences from the VSV genome using DNA primers derived from cDNA clones has been described previously (3). These experiments involved the following steps. 1) Isolation of cDNA clones containing sequences from the 3'-termini of VSV mRNAs. 2) Determination of the 3'-terminal mRNA sequences from the cloned DNAs. 3) Isolation from the cDNA clones of single-stranded DNA primers containing sequences derived from near the 3'-termini of each mRNA. 4) Extension of 5'-[^{32}P]-labeled primers through the intergenic regions into adjacent gene sequences on vRNA template using reverse transcriptase. 5) Sequencing of the extended primers by the Maxam-Gilbert procedure (30) and locating the adjacent gene sequences from the known sequences of the ribosome binding sites in VSV mRNAs (12,32).

The sequences of the intergenic region determined in this manner are shown in Figure 5 along with the corresponding mRNA sequences. The N-NS junction sequence is from McGeoch (24).

General Features of the Intergenic Regions. The extent of sequence homology is the most striking feature of the four vRNA regions which encode the boundaries of the five structural genes. The general structure can be represented as follows:

(3')AUACUUUUUUUNAUUGUCNNUAG(5')

with N representing positions at which the sequence varies. The first eleven nucleotides of this sequence probably signal polyadenylation because they are complementary to the common mRNA sequence, (5')UAUGAAAAAAA, which occurs at the mRNA-poly(A) junctions. The eleven nucleotide sequence is followed by a dinucleotide (underlined) and then immediately by the sequence complementary to the 5'-terminal mRNA sequence (5')AACAGNNAUC(3') beginning the next gene. Thus, the dinucleotides are the only vRNA sequences from these junctions that are not represented in the mRNA sequences. The intergenic dinucleotide is always GA except between the NS and M genes where it is CA (Fig. 5). The GA sequence also follows an identical polyadenylation signal in L mRNA (33), suggesting that it may be an important part of the signal. Correlating with this one sequence difference is the finding that anomalous polyadenylation is especially prevalent at the NS-M junction, generating NS mRNA linked to M mRNA by long poly(A)

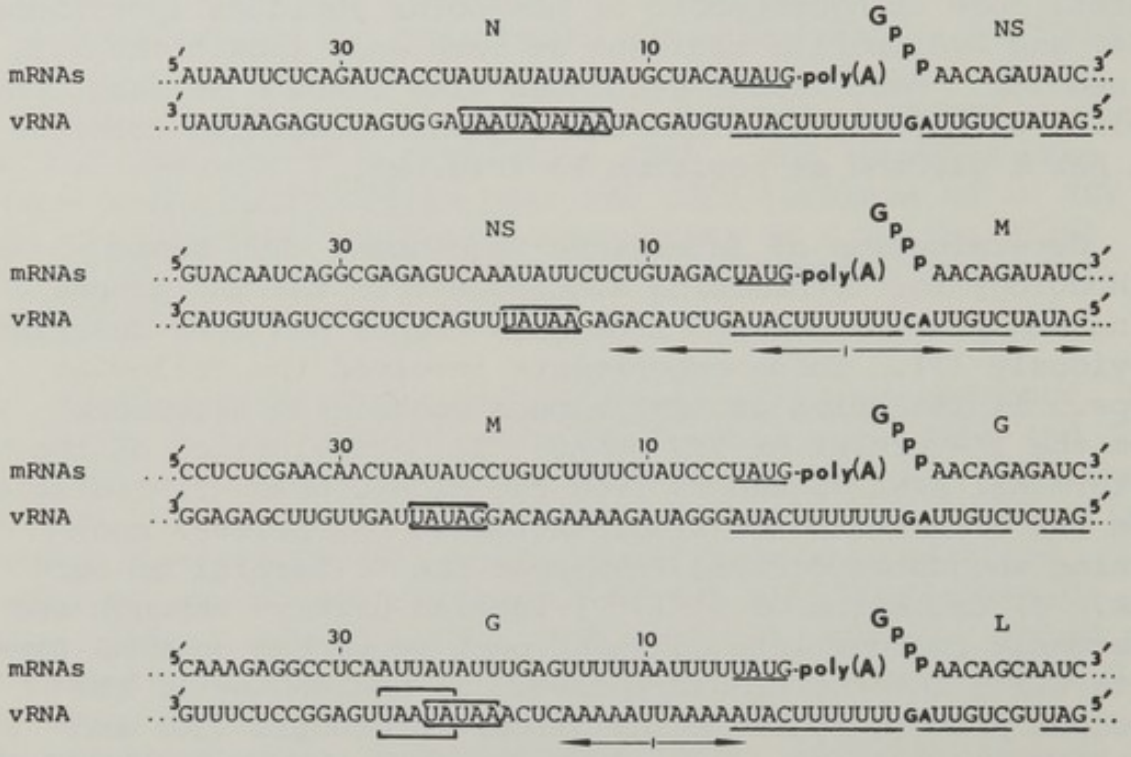

FIGURE 5. Nucleotide sequences of VSV vRNA at the junction of the five structural genes. The vRNA sequences at the junctions of the NS, M, G and L genes were determined as described previously (3). The mRNA sequences encoded are shown above the vRNA sequences.

One interesting feature of the vRNA sequence in each gene which precedes another gene (but not in L, 33) is the occurrence of related sequences (3')UAUAA(5'),(3')UAUAG(5') and (3')UAAUA(5') which begin 28 to 36 nucleotides before the start of the next gene (Fig. 5). These sequences resemble the putative eucaryotic "promoter" sequence (5')TATAAA(3') that has been found 27 nucleotides before presumed polymerase II initiation sites on eucaryotic DNA (35). The sequence (3')UAAUA(5') also occurs twice in the vRNA sequence encoding the leader RNA (36) at positions 29 and 32 nucleotides from the start of the N gene. The occurrence of these sequences preceding each VSV mRNA start site would thus be consistent with a role in promoting transcription initiation.

The vRNA regions encoding the last 40 nucleotides at the 3'-ends of the N and G mRNAs are extremely AU-rich (75%), but this is not a general feature of the 3'-ends of VSV mRNAs so its significance is uncertain. Regions of dyad symmetry are found centered around the junction of the NS and M genes and at the 3'-ends of the G mRNA (Fig. 5) but these also are not general features of the intergenic boundaries.

Transcription at the Intergenic Boundaries. The two general models to explain the sequential nature of VSV transcription involve either cleavage of a precursor (23) or multiple, sequential, transcription initiations.

In the cleavage model, the polymerase would transcribe through the intergenic regions. Two subsequent cleavage events would then be required to remove the intergenic dinucleotide, followed by capping and methylation of the5'-terminus. Poly(A) tails could be added by an enzyme that recognizes . . .UAUGAAAAAAA(3') at the 3'-end of the cleaved transcript. Alternatively, poly(A) could be added before cleavage if the polymerase copied the oligo(U) sequence repetitively before proceeding.

In the multiple initiation model, the polymerase would terminate in the oligo(U) sequence, perhaps after repetitive copying. Synthesis of the next mRNA species would then require a separate initiation event. The possible promoter sequence located in the 3'-end of the preceding gene might be part of a signal for this initiation. That internal initiation of transcription can apparently occur on the VSV genome was shown by Roy and Bishop (37) who found a γ-[^{32}P]-ATP labeled, 5'-terminal sequence pppAAPyNG on RNA synthesized in vitro. This sequence is different from the 5'-terminal leader RNA sequence (22) and could be a precursor of the common 5'-terminal mRNA sequence, m^{7}GpppAACAG (3,38).

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RIBOSOME BINDING TO POLIO VIRUS RNA

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ABSTRACT Three ribosome-binding sites have been found on polio RNA. The sites were located by comparing the T1 RNase digests of ribosome-protected fragments to T1 digests of RNase III fragments mapped on the polio genome. Ribosome binding sites at 0.02, 0.66 and 0.92 map units were confirmed by visualizing ribosome-RNA complexes in the electron microscope and measuring the positions of bound ribosomes. Furthermore, two subgenomic fragments of polio RNA which included the internal binding sites were translated *in vitro*.

INTRODUCTION

Polio virus RNA produces a complex polyprotein when translated *in vivo* which accounts for most of the coding capacity (1). Studies by Summers and Maizel (2) as well as Butterworth (3) detailed the map positions of protein cleavage products with some unaccounted for variation in expected molar ratios. Because of the precursor-product relationships and the ability to do pactamycin mapping, the concept of a single initiation AUG for translation of the polyprotein is well established (1,3).

Celma and Ehrenfeld (4) found two f-met dipeptides when polio virus RNA was translated *in vitro* with extracts of poliovirus-infected HeLa cells. Subsequent work by Knauert and Ehrenfeld (5) has also suggested the presence of two ribosome binding sites using reticulocyte lysates. They found that one f-met dipeptide was attached to a protein of 6-8000 MW and the other to a protein of 150,000 MW. They suggested that the latter could be the first polyprotein cleavage product for the coat protein NCVP-1. The other might be related to the protein attached to the 5' end of the RNA, VpG, which Lee (6) and

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Flanagan (7) have described.

There is precedence for internal initiator AUG's in several plant and animal viruses including Sindbis (8), Semliki Forest virus (9), Tobacco mosaic (10), and the RNA tumor viruses (11). However, these "cryptic" initiation codons are poorly or non-functional. The viral genomes are processed into subgenomic fragments with the AUG on the 5' end thus producing separate messenger RNAs. Kozak (12) has stated that there were no definitive examples of internal initiation. Although there seems to be no fixed sequence requirement 5' to the AUG for initiation, usually the most 5' AUG is the initiator and the M^7G enhances initiation. She proposed a "scanning" mechanism whereby the 40S ribosomal subunit binds on the 5' end and moves toward the 3' end until the AUG codon is encountered. To test this hypothesis, Kozak (13) constructed synthetic templates for ribosome binding which were tested as linear or circular molecules. One type of polymer was $UpGp(A)_n$. Two sizes of poly A were used: n=25 and n=110. The other polymer was poly(A,U,G). Wheat germ and reticulocyte ribosomes bound to linear molecules but not to the circular ones unless the latter were nicked with T1 ribonuclease. However, *E. coli* ribosomes did bind to the circular molecules. These experiments were presented as evidence that eukaryotic ribosomes must have a free 5' end of RNA onto which they thread. Also the eukaryotic ribosomes used in these experiments did not convert circular polymers to linear ones.

METHODS

The methods which were used can be found in the papers by McClain *et al* (14,15,16).

RESULTS

Identification and Location of Three Ribosome Binding Sites. Incubation of polio RNA and the reticulocyte lysate with puromycin and aurintricarboxylic acid, inhibitors of peptide bond formation and of initiation respectively, prevented the formation of an 80S peak of RNA protected by ribosomes after T1 RNase treatment (data not shown). However, there is a "background" of RNA which sedimented into the gradient even though no ribosomes were bound. This background was from oligonucleotides which are rendered T1 resistant either from non-ribosomal protein binding or from changes in the sensitivity of polio RNA to the T1 RNase at these conditions. The 80S peak was also absent when all available magnesium was chelated by EDTA (data not shown).

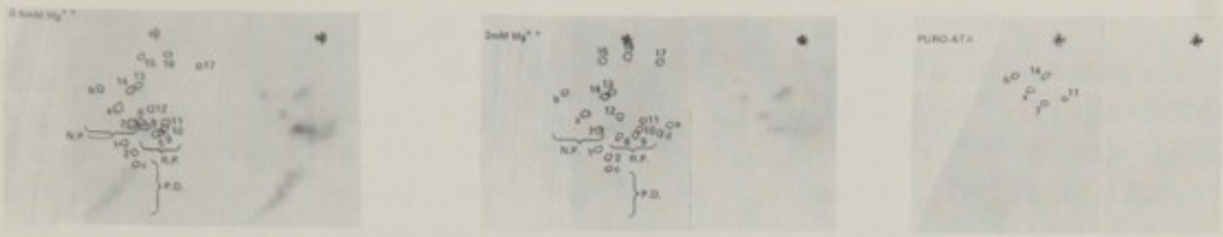

Figure 1. A series of autoradiograms from second dimension gels of ribosome binding reactions with whole lysate at 0.5 and 2 mM Mg^{++} plus a control with puromycin and ATA.

In Figure 1, Panel C illustrates that with the inhibitors of initiation, puromycin plus ATA, several oligonucleotides are still found in the 80S region. These oligonucleotides as well as partially digested RNA were more concentrated in the 40-60S peak (data not shown) and are thought to be caused by nonribosomal proteins which protect the RNA from T1 nuclease degradation as well as partial digests. Uninhibited ribosome binding reactions at 0.5 mM and 2 mM Mg^{++} result in additional oligonucleotide protection. At 0.5 mM Mg^{++} three new oligonucleotides are most prominent (ribosome-protected, RP numbers 8, 10, and 12). At 2.0 mM Mg^{++} oligonucleotide number 12 is practically absent and number 8 becomes more prominent relative to number 10. Additional oligonucleotides were found to be partial digests of 8, 10, and 12 or non-ribosomal protein protected.

Further identification of these oligonucleotides was accomplished by a second T1 digestion after elution of the RNA from the gels. The second treatment was necessary since the guanosine sites for T1 RNase could be protected by the protein. Since RP 8, 10, and 12 had very different products, it seemed likely that they represented distinct ribosome binding sites.

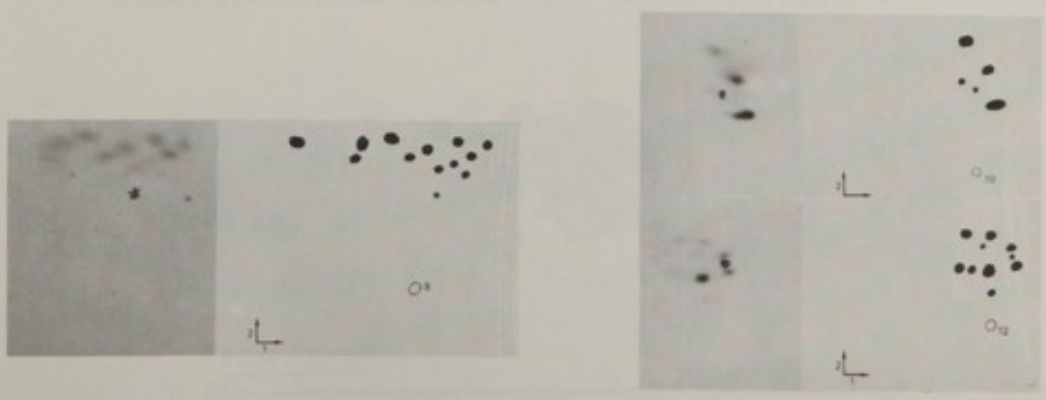

Figure 2. Products of the secondary T1 digestion of oligonucleotides 8, 10, and 12.

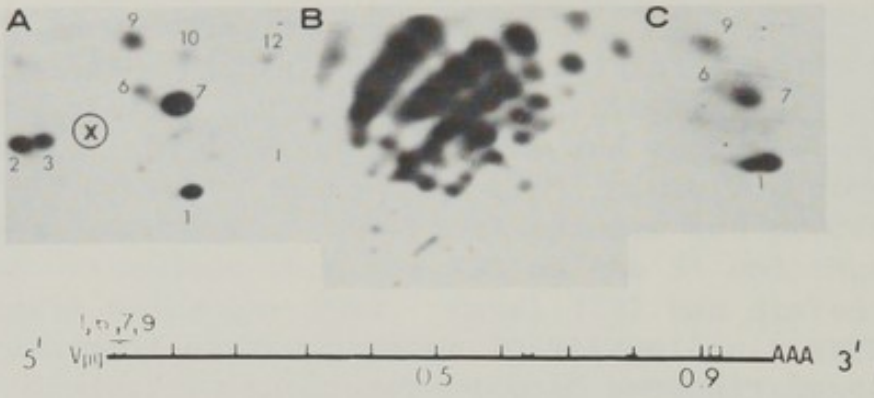

Figure 3. Autoradiograms of two dimensional gels showing separation of T1 RNase digest products of: A, RNase III fragment Vpg-115; B; Polio RNA; and C, RP 10.

Figures 3 and 4 illustrate that T1 RNase digests of the RP fragments have unique T1 oligonucleotides which match specific T1 digests of RNase III fragments mapped by Stewart et al (17). In Figure 3, the RNase III fragment containing Vpg and 115 bases of the 5' end is identified by a new position of an oligonucleotide X after protease treatment. The key pattern which emerges in this RNase III fragment is the spots for oligonucleotides 1, 6, 7, and 9. By visually comparing the pattern in RP 10, the same group is present. A T1 digest of ^{32}P-labeled polio RNA is included in the center panel to show the relative position of these unique oligonucleotides in the complete fingerprint.

RP 12 can be mapped to the 3' end of polio RNA. Figure 4 illustrates the presence of T1 products 46, 56a, 58, and 66a in both RNase III fragment E2 (530 bases from the 3' end) and in RP 12. RP 8 has a T1 digest fingerprint pattern very different from the other two (Figure 2). It cannot be precisely mapped with an RNase III fragment because it lacks large unique T1 products. However, it visually fits some of the RNase III fragment T1 digests from the middle of the genome.

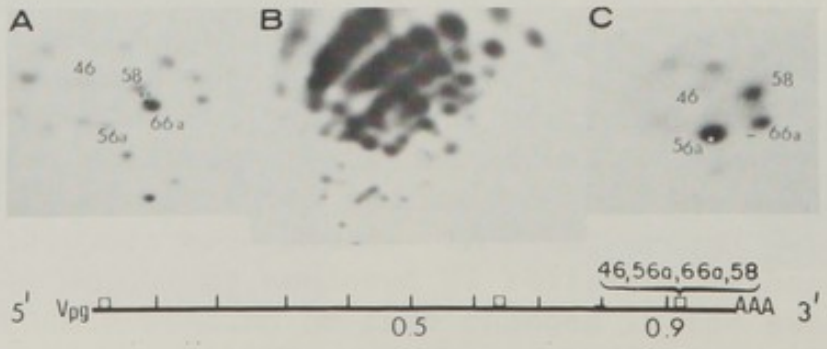

Figure 4. Autoradiograms of two dimensional gels for T1 digest products of: A, RNase III fragment E2; B, Polio RNA C, RP 12.

Localization of Ribosome Binding Sites by Electron Microscopy. Figure 5 illustrates representative electron micrographs of polio RNA bound with ribosomes and polio c-DNA. Since the c-DNA is 95% dependent on poly dT for synthesis (16), the majority of molecules will begin hybridization from the 3' end of the RNA. Thus we established two internal sites for ribosome binding. This procedure had limitations since few full length molecules were available, i.e., straight strands were rare and the extra incubation with c-DNA caused some breaking of RNA. Furthermore the optimal Mg^{++} concentration for ribosome binding with the fractionated lysate (4mM) selects against binding at the 5' site (15).

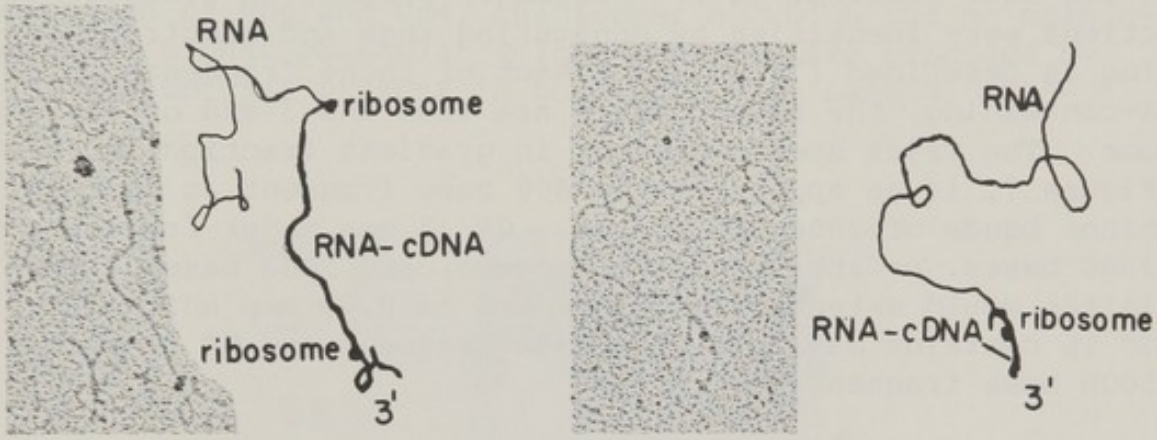

Figure 5. Electron micrographs of polio RNA with ribosomes bound by incubation in a purified reticulocyte lysate. The complexes were isolated by isopycnic centrifugation. c-DNA binding was as described (16).

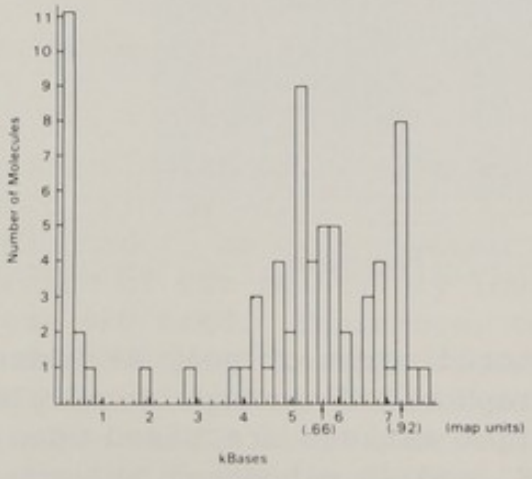

Figure 6. Histogram of ribosome binding sites on polio RNA.

With ribosome binding sites oriented by the previous procedure, binding experiments were repeated and the RNA straightened with gene 32 protein. Many more RNA molecules could be evaluated by this method and three distinct ribosome binding sites were found. Modal frequencies are highest at 0.02, 0.66 and 0.92 map units. These correspond to 100-200 bases, 5300 and 7100 bases from the 5' end. The wide frequency of binding sites at 4000-6000 bases may result from small differences in the lengths of evaluated molecules which were normalized for the histogram.

In Vitro Translation With Subgenomic Fragments of Polio RNA. Poly A-containing RNase III fragments were separated in a low salt gradient (17). Fragments present in gradient fractions were identified by denaturing them and electrophoresing as described (17). Since each of these fragments is poly-A-containing, the base numbers are from the 3'end of the genome. The major specie present in gradient fraction 10 (GF10) Figure 7, is an approximately 600 base fragment as well as minor bands of 2000-3000 bases. GF 23 has major fragments of 3500 bases, or about half the genome, and 2000 bases. The latter would extend from the 3' end to 0.74 map units (5'-3'). GF 26 contains mainly a 3500 base fragment and GF 32 mainly a 5000 base fragment.

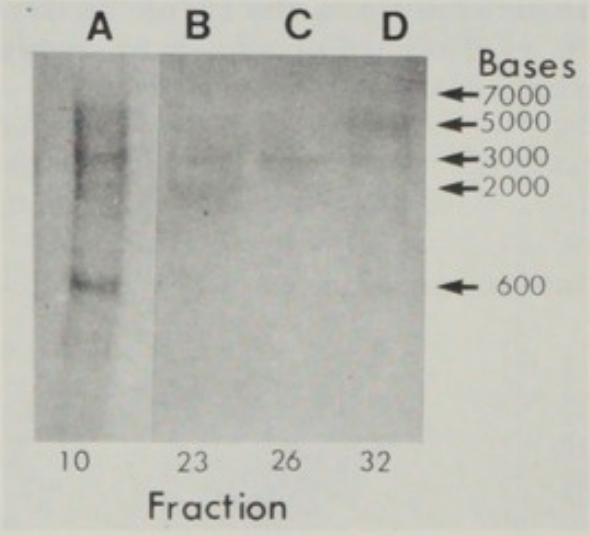

Figure 7. Autoradiogram of poly A+ RNase III fragments of polio RNA electrophoresed through an acrylamide-agarose gel. Molecular weight markers are based upon denatured whole polio RNA, 28S, 18S, and 4S ribosomal RNA run in the same gel. Lane A, GF 10 (Exposed longer because of fewer CPM); Lane B, GF 23; Lane C, GF 26; Lane D, GF32.

In Figure 8 *in vitro* translation products of 3' RNA fragments from the same gradient as Figure 7 are seen analyzed by SDS-PAGE. Fraction numbers refer to the gradient fractions from which RNA was taken for translation. All *in vitro* translation products have bands of endogenous proteins at 92, 40, and 25 kD. Lane 1 shows the products of polio mRNA translated *in vivo* with ^{35}S-methionine lable only. Lane 2 is from polio mRNA translated *in vitro* in the presence of ^{3}H-lysine and ^{35}S-methionine. Lane 3 is with no added mRNA. In Lane 4 the RNA from GF 11-14 (predominently the 600 base fragment) produced a 8-10 kD protein which is not present from the *in vivo* polio proteins labled with only ^{35}S (Lane 1). Since the 8-10 kD protein is present when polio RNA is translated *in vitro* with ^{3}H- lysine and ^{35}S-methionine, it may represent a combination of Vpg and Vp4, the former of which has no methionine (6). Higher molecular weight products are produced by larger RNA species present in this pool. In Lane 5, GF20-

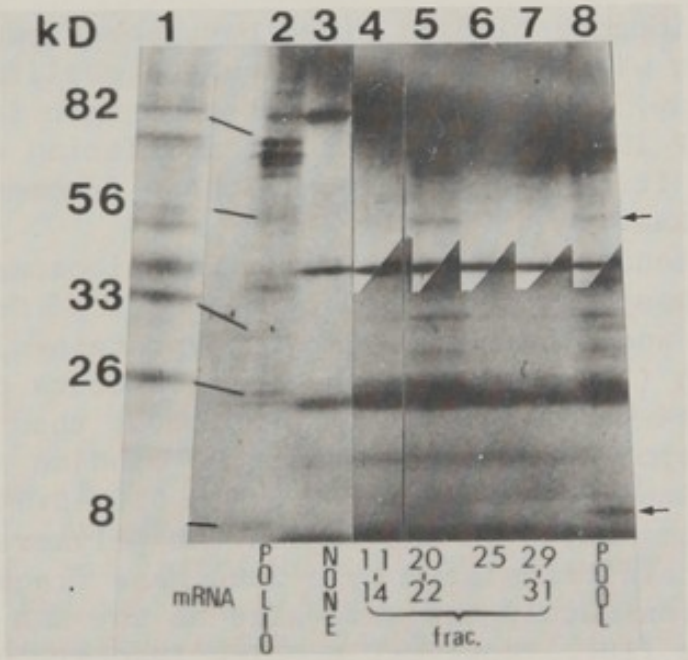

Figure 8. Autoradiogram of 7.5-25% acrylamide SDS protein gel of proteins translated *in vitro*. The lower one-half of the autoradiogram had to be over-exposed relative to the upper one-half because of the relatively lower amount of radioactivity in the 8kD band. Therefore, two photographic exposures have be "sandwiched" in this figure. Lane 1, cytoplasmic extract of polio virus-infectd cells; 2, Polio mRNA; 3, No added mRNA; 4, GF 11-14 (Correlate with RNA Lane A 5, GF 20-22 (Correlate with RNA Lane B); 6, GF25 (Correlate with RNA Lane C); 7, GF 29-31 (Correlate with RNA Lane D); 8, Pool of poly A^{+} RNA fragments. Equal CPM put on each lane.

23, the 2000-3500 base RNA fragments have been translated into precursor proteins of the 70-80 kD range and capsid proteins of 26-33 kD. A prominent band of 56 kD is enhanced relative to the same specie when intact polio RNA is translated *in vitro* (Lane 1) or when the pool of A^+ fragments is translated (Lane 8). Since GF 23 (Figure 7) is the only one with a 2000 base RNA fragment we suggest that the 56 kD protein is the product of that fragment. When GF 24 was translated it did not produce the 56 kD protein and very little of any other sizes as well. This region of the gradient contained mainly the 3500 base fragment. The protein bands of Lane 7 (GF 29-31, RNA 3500-5000 bases) are predominently of the 70-80 kD A^+ fragments (not fractionated on the gradient) translates into proteins of all size ranges. Most notable is the 8-10 kD band.

DISCUSSION

Three ribosome binding sites have been found on polio RNA by mapping with RNase III fragments, localization by electron microscopy, and translation of subgenomic fragments of polio RNA. RP 10 is most likely the initiation site for the polyprotein. It maps with a Vpg-115 base fragment at the 5' end of polio RNA.

The existence of at least one other ribosome binding site on polio RNA has been suggested by the data of Celma and Ehrenfeld (4) and others (5). The polyprotein cleavage data of Butterworth (3) showed that the molar amounts of noncapsid proteins 1b+2+4 were higher than predicted, thus this group was a likely spot for a second ribosome binding site. Indeed we have localized such a site, RP 8, to 0.62-0.74 map units on the polio genome which is the region the polymerase is encoded. The 56 kD protein translated by a 2000 base fragment from the 3' end is approximately the same size as the RNA polymerase of polio virus (18). However, precise identity of these two proteins awaits tryptic peptide mapping data.

As yet the origin of the 5' protein Vpg is unknown (6,7,). Earlier work on polyprotein cleavage patterns have not shown any unmapped 8-10 kD protein; perhaps because this migrates with the smallest capsid protein Vp4. It is thus intriguing that a third ribosome binding site has been confirmed by three methods at 0.92 on the polio genome. The 530 base RNA fragment mapping with RP 12 would be sufficient to code for a protein of 8-10 kD. This fragment translates such a protein *in vitro*. Interestingly the band of protein(s) at 8-10 kD is not well labled with methionine (Lane 1, Fig. 8). When ^{3}H-lysine

is also used for labeling in vitro products this region is more intensely radioactive. This may suggest that the methionine-free Vpg is labled with lysine (as one would expect for such a basic protein, 6) and migrates to the 8-10 kD region of the gel.

Although ribosomes were seen bound to the RNA at the internal sites, translation may not occur until the RNA is processed as stated by Hunter (10). We found that a 3000 base poly A-containing fragment would not produce the 56kD protein as did the 2000 base fragment. This suggests that the ribosome binding site may need to be some critical distance from the 5' end for translation to occur. Kozak (13) did not find cleavage of circular synthetic RNA polymers during ribosome binding. However, these molecules may not have the correct recognition sequences for cytoplasmic or ribosome-associated RNases.

ACKNOWLEDGMENTS

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SEQUENCE ANALYSIS OF THE POLIOVIRUS GENOME AND MAPPING OF THE GENOME-LINKED PROTEIN[1]

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ABSTRACT We are in the process of sequencing the poliovirus genome by an adaptation of Sanger's dideoxy method. This involves reverse transcription of virion RNA, followed by *E. coli* Pol I catalyzed DNA synthesis in the presence of cDNA, dNTPs, ddNTPs, and [5'-^{32}P]labeled primers (RNase T1 or RNase A resistant oligonucleotides of virion RNA). So far, we have sequenced segments of the RNA totalling 7,000 nucleotides. A sequence of 1,060 bases from the 3' end reveals that at least 562 nucleotides preceding the poly(A) remain untranslated. A base sequence in the 3'-terminal half of the genome corresponding to known amino acid sequences of the genome-linked protein (VPg) has been detected. These data suggest that VPg is part of the viral polypeptide NCVP1b. Thus, VPg and the viral RNA polymerase NCVP4 originate from the same polypeptide precursor. VPg is, at most, 27 amino acids long (max. mol. wt. 3,296).

INTRODUCTION

Poliovirus is a member of the Picornaviridae, a family of small, single-stranded positive RNA viruses. The virion RNA is covalently linked at its 5' end to a small protein (VPg) (1,2,3,4) and polyadenylated at its 3' end (5). The genome is approximately 7,800 nucleotides long (6) of which we have sequenced approximately 7,000 nucleotides.

Knowledge of the entire nucleotide sequence is important in understanding the biology of the virus. Combining techniques such as protein sequencing, ribosome binding, and

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secondary structure analysis with primary sequence information, will eventually lead to the determination of: the initiation and termination sites of translation, the entire genetic map, cleavage sites in protein processing and recognition site(s) for the RNA replicase, ribosomes, and capsid formation (RNA/protein binding), etc. The nucleotide sequence studies will also allow the exact determination of the nature of Defective Interfering (D.I.) particles.

Using a modification of Sanger's dideoxynucleotide method (7,8,9,10) to sequence virion RNA and automated protein sequencing we have already mapped the position of VPg to the 3' terminal half of the genome (9). This segment of the RNA codes for the viral replicase, which furthers our hypothesis that VPg may be involved in genome replication, most likely in the initiation of RNA synthesis (4,11).

We also present a sequence of 1,060 3'-terminal nucleotides of poliovirus RNA. Within this sequence an untranslated region of at least 562 nucleotides in the 3' end preceding the poly(A) tail has been found, with termination triplets in all 3 reading frames.

METHODS

Virus and RNA Preparation. Unlabeled, ^{3}H, ^{14}C and ^{32}P-labeled type 1 (Mahoney) poliovirus was isolated from infected suspension cultures of S3 HeLa cells as previously described (6,12).

Viral RNA was extracted from sucrose gradient purified virus using phenol/chloroform, precipitated with 2 volumes of ethanol and further purified by zonal sedimentation through sucrose gradients (6).

Enzymes, Nucleotides and Amino Acids. Reverse transcriptase from avian myeloblastosis virus was generously supplied by Dr. J.W. Beard (Life Sciences, Inc.; St. Petersburg, Florida), as arranged by The Research Resources Viral Oncology Program, National Cancer Institute. The enzyme was further purified as described (10).

E. coli DNA polymerase (Klenow fragment) and calf intestine alkaline phosphatase were obtained from Boehringer Co.; RNase T1 from Calbiochem; ^{3}H deoxy adenosine triphosphate, ^{14}C-, and ^{3}H-amino acids from Amersham; RNase A, RNase T2, and unlabeled deoxynucleoside triphosphates from Sigma; 2',3'-dideoxynucleoside triphosphates, oligo $(dT)_{12}$, and polynucleotide kinase from P.L. Biochemicals. Nearly carrier-free [γ-^{32}P]ATP was prepared from $^{32}P_i$ and ADP as described (13,14).

Preparation of cDNA. cDNA synthesis conditions were those described by Kacian and Myers (15). Specific preparation of cDNA to poliovirus RNA and subsequent sucrose gradient purification and selection of nearly full-length cDNA (10% of input RNA) is described elsewhere (10).

Preparation of [5'-^{32}P]labeled Oligonucleotides. ^{32}P-labeled virion RNA was digested with either RNase A or RNase T1 and calf alkaline phosphatase. Oligonucleotides were separated by two-dimensional gel electrophoresis, eluted from the gel with H_2O, and further purified by adsorption to and elution from DEAE-cellulose. The oligonucleotides were subsequently labeled with polynucleotide kinase and [γ-^{32}P]ATP (10).

DNA Synthesis in the Presence of Dideoxynucleoside Triphosphates. DNA synthesis using [5'-^{32}P]-labeled oligonucleotide primers, cDNA, dideoxynucleotide triphosphate chain terminators, deoxynucleotide triphosphates and DNA polymerase was carried out as described by Sanger et al. (7) with modifications (10). In short, we are using labeled primers and unlabeled triphosphates. The incubation is carried out at 37°C for 30 minutes, and stopped with a mixture of EDTA, urea, xylene cynol and bromophenol blue. A chase step is no longer needed. The mixture is heated and sequenced on 6% or 8% polyacrylamide gels as described (8).

Sequencing ^{3}H and ^{14}C-Labeled VPg. ^{3}H-or ^{14}C-labeled (one or more amino acids at a time) virion RNA was digested with RNase T2 in ammonium acetate. This mixture was directly applied to a polybrene coated cup of a Beckman Sequentator Model 890C and subjected to automated Edman degradation (16). Half of each fraction collected was monitored by scintillation counting, the remaining portion was converted to the phenythiohydantoin form (17), and analyzed by high pressure liquid chromatography (HPLC).

RESULTS

Sequence Studies of Poliovirus RNA. Our general strategy in sequencing poliovirus RNA, without the use of molecular cloning, is summarized in Figure 1. cDNA to purified poliovirus RNA is prepared by reverse transcription (15). This DNA is complementary to virion RNA and may now be sequenced by Sanger's chain termination method (7,10). In this procedure, the cDNA is used as template for DNA synthesis catalyzed by

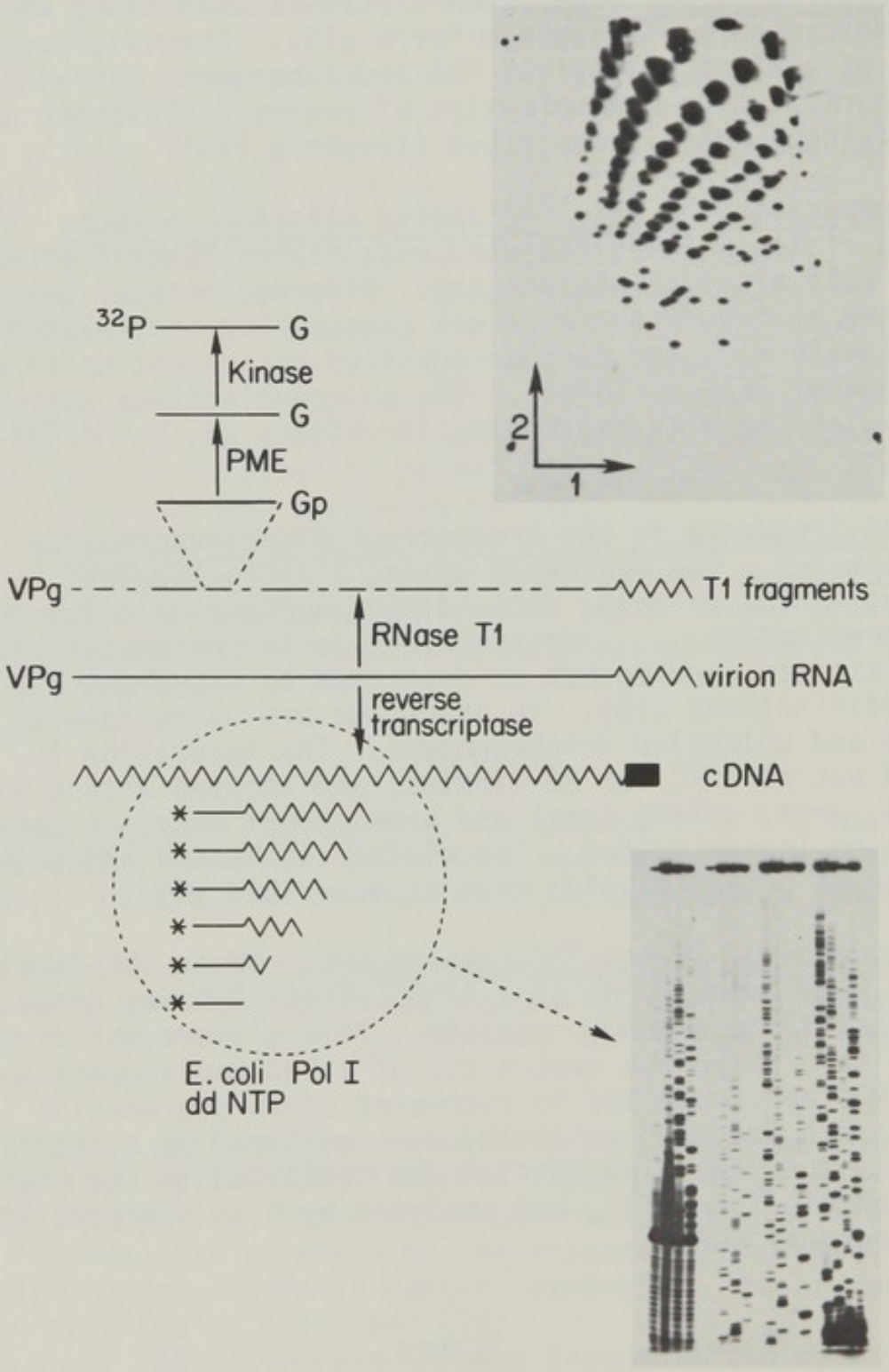

Fig. 1: Strategy to determine the primary structure of poliovirus RNA (10). Separation of RNase T1-resistant oligonucleotides of poliovirus is achieved by 2D-PAGE (upper right). An oligonucleotide is recovered from the 2D gel, dephosphorylated, labeled at the 5' end and used as primer for cDNA dependent DNA synthesis.in the presence of dideoxynucleoside triphosphates (ddNTP). DNA fragments are separated by PAGE (7). This method can be applied to the sequence determination of any high molecular weight RNA (10).

E. coli polymerase I (Klenow fragment) in the presence of dideoxynucleoside triphosphates (chain terminator nucleotides). DNA synthesis with the DNA polymerase I is primed by a [5'-^{32}P]-labeled oligonucleotide selected from a two-dimensional gel (6) whose position in the genomic RNA is known (18). The DNA chains are then separated by sequencing gels generating a pattern of bands that allows to read the nucleotide sequence of the cDNA. Our modification of using labeled primers and unlabeled triphosphates proved crucial for us in that it reduced the background of radioactivity in sequencing gels to near zero (10). The primer oligonucleotides were generated by digestion of virion RNA with RNase T1 or RNase A, purified by two-dimensional gel electrophoresis (6,19) and sequenced by the method of Donis-Keller et al. (20). Overlaps of DNA sequences and the oligoribonucleotide sequences served as controls for the sequence generated by the dideoxy method.

The 3' End of Poliovirus. Figure 2 shows a set of ladders generated by oligonucleotide #48. We find it impossible, as have others, to read 5-7 bases immediately following the primer. The reason for this phenomenon is not clear. Overlapping sequences, that must be generated to exactly order sequences, solve this problem.

Figure 3 shows the 3' terminal sequence of 1,060 nucleotides that we have elucidated with the aid of 8 oligonucleotide primers (10).

All sequences generated by oligonucleotides #1', #22, #6', #3, #13, #50, #48 and #8 overlapped, sometimes by as many as 200 bases. The sequence generated by #8 connected with the 3'-terminal poly(A). It overlaps, and is consistent with, a sequence of 156 nucleotides reported by Porter et al. (21) for poliovirus type 1 (Mahoney) RNA.

Partial Amino Acid Analysis of VPg. RNA was purified from poliovirus, labeled with [^{3}H] or [^{14}C] amino acids one or more at a time. VPg-pUp was generated from this RNA with RNase T2 and subjected to automated Edman degradation with subsequent analysis of radioactive fractions by scintillation counting and HPLC (16).

[^{3}H]Lys and [^{3}H]Arg were first used to label VPg since VPg was known to be a basic protein (1,2,4). Lysines were found at positions 9, 10 and 20 and arginine at 17. Subsequent analyses revealed Leu at position 6 and Val at position 11 (Figure 4). Using the genetic code this information was used to search for, and detect, a corresponding sequence in genome RNA (9).

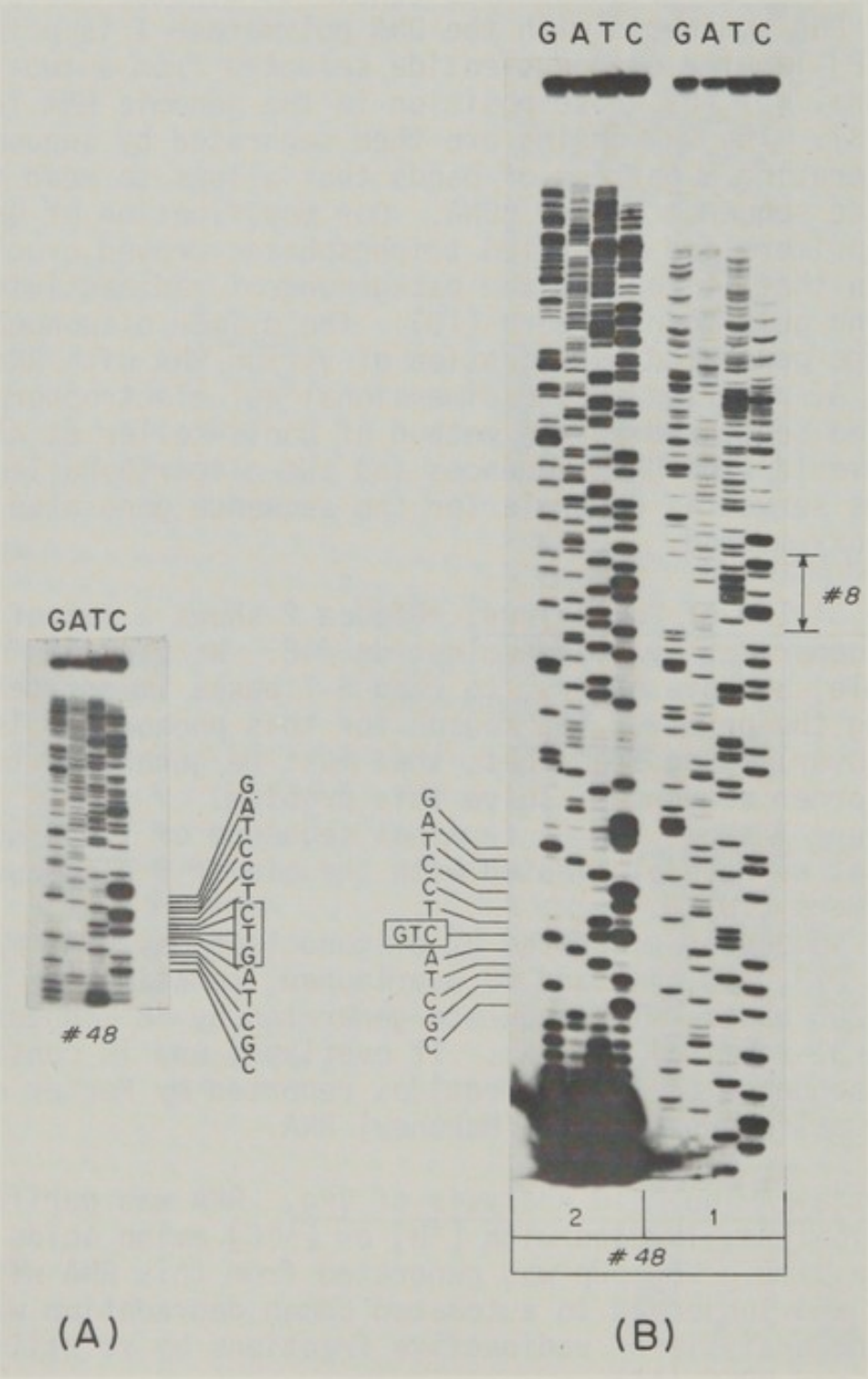

Fig. 2: Autoradiograms of sequencing gels. DNAs were synthesized in the presence of poliovirus cDNA and ddNTP as primed with [5'-^{32}P]-oligonucleotide #48 of polio virus RNA. (A) Separation on a 20% gel at 50-60^{o}C. (B) Separation on an 8% gel (12); 1: first application; 2: second application. G, A, T, C represent lanes of DNA chains terminated in ddG, ddA, ddT and ddC, respectively.

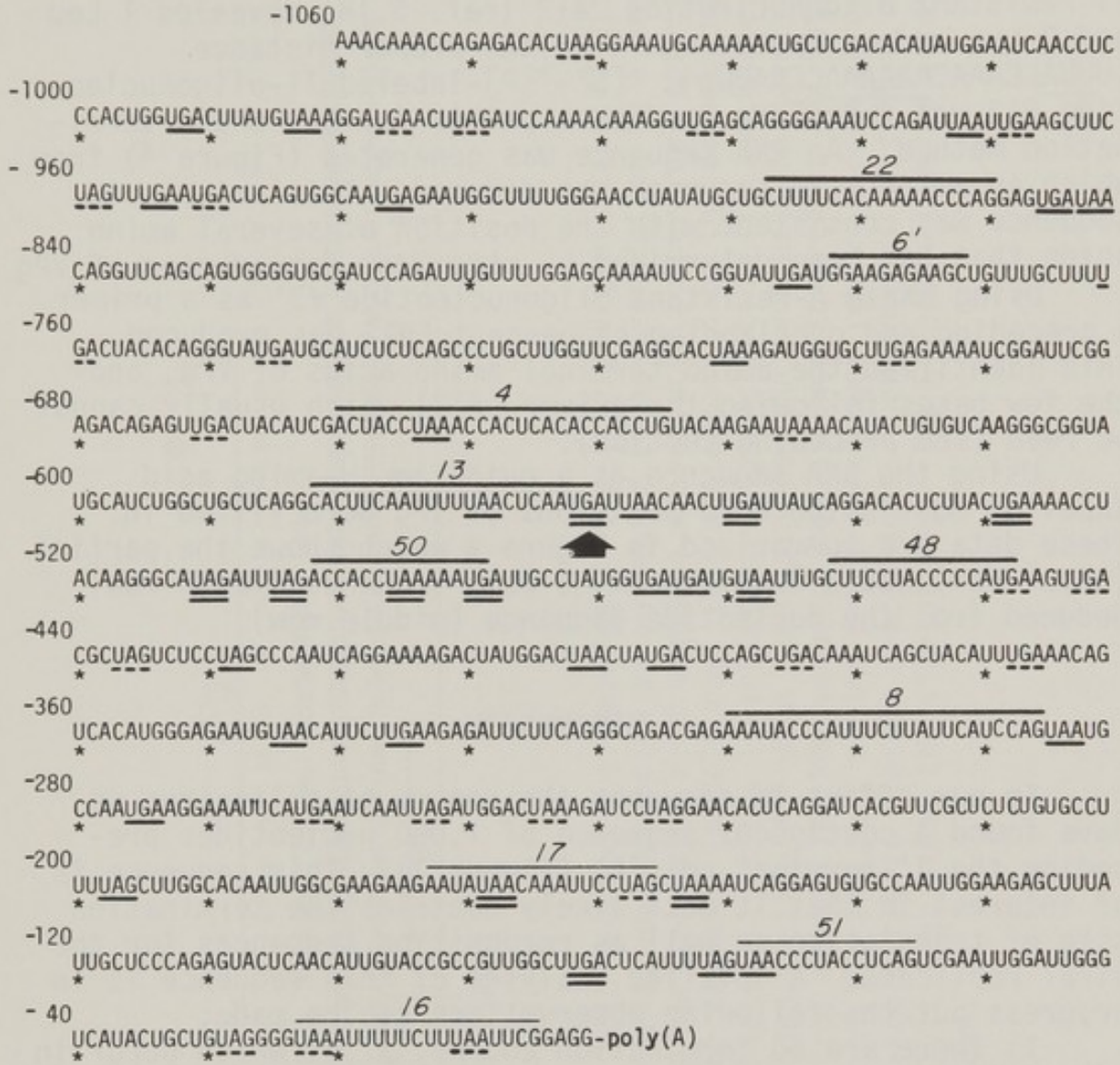

Fig. 3: 3'-Terminal nucleotide sequence of poliovirus, type 1 (Mahoney) RNA (10). Bars above the sequence correspond to oligonucleotides used as primers for the dideoxy method (see Fig. 1). Triplets underlined with a solid or broken line, or a solid double-line indicate termination codons in three reading frames. The black arrow marks the position (-562) at which termination codons begin to occur in all 3 reading frames. We suggest that this is the termination site of viral translation (10).

The double lysines were fortuitous in that the possible sequences of 6 nucleotides coding for Lys-Lys were limited to: 1) AAA AAA, 2) AAA AAG, 3) AAG AAA and 4) AAG AAG. RNase T1 and RNase A resistant nucleotides were searched for these sequences. There were several possibilities, but only RNase T1 resistant oligonucleotide #12 (ref. 6,18) revealed 1 Leu and 2 Lys codons spaced apart at the proper distance (UUUACCAAACAAAAAACCAACGp). [5'-^{32}P]-labeled T1-oligonucleotide #12 was therefore used as a primer for the chain termination method. An RNA sequence was generated (Figure 4) from which an amino acid sequence was deduced. This amino acid sequence was consistent with the position of several amino acids that had been determined by stepwise degradation of VPg.

Using RNase A-resistant oligonucleotide #3' as a primer a preceding and overlapping sequence to #12 was produced. This identified the amino terminal amino acids of VPg, and the few bases following the primer (#12) which usually cannot be read (see preceding section).

Using the RNA sequence as a guide to ^{3}H-amino acid labeling most amino acid positions of VPg were filled in. These data are summarized in Figure 4 which shows the partial (upper row) and a complete amino acid sequence (lower row) deduced from the nucleotide sequence (middle row).

DISCUSSION

In our effort to sequence the genome of poliovirus we have found a contiguous sequence of 1,060 nucleotides preceding the 3'-terminal poly(A) (Figure 3). This sequence is of interest in that it most likely contains the termination site of translation as well as recognition sequences for the viral replicase. A detailed analyses of this sequence is in progress but the following observations can be made:

1) There are 60 termination codons, 37 of which occur in all three reading frames between the poly(A) and position -562. We suggest that the termination codon at position -562 is the termination site of viral translation. Thus at least 562 nucleotides preceding the 3'-terminal poly(A) remain untranslated. It should be noted that long segments of non-coding nucleotides at the 3' end have been observed in other eukaryotic mRNAs (22,23).

2) The sequence does not contain an AAUAAA hexanucleotide that characteristically occurs in the vicinity of poly(A) (within 20 bases) in all untranslated regions of mammalian cytoplasmic mRNAs known to date (24). This is in agreement with the finding that poly(A) of poliovirus is genetically coded, and not added by post-transcriptional polyadeny-

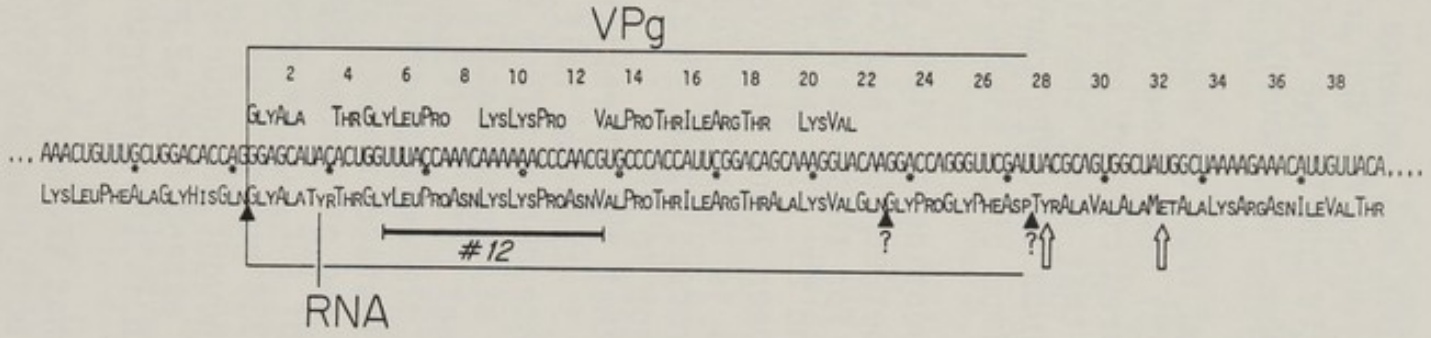

Figure 4: Partial amino acid sequence and mapping of VPg (16). VPg was labeled with one or several amino acids and subjected to automated Edman degradation in a protein sequenator. A partial amino acid sequence was deduced (upper row) corresponding to a nucleotide sequence (middle row) that was obtained by the dideoxy method described in Figure 1. RNase T1-resistant oligonucleotide #12 and RNase A-resistant oligonucleotide #3' were used to prime DNA synthesis on nearly full-length poliovirus cDNA. The RNA sequence has allowed to predict the total amino acid sequence of VPg and part of its precursor (lower row). Closed arrows: possible cleavage sites in protein processing. Open arrows: positions of Met and a second Tyr, that limit the size of VPg. The C-terminal amino acid of VPg is still unknown.

lation (25).

3) Preliminary computer analysis suggests that no large, stable secondary structures can be formed within the region of the 800 terminal bases (Jacobson, Nussinov, Kitamura and Wimmer, unpublished results).

A second interesting result of our RNA sequence studies so far is the finding of the coding region for VPg and part of its precursor. This observation is conclusive evidence that VPg is encoded by the viral genome, previously suggested by circumstantial evidence (2,26).

The size of VPg can be estimated from the encoding RNA sequence. Labeling studies (16,27,28) have shown that VPg does not contain Met, but a Met is predicted at position 32. Moreover, VPg contains only one Tyr that forms the linkage to the RNA (12,29). We therefore conclude that VPg contains a maximum of 27 amino acids and a minimum of 21 since the Val at position 21 has been confirmed.

Cleavage of the precursor polypeptide to yield the NH_2-terminus of VPg must have occurred between Gln and Gly residues. The protease responsible for this cleavage may also cleave the Gln/Gly residues at 22 and 23. It should be noted that cleavage at Gln/Gly pairs has been found in several picornavirus coat proteins (30). It is therefore feasible that the protease responsible for processing of viral coat proteins may also cleave the precursor to VPg.

We have previously determined that the T1-resistant oligonucleotide #12 maps within the 3'-terminal half of the genome (18). A more accurate estimate derived from sequence analyses places oligonucleotide #12 at approximately -2,100 $\pm$ 50 bases from the 3'-terminal poly(A) (Kitamura and Wimmer, unpublished results). This places VPg within NCVP1b, (reviewed in ref. 31) the primary cleavage product of the polio polyprotein encoded at the 3' end of genome RNA. NCVP1b with an estimated molecular weight of 85K, corresponds to a coding sequence of approximately 2,334 nucleotides. Since the termination of translation is thought to occur at position -562, the sequence of NCVP1b should extend from -2,896 to -562. This is significant as NCVP1b is the precursor for the viral RNA polymerase NCVP4 (32,33).

We have previously suggested that VPg may be involved in the initiation of RNA synthesis of both plus and minus strand RNA, possibly as a primer (4,11). Because no free, unbound VPg can be detected in infected cells, we have proposed that VPg may be cleaved from a precursor molecule, and linked to the first nucleotide at initiation (11). The map position of VPg makes likely the possibility that this precursor molecule is NCVP1b or a product thereof. These considerations might explain why initiation of polio RNA synthesis *in vitro*,

leading to VPg-linked RNA strands, has not been achieved to date.

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REOVIRUS GENOME RNA: COMMON 3'-TERMINAL NUCLEOTIDE SEQUENCES AND ASSIGNMENT OF mRNA RIBOSOME BINDING SITES TO VIRION GENOME SEGMENTS

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ABSTRACT All ten reovirus double-stranded genome RNA segments were radiolabeled at their 3'-termini to a similar extent by incubation with T4 RNA ligase and ^{32}pCp. Radioactivity was equally distributed between the separated plus and minus strands indicating that the 5'-cap in plus strands did not block 3'-end-labeling of minus strands. The 3'-termini of the four small size (S) and three medium size (M) segments included common but different sequences: ...U-A-G-C in minus strands and ...U-C-A-U-C in plus strands. By comparing the minus strand 3'-sequences with the 5'-sequences of reovirus mRNAs segments S2, S3 and S4 and M1, M2 and M3 were correlated with the previously reported initiation fragments s46, s45 and s54 and m30, m52 and m44 derived from small and medium class mRNAs, respectively. From these and other results the N-terminal amino acid sequences can be predicted and assigned to the nascent chains of particular reovirus proteins.

INTRODUCTION

The discovery that human reoviruses contain a double-stranded RNA genome (1) was followed by reports of other similar viruses that infect different animals and plants (2,3), yeasts and fungi (4) and bacteria (5). The prototype reovirus type 3 has been studied in great detail, and a number of its structural and replicative features have proven to be characteristic of other eukaryotic viral and cellular systems.

Purified reovirions contain a reproducible collection of genes organized into ten distinct segments (6), all required for infectivity (7). One strand of each of the duplex segments is transcribed to form the viral mRNAs by an RNA poly-

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merase activity that is packaged in virions (8-10). Accompanying the polymerase are other enzyme activities including nucleotide phosphohydrolase, guanylyl transferase and methyl transferases that modify the 5'-ends of nascent viral mRNAs to yield "capped" termini (11). Other RNA and DNA viruses contain similar transcriptase and mRNA modifying enzymes (12). Consequently they, like reovirus (13), are capable of synthesizing mRNA with a 5'-terminal cap structure, $m^7G(5')ppp(5')N$ identical to the 5'-cap present on most eukaryotic viral and cellular mRNAs (14).

Studies with reovirus mRNA have shown that the cap is involved in the initiation of translation (15-17) and led to a general model of how eukaryotic ribosomes may recognize mRNA initiator sites for protein synthesis (18). In reovirus-infected mouse L cells and in viral mRNA directed *in vitro* protein synthesizing systems, ten primary reovirus polypeptide products have been detected (3,19). Eight of these products are assembled into virions, three in the virion outer shell and five in the inner core particle. Which of the latter account for the several enzyme activities in viral cores remains to be determined. There is a good correspondence between the size of the virus-specific polypeptides and the coding capacities of the ten viral mRNAs, and the various polypeptides have been assigned to the individual reovirus genome segments which encode them on the basis of recent biochemical and genetic findings (20,21). Furthermore, nucleotide sequences have been established for the ribosome binding sites of six of the ten reovirus mRNAs by analyzing ribosome-protected mRNA fragments from RNase-treated 40S initiation complexes (22). A single initiation fragment including a cap and the 5'-proximal A-U-G codon was obtained for each mRNA (23), but the mRNA fragments were not assigned to their template genome segments.

Recently we observed that the 3'-ends of both strands of the ten reovirus genome RNAs can be radiolabeled by incubation with ^{32}pCp and T4 RNA ligase (24,25). We have used this method to prepare end-labeled RNA for nucleotide sequencing by ladder gel analysis after partial enzymatic digestion (26) or chemical hydrolysis (27). By comparing the 3'-end sequences of the separated minus strands with the 5'-terminal sequences of mRNA plus strands determined previously (28), we have related the ribosome binding sites of six mRNAs to genome segments in the medium (M) and small (S) size classes. Together with the protein coding assignments (20,21), it is now possible to predict the N-terminal amino acid sequences of reovirus nascent polypeptides.

RESULTS

Equi-Labeling of the 3'-Ends of All Genome RNA Segments. From analyses of reovirus genome RNA terminally labeled by incubation with α-^{32}P-ATP and polynucleotide kinase (29) or by reduction with ^{3}H-borohydride after periodate oxidation (30) it was established that the ends of the two strands of each duplex segment are base-paired in the structure:

(+) 5' m^7GpppG^m-C...A-U-C 3'
(-) 3' C -G...U-A-Gpp 5'

When a mixture of reovirus genome RNA segments was incubated with RNA ligase and ^{32}pCp (24) and analyzed by polyacrylamide gel electrophoresis (25), all ten duplexes were resolved as radiolabeled bands (Fig. 1). Consistent with 3'-ligation of ^{32}pCp, the ten RNAs ranging in molecular weight from 0.6 to 2.7 x 10^6 daltons (6) were labeled to a similar extent (Fig. 1, left lane). As an indication of their high purity, each of the recovered segments yielded a single band upon re-electrophoresis under the same conditions.

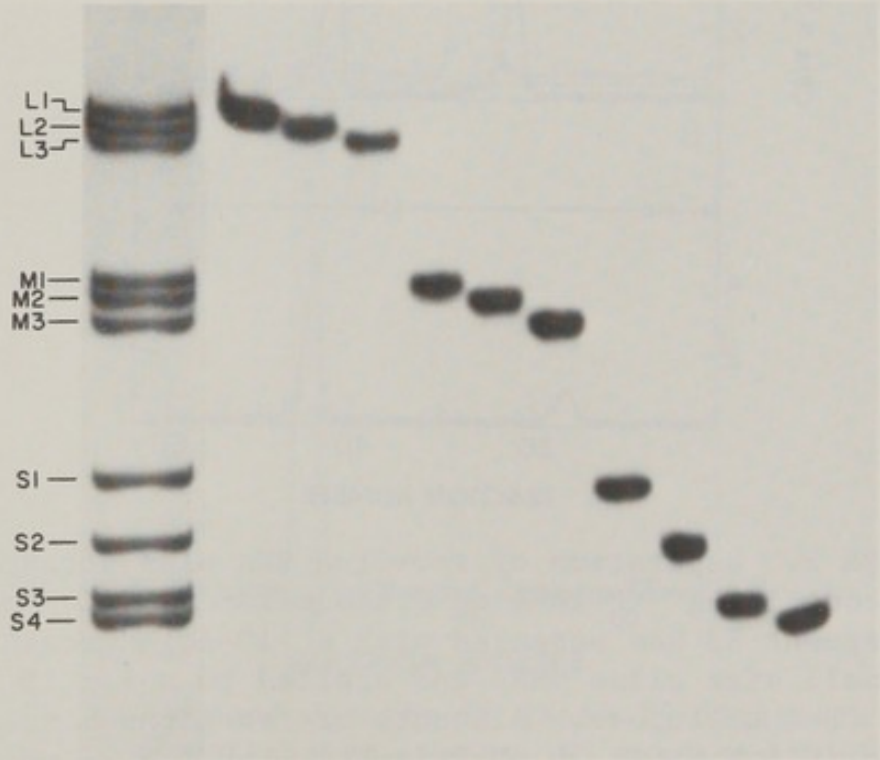

FIGURE 1. Resolution of 3'-labeled reovirus genome RNA segments. Double-stranded RNA from type 3 virions was labeled with ^{32}pCp and analyzed in a 5% polyacrylamide slab gel as described (25). The individual segments (L1-3, M1-3, S1-4) were located by autoradiography, eluted and re-analyzed.

Separation of 3'-Radiolabeled Plus and Minus Strands. Individual M and S segments were separated into their constituent plus and minus strands by denaturation with dimethylsulfoxide and annealing with an excess of viral mRNA which displaced the labeled plus strands from the duplexes (Fig. 2). After a single round of annealing, strand displacement was incomplete as determined by CF-1 cellulose chromatography, a procedure that resolves single-stranded and double-stranded molecules (31). However, after two rounds of annealing, more than 90% of the radioactivity remaining in the duplex segments was in minus strands, and approximately 50% of the total recovered counts were in single-stranded plus molecules. A typical experiment demonstrating the effectiveness of strand

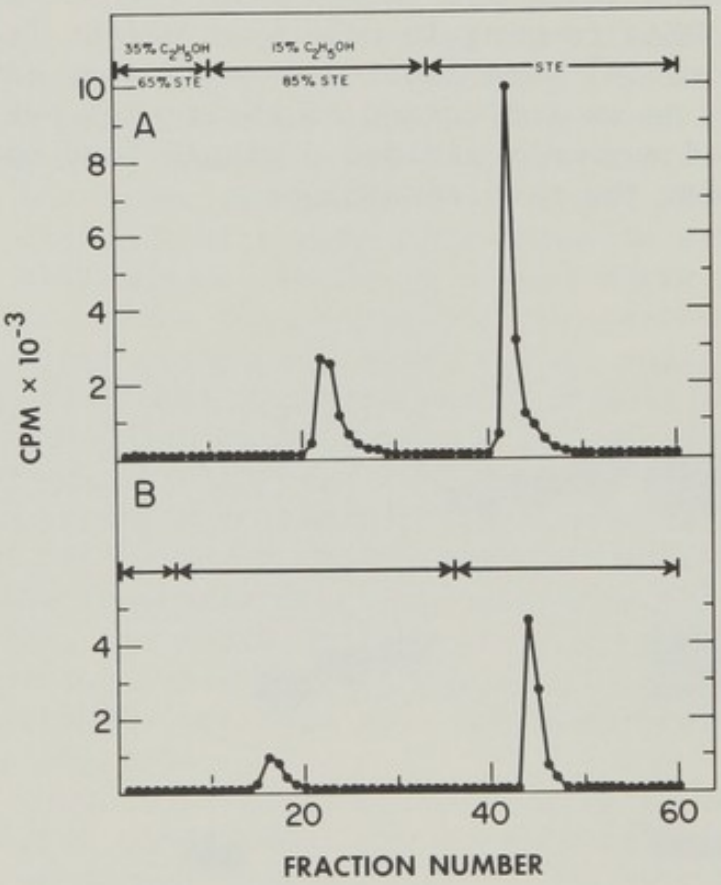

FIGURE 2. Separation of reovirus RNA plus strands from duplexes containing ^{32}P-labeled minus strands. A. ^{32}pCp-labeled segment S3 was annealed with a >10-fold excess of unlabeled small size class mRNA and applied to a 1 x 15 cm column of CF-1 cellulose. Aliquots of fractions eluted with C_2H_5OH/STE (0.1 M NaCl, 50 mM Tris-HCl - pH 7.4, 1 mM EDTA) were counted, and peak fractions containing plus strands (15% C_2H_5OH) or duplex RNAs (STE) were separately pooled and ethanol precipitated. B. The recovered duplex RNA was re-annealed with mRNA and re-analyzed. The ^{32}P distribution after two rounds of annealing was consistent with ∿95% displacement of labeled plus strands from double-stranded molecules (25).

displacement by one and two rounds of annealing of genome segment S3 with an excess of small size class viral mRNAs is shown in Fig. 2.

The extent of plus and minus strand separation was determined by DEAE-cellulose chromatography of RNase T_1 digests of single-stranded and double-stranded RNA fractions obtained from CF-1 cellulose after two rounds of annealing as in Fig. 2B. As shown for representative segment S3 in Fig. 3, essentially all of the radioactivity in the T_1 digest of single-stranded RNA eluted from DEAE-cellulose as a single peak in the position of a long oligonucleotide of net negative charge >6 (panel A). The bulk of the radioactivity in the double-stranded RNA (>95%) was released by RNase T_1 treatment as a fragment of net charge close to -3 (panel B). This is consistent with the 3'-sequence of ...GpC which is present in

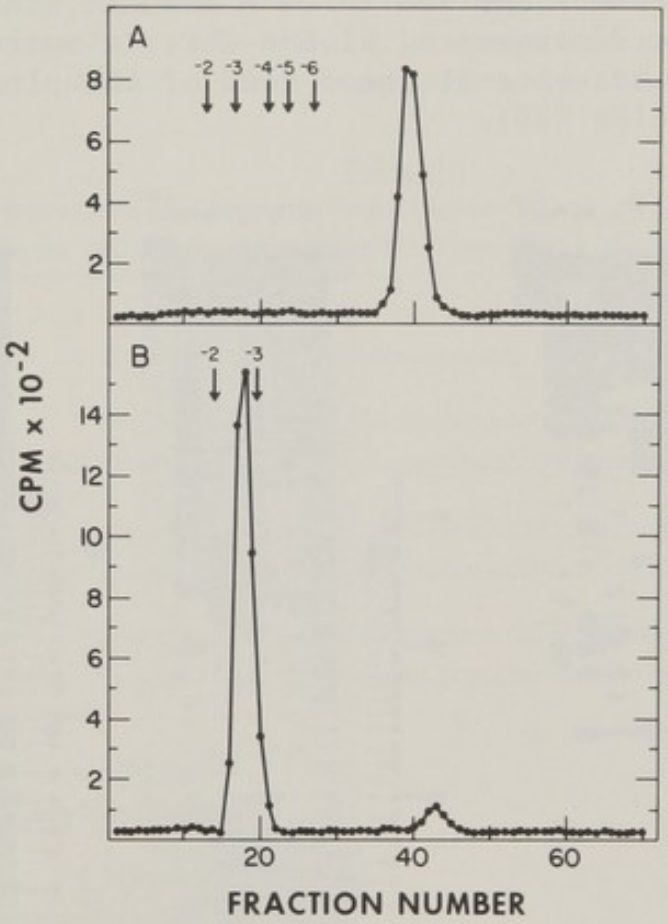

FIGURE 3. Chromatography of RNase T_1 digests of genome RNA strands. ^{32}pCp-labeled segment S3 was separated into plus strands and duplexes containing labeled minus strands. RNase T_1 digests of the plus strands (panel A) and of denatured duplexes (panel B) were analyzed by DEAE-cellulose chromatography (25). Arrows indicate the elution positions of marker oligonucleotides of increasing net negative charge obtained from the $A_{260\ nm}$ profile.

minus strands of reovirus genome RNA (30), since treatment of ...GpC^{32}pCp with RNase T_1 would yield C^{32}pCp (-3 charge) as the only radioactive oligomer. The small peak of radioactivity eluting as a longer oligonucleotide was in the position expected for material released from residual ^{32}P-labeled plus strands in the re-annealed duplexes.

Minus Strand 3'-Terminal Nucleotide Sequences. Individual genome segments that contained ^{32}pCp-labeled minus strands were denatured and partially digested by incubation with T_1, U_2, PhyI or pancreatic RNase or by treatment with $NaHCO_3$ at pH 9 (25,26). The digests were analyzed by electrophoresis in 20% polyacrylamide gels containing 7 M urea as described (26). Autoradiographs of partial digests of the minus strands of segments S1 and S2 are shown in Fig. 4 and the S segment 3'-sequences are summarized in Table 1. Four nucleotides were common and the three M segment minus strands contained the same 3'-sequence, ...U-A-G-C, in agreement with the complementary sequence at the 5'-end of the plus-stranded reovirus mRNA species (28).

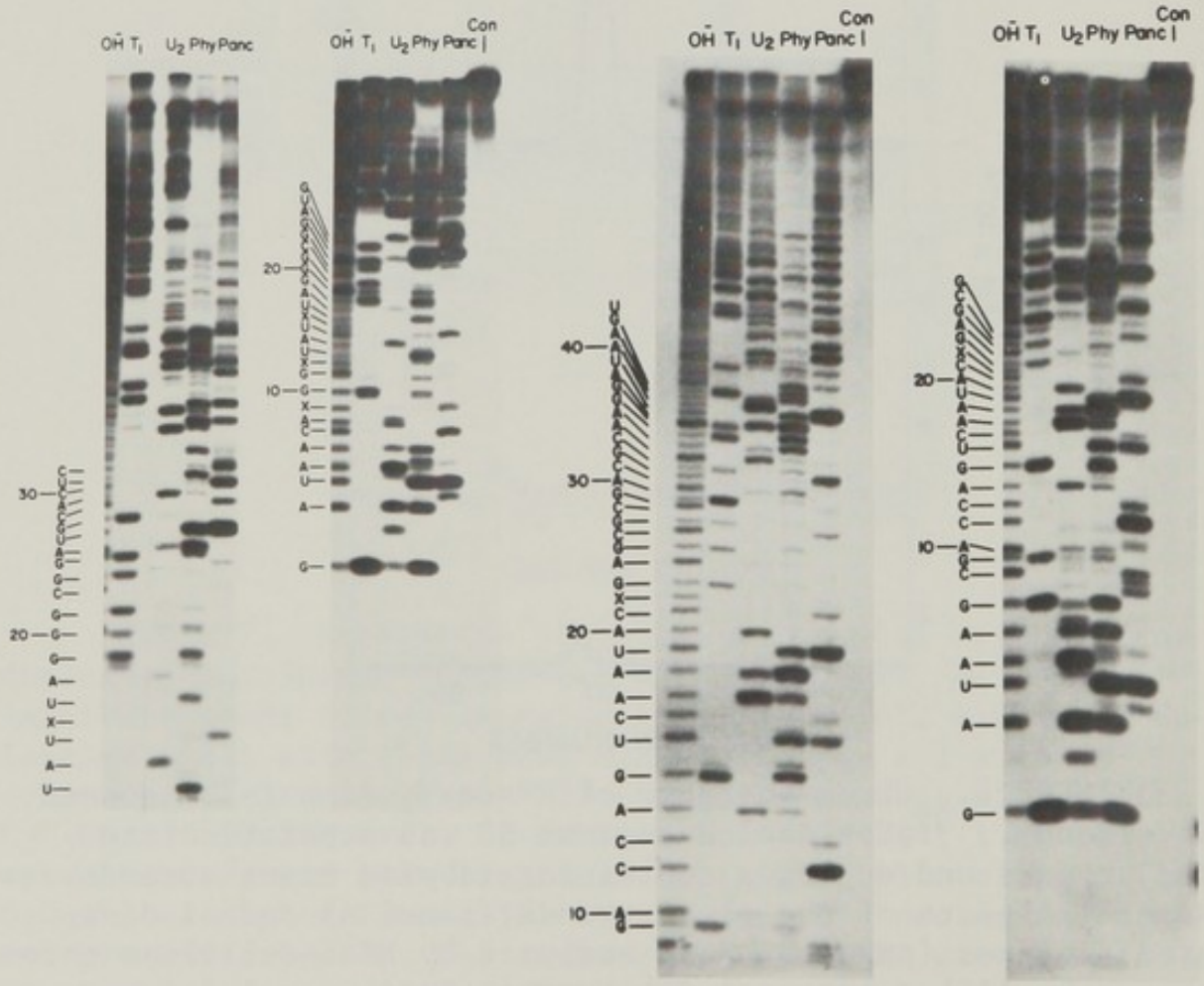

FIGURE 4. Autoradiogram of gel patterns of partial digests of segment S1 (left) and S2 (right) minus strands.

Table I

3'-Terminal Sequences of Reovirus Genome RNA Minus Strands

3'	10	20	30	40	50
S1	C G A U AACAXGGXUAUXUAGGGCGGAUGCACUC...				
S2	C G A U AAGCGACCAGUCAAUACXGAGCGCGACGCAAGGAUAAGU...				
S3	C G A U UUXAGUGXGGXCAGXAGCAGUGAUACCGXXGGAGUGAGUXXGACGXUAG...				
S4	C G A U AAAAACGAGGAUGGGUGUGXAXACAGCGUUACCAGUACACGAXCGGGU...				

Reovirus mRNAs apparently are complete copies of the genome segment plus strands (10) which are base-paired end-to-end with minus strands (30). Consequently the 3'-sequences of genome minus strands should be complementary to the 5'-ends of viral mRNAs. This comparison is shown in Table 2.

Table II

Comparison of Sequences Complementary to the 3'-Termini of Genome Minus Strand RNAs and 5'-Termini of S and M Class mRNAs*

5'	1	10	20	30	40	50
S1	G CUAUUGUXCCXAUAXAUCCCGCCUACGUGAG...					
S2	G CUAUUCGCUGGUCAGUUAUGXCUCGCGCUGCGUUCCUAUUCA...					
mRNA (s46)	m^7GpppG^mCUAUUCGCUGGUCAGUUAUGGCUCGCUGCGCGUUCCUAUUCAAG...					
S3	G CUAAAXUCACXCCXGUCXUCGUCACUAUGGCXXCCUCACUCAX...					
mRNA (s45)	m^7GpppG^mCUAAAGUCACGCCUGUCGUCGUCACUAUGGCUUCCUCACUCAG...					
S4	G CUAUUUUUGCUCCUACCCACACXUXUGUCGCAAUGGUCAUGUGCUXGCCCA...					
mRNA (s54)	m^7GpppG^mCUAUUUU-GCCUCUUCCCAGACGUUGUCGCAAUGGAGGUGUGCUUGCCCAACG...					
M1	G CUAUUCGCG					
mRNA (m30)	m^7GpppG^mCUAUUCGCG					
M2	G CUAAUCUGCXGACCGU					
mRNA (m52)	m^7GpppG^mCUAAUCUGCUGACCGU					
M3	G CUAXXXUGACCCUGAUC					
mRNA (m44)	m^7GpppG^mCUAAAGUGACCGUGGUC					

*mRNA sequences from Kozak, M. (1977) Nature 269, 390-394

The genome segment 5'-sequences were deduced from the ladder gel profiles of the 3'-labeled minus strands. mRNA sequences

were determined by fingerprinting initiation fragments derived from uniformly ^{32}P-labeled mRNAs by trimming 40S translation complexes with RNase (28). Although some nucleotides were not identified from the gel analyses, the sequences were sufficiently complete to show clearly correspondence of segments S2, S3 and S4 with the three ribosome binding sites s46, s45 and s54, respectively. Similarly, M1, M2 and M3 are templates for mRNA initiation fragments m30, m52 and m44.

The ribosome binding site in the mRNA species transcribed from segment S1 has not been determined. From the gel profiles of partial digests of S2, the order of the underlined residues, which was not established for the corresponding mRNA fragment s46, can be written as C-G-C-G-C-U. In the case of S4, the ladder sequencing gel patterns indicated the presence of five consecutive U residues near the 5'-end, as reported for one of the 5'-terminal T_1 oligomers from a mixture of reovirus mRNAs (32).

Plus Strand 3'-Terminal Sequences. Partial digests of ^{32}pCp-labeled plus strands displaced from genome segments by two rounds of annealing were also analyzed by gel electrophoresis. Enzymatic (25) and chemical (27) reactions were used, either with plus strands derived from individual genome segments (Fig. 5) or with mixtures of all ten plus strands, in each case derived from double-stranded segments by re-annealing with mRNA and CF-1 cellulose

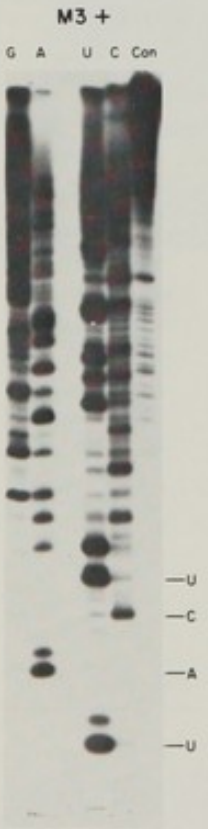

FIGURE 5. 3'-Sequence of plus single strands. Plus strands of genome segment M3 were ^{32}pCp-labeled and analyzed by gel electrophoresis after chemical cleavage.

chromatography. The oligonucleotide profiles were consistent with a common 3'-sequence, ...U-C-A-U-C.

DISCUSSION

Recent studies have shown that the species of mRNA transcribed from reovirus genome segment S1 codes for capsid polypeptide σ1 (20,21), a minor component of the virion outer shell that carries hemagglutinin activity and determines the virus host range (33). Segments S2, S3 and S4 code respectively for viral core polypeptide σ2, non-structural polypeptide σNS and the major outer shell protein of virions, σ3 (20, 21). With the exception of the medium size class polypeptide μ1 which is cleaved during virus maturation, all reovirus structural proteins have N-termini that are blocked (34). However, on the basis of the genome segment assignments of viral polypeptides (20,21), the 5'-sequences of viral mRNAs (28) and the correlation of mRNAs with genome segments, the amino acid sequences of nascent chains of the proteins can be predicted as: σ2=Met-Ala-Arg-Ala-Ala-Phe-Leu-Phe; σNS=Met-Ala-Ser-Ser-Leu; and σ3=Met-Glu-Val-Cys-Leu-Pro-Asn. Similarly, minor virion polypeptide μ2, the cleaved structural protein μ1 and μNS coded for by segments M1, M2 and M3 respectively are predicted to have the initiated N-terminal sequences: μ2=Met-Ala-Tyr-Ile-Ala; μ1=Met-Gly-Asn-Ala; and μNS=Met-Ala-Ser-Phe-Lys-Gly-Phe-Ser.

As in the case of the single-stranded RNA segments comprising the influenza virus genome (35-37), the reovirus duplex RNAs have common 3'-terminal sequences, ...U-A-G-C in the minus strands and ...U-C-A-U-C in plus strands. Common end sequences in genome RNA segments may be a characteristic of multi-segmented RNA viruses related to genome assembly during maturation. For reovirus the terminal sequences presumably are recognized by the viral transcriptase and replicase activities as the respective starting sites for the synthesis of mRNAs and the minus strands of genome duplex RNAs. Since the viral mRNAs are transcribed end-to-end on template genome segment RNAs and are not polyadenylated (38), the plus strand 3'-sequences also correspond to eukaryotic transcription terminators. It is of interest in this regard that the 3'-ends of the plus strands generally are rich in A and U residues and preceded by G clusters, i.e. similar to a variety of bacterial terminators (39).

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TERMINAL SEQUENCE HOMOLOGIES IN REOVIRUS GENES[1]

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Abstract. The 3'-terminal regions of the plus and minus strands of several genes of the three reovirus serotypes have been sequenced in order to investigate how genetic relatedness is distributed among them. The following results have been obtained so far:
1) The 3'-termini of the plus strands of the S1 genes of serotypes 1, 2 and 3 show extensive homology. There are four blocks of homology within the 40 terminal residues, including the common terminal sequence -UCAUC-3'.
2) The 3'-termini of the minus strands of the three S1 genes, and therefore the 5'-termini of their plus (and messenger RNA) strands, share six common residues (5'-GCUAUU-). Their coding sequences start at residue 13 or 14, and show no detectable homologies for the first 20 codons.
3) The three species of s1 messenger RNA contain regions of inverted homology near their ends. Sequences capable of base pairing with the 3'-terminal region of 18S ribosomal RNA are either adjacent to or overlap with these regions. The lengths of these homologous regions vary among the three s1 messenger RNA species.
4) Similar studies have been initiated for the S2 genes of the three reovirus serotypes. These genes possess the same 5'- and 3'-terminal sequences on their plus strands as the S1 genes; they possess additional regions of homology with S1 genes near their 3'-termini that are not the same as those shared by the three S1 genes; and the secondary structures possible for s2 messenger RNAs are similar to those possible for s1 messenger RNAs.

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INTRODUCTION

The mammalian reoviruses form a very interesting system for studying evolutionary relationships because (a) their genomes are segmented into their ten component genes, and (b) there are three major serotypes, two of which (serotypes 1 and 3) are closely related to each other (at least 80%, as judged by the ability of their total RNAs to hybridize with each other), while the third, serotype 2, is only distantly (no more than 10%) related to the other two (1,2). A variety of questions can be asked of this system, such as:

(a) How closely related are the coding sequences of the genes of serotypes 1, 2 and 3?

(b) Are there common sequences at the termini of the homologous genes of the three serotypes?

(c) Are there terminal sequences common to the ten genes of each serotype, and how closely are they related to sequences common to the genes of the other two serotypes?

(d) What secondary structure features can be discerned in the messenger RNAs of the various reovirus genes, that is, are there extensive hairpin loops, are there homologies among sequences at their 3' and 5'-ends, and do they possess sequences capable of binding to 18S ribosomal RNA?

(e) Is there any evidence that the larger reovirus genes are derived from smaller ones by gene duplication?

Evidence that genetic relatedness is distributed in an interesting manner among the reovirus genes has been obtained from an analysis of the antigenic determinants on reovirus-coded proteins (2). The basic experimental approach consisted of making antisera to each of the three serotypes, and then determining the efficiency with which they precipitated virus-coded proteins from extracts of cells infected with the homologous serotype and the two heterologous serotypes. Focusing on the closely related serotypes 1 and 3, it was found that, as expected, the antigenic determinants on most of the proteins encoded by these two serotypes were very similar, and that there were only two proteins the antigenic determinants on which could be readily distinguished, that is, they were type-specific. Very surprisingly, similar relationships were found when the antigenic determinants on proteins encoded by serotype 2 were compared with those encoded by serotypes 1 or 3; that is, the antigenic determinants on most serotype 2 proteins were very similar to those on the corresponding serotype 1 and 3 proteins, while once again the antigenic determinants on only a few serotype 2 proteins were significantly different from those on the cognate serotype 1 and 3 proteins. This is unexpected, since, as judged by the

ability of serotype 2 RNA to hybridize to serotype 1 or 3 RNA, the amino acid sequences of serotype 2 proteins should be quite dissimilar from those of the corresponding serotype 1 or 3 proteins. In other words, it seems that the antigenic determinants on most of the proteins encoded by the three reovirus serotypes have been highly conserved during evolution.

In order to elucidate further the evolutionary relationships among the various genes of the three serotypes of reovirus, we have initiated a program to sequence selected reovirus genes. One study along these lines has already been made: Kozak and Shatkin (3,4) have sequenced the ribosome binding sites on six reovirus type 3 messenger RNA species, three of the s class and three of the m class. In all cases they sequenced the region from the 5' terminus to and slightly beyond an initiation codon. Since they did not work with individual messenger RNA species but with unfractionated species of the s and m size classes, they could not assign sequences to individual genes. More recently Darzynkiewicz and Shatkin (5) sequenced the 3'-termini of the minus strands of the S and M size class genes of reovirus serotype 3, which enabled them to assign the previously sequenced ribosome binding sites to individual reovirus genes. Both Shatkin and coworkers (3,4,5) and Hastings and Millward (6) concluded that reovirus messenger RNAs shared the same 5'-terminal sequence $^{m}GpppG_{m}CUA-$.

METHODS AND RESULTS

The approach that we have adopted is to isolate individual reovirus genes by polyacrylamide gel electrophoresis, to ligate [^{32}p-5']pCp to the isolated genes, to isolate their component plus and minus strands, and to sequence them. Figs. 1 and 2 illustrate representative sequencing data. Fig. 1 shows the two-dimensional oligonucleotide fingerprint patterns of partial alkali digests of the plus and minus strands of the S1 gene of serotype 1. In each case there is only a single track, indicating complete separation of plus and minus strands. In each case the complete 3'-terminal sequence, starting with the first residue, is shown. The identity of individual spots was confirmed by elution and analysis by high-voltage electrophoresis on DEAE-paper at pH 3.5 and 1.7. Fig. 2 shows chemical sequencing gels of the plus and minus strands of the S1 gene of serotype 3. In each case, two loadings are shown. On the basis of data such as are shown in Figs. 1 and 2, stretches of RNA some 80 to 100 residues long were sequenced.

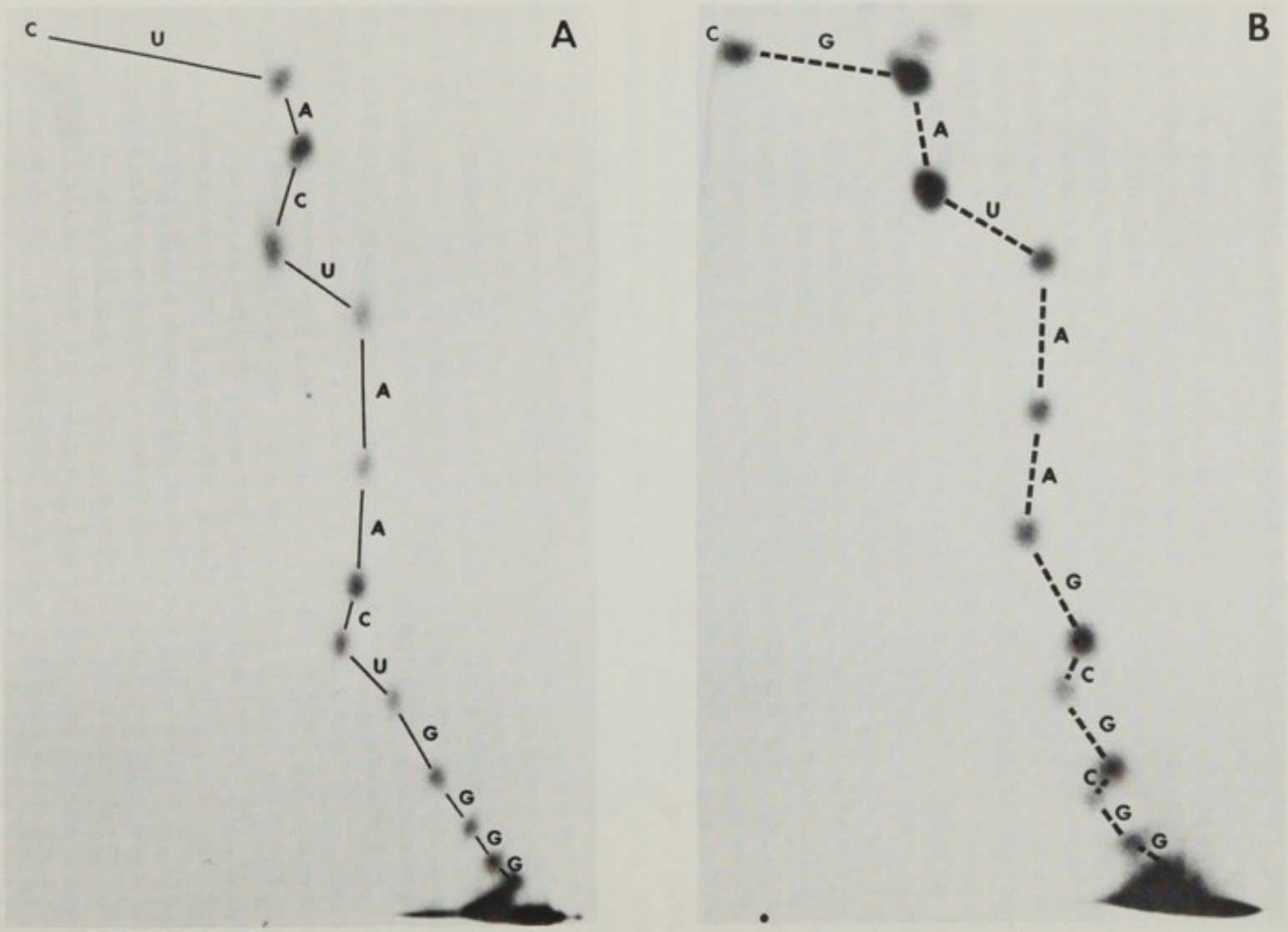


FIGURE 1. Two-dimensional oligonucleotide fingerprint patterns of partial alkali digests of the plus (A) and minus (B) strands of the S1 gene of reovirus serotype 1.

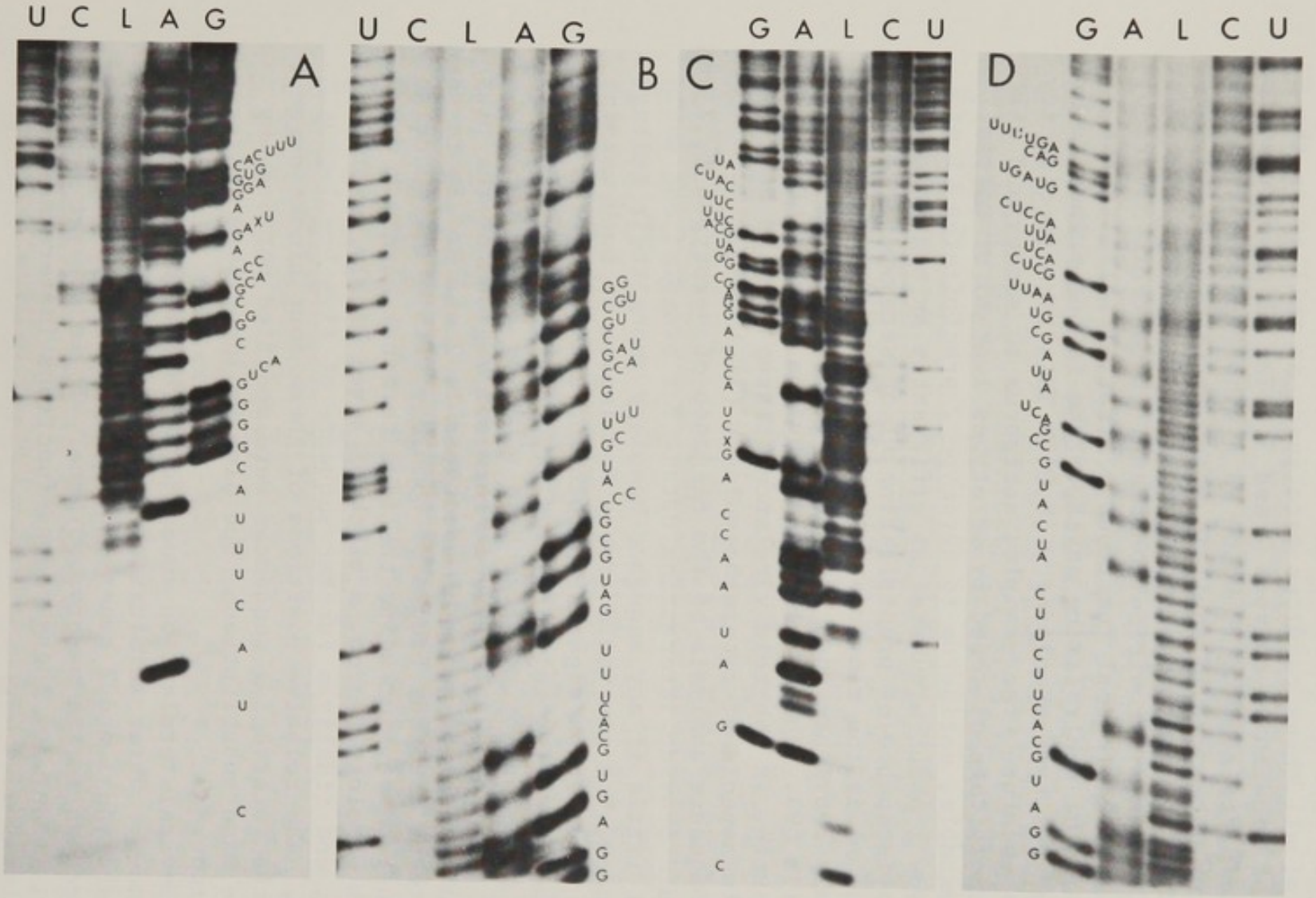


FIGURE 2. Chemical sequencing gels of the plus (A and B) and minus (C and D) strands of the S1 gene of reovirus serotype 3.

THE SEQUENCES OF THE S1 GENES

This type of analysis has been carried out on the S1 genes of all three reovirus serotypes, as well as on the S2 genes of reovirus serotypes 2 and 3. The S1 gene codes for a protein designated σ1, which is a minor component of the reovirus outer capsid shell in which it is present to the extent of no more than 24 copies (7). In spite of the fact that it is a minor virion component, it controls a variety of crucial biological functions. First, it elicits the formation of neutralizing antibody and is the primary source of reovirus type-specificity (8,9). This type-specificity can be absolute (for example, antiserum to serotype 3 will not precipitate σ1 coded by serotypes 1 or 2) or partial (for example, antiserum to serotype 2 precipitates σ1 of serotype 1 about a quarter as efficiently as homologous σ1, but is incapable of precipitating σ1 of serotype 3 (2)). Second, σ1 is the reovirus cell attachment protein; it is the only reovirus-coded protein that is capable of attaching to cells (9). Third, σ1 is the reovirus hemagglutinin (10); and fourth, it controls virulence, tissue tropism and certain aspects of cytopathogenicity (11,12,13).

Sequence analysis of the S1 genes of serotypes 1, 2 and 3 should provide interesting data. For example, one would expect little if any sequence homology among their coding sequences, but one would expect homologies at both termini, where recognition signals for binding RNA polymerase as well as ribosomes, for encapsidation, and perhaps for recognizing other genes during morphogenesis would be expected. Fig. 3 shows the sequences of the 3'-terminal regions of the plus strands of the S1 genes of serotypes 1, 2 and 3. It is seen that there are four blocks of homology within the 40 or so terminal bases, including the five terminal bases, a very G-rich homology region, a block of seven highly homologous bases, and finally a further sequence of five homologous bases. The relative positions of these four blocks suggest that the intervening sequences were modified not only by single base substitutions, but also by insertions and/or deletions.

Several termination codons are present in each of these sequences. Three termination codons in phase are present in the serotype 1 and 2 genes, and three out-of-phase termination codons in the serotype 3 gene. It is not yet known which, if any, of these codons functions in terminating translation.

The most obvious functions of homologies at the plus strand 3'-terminal ends of reovirus genes include (i) RNA polymerase binding sites for transcribing plus strands into

FIG. 3. 3'-TERMINAL SEQUENCES OF THE PLUS STRANDS OF THE S1 GENES OF REOVIRUS SEROTYPES 1,2 & 3

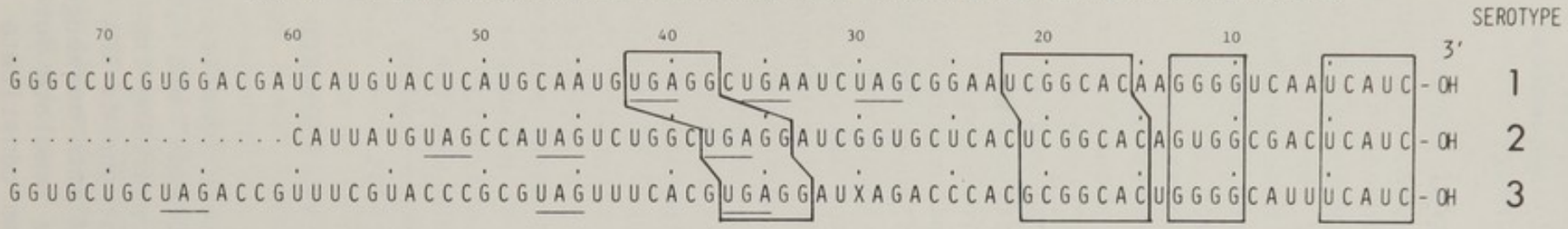
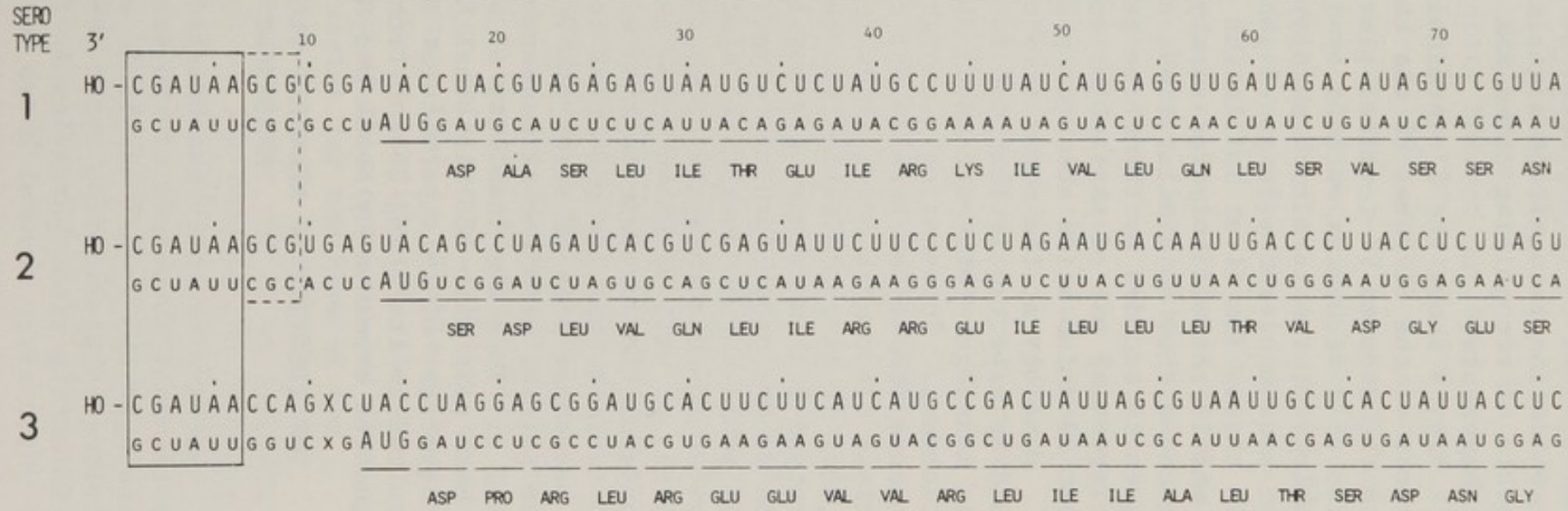


FIG. 4. 3'-TERMINAL SEQUENCES OF THE MINUS STRANDS OF THE S1 GENES OF REOVIRUS SEROTYPES 1,2 & 3



minus strands to form progeny ds RNA molecules; (ii) encapsidation recognition signals; and (iii) sequences capable of recognizing other RNA strands during morphogenesis.

Fig. 4 shows the 3'-terminal sequences of the minus strands of the S1 genes of the three serotypes, together with the deduced 5'-terminal plus strand sequences and the amino acid sequences encoded by them. There is extensive homology at the termini: serotypes 1 and 2 share a nine base sequence, and serotype 3 shares a six base sequence with them. The first initiation codon starts at residue 14, 14 and 13 respectively for serotypes 1, 2 and 3. The coding sequences show no similarities, nor do the amino acid sequences encoded by the three genes. There is no preferential use of particular codons; and about 50% of the first 20 amino acids are hydrophobic in all three cases.

Homology at the minus strand 3'-terminal end of genes would be expected to function in the transcription of ds RNA into messenger (plus-stranded) RNA and for ribosome attachment. Interestingly enough, the s1 messenger RNA sequence identified here is not represented among the three s class ribosome-binding sites reported by Kozak and Shatkin (3,4). It is conceivable that the reason for this is that serotype 3 s1 messenger RNA has less affinity for ribosomes than do the other three serotype 3 s class messenger RNAs. This would not be unexpected since σ1 is formed in much smaller amounts than the other three σ class proteins. Indeed, it will be shown below that the potential of 18S ribosomal RNA for base pairing with serotype 3 s1 messenger RNA is less than its potential for base pairing with the s1 messenger RNAs of serotypes 2 and 3, or its potential for base pairing with the s2 messenger RNA species of serotypes 2 and 3. Thus the inability of serotype 3 s1 messenger RNA to base pair with 18S ribosomal RNA may be a trivial idiosyncrasy of this particular messenger RNA species (which resulted in its being absent from the ribosome-binding sequences examined by Kozak and Shatkin (3,4)).

THE IDENTITY OF REOVIRUS PLUS STRANDS AND REOVIRUS MESSENGER RNA

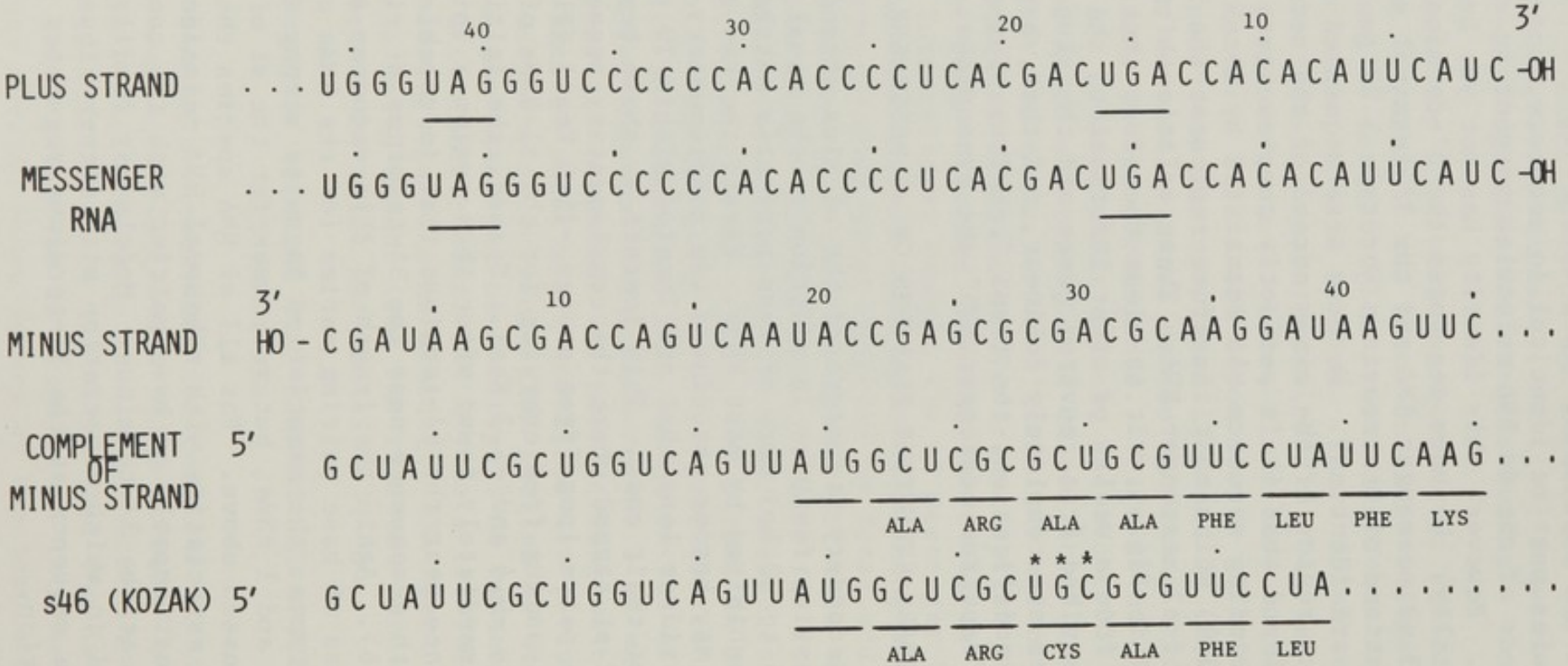
We have recently established the complete identity of the terminal sequences of reovirus "plus-strands" and "messenger RNA". All available evidence concerning the reovirus multiplication cycle indicates that the ds RNA of infecting virus particles is transcribed into messenger RNA which after several hours serves as the template for the formation of minus strands; together the two constitute

progeny ds RNA molecules. This scheme implies that messenger RNA molecules must be identical in sequence content to the plus strands of the ds RNA molecules present in virus particles. However, this identity has not yet been proved experimentally. We have sequenced the 3'-terminal region of serotype 3 s2 messenger RNA and the 3'-terminal sequence of the plus strand of the reovirus serotype 3 S2 gene and found that they are identical. We have also sequenced the 3'-terminal region of the minus strand of the serotype 3 S2 gene and found that it is perfectly complementary to ribosome binding sequence s46 identified by Kozak and Shatkin (3,4) which must therefore represent the 5'-terminal sequence of s2 messenger RNA. These relationships are summarized in Fig. 5. It is clear therefore that, as predicted from a wealth of other information, the messenger RNAs transcribed from reovirus genes and the plus strands of these genes are completely colinear, and that, barring secondary modification, the terms "reovirus plus-stranded RNA" and "reovirus messenger RNA" are synonymous.

SECONDARY STRUCTURE FEATURES OF s1 MESSENGER RNAs

The secondary structure of the s1 plus strands reveals two interesting features in addition to the usual short-stemmed (5 to 10 bp) more or less perfectly matched hairpins that are exhibited by most RNAs. First, the two ends of the molecules may become associated via complementary sequences 6 to 10 residues long that are located within 10 to 15 residues of their ends. This feature is shown best by the serotype 2 s1 strand where the complementary sequence is seven base pairs long (free energy -15.0 Kcal) (Fig. 6). The corresponding free energies for the s1 RNAs of serotypes 1 and 3 are -9.4 and -5.3 Kcal. Second, the initiation codon is immediately found after this region. Third, there is a sequence near the 5'-terminus that is capable of base pairing with a sequence near the 3'-terminus of ribosomal RNA (Fig. 6). Again the length of this sequence and the faithfulness of base pairing varies for the RNAs of the three serotypes; the association is quite strong for the serotype 2 and 1 RNAs, but very weak for the s1 of serotype 3, as discussed above. For all s1 RNA species the sequence capable of associating with ribosomal RNA coincides with or overlaps that capable of base-pairing with the complementary sequence near the 3'-terminus. Models may readily be constructed in which association with either ribosomal RNA or with the sequence at the 3'-terminus regulates efficiency of translation.

FIG. 5. TERMINAL SEQUENCES OF THE PLUS AND MINUS STRANDS OF THE S2 GENE OF SEROTYPE 3 AND OF ITS MESSENGER RNA



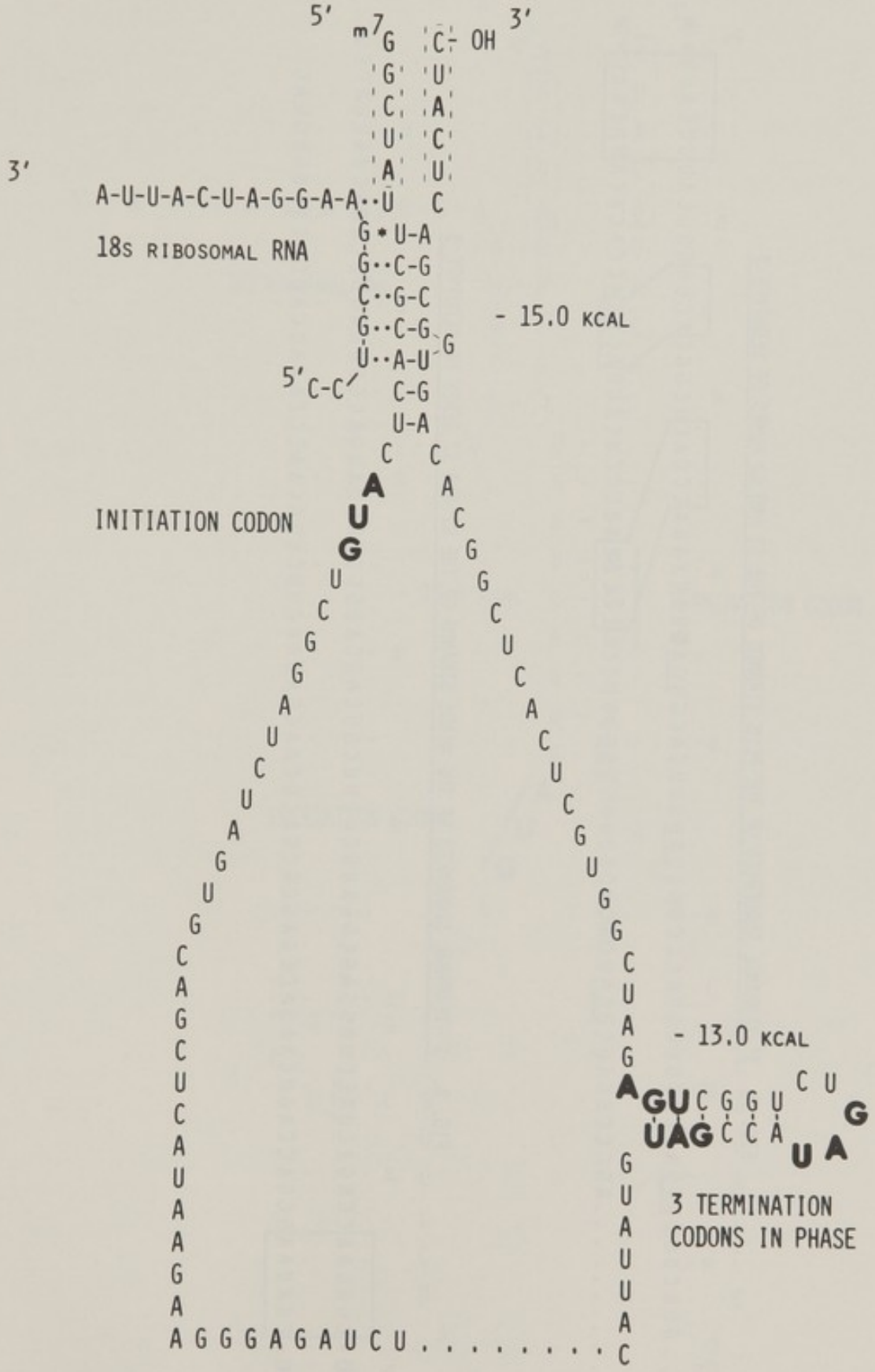


FIGURE 6. Putative secondary structure of serotype 2 s1 messenger RNA.

FIG. 7. 3′-TERMINAL SEQUENCES OF THE PLUS STRANDS OF THE S1 AND S2 GENES OF SEROTYPE 3

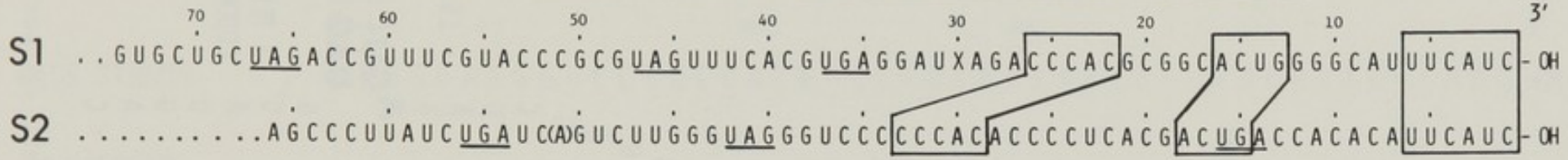
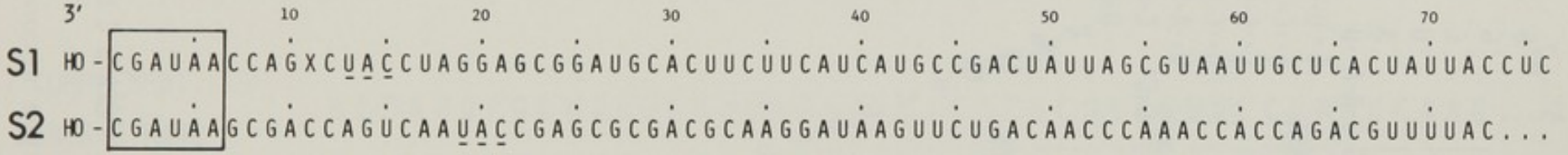


FIG. 8. 3′-TERMINAL SEQUENCES OF THE MINUS STRANDS OF THE S1 AND S2 GENES OF SEROTYPE 3



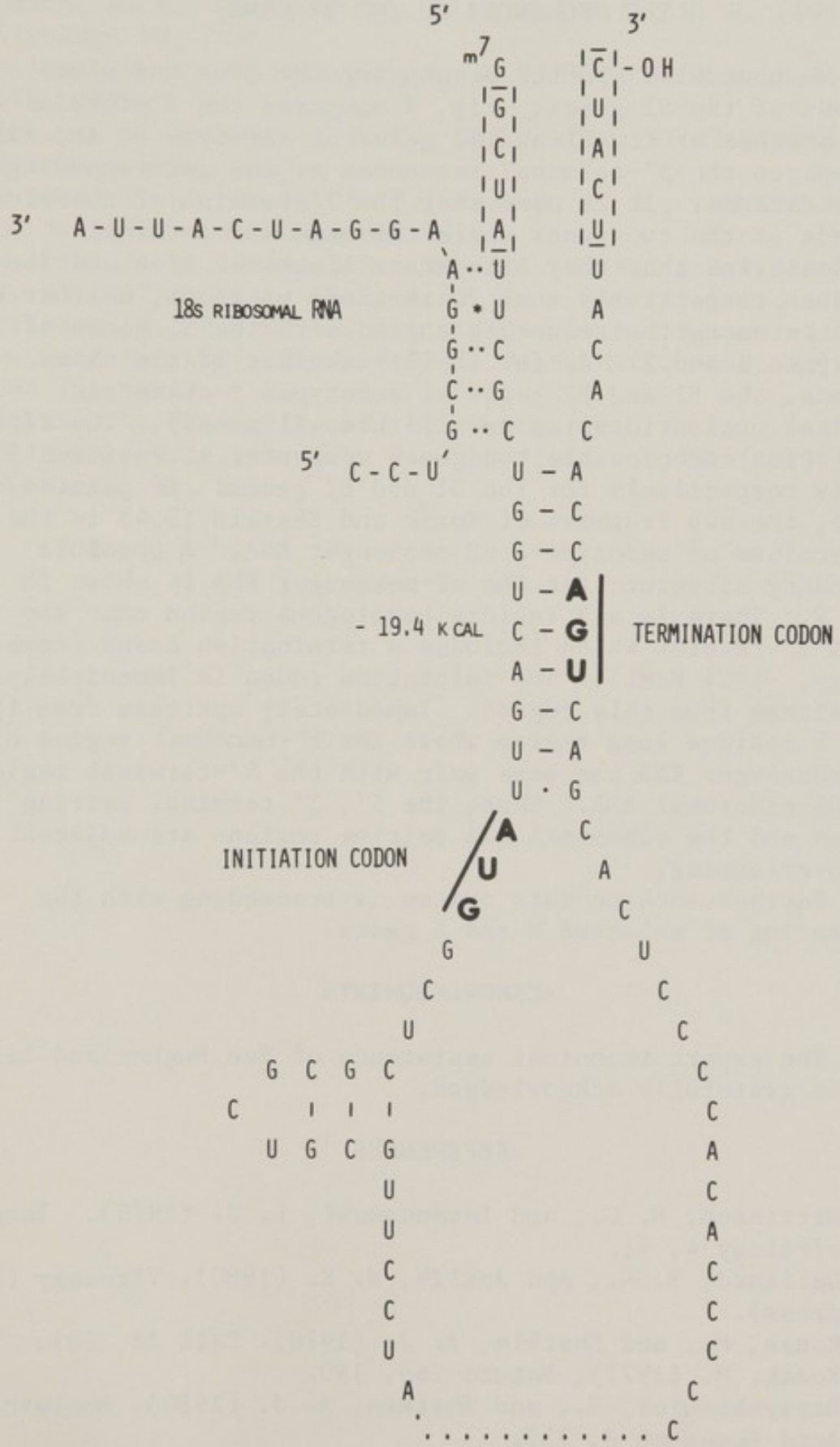


FIGURE 9. Putative secondary structure of serotype 3 s2 messenger RNA.

THE SEQUENCES OF THE S2 GENES

We have also started sequencing the plus and minus strands of the S2 genes. Fig. 7 compares the 3'-termini of plus strands of the S1 and S2 genes of serotype 3, and Fig. 8 compares the 3'-terminal sequences of the corresponding minus strands. It is seen that the 3'-termini of the plus strands of the two genes share the same six terminal residues, and that they also share blocks of five and four residues respectively some 20 residues upstream, neither of which is among the sequences shared with the S1 genes of serotypes 1 and 2. As for the 3'-terminus of the minus strands, the S1 and S2 genes of serotypes 3 share six terminal nucleotides (as do all three S1 genes). The first initiation codon on the two genes commences at residues 13 and 19 respectively for the S1 and S2 genes. As pointed out above, the s46 fragment of Kozak and Shatkin (3,4) is the 5'-terminus of serotype 3 s2 messenger RNA. A possible secondary structure for the s2 messenger RNA is shown in Fig. 9. There is a 9 residue homologous region near the 5'- & 3'-termini which includes a termination codon (free energy, -19.4 Kcal). The initiation codon is immediately downstream from this region. Immediately upstream from it is a 5 residue long region where the 3'-terminal region of the messenger RNA can base pair with the 5'-terminal region of 18S ribosomal RNA. Thus, the 5', 3' terminal pairing region and the ribosomal RNA pairing regions are adjacent, not overlapping.

Further work on this system is proceeding with the sequencing of selected M and L genes.

ACKNOWLEDGMENTS

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ATTEMPTS AT THE MOLECULAR CLONING OF A TRANSFORMING ALLELE FROM CHEMICALLY TRANSFORMED MOUSE CELLS[1]

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ABSTRACT DNA has been extracted from a series of chemically transformed mouse fibroblast cells. As reported previously (1), DNA of some of these cell lines will induce foci when applied to NIH3T3 mouse fibroblast monolayer cultures. The data suggest the presence of discrete, dominantly acting, transmissible alleles for transformation present in the chemically-transformed cells but not in their normal counterparts. The transfection technique allows one to use the transforming alleles as selectable markers in procedures designed to generate molecular clones of these genes. An experimental strategy, currently underway, is described by which the transformation allele and other single-copy, selectable marker genes may be cloned.

INTRODUCTION

The development of novel techniques of gene transfer into eukaryotic cells has made it possible to resolve a number of experimental questions which were inaccessible by earlier techniques. Among the gene transfer techniques currently available is the DNA transfection technique of Graham and van der Eb (2) which makes possible the relatively efficient introduction of genes in the form of naked DNA or metaphase chromatin. Although this technique was originally developed for the introduction of sub-genomic fragments of adenovirus DNA, it could quickly be applied to a series of other viral and nonviral DNAs (3,4,5,6,7,8).

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Among the viral DNAs studied using this transfection was the DNA of avian and murine retroviruses (3,4,5). These retroviral DNAs were discovered using a series of nucleic acid hybridization techniques, which were subsequently used to elucidate the detailed structure of unintegrated and integrated viral genomes (9, 10, 11). These characterizations left a series of questions unanswered, among them the biological activity potentially attributable to the various molecular configurations of the viral DNA. Such biological activity was first detected in experiments using DNA of avian sarcoma transformed cells (3). Graham-van der Eb procedure was subsequently applied to the study of mammalian retrovirus DNAs beginning in 1974 (5).

These initial experiments depended upon our ability to prepare relatively large amounts of viral DNA from mass cultures of cells recently infected with Moloney murine leukemia virus. The deproteinized DNAs were suspended in a phosphate buffer and calcium was added to induce the formation of calcium phosphate crystals with which the DNA was co-precipitated. Application of the co-precipitate to NIH3T3 fibroblast cultures resulted in the generation of infectious centers releasing wild type virus (5).

These experiments were later extended to study of analogous DNAs deriving from cells infected by Moloney and Harvey sarcoma viruses. Because of the replication defectivity of these sarcoma viruses, no virus was released from the successfully transfected cells. Rather, regions of the transfected cultures developed into foci of transformed cells whose sarcoma viral genomes were demonstrable by subsequent infection with replication-competent helper viruses. These helper viruses induced the release of sarcoma virus genomes enveloped by the virion proteins provided by the helper genome (12, 13).

One type of biologically active DNA which yielded foci upon transfection was the DNA of cells containing one or several sarcoma proviruses per diploid cell DNA complement. This result suggested the ability to transfer DNA sequences, including cellular genes, which were present in low or unique copy number in the cell genome a conclusion compatible with the work of others using genes which specify well-characterized enzyme markers (14).

EXTENSION OF THE TRANSFECTION PROCEDURE TO STUDY OF DNAS FROM CHEMICALLY TRANSFORMED CELLS

The marker which we had been using was that of oncogenic transformation. We began experiments designed to inquire whether non-viral transforming elements present in low copy number, might also be similarly transmissible. To this end, DNAs were prepared from a series of mouse fibroblast lines which had been transformed by exposure to chemical carcinogens during their passage *in vitro*.

The results of these experiments have been reported recently (1). In brief, DNAs were prepared from 15 different transformed rodent fibroblast lines and from several non-transformed lines. The DNAs were transfected and 5 out of the 15 DNAs tested yielded significant numbers of foci when the DNAs were applied to NIH3T3 monolayers. DNAs from non-transformed cells yielded few or no foci. The few foci may derive from spontaneous overgrowths of the transfected monolayer unrelated to the transfection process. Alternatively, these few foci may derive from genes present in the DNA of normal, untransformed cells which become activated by shearing as has been reported by others (15). The foci which derived from transfection of DNA from the chemically-induced transformants yielded cells which grew in 0.3% agar and were tumorigenic in newborn mice.

Several additional controls were performed to preclude certain types of spurious results. Thus, foci were frequently counted via double-blind protocols to prevent the intrusion of subjective factors in the evaluation of foci. DNAs were analyzed for their endogenous leukemia proviruses to assure that they derived from those strains of inbred mice from which their parent cell lines had originally been prepared. Finally, both the donor cells yielding DNAs and the transfected, transformed cells were exposed to helper murine leukemia virus in an attempt to elicit transmissible C-type sarcoma viruses. No sarcoma viruses were detected and we tentatively concluded that the observed transformation potency of these DNAs is not associated with known types of transforming retroviruses (1).

We consider it likely that these transforming alleles derive from alteration of normal cellular gene sequences. The proof of this will be available only upon successful molecular cloning of the biologically active sequences.

Several other tentative conclusions derive from the existing data. Firstly, these genes behave as if they were dominant *vis a vis* those genes which exist in the NIH3T3 cells prior to the transfection. Such a conclusion is tempered by some qualifications. It is not proven that the

"normal" sequences which are genetically allelic to the transforming gene(s) are present in diploid or even haploid amounts in the untransformed NIH3T3 prior to transfection. Additionally, it remains unproven whether these transforming alleles are capable of transforming a variety of other non-transformed cells in culture. Thus, the apparent dominance of these alleles may be relevant only to the genetic background of the NIH3T3 cells into which they have been introduced.

A second conclusion derived from these experiments concerns the physical structure of the alleles which are inducing the transformed phenotype. It might be possible that the information encoding the transformed phenotype is located in two or more unlinked genes, all of which are required to cooperate in expressing the transformed phenotype. In this case, these genes, each of which would be likely present in low or unique copy number, would by necessity be required to be co-transferred into the recipient cells in order for the induction of transformation. In view of the low efficiency of transfer of even a single contiguous gene segment via transfection, we consider it unlikely that two or more unlinked, unique genes are co-incidentally introduced into the same cell as a consequence of a single transfection procedure. Rigorous proof of the presence of the transformation allele in a single, contiguous segment of DNA will also await the molecular cloning of the gene.

A STRATEGY FOR THE MOLECULAR CLONING OF THE ALLELE FOR TRANSFORMATION

As discussed above, the resolution of a number of questions surrounding the chemical transformation allele dependson the successful molecular cloning of its DNA sequence. Such cloning is not achievable through many of the available strategies which have been exploited in the past several years for the cloning of structural genes from animal cell DNA. The successes of these cloning experiments generally depend upon the presence of a relatively high concentration of a specialized gene product and its template mRNA in appropriate differentiated tissues or cell lines. In the present instance, the gene product remains unidentified and there is little reason to believe that it is made in particularly high amounts, even in chemically transformed cells.

This has forced us to attempt the molecular cloning using an alternative strategy developed by others for the cloning of genes from the yeast genome (16 , Figure 1). This novel strategy depends only on the ability of the gene of interest to serve as a selectable marker. In the present case the

selectable marker is the focus inducing ability of the transforming allele.

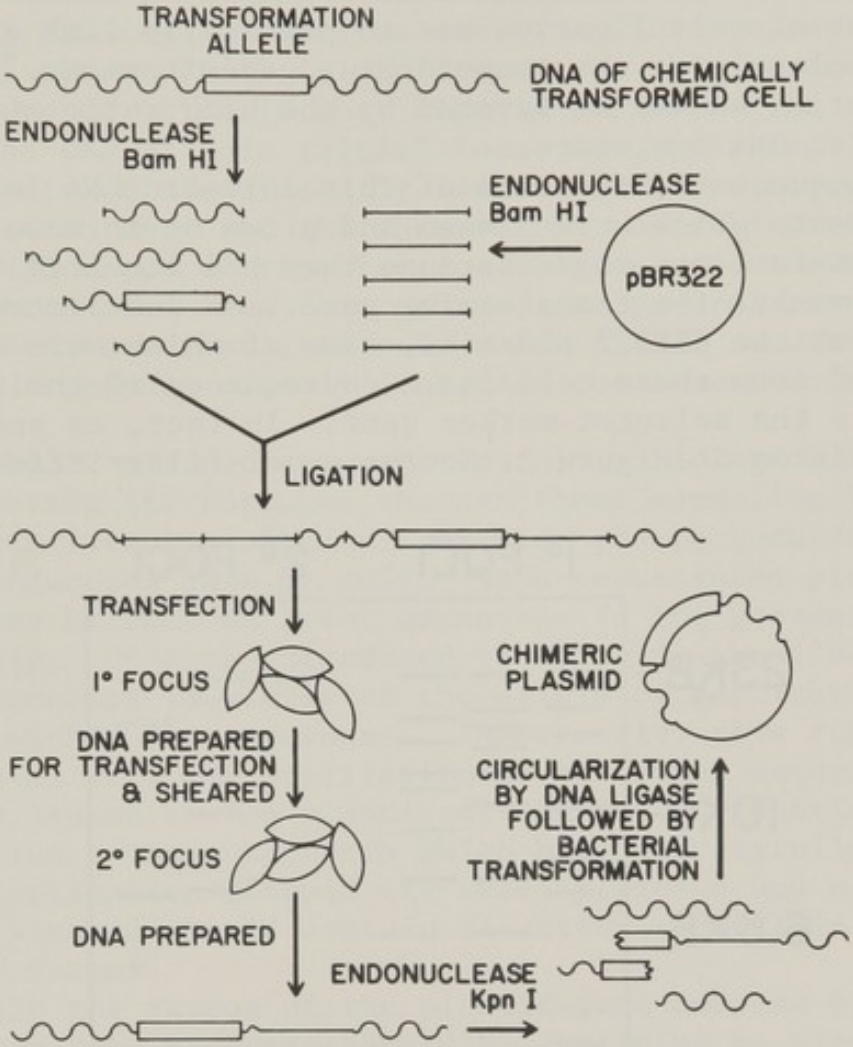

FIGURE 1. Scheme of the Strategy used for Molecular Cloning of the Transforming Allele.

DNA was prepared from a cell line descended from a transfection-derived focus. The original focus had been induced by application of DNA from a methyl-cholanthrene transformed C2H10T1/2 cell. DNA was used from the focus, and not from the original donor, since it was seen to have a higher biological activity (focus forming units per microgram) than the original donor. This DNA was treated with one of a series of restriction enzymes prior to transfection. Endonuclease BamHI was found to spare the biological activity of the DNA. This suggests to us that the biological activity is encoded within a contiguous sequence found entirely within a BamHI segment.

The cleavage of cellular DNA with this endonuclease leaves about 10^6 unique cellular DNA fragments. A two fold

molar excess of BamHI-cleaved pBR322 plasmid DNA was added to the cleaved cellular DNA and the mixture treated with DNA ligase. This resulted in the creation of a long tandem array of cellular and plasmid sequences arranged in randem order. The intent of this ligation was to physically link a pBR322 sequence closely to the transforming gene fragment. Such juxtaposition should be favored by the high ratio of pBR322 to cellular DNA fragments.

Subsequent transfection of this chimeric DNA led to focal transformants which were picked and grown up to mass culture. These transformants arose because they had acquired the selected marker-the transforming gene. An unselected sequence was that of the pBR322 plasmids, some of which were also introduced into these cells as a consequence of their physical linkage to the selected marker gene. In fact, as seen in the schematization of Figure 2, Southern gel-filter transfer

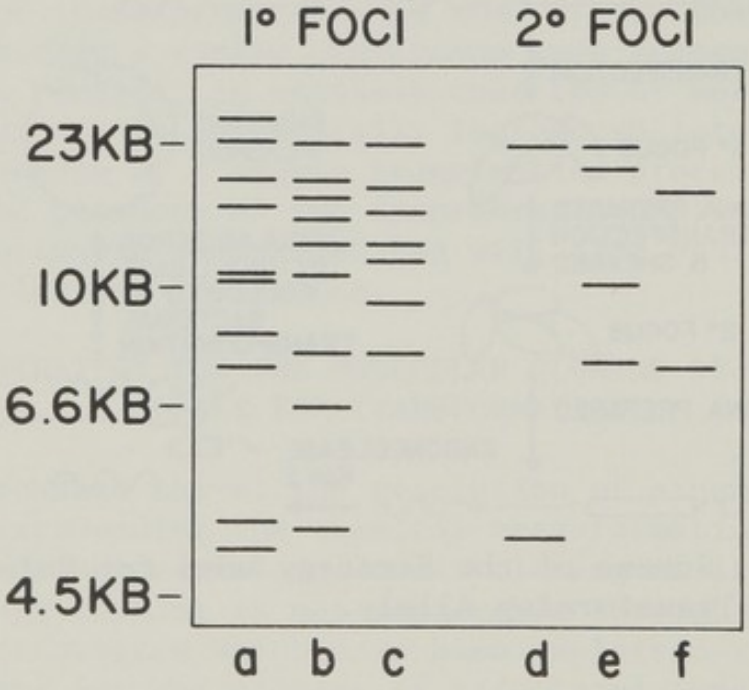

FIGURE 2. Scheme of Results of Southern Blot Analysis of Primary and Secondary Foci. DNA of a 3-methyl cholanthrene transformed C3H10T1/2 cell (MCA16) was cleaved with endonuclease BamHI ligated to BamHI cleaved pBR322 and transfected into NIH3T3. DNAs of 3 primary foci were cleaved with XbaI (lanes a-c) and analyzed by Southern blotting, using ^{32}P labelled nick translated pBR as a probe. Secondary foci derived from transfection of the DNA of the primary foci were analyzed in a similar way. d,e -XbaI digest of secondary foci derived from the primary focus shown in lane a. f - KpnI digest of a focus derived from the DNA of the primary focus shown in lane c.

analysis indicates that such transfectants carry multiple pBR322 sequences deriving in part from the large array of fragments to which the gene became linked during ligation, and in part from other DNA molecules which were independently acquired by the cell during the same transfection event.

One of these pBR322 is of interest in this protocol- that plasmid sequence which is most closely linked to the selected marker. The DNA of this initial focus was mechanically sheared to dissociate distantly linked pBR322 sequences from the selected marker, and the sheared DNA was subjected to a second serial round of transfection to generate secondary foci. As predicted from this scheme, the several resulting secondary foci now contained only a few pBR322 sequences. These were hopefully closely linked to the transforming marker gene.

The pBR322 plasmid represented only a biologically silent sequence during its passages through these mammalian cells. However, its latent biological activity can be resurrected by re-introduction into *E. coli*. This reactivated biological activity may be used to great advantage in the present cloning strategy. Once re-introduced to *E. coli*, the plasmid can begin replicating by virtue of the origin of replication contained within its sequences. Importantly, this replication will drive as well the replication of any other sequences which were linked to the plasmid during re-introduction into the bacterium. Those bacteria which have successfully acquired replication plasmid are readily scored and selected since the plasmid should contain an active ampicillin resistance marker.

Ideally the rescue of the plasmid-gene complex by re-introduction into bacteria should be initiated by cleavage of the DNA of the secondary focus by an enzyme which cuts neither pBR322 nor the transforming gene and which leaves the linkage between them intact. In our case, we did not have an endonuclease which fulfilled these requirements and additionally yielded reasonably small (15 kb) fragments of DNA. We chose therefore endonuclease KpnI which cleaves within the gene. Having treated the DNA of the secondary focus with this enzyme, we then added DNA ligase to the resulting DNA fragments under conditions which would favor intra-molecular circularization instead of end-to-end tandemization. The resulting circles were introduced to a culture of χ1776 strain of *E. coli*. Seven plasmid-positive colonies derived from one of the five attempts at reintroduction of these DNAs into bacteria.

The resulting chimeric plasmid was amplified, and its structure corresponded exactly to the structure predicted from Southern blot analysis of the DNA of the secondary focus.

The cellular DNA cloned as part of this chimera did not contain biological activity i.e. the ability to transform mammalian cells. This was expected because the derivation of this cloned sequence utilized an enzyme known to cleave the transforming gene. Nevertheless, the presence of this gene could be tested by using the cloned cellular sequences as a nucleic acid hybridization probe in Southern blot analysis of the DNAs of normal and transfected mouse cells. Normal NIH3T3 cells should contain one or a small number of DNA fragments reactive with this probe and transfected. Transformed cells should have acquired an additional copy if their transformation indeed resulted from the acquisition of this gene. (This diagnostic test can be made under conditions in which the newly acquired allele will reside in a differently-sized restriction fragment than the fragment carrying the normal, resident allele.) Such analysis indicated that this initial clone of cellular DNA was unrelated in sequence to the transforming gene. In fact, further analysis of the DNA of this secondary focus indicated that the transforming gene is likely to be only distantly linked (20-30 kb) to the nearest pBR322 sequence.

CONCLUSIONS

We have discussed in some detail the as yet unsuccessful cloning of the DNA containing an allele for carcinogen-induced transformation. These cloning experiments are of potential interest not only because of the biological relevance of the allele, but also because of the methodological importance of the cloning strategy. Such a strategy has succeeded in one instance using animal cell DNA and is mentioned in the paper of M. Wigler in this volume. This work of Wigler resulted in the successful cloning of a chicken thymidine kinase gene.

The initial lack of success of our cloning procedure is in part attributable to a trivial factor. This factor was the absence of close linkage between the selected gene and pBR322. Such lack of adjacency is likely to be remedied by altering the relative amounts of pBR322 and cellular DNA fragments during the initial ligation.

An additional, more basic consideration has forced us to undertake alternative cloning strategies. This factor is the low efficiency of passage of pBR322 DNA from mammalian cells back into *E. coli*, a phenomenon also described here by Wigler. Although we are unable to explain the mechanistic basis of this low efficiency, its existence precludes general use of the cloning strategy as described.

An alternative strategy currently being undertaken does not rely on the reactivation of the biological activity of pBR322. Rather, it depends only on the pBR322 sequence serving as an identifiable tag detectable by nucleic acid hybridization. Following this alternative plan, a "library" is made of those DNA sequences present in the secondary focus, via introduction of large DNA fragments (ca. 15 kb) into the genomic ends of one of the lambdaphage vectors of the Charon series (17). Those few components of the library containing the pBR322 sequences can be detected by standard techniques (18) and these chimeric phages can be virally cloned and amplified. The pBR322 is serving in this case only as a sequence tag. The amplified lambdaphage should contain in its genome pBR322 sequences along with the desired adjacent cellular sequences.

When the transforming allele is successfully cloned, several questions can be immediately addressed. Firstly, is the transforming gene related to any of the cellular sequences which have been acquired by retroviruses during the formation of the defective, focus-forming, acutely-oncogenic virus strains? Secondly, have one or more different transforming genes been activated during the independent generation of the five different lines of transformed mouse cells which we have been studying? Thirdly, what are the structural differences between the transforming sequence and the DNA sequences of normal cells from which it was derived? We look forward to the answering of these questions with great anticipation.

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MECHANISMS OF DNA-MEDIATED TRANSFORMATION IN ANIMAL CELLS [1]

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ABSTRACT. Certain cultured cell lines can readily be transformed by exogenous DNA when given as a calcium phosphate coprecipitate. Although stable transformants, detected by biochemical selection, can arise from exposure of 10^6 cells to as little as 10 pg of a purified gene such efficient gene transfer requires the use of vast excesses of carrier DNA. Cells which incorporate a selectable marker, such as thymidine kinase, are also likely to be cotransformed with significant amounts of carrier DNA. By adding well defined DNA sequences to the carrier it is possible to construct cell lines containing virtually any defined DNA sequence. Genetic and biochemical data indicate that selectable markers and carrier DNA become covalently linked in the transformed cell.

INTRODUCTION

Methods for the stable introduction of exogenous DNA sequences into cultured cells are providing new approaches to the study of higher vertebrate cells. To date, the most efficient and general method of DNA transfer is that developed Graham and van der Eb, in which DNA is coprecipitated with calcium phosphate and the coprecipitate is added directly to cell monolayers (1). The stable incorporation and expression of added viral genes coding for such functions as growth transformation (2) and thymidine kinase (3-5) as well as cellular genes coding for phenotypically selectable functions (6-11) can be demonstrated by the acquisition of the recipient cell of the ability to grow under new selective conditions. Although only a small proportion of an exposed cell population actually

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becomes genetically transformed[2], it is nevertheless possible to introduce unselected genetic elements into cells by cotransformation.

In a manner analogous to bacterial systems (12-13), cotransformation may be effected by either direct linkage of an unselected marker to a selectable one (14) or by taking advantage of the observation that cells which incorporate a selectable marker also take up additional unlinked DNA sequences present in the calcium phosphate precipitate.(15) For this purpose, the HSV-1 thymidine kinase gene serves as an excellent selectable marker when used in combination with tk^- recipient cells. Resulting tk+ transformants may be readily selected in medium containing hypoxanthine, aminopterin, thymidine and glycine (HAT medium). Genes of prokaryotic origin (15) as well as cloned eukaryotic genes such as rabbit globin (14-16) and chicken ovalbumin (17,18) have thus been introduced into Ltk^-, a tk^- mouse cell line. Other selectable markers will eventually be generally available which do not require the use of mutant recipient cells (see Berg, this volume).

One opportunity opened by the gene transfer methodology is the study of gene regulation. Examination of the expression of developmentally regulated genes introduced into different host cells can provide some information as to the nature of developmental controls. Study of the consequences of *in vitro* modification on the expression of transferred genes may identify DNA sequences which are targets for regulatory controls. In principle, it may also be possible to study chromosomal position effects on expression. These application of gene transfer presuppose that we understand something of the mechanisms by which exogenous sequences become stably incorporated into the host cell.

Efficient transfer with the calcium phosphate method requires the addition of carrier DNA as a component of the precipitate (1), with at least 100 fold lower efficiencies if carrier DNA is omitted (M.W., unpublished). Presumably carrier DNA is required in part to form a precipitate suitable for cellular uptake. As mentioned above, cells which demonstrably incorporate a selectable marker often incorporate other sequences present in the carrier. These sequences are found within the high molecular weight nuclear DNA of transformants (15). We previously proposed that each exogenous sequence present in transformants was independently integrated into host chromosomal DNA (15).

[2]By transformation we mean the stable incorporation of exogenous DNA by cells.

Subsequent studies now indicate this hypothesis is untenable: exogenous sequences segregate and amplify coordinately, and their flanking sequences derive in general from carrier sequences and not from the host. These observations will be detailed below, and a new model proposed for the major pathway of transformation.

RESULTS

Coordinate segregation of markers. Although the arrangement of exogenous sequences present in tk+ transformants is stable upon subcloning transformants in HAT medium (15), the stability in tk$^-$ revertants has not been previously characterized. Most transformed clones of Ltk$^-$ expressing the HSV tk gene plate in medium containing Bromodeoxyuridine (BUdR) with 1.0-0.01% the efficiency observed in HAT medium (5). Most of these BUdR resistant clones are tk$^-$ revertants: they cannot grow in HAT medium and have lost the HSV tk gene sequences (data not shown). We have now examined the retention of exogenous sequences upon reversion of several different transformants.

One tk$^+$ transformed line which we have studied extensively is øX-4. øX-4 is an HSV tk$^+$ Ltk$^-$ derivative containing approximately 50 integrated copies of bacteriophage øX174 RF DNA (15). øX174 sequences in øX-4 can be visualized as discrete bands by blot hybridization after digestion of host DNA with restriction endonucleases which do not cleave within the bacteriophage genome. tk$^+$ subclones of øX-4 display the same blot pattern as the parental line from which they derive. 20 tk$^-$ revertant subclones of øX-4 were examined, all of which had lost the HSV tk gene sequences: eight of these lost all traces of øX174 sequences and the remaining twelve subclones each lost some of the sequences which were formerly present. Blot hybridizations of representative tk$^-$ revertants are shown in Figure 1. We note that loss of particular integrated copies of øX174 sequences in revertants is not random. Some integrants have a higher probability of retention than others.

The pattern of loss of øX sequences we observe upon reversion is consistent with the following propositions: 1) Reversion occurs by deletion about the tk gene; 2) The size of the deletion may vary from revertant to revertant; 3) øX174 sequences are arrayed about the region flanking the tk gene; 4) Large deletions result in the loss of all øX174 copies while small deletions result in only partial losses; 5) The øX174 sequences most distant from the tk gene are more likely to be retained after a small deletion.

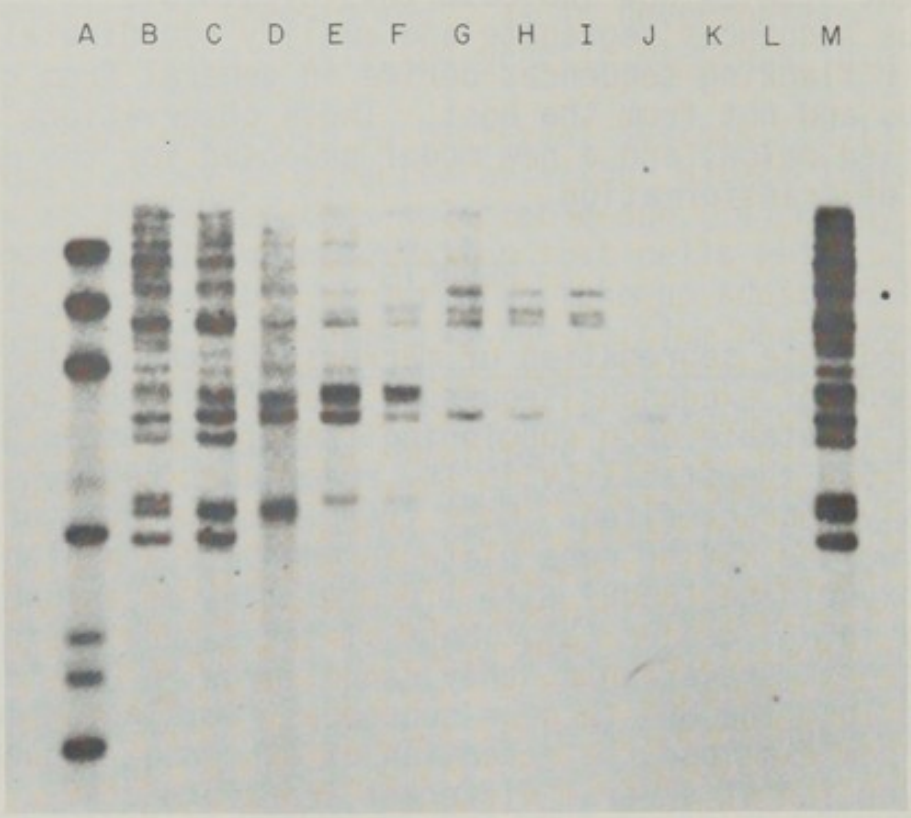

Figure 1

FIGURE 1. øX sequence pattern in tk$^-$ revertants. 10^3 cells from the line øX-4 were plated in DMEM with 10% calf serum and 30 µg per ml of BUdR. Resistant clones were isolated, grown into mass culture, and high molecular weight DNA extracted (24). 10 µg of DNA from each subclone was digested with Hind III, electrophoresed through 0.8% agarose gels, and analyzed by filter hybridization using ^{32}P-labeled nick translated øX 174 DNA (4 x 10^8 cpm/µg) as probe (15). Lane A: mixture of Eco R1 and Bam H1 digests of ^{32}P-adenovirus 2 DNA. The seven bands are 20.3, 14.4, 10.2, 6.3, 4.5, 4.2 and 3.6 Kbp. Lanes B and M: øX-4 parental line. Lanes C-L: Ten different subclones resistant to BUdR.

We have analyzed many other transformants in this manner, including Ltk- derivatives transformed with other selectable markers (cellular genes coding for tk and adenine phosphoribosyl transferase), cells cotransformed with other hybridization markers and other carriers as well as transformants derived from other tk$^-$ cell lines. In all these cells, the sequence content and arrangement of cotransformed markers were consistently stable under positive selective pressure while their pattern of segregation in revertants was as described for the øX174 sequences

in øX-4. Thus, in general, cotransformed markers and presumably other carrier sequences, may all be arranged about the sequences flanking the selected marker.

Coamplification of markers. Genetic linkage between cotransformed markers can also be demonstrated using an amplifiable vector. Wild type cells are exquisitely sensitive to the folic acid analog methotrexate (mtx) or amethopterin, which binds with high affinity to dihydrofolate reductase (dhfr). Wild type cells can be rendered resistant to low levels of mtx by transformation using total genomic DNA from A29 cells, a CHO derivative encoding a mutant dhfr with lowered affinity for mtx (19). When initial mtx resistant transformants are exposed to still higher concentrations of mtx, resistant cells emerge which have increased amounts of the exogenously acquired mutant gene (19). Gene amplification as a mechanism of resistance to selective pressure was first demonstrated by Schimke and coworkers for the endogenous dhfr gene of mouse (20). The size of the amplification unit is not known, but in mouse it must exceed 50 kb, the minimum size estimate of the dhfr gene itself (21). If the amplification unit extends beyond the gene into flanking sequences, these sequences would be amplified as well during selection. We therefore tested if sequences cotransformed into mouse cells using the hamster A29 dhfr gene as a selectable marker are amplified in cells resistant to increased levels of mtx.

Ltk$^-$ cells were transformed with A29 DNA and cotransformed with Tgl8 a derivative of the E. Coli plasmid pBR322. Selection for transformants resistant to 0.1 μg per ml of mtx was as previously described (19). Several transformed clones were grown into mass culture and exposed to increasing levels of mtx. Mass cultures resistant to 0.1, 2.0 and 10.0 μg per ml of mtx were derived. DNAs from these cultures were analyzed for the presence of Tgl8 sequences by blot hybridization: DNAs were cleaved with the restriction endonuclease Hind III, which cleaves Tgl8 once, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with nick translated, ^{32}P labeled Tgl8. Four independent transformants initially resistant to 0.1 μg/ml mtx were examined in this manner (Fig. 2). Each transformant contained Tgl8 sequences in a unique blotting pattern. Two lines, 1b1 and 1b2, showed no significant changes in band intensities as cells resistant to high levels of mtx were selected. The Tgl8 sequence content of two other tranformants, 1b3 and 1b6 increased following selection in higher concentration of mtx. In the line 1b3, the only visible band present in the initial transformant increased

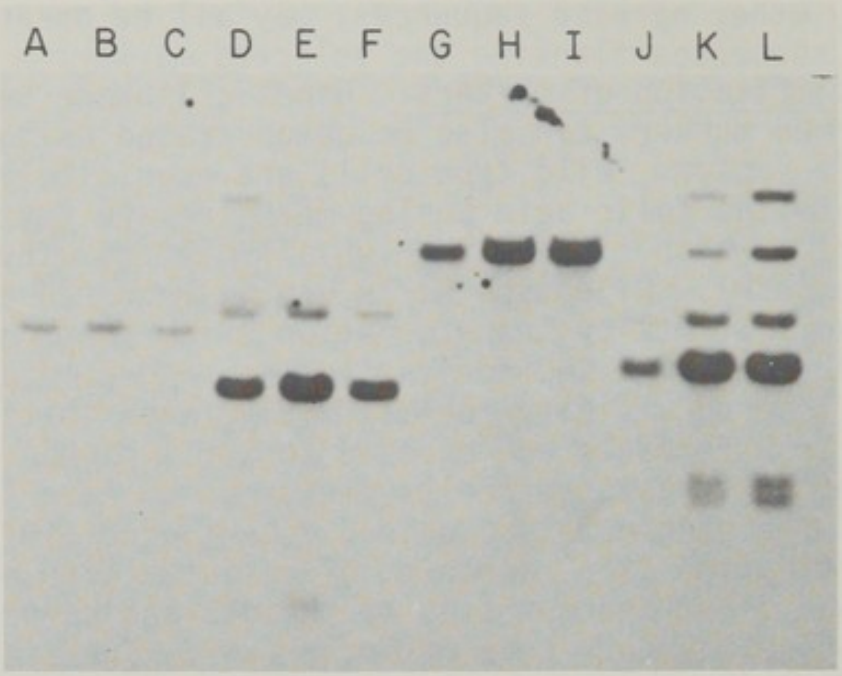

Figure 2

FIGURE 2. Amplification of cotransformed Tg18 sequences. Ltk$^-$ cells were transformed to mtx resistance with a mixture of 1 μg of Tg18 DNA and 20 μg of A29 DNA per dish. Transformants were selected initially for resistance to 0.1 μg per ml of mtx. Individual colonies were isolated using cloning cylinders and cultures were exposed to increasing concentrations of mtx. High molecular weight DNA was extracted, cleaved with Hind III, electrophoresed through 0.8% agarose gels and analyzed for the presence of Tg18 sequences by filter hybridization using ^{32}P-labeled nick translated Tg18 (2 x 10^8 cpm/μg) as probe. Lanes A-L: 10 μg of DNA from cell lines 1b1 (A, B, C), 1b2 (D, E, F), 1b3 (G, H, I) and 1b6 (J, K, L), grown in 0.1 (lanes A, D, G and J), 2.0 (lanes B, E, H and K) and 10 (lanes C, F, I and L) μg per ml of mtx.

several fold in intensity in cells resistant to 2 μg per ml of mtx, although no further increase in intensity was observed in cells selected at 10 μg per ml. In 1b6, two Tg18 homologous bands were present in DNA from cells initially selected at 0.1 μg per ml of mtx. After selection in 2 μg per ml, the intensity of the bands increased and new bands appeared whose intensities also increased in cells resistant to 10 μg per ml. These results imply that at least some of the cotransformed Tg18 sequences have integrated within the amplification unit of the exogenous dhfr gene.

Flanking sequences of cotransforming elements derive from carrier. The previous results have demonstrated the genetic linkage between transforming elements. Two plausible models of linkage can be imagined. In the first, exogenous sequences become linked together by the cell during the transformation process into large concatemeric structures. In the second, exogenous sequences become integrated independently into one host chromosome or one region of a chromosome. If the first model is correct, the DNA flanking an exogenous sequence should in most cases derive from the carrier. If the second model is correct, flanking DNA will derive from the host.

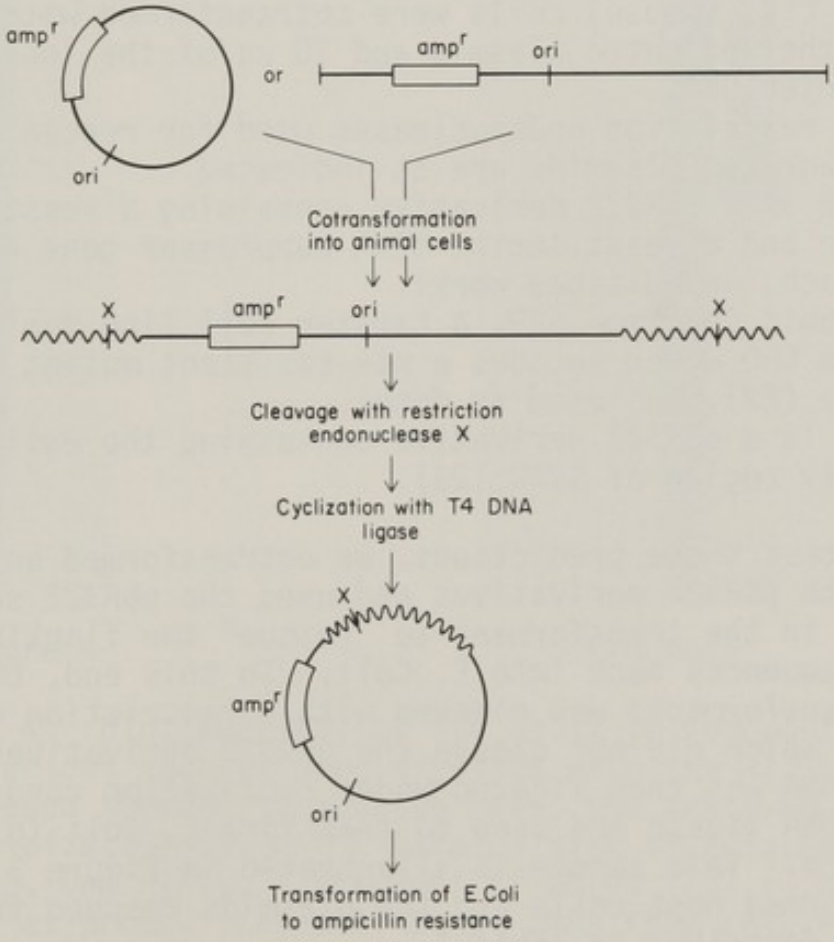

FIGURE 3. Plasmid rescue schema. pBR322 derivatives coding for ampicillin (amp^r) resistance are cotransformed into cells either as linearized or circular molecules. The integrated plasmid sequences and their flanking sequences are rescued by cleaving host DNA with restriction endonuclease X, cyclizing and transforming E. coli to ampicillin resistance. Note that the rescued plasmid linearized with X is colinear with its integrated structure in the host cell from which it derived.

TABLE I
ORIGINS OF RESCUED PLASMIDS

Rescued Plasmid	Host Cell	Carrier Species(a)	Rescuing Enzyme(b)	Cotransformed Plasmid(a)	Selectable Marker
plb6R	1b6	Hamster	Xba I	Tg18 (c) (uncut)	A29/dhfr(d)
plb3R	1b3	Hamster	Xba I	Tg18 (uncut)	A29/dhfr
pC10S	poTC10	Salmon	Sst I	poT (e) (Sal I cut)	HSV-1/tk

(a) 10^6 Ltk^- (mouse) cells were cotransformed with 1 μg of the indicated plasmid and 20 μg of the indicated carrier DNA.
(b) The restriction endonucleases used for rescue of integrated plasmids are as indicated.
(c) Tg18 is a pBR322 derivative containing a yeast Leu 2 gene and a yeast serine tRNA suppressor gene (J. Broach, unpublished work).
(d) Genomic DNA from A29, a hamster cell line derived from CHO which encodes a mtx-resistant mutant dhfr gene (22), was used as donor.
(e) poT is a pBR322 derivative containing the entire early region of SV40 (23).

To test these predictions, we cotransformed animal cells with pBR322 derivatives and used the pBR322 sequences residing in the transformant to "rescue" the flanking animal sequences back into E. Coli. To this end, DNA from the cotransformants was cleaved with a restriction endonuclease which did not cleave the pBR322 derivatives. Cleaved DNA was then ligated under cyclization conditions with T4 DNA ligase and used to transform E. Coli to ampicillin resistance. This scheme is illustrated in Figure 3. The cotransformed host cells and the plasmids rescued from them are described in Table I.

Restriction maps of all rescuants were prepared which confirmed their derivation from the original cotransforming plasmid. Blot hybridization confirmed that the rescued plasmid, when linearized with its rescuing enzyme, was colinear with sequences present in the host from which they derived. The rescued plasmids contained from 1kb to 8kb of flanking sequences.

To identify the origin of the flanking sequences, we nick translated the rescuants and used them as probes in blot hybridization experiments to restriction endonuclease

cleaved DNA from several species. As seen in Figure 4 and Figure 5, nick translated probes derived from plb6R and pC10S hybridized intensely and diffusely with DNA from the species used as carrier but not with host mouse DNA.

Figure 4

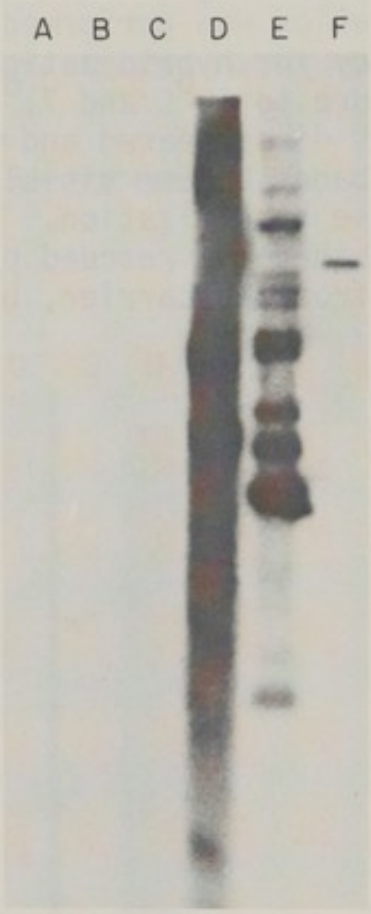

Figure 5

FIGURE 4. Blot analysis of the PoT C-10 rescuant. DNAs from various sources were digested with Bam H1/Sst I, electrophoresed through 1% agarose gels, and analyzed by filter hybridization using ^{32}P-labeled nick translated, gel purified, Bam H1/Sst I fragment of pC10S (23) (1 x 10^8 cpm/μg) as probe. Lanes A, B, C: DNAs from chicken, salmon, and mouse respectively (10 μg each). Lane D: pC10S (50 pg with 5 μg of chicken DNA carrier).

FIGURE 5. Blot analysis of the 1b6 rescuant. DNAs from various species were digested with Xba/Eco R1, electrophoresed through 1% agarose gels, and analyzed by filter hybridization using ^{32}P-labeled nick translated plb6R (2 x 10^8 cpm/μg) as probe. Lanes A through E: DNAs from chicken, salmon, mouse, hamster and yeast respectively (10 μg each). Lane F: plb6R digested with Xba (50 pg with 5 μg chicken DNA carrier).

Under our conditions, hybridization to unique sequence DNA would be visible but might be masked by a more intense and diffuse hybridization due to a component in the probe annealing to repetive DNA. Nick translated plb3R also hybridized intensely and diffusely to hamster DNA although faint hybridizing bands were observed in mouse DNA when hybridization was performed at 65°C (Fig. 6). When the stringency for hybridization was increased by raising the temperature to 68°C and 71°C, the hybridization to mouse sequences dissappeared and hybridization to specific hamster bands became visible against a less intense background of diffuse hybridization. These experiments provide evidence that the rescued plasmids contain sequences derived from the carrier, but not from the host.

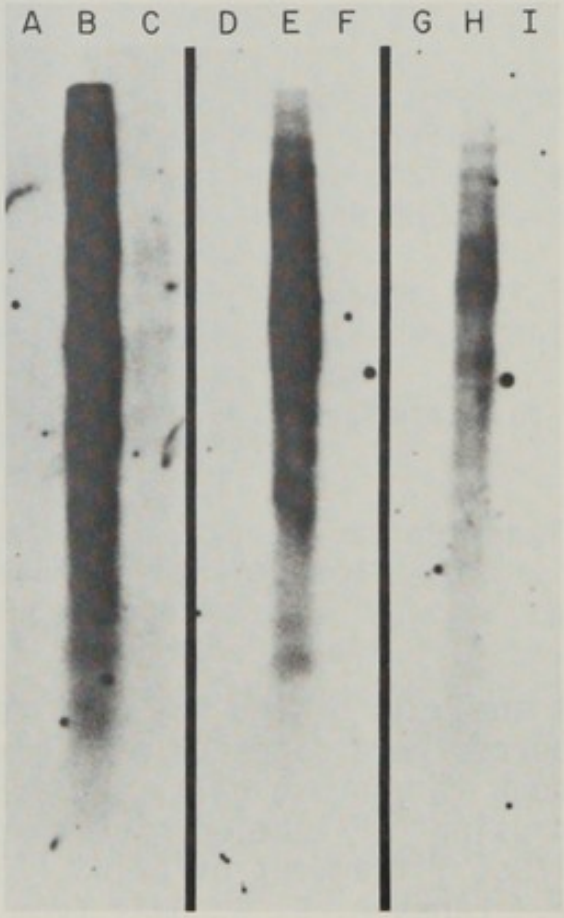

Figure 6

FIGURE 6. Blot analysis of the lb3 rescuant. DNAs from salmon (Lanes A, D and G), hamster (lanes B, E and H) and mouse (lanes C, F and I) were digested (10 μg each) with Xba/Eco Rl, electrophoresed through 1% agarose gels and analyzed by filter hybridization using as probe ^{32}P-labeled nick translated plb3R (2 x 10^8 cpm/μg). The hybridizations were carried out in 6 xSSC for 24 hours at 65°C (lanes A, B and C), 68°C (lanes D, E and F) and 71°C (lanes G, H and I).

More direct evidence for this conclusion comes from solution hybridization experiments. Restriction endonuclease fragments from rescuants were purified which contained primarily flanking sequences. These were nick-translated and the kinetics of reannealing in the presence of vast

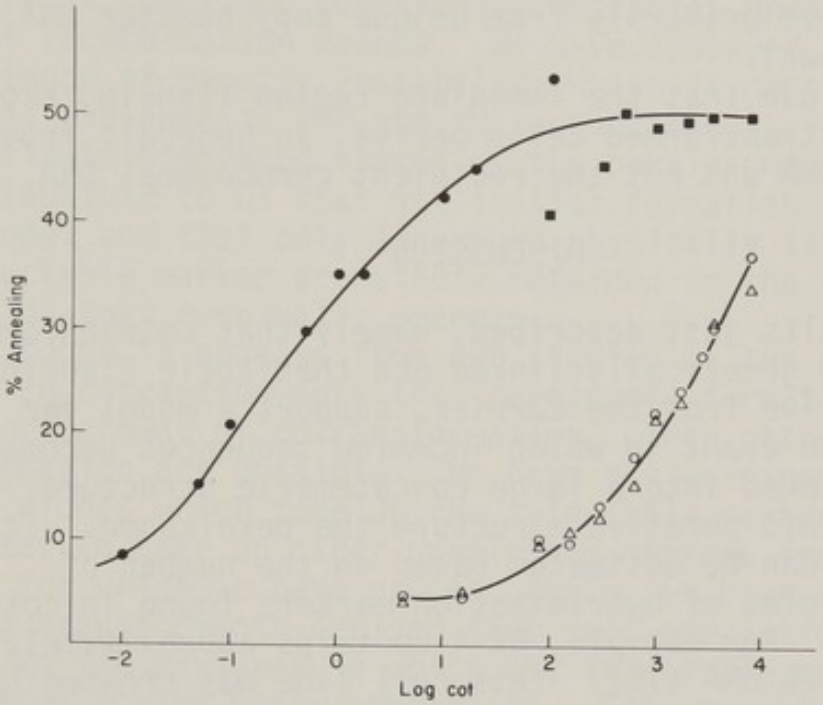

Figure 7

FIGURE 7. Kinetics of hybridization of the Bam Hl/Sst I fragment of pClOS to mouse, hamster and salmon DNAs. The Bam/Sst fragment of pClOS comprising flanking sequences (23) was purified after agarose gel electrophoresis and labeled by nick translation (5×10^7 cpm/μg). The probe was denatured, allowed to renature to a Cot of 10^{-4} and the remaining single stranded sequence purified through hydroxylapatite chromatography (24). 0.5 ng of this DNA was annealed with 6 mg of salmon (■-■), mouse (o-o), or hamster (Δ-Δ) sonicated DNAs, in 0.4 ml of lmM Tris (pH 7.6), lmM EDTA, 0.4 M NaCl, at 68°C under parafin oil. Aliquots, (40 μl) were removed at different times, and diluted in 1 ml of 30 mM NaOAc (pH 4.5), 0.1 M NaCl, 1 mM $ZnCl_2$ and 50 μg/ml bovine serum albumin. The samples were assayed for duplex formation by digestion with 2000 units of S1 nuclease as described (24). For the lower Cot values of salmon DNA (●-●), 0.05 ng of the probe was annealed with different amounts of salmon DNA in 0.1 ml reaction volumes for 1 hour at 68°C, and processed as before.

excesses of genomic DNA from various species was measured by S1 protection (24). Figure 7 demonstrates that annealing of flanking sequences derived from pC1OS is accelerated in the presence of salmon DNA but not in the presence of mouse or hamster DNA. These particular flanking sequences must contain a highly repetitive component of salmon DNA. A similar study showed that the flanking sequences contained in p1b6R derive primarily from unique copy hamster DNA (data not shown).

We conclude that the immediate region flanking exogenous sequences in transformed cells derive, in general, from the carrier DNA and not the recipient chromosomal DNA.

DISCUSSION

The results just described, namely that cotransformed sequences are genetically linked and that their flanking sequences derive from the carrier, support a model for the transformation event in which incoming sequences become physically linked into a large concatemeric structure. We have termed this putative structure the pekelasome. Its maximum size can be estimated based on the number of integrated copies of hybridization markers found in cotransformed cells. For example, øX-4 contains approximately 50 copies of øX174 DNA (15). This cell line was created by transformation in the presence of 20 µg of salmon DNA, 1 µg of øX174 RF DNA and 1 ng of the HSV tk gene. If the carrier DNA were incorporated in øX-4 with the same efficiency on a mass basis as øX174 DNA, the transformant would contain 50×20 øX174 genome equivalents or approximately 4×10^9 base pairs of carrier DNA. The ligation of sequences acquired by a recipient cell could be mediated by DNA repair enzymes. Ligation of nonhomologous fragments might be a function necessary for the repair of double stranded chromosome breaks. Intramolecular ligation of linearized SV40 molecules is apparently an efficient process (25).

The model we have proposed is of course incomplete. It is unclear whether the pekelasome is integrated into a host chromosome or replicates independently as an extrachromosomal unit. Some transformants are genetically stable in the absence of selective pressure, while others not (6). The former class may represent instances in which the pekelasome becomes chromosomally linked. In a study of one stable transformant, the HSV-1 tk gene was localized to a mouse chromosome (26). The genetically unstable transformants may represent instances in which the pekelasome resides extrachromosomally and is lost with

high frequency through asymmetric segregation during mitosis. Other explanations of highly unstable genotypes are possible. For example, although the pekelasome may have the physical dimensions of a small chromosome, its local structure is probably quite strange, being a patchwork of genomic fragments derived from the carrier. It would not be too surprising, then, to find that this patchwork structure is sometimes genetically unstable, undergoing frequent recombination events. We have observed at least one instance of genetic instability which is most compatible with this explanation (MP and MW, unpublished). Regardless of the final integrated status of the pekelasome, it seems quite plausible to us that its initial formation is extra-chromosomal and that only sequences physically linked to the selectable marker are stably retained in the transformant population that eventually emerges.

Although it appears from our studies of the genetic linkage in cotransformants that pekelasome formation is the major pathway of cotransformation, other pathways for transformation are possible. We have not examined transformant clones which take up the selectable marker but do not appear to take up other unselected markers from the carrier for which we have probes. Again, we have in general examined transformants derived from cell populations exposed to DNA mixtures in which the selectable marker represented a very minor proportion. This may bias transformation to a particular pathway in a subpopulation of cells. Evidence that direct chromosomal integration of a transforming gene can occur comes from the work of Botchan et al. (27) who examined the sequences flanking an SV40 integrant in a rat cell transformed with EcoR1 linearized SV40 DNA. In that instance, calf thymus DNA had been used as carrier yet one end of the SV40 integrant was shown to be embedded in rat sequences.

One hope for the DNA transfer methods is that they will provide a method for studying chromosomal position effects. In view of our findings, the hope of achieving this with the calcium phosphate method appears to be complicated.

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CHROMOSOMAL MAPPING OF ECOTROPIC AND XENOTROPIC LEUKEMIA VIRUS-INDUCING LOCI IN THE MOUSE

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ABSTRACT Classical mendelian segregation analysis and somatic cell hybridization techniques were used to map chromosomal genes for the induction of ecotropic and xenotropic retroviruses in high and low leukemic mouse strains. This work has resulted in the genetic mapping of 6 ecotropic virus-inducing loci in 5 mouse strains to nonallelic positions in 4 mouse chromosomes. In contrast, all 5 mouse strains examined were shown to carry a single genetic locus for xenotropic virus induction on chromosome 1. These studies provide further evidence that endogenous viral loci are present at different chromosomal sites in various mouse strains and emphasize the difference in the chromosomal distribution of ecotropic and xenotropic induction loci.

All mice have cellular DNA sequences complementary to the RNA genomes of the mouse leukemia viruses (MuLVs). However, inbred strains show differences in the expression of viral phenotypes, and breeding experiments have shown that virus induction in high and low lymphoma strains is controlled by mendelian genes. The identification and characterization of these loci represent part of our efforts to elucidate the genetic control of viral gene expression and leukemic transformation. In this paper we describe experiments using sexual crosses and interspecific somatic cell hybrids to map a number of these chromosomal genes for ecotropic and xenotropic virus induction in high and low leukemic mouse strains.

The AKR mouse, the prototype for high virus, high leukemic mice, was the first strain used in the study of the genetic control of ecotropic MuLV production (1). In crosses with NIH Swiss mice, a virus induction negative strain, two independently segregating loci were defined for ecotropic virus inducibility. One of these, designated Akv-1, was mapped to mouse chromosome 7 following the observation that virus expression was loosely correlated with inheritance of

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the albino locus, c. Analysis of subsequent backcross generations confirmed this assignment and defined the gene order: Akv-1 - Gpi-1 - c (2). Akv-1 is now known to contain a set of AKR specific viral sequences and therefore represents the integrated provirus rather than a cellular gene that governs virus expression (3).

More recent genetic studies have been done to map the second AKR locus, Akv-2, using a combination of somatic cell hybridization and standard breeding techniques. A previous attempt to map this locus with NIH Swiss mice partially congenic for Akv-2 showed that this locus was not linked to a number of morphological markers on 7 chromosomes (Risser and Rowe, unpublished data). In our studies, analysis of Chinese hamster/mouse somatic cell hybrids showed that virus inducibility was coordinately expressed with the mouse isozyme marker, Sod-1, on chromosome 16. A mendelian cross was then used to position Akv-2 at the centromeric end of chromosome 16 near the md locus.

Subsequent genetic studies have mapped viral loci in other high virus, high leukemic mouse strains. One of the high virus inducibility genes identified in C3H/FgLw mice showed linkage to markers on chromosome 7. However, results of a 3 point cross demonstrated that this locus, designated Fgv-1, is distinct from Akv-1 (4). A third high virus strain, C58, was shown to carry 4 unlinked viral loci (5), and one of these genes has now been mapped to a position on chromosome 8 distal to the Es-1 locus. Thus, 3 high leukemic strains carry multiple high virus inducing genes, and mapping studies with 4 of these loci revealed no viral loci common to all strains.

Other studies have examined ecotropic virus inducibility loci in 3 strains with a lower incidence of leukemia-BALB/c, C3H/HeJ, and C57BL/10. Spontaneous virus expression in these strains occurs sporadically and late in life. However, virus can be efficiently induced from cultured cells with 5-iododeoxyuridine. Genetic crosses with virus negative mice identified single loci for virus inducibility in BALB/c (6) and C57BL/10, and a single locus was identified serologically in C3H/HeJ mice (7). We mapped the BALB/c inducibility locus, designated Cv, to chromosome 5 by means of somatic cell hybridization techniques, and established the chromosomal position of this gene in a mendelian cross (8). In an independent study, Ihle and coworkers showed that the ecotropic viral locus in C3H/HeJ mice was also on chromosome 5 and that this locus was allelic with the endogenous viral sequences present in BALB/c mice (7). However, not all low leukemic mouse strains carry this same virus inducibility locus. We have now mapped the single locus for ecotropic virus inducibility

in C57BL/10 mice to a position on chromosome 8. The demonstration that BALB/c and C3H/HeJ but not C57BL/10 carry the same chromosome 5 locus can be attributed to the fact that these two strains share common ancestors in their breeding history. In this regard it should be noted that C58, a high virus strain, and C57BL/10, a related low virus strain, carry virus loci in the same region of chromosome 8.

The dramatic differences in the chromosomal distribution of ecotropic loci among inbred strains emphasizes the fact that these loci constitute a unique gene system. Since the products of these chromosomal genes are viruses infectious for mouse cells, the existence of multiple viral loci in strains such as AKR and C58 is not surprising and may in fact be the result rather than the cause of the high virus phenotype. Evidence from a number of sources now indicates that new loci for viral phenotypes can be acquired following infection of germ line cells and the reinsertion of the provirus into mouse chromosomal DNA. Experiments by Jaenisch have demonstrated that such stable reinsertions can be generated *in vitro* by the infection of preimplantation embryos (9). In our own lab, recent studies have identified several new genes for virus induction which appeared spontaneously in NIH Swiss mice carrying the *Akv-1* locus. Another type of reinsertion we have observed is the appearance of a novel virus inducing locus for B-tropic ecotropic virus in the H-2 congenic strain B10.BR. Expression of this viral locus *in vitro* and *in vivo* is detected as B-tropic virus (10). This insertion is of particular interest since the B-tropic virus genome is not normally an endogenous retrovirus but presumably arises from the N-tropic ecotropic virus by recombination in the viral gene for the major internal virion protein p30. Our mapping studies show that this locus represents yet another inducibility gene, on chromosome 11.

Molecular and genetic studies on the chromosomal distribution of endogenous xenotropic viral genes show a markedly different pattern. While complete ecotropic viral genes are present in some inbred strains but not others, all mice have sequences homologous to the xenotropic viral genome (11). However, the induction of infectious virus is not characteristic of all strains, and we have used this phenotype as the basis for classical mendelian segregation analysis.

The most complex genetic control of virus expression is seen in NZB mice, the prototype for high virus strains. Two independently segregating loci have been identified for production of xenotropic virus in this strain (12). These loci show distinct patterns of high or low virus expression, and the 2 viruses isolated from this strain are different by fingerprint analysis of viral gp70s (13). Neither of these

loci have been mapped to mouse chromosomes.

A single locus for inducibility has been identified in low xenotropic viral strains (14). The tryptic digest pattern of gp70s of viruses isolated from these strains resembles one of the NZB types (13). Our initial genetic studies showed that the single virus locus carried in C57BL/10 and BALB/c are both linked to the Pep-3 (formerly Dip-1) locus on chromosome 1 (15). We have since examined additional strains with inducible xenotropic loci. Our data demonstrate that 5 mouse strains (AKR, C57BL/10, C57L, C57BR, BALB/c) carry a single genetic locus for xenotropic virus inducibility on chromosome 1. The position of this locus, designated Bxv-1, is Id-1 - Pep-3 - [Bxv-1 - Lp] (16). Thus, endogenous xenotropic virus inducing loci have a more limited chromosomal distribution than ecotropic loci in these same strains. Therefore, although these 5 strains represent 3 unrelated lines of descent, the presence of the same viral locus suggests that this locus was widely distributed in mouse colonies before the separation of inbred lines. The absence of additional loci in these strains can be attributed to the inability of this virus to infect mouse cells.

As a result of these studies, loci for induction of infectious MuLVs have been mapped in high and low leukemic strains. These data should facilitate an analysis of the chromosomal distribution of viral genes carried in other mouse strains and wild mouse populations. Knowledge of these map positions may also help identify and characterize the noninducible or partial viral genomes present in mouse chromosomes. Finally, the identification of integration sites for viral loci can help unravel the complex genetic control of virus expression and leukemogenesis. It has long been known that spontaneous leukemia in the mouse is influenced by a large number of genetic factors and a number of cellular genes have been identified and mapped which alter the expression of endogenous viral loci or the persistence of infection such as Fv-1, Fv-2, Rgv-1. The chromosomal map position of specific viral loci and the identification of closely linked markers with easily recognized phenotypes can be used to efficiently transfer such loci to other inbred strain backgrounds. In this way, one can assess the role of cellular genes in the regulation of virus expression. For example, the different patterns of virus expression in strains such as AKR and BALB/c may simply reflect the differences in the number and chromosomal distribution of endogenous viral loci. However, the allelic ecotropic loci of BALB/c and C3H/HeJ also show different patterns of virus expression; inheritance of the C3H/HeJ locus results in the earlier appearance of antibody (7). It remains to be determined whether such differences can ulti-

mately be attributed to differences within the integrated proviral genes, differences in adjacent chromosomal DNA at the site of integration, or to other unlinked genetic factors in these strains which affect viral gene activity. We hope to develop additional congenic lines for some of the viral loci to analyze this problem.

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CORRELATION BETWEEN THE DEVELOPMENT OF MURINE MAMMARY CANCER AND THE SEGREGATION OF ENDOGENOUS GENES[1]

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ABSTRACT We have investigated the mouse mammary tumor virus (MMTV) specific proviral DNA content of the C57BL and GR strains of mice by restriction endonuclease digestion of cellular DNA and Southern transfer of DNA fragments to nitrocellulose filter paper. GR mice develop 100% mammary tumors at an early age whereas C57BL mice have less than a one percent incidence. The MMTV containing DNA fragments were identified by hybridization to MMTV [^{32}P] complementary DNA. Digestion of cellular DNA with the restriction endonuclease Pst-1, which generates multiple internal MMTV specific DNA fragments, has identified viral DNA fragments at 2.5 and 0.6 megadaltons in the GR DNA, which are absent in the C57BL DNA. The GR and C57BL mice share Pst-1 restriction fragments at 3.3, 1.1, 0.9 and 0.5 megadaltons. The C57BL mouse has a Pst-1 restriction fragment at 3.1 megadaltons which is absent in DNA from GR mice. Genetic analyses of GR and C57BL crosses has determined that the Pst-1 fragments specific for the GR MMTV are inherited as a single Mendelian unit in [C57BL x (C57BL x GR)F_1] backcross mice, i.e., 50 percent of the livers examined contain Pst-1 fragments at 2.5 and 0.6 megadaltons. We have been able to correlate the presence of these MMTV(GR) specific Pst-1 fragments with the occurrence of early mammary tumors in the [C57BL x (C57BL x GR)F_1] backcross mice. These studies thus demonstrate the utility of restriction mapping in the diagnosis of a naturally occurring neoplasm.

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INTRODUCTION

Several studies have demonstrated that members of certain families exhibit a susceptibility to mammary cancer that follows a single Mendelian gene segregation pattern (1). An analogous situation exists in offspring of crosses of the GR mouse strain. Bentvelzen et al. (2) have shown that when GR mice (100% mammary tumor incidence in females) are crossed with C57BL mice (0% incidence), the resulting (C57BL x GR)F_1 mice develop 100% mammary tumors. When the F_1 mice are backcrossed to C57BL, the resulting female BC_1 mice, i.e., [C57BL x (C57BL x GR)], have a 50% mammary tumor incidence before one year of age. It was therefore postulated that susceptibility to early mammary tumorigenesis in this strain is segregating as a single Mendelian unit; the inference was that this was the result of an MMTV gene. Heston, Smith, and Parks (3), however, did not observe a 50% segregation of viral antigens when testing milks of similar BC_1 mice by radioimmunoassay for MMTV proteins. A controversy has therefore existed as to the existence and the nature of the gene that is responsible for the susceptibility to development of mammary cancer.

Several studies have demonstrated that both GR and C57BL mice contain MMTV proviral DNA. Our studies with unique MMTV tumor-associated sequence (TAS) probe (4), however, demonstrated that C57BL DNA is devoid of these particular sequences, whereas the GR strain possesses them as endogenous genetic elements (5). Restriction endonuclease studies have also revealed distinct differences between GR and C57BL mice (6). The possibility was thus explored that the Mendelian segregation of specific endogenous genes of GR mice may correlate with the occurrence of early occurring mammary cancer.

RESULTS

C3H/He mice contain at least two mouse mammary tumor viruses (MMTVs), one of which [MMTV(C3H)] is highly oncogenic and readily isolated from milk or from mammary tumors arising before one year of age. To determine if there are MMTV proviral sequences present in early C3H/He mammary tumors that are not present in the DNA of late occurring mammary tumors and in apparently normal C3H tissues, recycling experiments were conducted (4). Iodinated MMTV(C3H) 60-70S RNA was first hybridized to a vast excess of apparently normal C3H liver DNA. The unhybridized single stranded [^{125}I]-RNA was eluted from hydroxylapatite and concentrated.

This recycled MMTV(C3H) RNA failed to hybridize to DNA of normal C3H/He tissues but did hybridize extensively to DNA from early occurring C3H/He mammary tumors; this probe has thus been termed tumor associated sequences (TAS). To determine the location of the sequences represented in the TAS-RNA on the MMTV(C3H) 30S RNA genome, unlabeled MMTV RNA was fragmented by sonication, and the fragments selected by poly (U) sepharose chromatography. The 3'-RNA subunit fragments of varying lengths were then analyzed for their ability to compete for the hybridization between labeled TAS-RNA and C3H mammary tumor DNA. Results indicate that TAS-RNA represents sequences somewhat spread throughout the genome, but enriched at the 3' end of the molecule.

Studies were conducted to determine the natural distribution of proviral sequences homologous to TAS. Complementary sequences were found in the DNA of all "early" mammary tumors tested of C3H/He, RIII, and DBA mice but not in the DNA of normal tissues of those same mice. Complementary sequences were found, however, in the DNAs of both GR mammary tumors and all apparently normal GR organs tested. Livers of BALB/c, C57BL/6N, C57BL/10SCN, A/He, NIH Swiss, NZB/N, and NZW/N were shown to contain some MMTV proviral information; they do not, however, contain, sequences homologous to TAS.

Several laboratories, including our own have employed restriction endonuclease digestion and the Southern DNA transfer techniques to determine the integration pattern and distribution of MMTV provirus in murine cellular DNAs (7,8). DNAs of apparently normal C3H tissues, digested with the restriction enzyme Pst-1 and hybridized with ^{32}P-labeled MMTV cDNA probe, revealed internal fragments of 3.3, 1.1, 0.9, and 0.5 x 10^6 d. When DNAs from early occurring mammary tumors of C3H/He mice were digested with Pst-1, however, unique internal restriction fragments of 2.5 and 0.6 megadaltons are observed. These fragments were always present in tissues positive for TAS and always absent in tissues which do not contain TAS in their DNA. Both these markers thus appear to be present in DNA of early occurring murine mammary tumors. They are absent in apparently normal tissues of C3H, RIII and C57BL mice, but are present as endogenous provirus in GR mice. It thus appeared feasible to determine if these unique sequences are segregating in a Mendelian fashion in GR mice; and if so, is their presence correlated with those animals that will develop mammary cancer?

We first set out to reproduce the experiments in which C57BL females were crossed with GR males. The male (C57BL x GR)F_1 offspring were then crossed with C57BL females. The

resulting [C57BL x (C57BL x GR)F_1]BC_1 females were then force bred and monitored for the occurrence of mammary tumors. Approximately 95% of F_1 mice developed mammary tumors; 50% of BC_1 females developed mammary tumors before one year of age. Experiments were then undertaken to determine if specific MMTV genes are segregating as a single Mendelian unit in [C57BL x (C57BL x GR)F_1]BC_1 mice, and if the presence of specific nucleic acid sequences in the DNA of normal tissue of a given BC_1 animal is associated with the occurrence of early mammary tumorigenesis. These studies were conducted employing both liquid hybridization and restriction endonuclease techniques.

All of the DNAs of apparently normal tissues (such as livers) of GR mice tested individually with radioactive MMTV TAS RNA probe were positive, and all the DNAs of C57BL mice were negative; likewise, all of the apparently normal tissues of (C57BL x GR)F_1 mice contained proviral sequences homologous to the TAS. The [C57BL x (C57BL x GR)F_1]BC_1 mice, however, segregated into two populations: seventeen out of the 33 mice tested contained the TAS sequences in normal cellular DNA, and sixteen did not contain the sequences. The presence of the TAS in DNA of 51.5% of the first backcross generation therefore indicates transmission as a single Mendelian unit.

$C_o t$ analyses were performed using radioactive TAS probe, on DNAs of normal tissues of GR, (C57BL x GR)F_1, and TAS positive BC_1 mice; $C_o t$ 1/2 values demonstrated approximately six copies of TAS in GR mice and three copies in F_1 and positive BC_1 mice. To further rule out the possibility that copy number effects were contributing to the segregation of TAS genes, DNA saturation curves were performed with the DNA from GR, C57BL, F_1, and BC_1 (pools of TAS positive and TAS negative) mice. Increasing the DNA input levels to as high as 1,200 ug for C57BL mice or TAS negative BC_1 mice, did not result in hybridization above background levels.

Spontaneous mammary tumor incidence at 42 weeks was approximately 95% in F_1, and 50% in BC_1 females. When 33 of the BC_1 mice were randomly sacrificed as weanlings (two mice per litter), 51.5% of mice (8 of 16 females and 9 of 17 males) contained proviral sequences homologous to TAS. To determine if there was indeed a correlation between the occurrence of an early mammary tumor and the presence of TAS provirus, the first fourteen females to develop mammary tumors were sacrificed and DNAs from normal tissues (both liver and kidney) were hybridized to radioactive TAS probe. As shown in Table 1, all were positive for TAS. The probability of randomly segregating sequences (i.e., TAS) being present in the DNA of apparently normal tissues of 100%

of the first fourteen tumor bearing mice tested is 3×10^{-5}. Mammary tumors of these mice were also all positive for MMTV antigens by immunoperoxidase. These studies thus demonstrate (a) the segregation of specific MMTV sequences (homologous to TAS) as a single Mendelian unit in GR and C57BL crosses, and (b) an association between the acquisition of these specific proviral sequences, via normal processes of Mendelian segregation of genes, and the occurrence of early mammary tumors in individual animals.

TABLE 1
HYBRIDIZATION OF MMTV(C3H) TUMOR ASSOCIATED SEQUENCES RNA WITH DNA FROM GR, C57BL, (C57BL X GR)F_1 AND C57BL X [C57BL X GR)F_1]BC_1 MICE[a]

SOURCE OF DNA	NO. POSITIVE/ NO. TESTED	% POSITIVE
GR	11/11	100
C57BL	0/10	0
C57BL X GR	23/23	100
C57BL X (C57BL X GR) random selection	17/33	52
C57BL X (C57BL X GR) tumor bearers	14/14	100

[a]500 μg of DNA from livers of individual mice were hybridized to 1,000 cpm of ^{125}I-labeled TAS RNA to a $C_o t$ of 20,000.

Restriction endonuclease studies have demonstrated that Pst-1 cuts MMTV genomic sequences internally. We have employed this enzyme to examine GR, C57BL, F_1 and BC_1 mice. There is a 100% correlation between the existence of Pst-1 bands at 2.5 and 0.6 megadaltons and TAS in DNAs of BC_1 mice. Table 2 and Figure 1 exemplifies some of these results.

TABLE 2

Pst-1 RESTRICTION FRAGMENTS CONTAINING MMTV INFORMATION OF GR, C57BL, (C57BL X GR)F_1 AND [C57BL X (C57BL X GR)F_1]BC_1 MICE[a]

MOLECULAR WEIGHT X 10^6	C57BL	C57BL	GR	GR	F_1	F_1	BC_1 (TAS NEG)	BC_1 (TAS NEG)	BC_1 (TAS +)	BC_1 (TAS +)	Feline: Uninfected	Feline: MMTV Inf.
3.3	+	+	+	+	+	+	+	+	+	+	NEG	NEG
3.1	+	+	NEG	NEG	+	+	+	+	+	+	NEG	NEG
2.5	NEG	NEG	+	+	+	+	NEG	NEG	+	+	NEG	+
1.1	+	+	+	+	+	+	+	+	+	+	NEG	+
0.9	+	+	+	+	+	+	+	+	+	+	NEG	+
0.6	NEG	NEG	+	+	+	+	NEG	NEG	+	+	NEG	+
0.5	+	+	+	+	+	+	+	+	+	+	NEG	NEG

[a]TAS denotes the presence of MMTV(C3H) tumor associated sequences in liquid hybridization. DNAs were restricted with Pst-1 and the fragments separated according to size on a 0.8% agarose gel. The fragments were transferred to nitrocellulose filter paper by the technique of Southern. MMTV specific bands were identified by hybridization to MMTV ^{32}P-labeled cDNA.

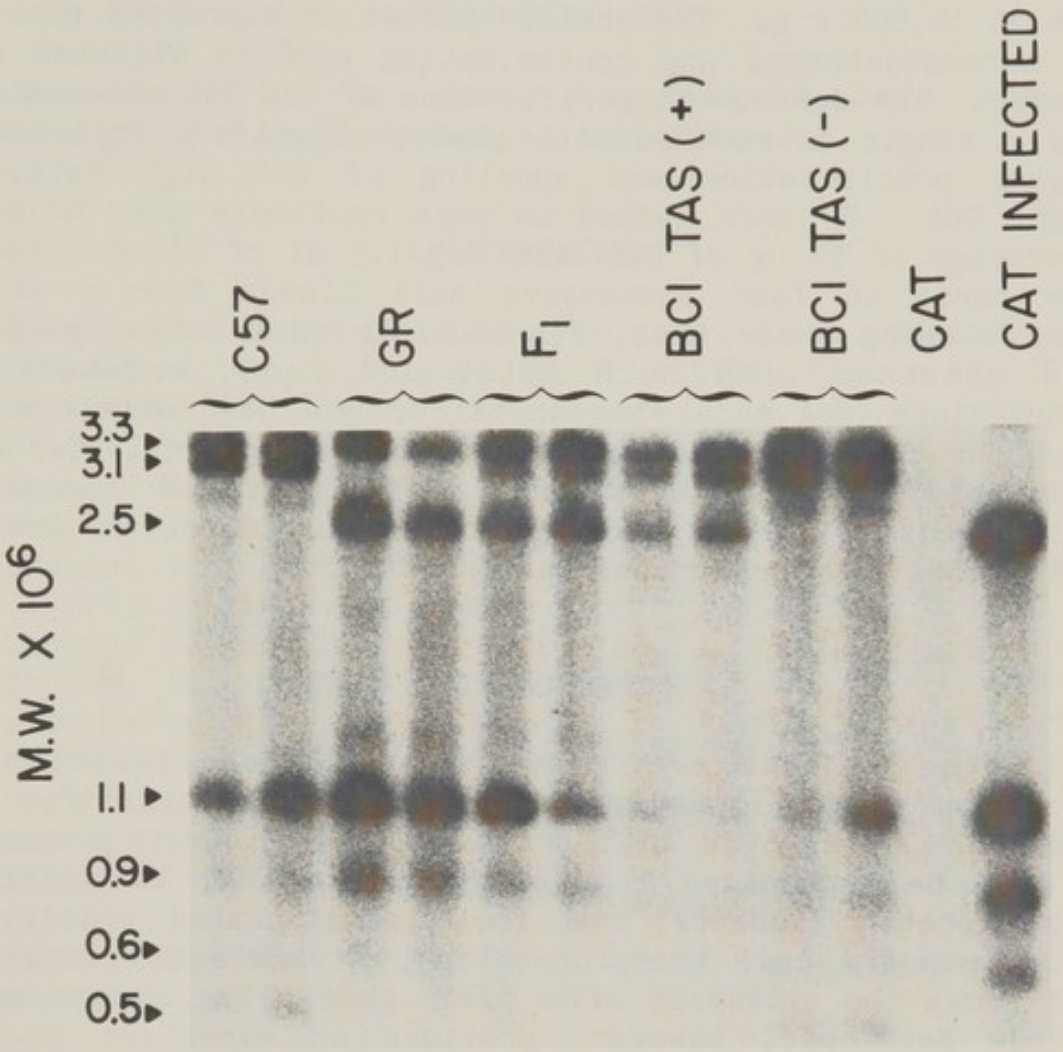

FIGURE 1. See legend to Table 2. The 0.6 x 10^6d restriction fragment is better resolved on 1.5% agarose gels.

We then set out to develop a system in which small amounts of DNA, obtained from normal peripheral blood leukocytes, could be assayed to predict which BC_1 mouse would eventually develop mammary cancer. Initial experiments using standard extraction procedures (9) to spool out high molecular weight DNA from 1-5 ml of fresh or frozen mouse blood failed to consistently yield quantities of high molecular weight DNA suitable for restriction analyses. However, a method developed using NH_4Cl wash to remove erythrocytes and accompanying protein led to obtaining reproducible amounts of spoolable DNA. The method involves: adding five times the blood volume of a 0.83% NH_4Cl solution, disruption by a Silverson homogenizer for ten seconds, pelleting for 15 minutes at 10,000 x g, resuspending in the original volume of 0.83% NH_4Cl and pelleting again for one

hour at 10,000 x g. The nuclear pellet is disrupted with SDS and mercaptoethanol and contaminating protein digested with pronase. The subsequent purification of the DNA necessitates only a single extraction with phenol-chloroform followed by ethanol precipitation and spooling of the high molecular weight DNA. By this method we were routinely able to spool an average of 50 ug of DNA from 1.3-1.5 ml of blood obtained from three to four successive tail bleeds from a single mouse. Using these DNAs, reproducible restriction patterns were obtained with both Pst-1 and EcoR$_1$ endonuclease restrictions. A model has therefore now been developed in which one can determine which siblings of a given litter will ultimately develop mammary cancer. Three to four successive tail bleeds of young adult mice are sufficient to provide quality DNA for such an evaluation.

DISCUSSION

These studies are aimed at the identification of endogenous genetic elements that are responsible for the Mendelian segregation of susceptibility to mammary cancer. It must be emphasized that only the physical isolation of these genetic elements, and their demonstrated ability to induce mammary cell transformation by transfection studies, will prove the existence of a "mam" gene. The system as it is now developed, however, provides an excellent in-vivo model to differentiate tumor initiation from promotion. DNA of blood samples obtained from weanling or young adult BC$_1$ mice will now reveal (by Pst-1 restriction analyses) which siblings of various litters will develop mammary cancer before the age of one year. This provides an excellent opportunity to determine which substances will initiate additional tumor formation (in mice negative for TAS or a 2.5 megadalton Pst-1 cut) versus those substances which will promote an earlier occurrence of mammary tumors in those animals positive for these specific genomic elements. Molecularly cloned MMTV fragments can now also be employed in this model as probes to determine differential degrees of transcription of specific sequences in the two BC$_1$ populations. Finally, these studies begin to shed some insight into the molecular events that manifest themselves in the Mendelian segregation of susceptibility to mammary cancer.

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A STUDY OF THE ENDOGENOUS MOLONEY RELATED SEQUENCES OF MICE[1]

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ABSTRACT Mouse cells carry DNA sequences related to murine C-type viruses in multiple copies. If mouse cell DNA is cleaved with EcoRI (which cleaves most known MuLV DNA's none or only a few times), a large number of MuLV-related restriction fragments are observed, indicating that the endogenous MuLV-related sequences consist of many copies in a large number of chromosomal sites. Digestion of uninfected mouse cell DNA with Kpn I resulted in a greatly simplified pattern of MuLV-related fragments, suggesting that the endogenous MuLV-related sequences consist of a small number of retrovirus-like organizations integrated into many different chromosomal sites. Sequential digestion with Kpn I and EcoRI indicated that three major classes of MuLV-related sequence organizations exist. Comparison of several inbred mouse strains as well as a feral isolate suggest that most of the MuLV-related sequence organizations were introduced into the germline of mice prior to the divergence of the animals studied.

INTRODUCTION

The presence of sequences related to C-type RNA viruses in the normal cellular DNA has been demonstrated for a wide variety of animal species (1). In the results presented here, such sequences in mouse DNA were analyzed.

METHODS

Preparation of DNA's. Cellular DNA was prepared either from the livers, spleen, kidneys, testes or brain of individual inbred mice (2) or from tissue culture cell lines as described previously (3). Radioactive Moloney murine leukemia virus (M-MuLV) cDNA probe (^{32}P-labeled) was prepared by

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reverse transcription of M-MuLV virions as described previously (4). Restriction enzyme fragments of M-MuLV DNA were prepared from a molecular clone of M-MuLV unintegrated DNA and Bam-HI restriction endonucleases. The fragments were purified by preparative agarose gel electrophoresis and electro-eluted from gel slices. The purified fragments were then labeled with ^{32}P by nick-translation (5).

Blot-transfer hybridization. Restriction enzyme digestion followed by agarose gel electrophoresis and blot-transfer to nitrocellulose according to Southern (6) and hybridization were performed as described previously (3). In some experiments, transfer to diazotized (aminothiophenol) cellulose paper (Brian Seed, personal communication) and hybridization in solutions containing dextran sulfate were performed (7).

RESULTS

Identification of MuLV-related sequence organizations in uninfected mouse DNA. Previous work has shown that DNA from normal mouse cells contains multiple copies (20-50) of sequences related to murine leukemia viruses (MuLV's) (8). Some of these MuLV-related sequences may represent endogenous mouse C-type viruses (9), although the number of biologically identifiable endogenous C-type viruses in a given mouse strain are generally less than the number of MuLV-related sequences present. These MuLV-related sequences are in a large number of cellular loci as shown previously in Figure 1A (10,11,3). DNA from Balb/c mice was digested with EcoRI restriction endonuclease and analyzed by agarose gel electrophoresis and Southern blot-transfer hybridization using radioactive cDNA from Moloney MuLV (M-MuLV, an exogenously infecting MuLV). EcoRI does not cleave M-MuLV DNA (12), and it might be expected that it would cleave the endogenous M-MuLV-related sequences none or only a few times as well. In this case, the number of distinct MuLV-related EcoRI fragments (visible as hybridizing bands in the blot) would give an approximate estimate of the number of different loci containing MuLV-related sequences. As shown in the figure, a large number of MuLV-related EcoRI fragments were detected. The complexity of this pattern could be due to heterogeneity of the EcoRI sites in the MuLV-related sequences, it could be due to heterogeneity in the DNA sequences adjacent to the MuLV-related sequences, or both.

In contrast to the EcoRI digestion pattern, a greatly simplified pattern of MuLV-related fragments resulted from digestion of the same Balb/c DNA with Kpn I. As shown in Figure 1B, four major MuLV-related fragments resulted, of 3.5, 2.5, 1.8 and 1.0 md. Kpn I cleaves exogenous M-MuLV

viral DNA at several places, including sites close to both termini (within the terminal redundancy) (13). Thus, cleavage of an integrated M-MuLV provirus with Kpn I would largely free the viral DNA sequences from adjacent cellular DNA sequences. The degree of simplification of the endogenous MuLV-related fragments after Kpn I digestion suggests that these sequences may consist of a small number of virus-like organizations attached to many different adjacent sequences, and that like M-MuLV DNA, Kpn I sites may exist near the termini. Other restriction endonucleases in addition to Kpn I also cleave M-MuLV proviral DNA in its terminal redundancy. If the endogenous MuLV-related sequences consist of organizations similar to M-MuLV viral DNA, then cleavage of uninfected cell DNA with these enzymes might also yield a simple pattern of MuLV-related fragments. When uninfected cell DNA was cleaved with three of these endonucleases (Sac I, Pvu II and Xba I), simple patterns of MuLV-related fragments resulted in all cases (data not shown).

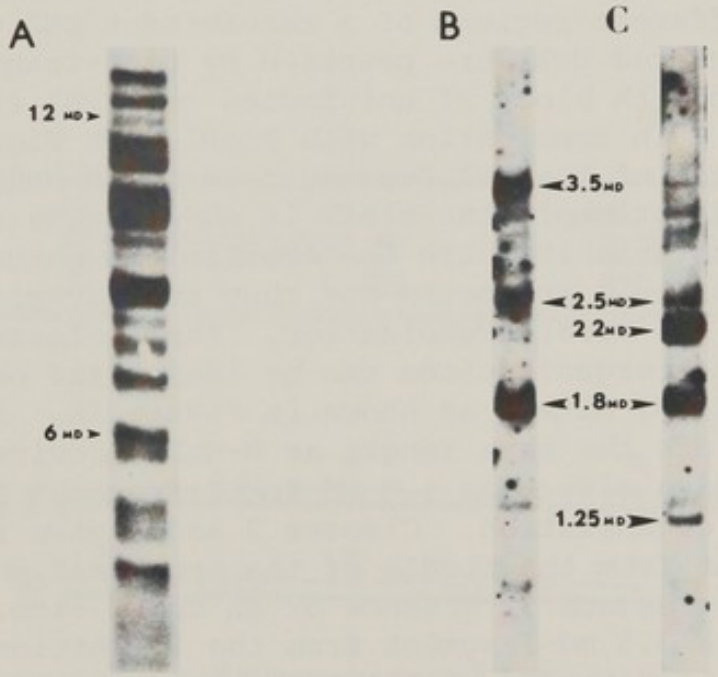

FIGURE 1. DNA from Balb/c mice was cleaved with (A) EcoRI, (B) Kpn I, or (C) sequentially with Kpn I and EcoRI. The digests were run on either a 0.5% (A) or a 0.6% (B and C) agarose gel, transferred to nitrocellulose (6) and hybridized with representative ^{32}P-labeled Moloney MuLV cDNA. Molecular weights were determined by co-electrophoresis of restriction enzyme fragments of lambda DNA and are given in mega daltons (md).

The internal Kpn I fragments of M-MuLV DNA give a combined molecular wieght of 5.4 md, while the combined molecular weight of the endogenous MuLV-related Kpn I fragments is 8.8 md, a value too large to represent one virus-like organization. This suggests that more than one class of MuLV related virus-like organization may exist in uninfected cells. In Figure 1B and 1C, Balb/c cell DNA was digested with Kpn I or Kpn I in combination with Eco RI and analyzed. The 1.8 md MuLV-related Kpn I fragment was not affected by digestion with EcoRI; in contrast, essentially all of the 3.5 md and some of the 2.5 md fragments were cleaved by EcoRI. A new fragment of 2.2 md resulted from the combined Kpn I and EcoRI cleavage, and intensities of hybridization suggest that both the 3.5 md and 2.5 md Kpn I fragments gave rise to this fragment upon EcoR I cleavage. This suggests that the 3.5 and 2.5 md MuLV-related fragments may contain overlapping sequences, and they may represent different virus-like organizations.

In order to further define the organizations of the endogenous MuLV-related sequences, ^{32}P-labeled restriction fragments from different regions of a recombinant DNA clone of M-MuLV unintegrated DNA were prepared by nick-translation and hybridized with blots of uninfected cell DNA cleaved with Kpn I and Kpn I in combination with EcoRI. In Figure 2A, the location of each of the DNA fragments in the M-MuLV genome as well as hybridization to the blots is shown. The results of these hybridizations indicate the overlapping nature of the 3.5 and 2.5 md Kpn I fragments and that they overlap toward the 5'-end of the viral organization. Three classes of MuLV-related sequence organizations can be identified on the basis of Kpn I and EcoRI sites, as shown in Figure 2B. All three organizations are the same length as M-MuLV proviral DNA, and like M-MuLV, give rise to a 1.8 md Kpn I fragment from the 5'-end of the organization. Classes 2 and 3 give rise to a 2.5 md fragment from the middle of the organization, and differ by the presence or absence of an EcoRI site. Class 1 gives rise to a 3.5 md fragment from the 3'-portion of the genome; most, if not all, of the members of this organization contain an EcoRI site at the same location as in Class 2.

The presence of these endogenous MuLV-related sequences in the germline of mice could have resulted from the evolution of endogenous C-type viruses present in the germline of early vertebrates (14), alterations of cellular sequences (15), or from the germline infection of M. musculus. Cohen and Varmus (16) have recently suggested that endogenous mouse mammary tumor viruses (MMTV) have been acquired by the germline infection of M. musculus. They based their conclusion on the fact that both the internal fragments of MMTV-related sequences and the genomic integration sites varied among feral isolates as well as inbred strains.

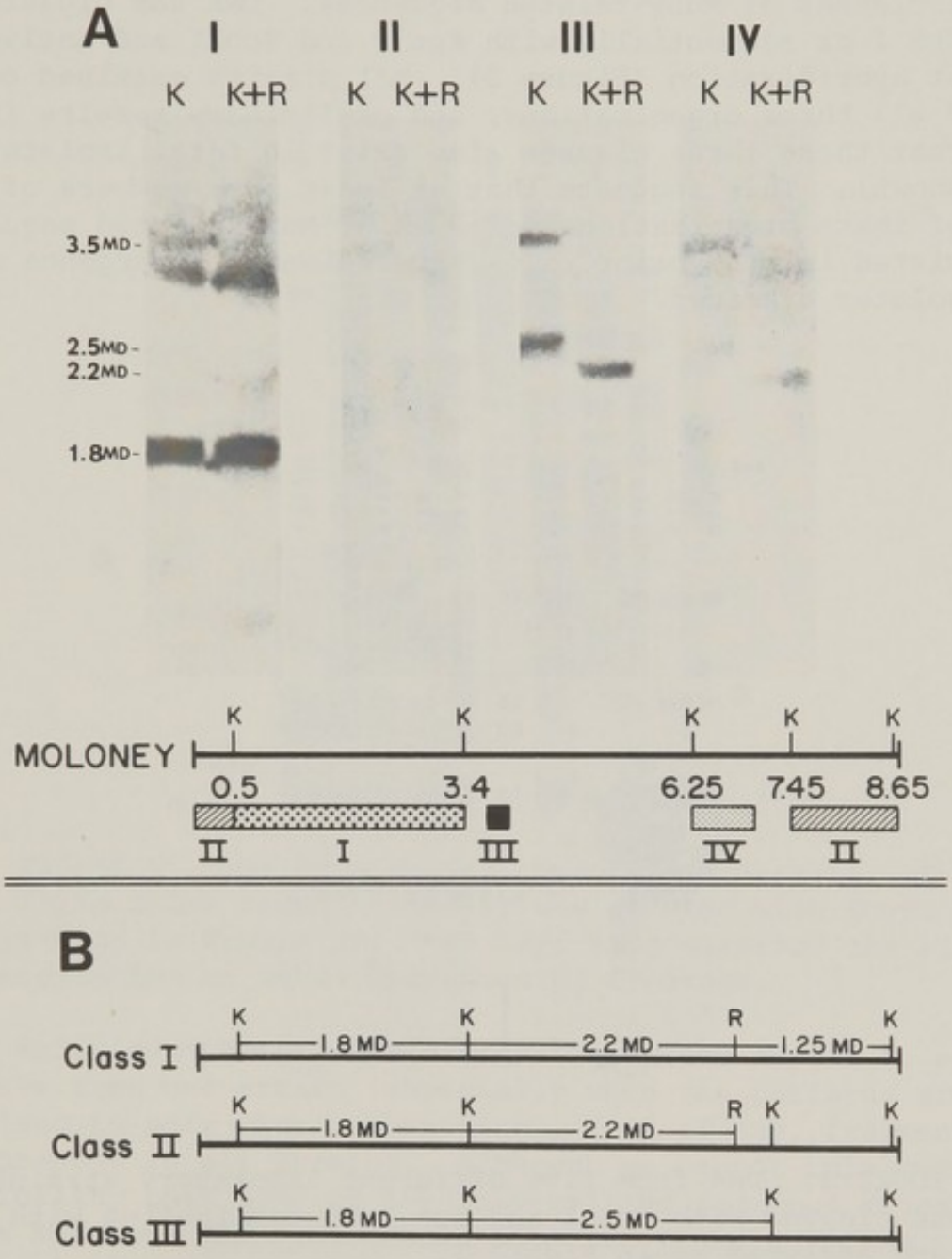

FIGURE 2. (A) Restriction fragments of a recombinant DNA clone of unintegrated M-MuLV DNA (provided by Dr. I. Verma) were labeled by nick-translation and represent a specific region of Moloney viral DNA as shown (13). Balb/c DNA, digested with Kpn I (K) and Kpn I and EcoRI (K and R), was run on a 0.6% agarose gel and transferred to strips of diaziotyzed ATP paper (I-IV). Each strip was hybridized with a nick-translated fragment as indicated. (B) Proposed classes of organization of endogenous MuLV-related sequences.

In a similar fashion, we examined DNA from several inbred strains of mice for the presence of the three organizational classes of MuLV-related sequences. DNA was digested with Kpn I or sequentially with Kpn I and EcoRI and analyzed by blot hybridization (Figure 3). All strains examined contained all three organizations, and preliminary results indicate that these three classes also exist in feral isolates (not shown). This suggests that at least some members of each of these organizational classes of MuLV related sequences existed in mice prior to the evolutionary divergence of the isolates examined.

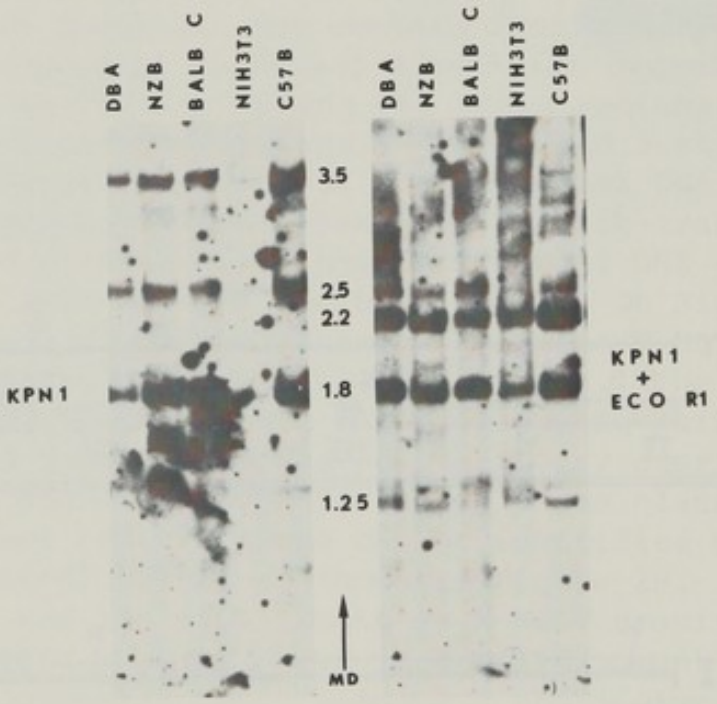

FIGURE 3. DNA from five different laboratory strains of mice was cleaved with Kpn I and Kpn I in combination with EcoRI and analyzed as in Figure 1.

We have also examined the sites of integration of these sequences by cleavage with EcoRI. Since this enzyme cleaves the three organizational classes no more than once, MuLV-related fragments also contain adjacent cellular DNA and can, therefore, be used to identify the location of these sequences in the genome. If MuLV-related sequences evolved from early vertebrate progenitors or from certain rearrangements of cellular sequences, EcoRI digestion of DNA from different isolates should give rise to MuLV related fragments of identical size. In Figure 4, the DNA of several laboratory

strains of mice as well as a feral isolate (SC-1) was digested with EcoRI and analyzed by blot hybridization.

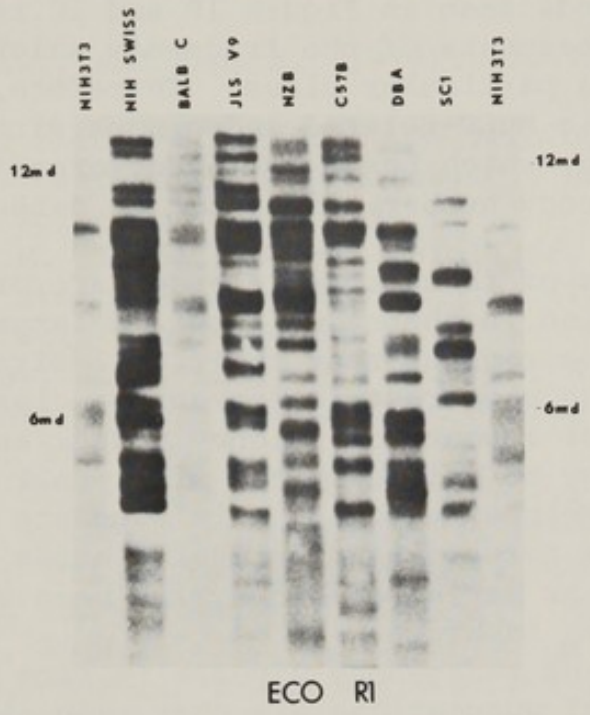

FIGURE 4. DNA from several laboratory strains, as well as a feral mouse isolate (SC-1), was cleaved with EcoRI and analyzed as in Figure 2A. We have also examined the sites of integration of these sequences by cleavage.

While some MuLV-related EcoRI fragments appear to exist in more than one strain (especially when the patterns are examined in pair-wise combination), few, if any, fragments are present in all strains. Although we cannot rule out the possibility that some of the MuLV-related sequence organizations may have been introduced into the mouse germline early in speciation, many of these sequences have been integrated into their present sites more recently. Thus, integration of MuLV-related sequences into the germline of mice appears to be an ongoing process.

DISCUSSION

We have described three major classes of endogenous MuLV related sequence organizations in mice. These organizations are distinguished by their Kpn I and EcoRI cutting patterns and by the homology of fragments generated by these

enzymes to specific regions of the Moloney viral DNA genome. We cannot rule out the possibility that other organizations do exist. Since Kpn I generates the same internal fragments from each member of a given class of virally-related sequences, the gel bands seen in Figure 1B and 1C represent multifold image enhancements of the fragments which are common to all members of a particular class. Therefore, it is possible that any specific MuLV-related endogenous virus or virally-related sequence, which has a different organization and is present in low copy number, might not be detected by this method.

The members of these three classes are organized in a virus-like fashion in that they occur in large contiguous blocks; they have regions near their cell-viral boundaries which contain the same restriction enzyme cleavage sites as the long terminal repeats of Moloney virus; and they have sequence homology to Moloney virus within all the functional regions of the Moloney genome. This suggests that many members of these three organizational classes may, in fact, be true viruses or may have at one time been viruses, but have since become inactive.

The origin of endogenous viruses in mice is unclear. The two principal suggestions are that they evolved from the endogenous viruses of early vertebrates (14) or they have been generated by the alteration of cellular sequences (15). We would argue that, if the majority of MuLV-related sequences arose by either of these two mechanisms, it is likely that these sequences would have been present quite early during the speciation of mice and would, therefore, appear as identical EcoRI fragments in the isolates we have examined. As shown in Figure 4, some of the fragments generated by EcoRI digestion of various mouse cell DNA's are common to many of the strains. Since each track has a very high density of bands, it is difficult to clearly distinguish common fragments. While some integrations may be common to all mice, the majority of integration sites appear to vary among different isolates. On the other hand, all isolates that we have examined do contain representatives of all three classes of MuLV-related sequences (Figure 3). This suggests that these sequence organizations were acquired prior to the evolutionary divergence of the mice we examined.

Thus, we conclude that the three classes of virus-like organizations have been present in mice throughout speciation. The sites of integration of these organizations vary among the mice we have examined. These integrations may have been generated by germline infection and we suggest that these infections are an ongoing process.

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STRUCTURAL AND GENETIC RELATIONSHIPS BETWEEN AN ENDOGENOUS RETROVIRUS (M432) OF MUS CERVICOLOR AND INTRACISTERNAL A-PARTICLES OF MUS MUSCULUS

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ABSTRACT A novel class of endogenous retroviruses has been isolated from the Asian murine species Mus cervicolor; the genome of the prototype virus, M432, has recently been shown by molecular hybridization to be related to the genome of intracisternal type-A particles (IAP); retrovirus like entities seen in many tumors of M. musculus. We have now shown that the major internal protein of the M432 virus (p24) is immunologically related to the respective protein of IAP (p73).

Nucleic acid sequences related to the M432 and IAP genomes are found in multiple copies in cellular DNA from members of the genus Mus. The organization of these sequences in the DNA of individuals of a feral population of M. cervicolor and various inbred strains of M. musculus has been studied using restriction endonucleases and the Southern blotting technique. The results indicate that the pattern of cellular DNA fragments containing viral related sequences is well-conserved within members of each species, whereas major differences are seen when different species are compared.

INTRODUCTION

A novel class of endogenous retroviruses, designated the M432 group, has been identified (1,2). The prototype virus, M432, was initially isolated by the long term cocultivation (9 months) of a normal M. cervicolor cervicolor spleen with NIH Swiss tissue culture cells. The host range of this group of retroviruses is restricted to cell cultures derived from M. musculus. Cells releasing the virus contain intracytoplasmic A particles. The mature viral particles were shown by electron microscopy to have a novel morphology, distinct from known type B, type C and type D retroviruses. The M432

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virus, contains an RNA genome which shares no nucleic acid sequence homology with the genomes of other Retroviridae (2). The viral associated proteins include a magnesium-dependent reverse transcriptase (70,000 daltons), two glycosylated envelope proteins (gp69 and gp33), a major internal protein (p24), a second internal protein (p16) and a phosphoprotein (p12)(2). A radioimmunoassay developed for the p24 protein detects this antigen in cultures infected with the virus. In this assay other mammalian retroviruses including type B and C viruses from M. cervicolor and M. musculus fail to compete with the labeled antigen. Similar immunological data have been obtained using sera prepared against the M432 reverse transcriptase. Recently, the RNA genome of intracisternal type-A particles (IAP) of M. musculus were found to share 25-30% sequence homology with the M432 viral genome (3). IAP are endogenous retroviral-like entities observed in a variety of tumors and in early embryos of M. musculus (4). IAPs do not have a typical retroviral structure (5,6,7). Their outer shell is derived from the endoplasmic reticulum; whether any components of this shell are IAP-specific is not known. The particle core is composed of a major protein (p73)(5,6) which is phosphorylated (E. L. Kuff and J. Fewell, unpublished results). This protein carries particle specific antigenic determinants and is coded for by the IAP high molecular weight RNA (8). The core also contains a reverse transcriptase (9) as well as other minor proteins that are related to the p73 (6,10). To further characterize the relationship between IAPs of M. musculus and the M432 group of endogenous retroviruses, we have undertaken a comparative immunological study of their respective structural proteins (11). We find a strong cross-reaction between the major internal structural proteins of these two types of particles, i.e. between p24 of M432 and p73 of IAPs. Other M432 associated viral proteins do not share antigenic determinants in common with the IAP p73. Using interspecies radioimmunoassays, based on the cross-reactivity of the major internal proteins of these particles, we have been unable to detect related antigenic determinants on the respective proteins of mammalian type B, type C or type D endogenous retroviruses. These results taken together with earlier studies (2) using homologous radioimmunoassays and molecular hybridization support the conclusion that the M432 group of endogenous retroviruses represent a new class of Retroviridae.

In addition to the unique physical and biochemical properties of the M432 group of endogenous retroviruses, sequences related to the M432 viral genome are highly conserved in the cellular DNA of members of the genus Mus (1,2,12). These sequences have a reiteration frequency of 25 copies per

haploid genome in M. cervicolor (1). In M. musculus, M432 related DNA sequences are represented in the IAP genes, which have a reiteration frequency of 500 to 1000 copies per haploid genome (3,13). The conservation of the viral related DNA sequences, using M. cervicolor as the point of reference, is consistent with the evolutionary divergence of the different murine species as determined by the degree of sequence homology between their non-repetitive cellular DNAs (2,12). We have examined the conservation of M432 related sequences in the cellular DNA of individual mice of outbred colonies of M. cervicolor and different strains of M. musculus, using restriction endonucleases and the Southern blotting technique. These experiments suggest that the arrangement of viral sequences is well conserved within members of each species, whereas major differences are observed when different species are compared.

RESULTS

Immunological Relationship between the M432 Virus and Intracisternal Type-A Particles (IAP). The potential immunological relationship between one or more of the M432 viral associated proteins and those of IAP from M. musculus was initially explored by immunoprecipitation of in vivo (^{35}S methionine) labeled virus and partially purified IAP. The results of these experiments (Figure 1A) using antisera prepared against the M432 viral proteins and the IAP p73 show that the pattern of IAP proteins precipitated by Anti-M432 virion or Anti-M432 p24 sera are identical to those precipitated by Anti-IAP p73 (11). In contrast, the Anti-IAP p73 sera precipitated only the M432 p24 protein and a minor 45,000 dalton protein in the virus preparation. Preliminary evidence suggests that the latter protein, which is observed infrequently in virus preparations, is a precursor to p24. (R. Callahan, unpublished results).

Since the IAP p73 is phosphoprotein it was of interest to determine its immunological relationship to the M432 viral phosphoprotein p12. The viral p12 is poorly labeled with ^{35}S methionine, so M432 virus and IAPs were prepared from cultures labeled with (^{32}P) - orthophosphate. Analysis of the ^{32}P labeled M432 proteins reveals two labeled viral proteins with apparent molecular weights of 16,000 and 12,000 daltons (Figure 1B). Anti M432 virion serum reacted with both of the homologous phosphoproteins and also with IAP p73, whereas, Anti p73 reacted only with its homologous antigen. In summary these studies, using the available antisera, suggest a specific cross reactivity between the major internal structural proteins (respectively, p24 and p73) of the two types

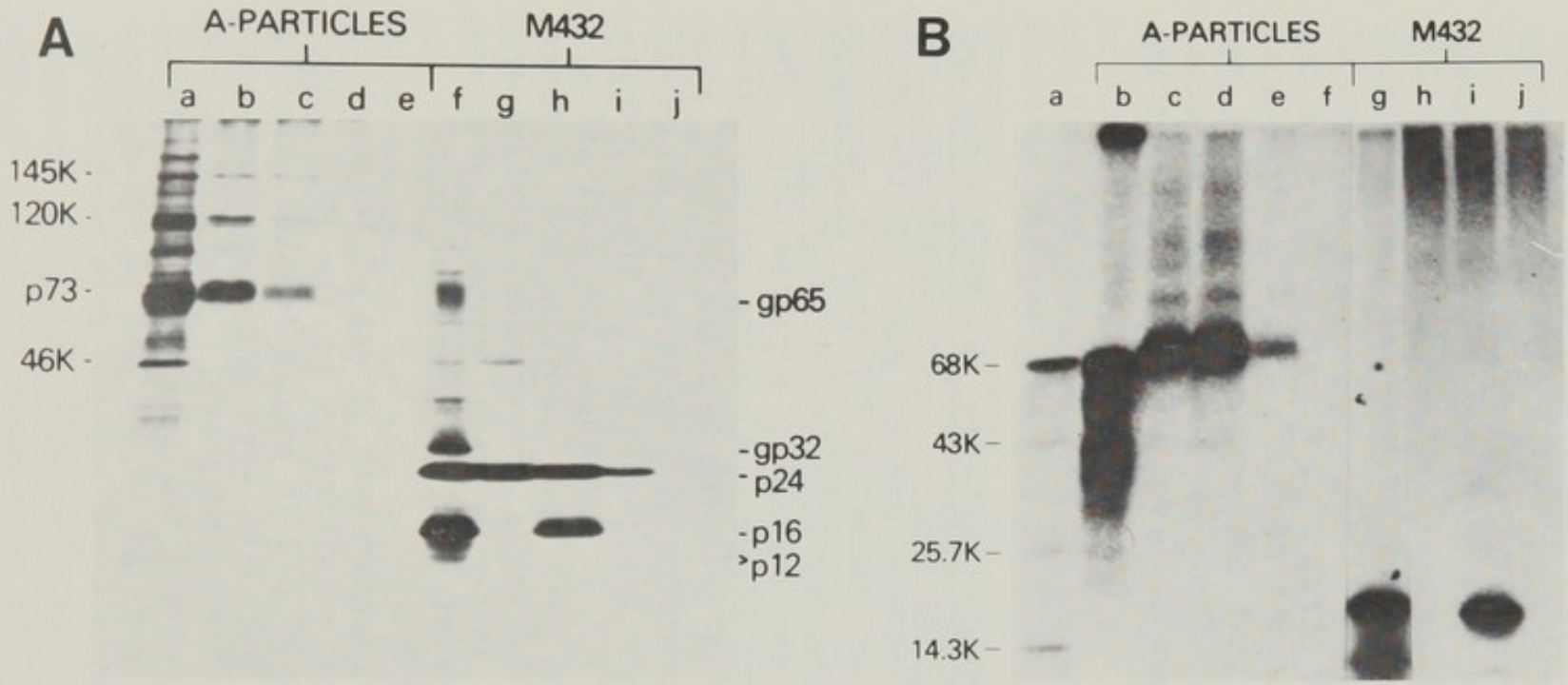

Figure 1. Immunoprecipitation of (A) [^{35}S] methionine-labeled and (B) [^{32}P]-labeled IAP and M432 associated proteins. Procedures for labeling in vivo, particle purification, immunoprecipitation, and analysis by SDS polyacrylamide gel electrophoresis followed by autoradiography have been previously described (11). (A) Lanes a and f, aliquots of the whole IAP and M432 preparations, respectively; b and g, immunoprecipations with Anti-p73 serum (Anti-IAP p73); c and h, with (Anti-M432 viron serum); d and i, with (Anti-M432 p24 serum); e and j, with non-specific rabbit serum (antigoat). (B) Lane a, [^{14}C]-labeled protein standards (bovine serum albumin, 68,000 daltons; ovalbumin 43,000; α-chymotrypsinogen 25,700; and lysozyme, 14,300). Lanes b and g, aliquots of the whole IAP and M432 preparations, respectively; c, d, and h, immunoprecipitations with Anti-p73, 2 µl, 4 µl, and 4 µl, respectively; e and i; immunoprecipitations with Anti-M432 viron, 4 µl, f and j, reaction with nonspecific rabbit serum, 4 µl.

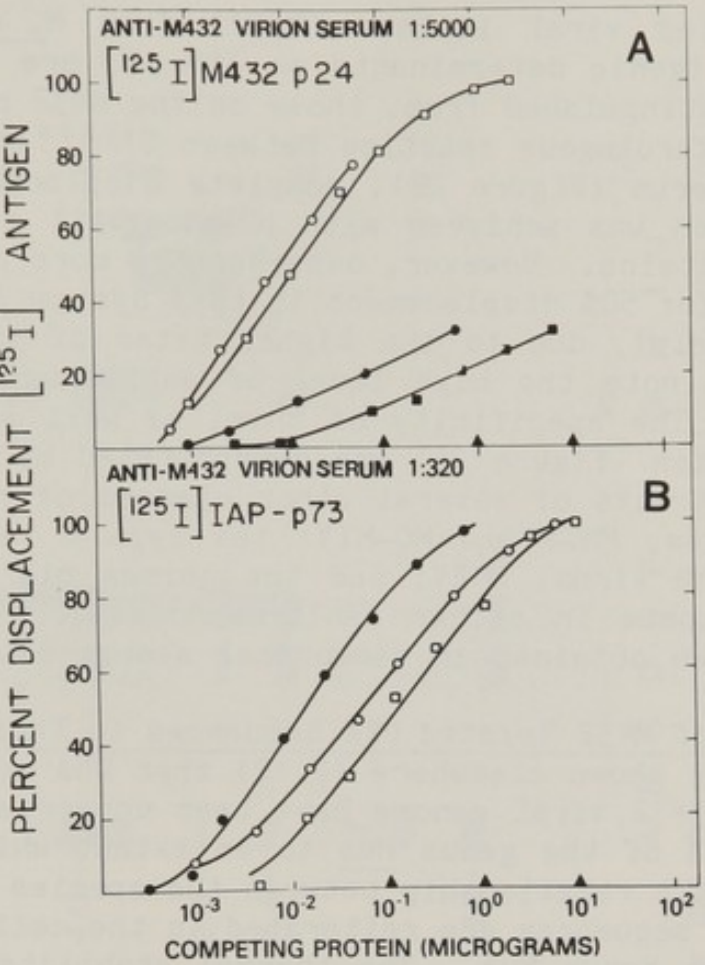

Figure 2. Competitive radioimmunoassay for IAP and M432 viral proteins using Anti-M432 virion serum with (A) [^{125}I] - M432-p24 and (B) [^{125}I]IAP-p73 as antigens. Components of the reaction mixtures and reaction conditions have been described elsewhere (2,11). Nonradioactive competing proteins were: (O) M432-p24; (□) whole M432 virus; (●) IAP-p73; (■) whole MOPC-104E myeloma IAPs; ( ) murine mammary tumor virus (MMTV); M. cervicolor mammary tumor virus (MC-MTV); guinea pig leukemia virus (B-GLV), squirrel monkey retrovirus (SMRV) and Raucher leukemia virus (RMuLV). In the absence of competing antigen 38% of input [^{125}I]-p24 was bound (A) and 21% of [^{125}I]-p73 of input was bound (B).

of particles.

On this basis, competition radioimmunoassays were developed to further analyze the nature of this relationship. Anti-M432 virion sera and [^{125}I]-p24 were used in a homologous assay as previously described (2). As shown in Figure 2A 50% displacement was achieved with 6 ng of competing purified p24 or 12 ng of whole M432 virus. Both purified p73 and whole IAP compete with the [^{125}I]-p24; however, the slope of the competition curve is reduced when compared to the homologous reaction. Similar results were observed in this assay

using a related viral isolate (M832) from M. caroli (2). Thus, the antigenic determinants on IAP p73 are related to, but can be distinguished from, those on the M432 p24.

In the heterologous reaction between [^{125}I] p73 and Anti M432 virion serum (Figure 2B), complete displacement of the labeled antigen was achieved with p24 as well as the whole M432 virus proteins. However, considerably more p24 than p73 was required for 50% displacement in this system (55 ng vs 15 ng, respectively), due to the higher titer of the sera for this antigen (note the high level of antiserum employed in this assay). The specificity of this, as well as, the homologous reaction (Figure 2A) was demonstrated by the failure of protein extracts of several other classes of retroviridae (type B viruses, MMTV and MC-MTV; the type C virus, RMuLV, and the type D virus, SMRV; and the guinea pig retrovirus, B-GLV) to compete in either radioimmunoassay. Similar results have been obtained in reciprocal assays using Anti p73 (11).

Conservation of M432 Related DNA Sequences in Individual Mice. We have shown elsewhere (2,12) that DNA sequences related to the M432 viral genome have been conserved in a number of species of the genus Mus to an extent which reflects the phylogenetic relationship between the species (2). M432 viral related sequences are reiterated in the cellular DNA of members of the genus Mus. The thermal stability (2,3) and, more recently, the molecular cloning of M432 (14) and IAP related cellular DNA sequences (15) suggests that they represent families of partially related sequences. Based on these observations, it was of interest to determine the genetic stability of the viral related sequences within the cellular DNA from individuals of a given species using restriction endonuclease and the Southern blotting technique (16). Recently we have established separate outbred colonies of M. cervicolor representing different provinces or regions of Thailand (M. cervicolor cervicolor: Chantaburi, Lampang, Lampang Forest, and Loei; M. cervicolor popaeus: Chantaburi, Khorat, Saraburi, and Tak)(12). DNA was extracted from liver tissue of individual mice representing each of these colonies and digested with the restriction endonuclease Bgl II. Preliminary studies have shown that digestion of M432 linear unintegrated viral DNA with this enzyme results in the appearance of three DNA fragments (from 5' to 3') 1.1 Kb, 4.3. Kb. and 2.7 Kb (14). Analysis of Bgl II digests of M. cervicolor cervicolor and M. cervicolor popaeus cellular DNA with [^{32}P] M432 cDNA (representative of the entire viral genome) reveals a number of viral related fragments, some of which correspond in size with those observed with digests of unintegrated viral DNA (Figure 3A). However, the striking aspect of this

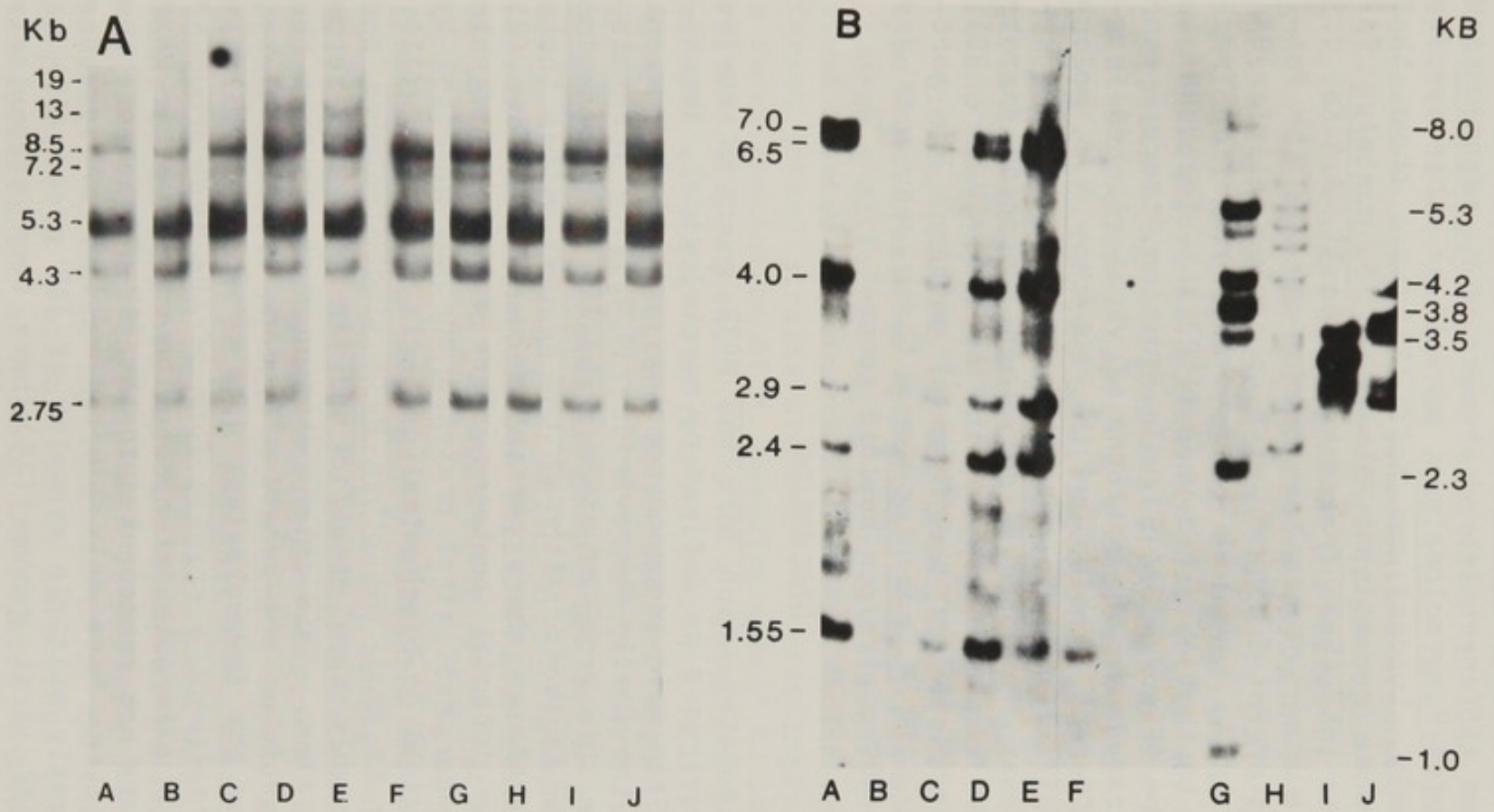

Figure 3. Analysis of M432 related cellular DNA sequences by restriction enzyme mapping. Mouse liver DNA (5 µg) was digested with (A) Bgl II or (B) Pst I and fractionated by electrophoresis on a 0.8% agarose gel, transferred to cellulose nitrate filters (16) and hybridized with [^{32}P] M432 cDNA (14). The digested samples were from (A), M. cervicolor cervicolor mice (a and b) Chantaburi, mice #1 and #2; (c) Lampang, (d) Lampang Forest, (e) Loei provinces and M. cervicolor popaesus from: (f and g) Tak, mice #1 and #2; (h) Chantaburi, (i) Khorat, (j) Saraburi provinces. (B) M. musculus strains (a-e); (a) Balb/c, (b) AKR, (c) C3H, (d) NIH Swiss, (e) GR, (f) M.m. molossinus and other species, (h) M. cervicolor, (i) M. cookii, (j) M. caroli and (k) M. dunni.

study is that the pattern of viral related fragments from each animal appear identical.

Similar studies were carried out with Pst-1 digests of cellular DNA from different inbred strains of M. musculus and an Asian subspecies M.m. molossinus, as well as other species of Mus. Recently, Lueders and Kuff (15) have shown that Pst-I cleaves some recombinant clones of M. musculus IAP related sequences into three fragments (5' to 3', 2.3 Kb, 1.3 Kb. 2.7Kb) while other clones lack one or more of the Pst-I sites. Analysis of Pst-I digests of M. musculus cellular DNA with [^{32}P] M432 cDNA, reveals a pattern of restriction fragments identical to that observed when [^{32}P] IAP cloned DNA is used as the probe (Figure 3B)(15). In addition, there are no apparent diffferences in the patterns of viral related fragments among different strains or the subspecy M.m. molossinus. Major differences in patterns of viral related restriction fragments are observed only when comparing digests of cellular DNA from different murine species (Figure 3B). The relative simplicity of the fragment patterns are particularly striking in M. musculus where these sequences are present in 500-1000 copies per haploid genome.

DISCUSSION

The viral isolates which form the M432 group of murine endogenous retroviruses as defined by morphological, immunological and nucleic acid sequence homology criteria represent a new class of the family Retroviridae (1,2,11). The relationship between this group of viruses and IAP of M. musculus is at present unresolved. Although the genomes within these two types of particles share partial sequence homology (3) and their major internal proteins share related antigenic determinants, other M432 viral proteins are not detected by either immunological or biochemical criteria in the IAP (11). one possibility is that the other M432 viral proteins are antigenically highly type specific and the Anti p73 serum does not detect them. This interpretation suggests that the two types of particles are genetically more broadly related than revealed by the immunological data presently available and that IAPs represent a cryptic form of the M432 group of retroviruses. An alternate interpretation is that other M432 viral proteins are not represented in the IAP-p73 because the two genetic entities have only limited regions of sequence homology. Recently multiple examples of IAP genes (15) as well as the M432 viral genome (14) have been molecularly cloned in bacteriophage lambda. In addition, IAPs have recently been observed in a tissue culture cell line derived from M. cervicolor mammary adenoacanthoma (R. Callahan,

unpublished results). Currently, studies are in progress to distinguish between these possibilities.

Previously we have shown that sequences related to the M432 viral genome are conserved in the cellular DNA of species of the genus Mus (1,2,14). This property is in sharp contrast to the lack of conservation of endogenous type B (17) and type C viruses of M. musculus (18) or type CII (12,16) viruses of other murine species. The M432 viral genome is represented in murine cellular DNA as a family of partially related sequences (1,2,12). Although the organization of M432 viral related sequences in cellular DNA is as yet unknown, we have shown by restriction enzyme mapping that the pattern of cellular DNA fragments containing M432 viral related sequences appears genetically stable among individuals of an outbred populations of M. cervicolor, as well as various inbred strains of M. musculus. Moreover, unique restriction patterns were observed only when comparing different species.

Similar studies on the organization of type B (19) and type C (20) related endogenous retrovirogenes of M. musculus present a different picture. Here the restriction patterns of viral related sequences in cellular DNA varied among the different inbred strains studied. Interestingly, a few mice of a feral population of M. musculus appear to lack type B viral related DNA sequences (19). At a biological level, these two classes of endogenous retroviruses are readily isolated from mice or tissue culture cells and are capable of infectious growth in M. musculus. In contrast, the host range of the M432 group of viruses is restricted to M. musculus. Moreover, the frequency with which M432 related viruses are released from tissue culture cells or expressed in tissues, in an infectious form, is apparently rare. After repeated attempts, only two additional isolates have been obtained: one from M. cervicolor popaeus (R. Callahan, unpublished results) and another from a M. caroli heart tissue culture cell line (2). Similarily, IAPs of M. musculus have never been found to have an infectious extracellular form. Attempts to induce either the M432 viruses (R. Callahan, unpublished results) or IAPs from virus or IAP particle-free tissue culture cell lines with pyrimidine analogues have been unsuccessful. Thus, it is possible that the apparent genetic stability of M432 and IAP related sequences in cellular DNA reflects their inability to escape the constraints of cellular DNA replication which is available to an infectious virus. Moreover, the conservation of M432 or IAP related sequences in cellular DNA suggests that they have some selective value for the species.

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GENETIC CONTROL OF MuLV EXPRESSION AND SPONTANEOUS LYMPHOMA IN CROSSES OF HIGH- AND LOW-LYMPHOMA STRAINS[1]

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ABSTRACT Previous studies in the crosses, AKR × BALB/c and AKR × RF, showed that alleles at the *Fv-1* locus were the major factors determining the resistance of F_1 mice to spontaneous lymphoma, and that these *Fv-1* alleles acted by suppressing the expression of endogenous ecotropic and/or xenotropic MuLV expression. We now report studies in three new crosses, AKR × C57L, AKR × DBA/1 and C58 × DBA/2. Our findings show that *Fv-1* is not a factor in the low-lymphoma phenotype of F_1 mice of these crosses. Rather, one or more new genes appear to govern this resistance to lymphoma, but present evidence indicates that the mechanism of resistance in these cases also involves suppression of endogenous MuLV expression.

Mice of virtually all laboratory strains develop some incidence of lymphoma, but in most strains the disease occurs only occasionally and in animals of an advanced age, *i.e.*, more than one year old. There exist a few strains which are characterized by a high incidence of the disease, usually beginning at six to eight months of age. The best known high-lymphoma strains are AKR, C58, PL, C3H/Fg and SJL, the last of these differing from the first four in that the disease is a Hodgkin's-like reticulum cell sarcoma rather than thymic lymphoma.

The discovery by Gross that cell-free extracts of lymphomatous tissues of mice of high-incidence strains could induce the disease in mice of certain low-incidence strains (1) provided the first major tool for investigation of the etiology of lymphoma. However, it was a great disadvantage that the

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lymphomagenic agent in these tissue extracts, called Gross virus, could be studied directly only by the extremely lengthy assay based on its capacity to induce the disease in susceptible mice.

Eventually assays were developed which detected viruses derived from lymphomatous tissues AKR mice, and these retroviruses proved to be structurally similar to those identified as etiologic agents in avian leukosis. A picture has gradually emerged from intensive studies in several laboratories according to which several related but nonidentical viruses can be demonstrated in mice of high-lymphoma strains, and the genesis of the disease depends upon an interaction among these viruses.

It appears that the genomes of at least two different types of retroviral genomes are integrally included in the form of DNA in the chromosomal complement of AKR mice (the most intensively studied of the high-lymphoma strains), and these endogenous genomes are transmitted by the mice as simple dominant Mendelian traits (2,3). One type, referred to as ecotropic murine leukemia virus (MuLV), is highly infectious for cells of many mouse strains but is poorly infectious at best for cells of other animal species. Another, called xenotropic MuLV, shows a different host range, being capable of infecting certain cells of nonmurine species but not mouse cells. AKR mouse tissues show high levels of infectious ecotropic MuLV throughout life from around the time of birth (4), and xenotropic MuLV can be detected in their lymphoid tissues much later in life, from around six months of age (5). These viruses can be isolated and inoculated into mice of Gross virus-susceptible strains, but such experiments have indicated that neither virus alone has the capacity to induce lymphoma. Rather, lymphomagenicity currently appears to be a property of a third variety of retrovirus recoverable from older AKR mice (6). The genome of this type of virus does not appear to exist as such in the AKR chromosomal complement. Instead, it occurs as a result of genetic recombinations between the genomes of ecotropic and xenotropic MuLV; it shares the host range of both parental viruses, giving rise to the name, polytropic or dual-tropic MuLV. It is likely that viruses of this latter category might prove to be the active lymphomagenic principle in preparations of Gross virus.

ECOTROPIC MuLV AND LYMPHOMA

The Mendelian transmission in mice of endogenous MuLV genomes is a major factor to be considered in any attempt to

analyze mouse lymphoma as a genetically determined trait. However, these viral genomes are transmitted as *dominant* traits in crosses, whereas in crosses of high-lymphoma and low-lymphoma strains the high-lymphoma phenotype is a *recessive* trait in most cases. This observation implies that mice of most low-lymphoma strains possess genes capable of suppressing the emergence of the disease. Such genes may be regarded as regulatory genes, and studies of their nature have revealed that they are a diverse set and exert their influence in different ways.

One important way in which a host gene can confer relative resistance to lymphoma is by increasing the capacity of the animal to respond immunologically to virus-induced, tumor-associated antigens on the surfaces of lymphoma cells. A major example of this type of genetic factor in mice is the *H-2* major histocompatibility complex, a group of closely linked genes in chromosome 17 (7). Mice of different *H-2* genotypes show different patterns of lymphoma occurrence, as best illustrated perhaps in the comparison of AKR and AKR-$H\text{-}2^b$ mice (8), the latter of which differ from the former only with respect to the chromosomal segment bearing the *H-2* complex and which nevertheless show a markedly delayed occurrence of lymphoma by comparison with AKR.

Another way that host genes can influence the occurrence of lymphoma is by a direct effect on the expression of endogenous viral genomes. The most intensively studied gene of this category in mice is *Fv-1*. Present evidence suggests that, although the genome of ecotropic MuLV is present in every nucleated cell of AKR mice, this genetic material is expressed in the form of infectious virus only rarely. It is not yet known if this apparent inactivity is due to active repression of the viral genome by the host cell or more simply to failure of the genome to be activated in some manner. Once the viral genome becomes active in a cell, however, infectious particles released from the cell as a result may pass to other cells of the individual and establish a nonlethal virus-producer state in the recruited cells. It is at this level of infectious cell-to-cell spread of virus that *Fv-1* exerts its influence (9), since cells of certain *Fv-1* types are relatively resistant to exogenous infection by particular types of ecotropic MuLV.

The relation between *Fv-1*-controlled resistance to exogenous MuLV infection and resistance to spontaneous lymphoma was first demonstrated in the cross, AKR × BALB/c (10). In this example of a high- × low-lymphoma cross, F_1 mice show

only a low and late incidence of the disease, similar to that in the parental BALB/c strain. Furthermore these F_1 mice show a markedly reduced level of expression of the ecotropic MuLV found at high levels in the tissues of their AKR parents. Studies of mice of the backcross generation (AKR × BALB/c)F_1 × AKR, showed that low virus expression, attributable mainly to the presence of the *Fv-1*^{b} allele inherited from the BALB/c ancestor, and resistance to spontaneous lymphoma were closely associated traits; lymphoma occurred at a mean age of ten months with increasing frequency in direct proportion to the levels of virus expression detected in the tissues of six-week-old mice. The paradox of this finding, made before the identification of xenotropic MuLV in AKR mice, lay in the fact that, though the experiment strongly implicated ecotropic MuLV as a major factor in the genesis of the disease, it had not been possible to induced lymphoma by injecting the virus into mice.

XENOTROPIC MuLV AND LYMPHOMA

Soon after completion of the studies in the AKR × BALB/c cross summarized above, we noted that F_1 mice of the cross, AKR × RF, also showed a low-lymphoma phenotype, and yet in this case the F_1 mice showed levels of ecotropic MuLV approximating those in parental strain AKR mice. Since AKR and RF mice were presumed to be identical in their *Fv-1* types at that point in time, it was surprising to find that, in the backcross (AKR × RF)F_1 × AKR, resistance to lymphomagenesis was seen mainly in those animals which had inherited the *Fv-1* allele of the RF grandparent (11).

Thus in two different crosses AKR lymphoma was suppressed by non-identical alleles at the *Fv-1* locus, but in one case the resistance was associated with suppression of ecotropic MuLV and in the other case suppression of this virus was weak, at best. Further studies in this cross then showed that *Fv-1* did indeed exert a major influence on the expression of the newly demonstrated xenotropic MuLV of AKR mice. Animals of the AKR × RF cross which had inherited an *Fv-1* allele from the RF strain, unlike their AKR ancestors, failed to express xenotropic MuLV in their lymphoid tissues at six to eight months, but rather did so only well after one year of age, and this suppression of xenotropic MuLV expression was associated with a marked suppression of lymphoma. From these experiments it appears that both viruses, ecotropic and xenotropic MuLV, play a role in the etiology of lymphoma in high-incidence mouse strains.

FURTHER HIGH- X LOW-LYMPHOMA CROSSES: LYMPHOMA INCIDENCE

Given the unexpected finding that a cross of mouse strains previously believed to share the same *Fv-1* type (AKR and RF) in fact differed at *Fv-1* in a way that was relevant to lymphomagenesis, we began a study of three additional crosses in which the high-lymphoma type was recessive but the parental strains were not known to differ at the *Fv-1* locus. These crosses were: AKR × C57L, AKR × DBA/1 and C58 × DBA/2.

Table I summarizes the occurrence of spontaneous lymphoma by 500 days of age in mice of these three crosses. Mice of each of the three F_1 populations showed, as expected only a low (10-12%) and late incidence of the disease, confirming the recessive nature of its transmission in the crosses. However, when these low-lymphoma F_1 mice were backcrossed to the high-lymphoma parental strain, AKR or C58, mice of each of the backcross populations showed a high incidence of the disease, ranging from 71 to 77% in the three populations. The maternal parent in each cross was a mouse of the high-lymphoma parental strain, in order to avoid possible "maternal resistance factors" that might be transmitted by mothers of the low-lymphoma strains (12).

TABLE I
DOMINANT RESISTANCE TO SPONTANEOUS LYMPHOMA
IN CROSSES OF HIGH- AND LOW-LYMPHOMA MOUSE STRAINS

Mice	No. of mice	Cumulative lymphoma incidence at 500 days of age
AKR	30	97%
(AKR × C57L)F_1	45	11%
AKR × F_1	115	77%
(AKR × DBA/1)F_1	45	15%
AKR × F_1	141	76%
C58	40	85%
C58 × DBA/2)F_1	47	10%
C58 × F_1	115	71%

The lymphoma incidences in these backcross populations can be used to arrive at an estimate of the number of genes involved in the occurrence of the disease in each cross. The simplest interpretation of the data indicates that each low-lymphoma parent possesses two dominant genes, both of which must be present simulataneously in order to confer the lymphoma-resistant phenotype. To illustrate this interpretation, assume the existence of two unlinked loci, *A* and *B*. Mice of a high-lymphoma strain possess recessive alleles for susceptibility at both loci (*aabb*); mice of a low-lymphoma strain possess dominant alleles for resistance at both loci (*AABB*); and F_1 mice are heterozygous at both loci (*AaBb*) and are resistant because they have one allele for resistance at each locus. Mice of the backcross population, resulting from an *aabb* × *AaBb* mating, should then be of four different types, occurring with equal frequency in the population; *AaBb*, *Aabb*, *aaBb* and *aabb*. Of these four types, only the first possesses dominant alleles for resistance at both loci, and only these mice would fail to develop the disease, whereas the other three types, representing about 75% of the population, would develop lymphoma.

Having inferred by this reasoning that each of the low-lymphoma parents in the three crosses studied transmitted two dominant genes for resistance, the next question concerned the identity of these genes. Backcross mice were tested individually for their phenotypes at the *H-2* and *Gpd-1* loci; *Gpd-1* was used as a marker for the *Fv-1* locus, since these two loci are very near each other on chromosome 4 of the mouse (13) and *Gpd-1* type should accurately reflect *Fv-1* type about 99% of the time. If either *H-2* or *Fv-1* were one of the two genes involved in resistance (*i.e.*, the *A* or *B* in the illustration above), then there should be a significant difference in the incidence of lymphoma according to either *H-2* or *Gpd-1* type in the backcross mice.

Analysis of the data according to this scheme showed that the *Fv-1* locus was irrelevant to lymphoma resistance in each of the three crosses, since mice of the different *Gpd-1* phenotypes showed approximately equal incidences of the disease. *H-2* type proved to be relevant to lymphoma resistance in only one of the three crosses. In the AKR × (AKR × C57L) backcross population, 95% of $H\text{-}2^k/H\text{-}2^k$ homozygotes developed the disease, whereas only 55% of $H\text{-}2^b/H\text{-}2^k$ heterozygotes did so. In the other two crosses studied, mice heterozygous at the *H-2* locus ($H\text{-}2^d/H\text{-}2^k$ in the C58 × DBA/2 cross, $H\text{-}2^q/H\text{-}2^k$ in the AKR × DBA/1 cross) showed about the same incidences as their $H\text{-}2^k/H\text{-}2^k$ homozygous littermates.

In two of the three backcrosses the albino locus was also segregating, with half of the mice being albinos and the other half colored; in neither case did these populations differ significantly in lymphoma incidence.

Thus only one of the two resistance genes was identified in one of the crosses studied: $H\text{-}2^b$ in the C57L cross. It is therefore clear that genes other than *H-2* and *Fv-1* that have major effects on lymphomagenesis remain to be identified in future studies. It should be noted that the $H\text{-}2^b$ haplotype had been shown to be involved in lymphoma resistance in earlier studies. The fact that the second gene for resistance in the AKR × C57L cross is not *Fv-1* may be relevant to the interpretation of early studies of genetic factors in resistance to Gross virus-induced lymphoma (7). In this study of C3H × (C3H × C57BL/6) backcross mice inoculated neonatally with Gross virus, the results implied an interpretation entirely analogous to that of the AKR × C57L cross reported here: two genes for resistance were transmitted by the C57BL/6 parent; one of these was associated with the $H\text{-}2^b$ haplotype, and the other remained unidentified. These two genes were given the designations, *Rgv-1* (associated with the *H-2* locus) and *Rgv-2* (independent of *H-2*). With the subsequent identification of *Fv-1* as a factor in lymphoma resistance in certain crosses, it has always seemed likely that the gene designated *Rgv-2* might have been *Fv-1*. This is still a likely hypothesis, but the present finding that other such genes also exist makes the hypothesis, *Rgv-2* = *Fv-1*, somewhat less imposing than before.

FURTHER HIGH- X LOW-LYMPHOMA CROSSES: MuLV EXPRESSION

Since the analysis of lymphoma incidence in these three new crosses indicated the effect of one or more new genes, not linked to *Fv-1*, as a major factor in resistance to the disease, the next question was: did this new gene, like *Fv-1*, confer lymphoma resistance by means of an effect on expression of endogenous ecotropic or xenotropic MuLV? Extensive studies of virus expression in mice of various ages were carried out in all three crosses, and these results will be published in detail elsewhere. Here we summarize only the findings in the AKR × C57L cross.

Only a modest difference in the levels of ecotropic virus expression was detected in comparing high-lymphoma AKR mice and low-lymphoma (AKR × C57L)F_1 mice: cell-free extracts of spleens and of thymuses showed about one log less activity in the hybrids than in the parental strain mice. Xenotropic

virus activity, for which our assay was basically qualitative and only very poorly quantitative, showed a markedly different expression in the two populations. Whereas almost all AKR mice were positive when tested for this virus at six to eight months of age, no F_1 mice shoed detectable virus activity at this age. Among backcross mice tested at about the same age, about half showed detectable xenotropic MuLV (14/28 in the spleen, 11/28 in the thymus); no association between the presence or absence of virus expression and *Gpd-1* (*Fv-1*) type was detected. When tissues from lymphomatous mice of this backcross population were tested, all were virus-positive.

These findings are strongly reminiscent of those in the AKR × RF cross studied earlier (11). However, in that previous study, the *Fv-1* allele of RF origin appeared to be responsible for both xenotropic virus suppression and lymphoma resistance. By contrast, in the present AKR × C57L cross, neither xenotropic virus suppression nor lymphoma resistance was associated with *Fv-1* type. It thus appears that C57L mice possess a previously unidentified dominant gene unlinked to *Fv-1* which suppresses AKR xenotropic MuLV expression and therefore favors resistance to lymphoma in the presence of an $H\text{-}2^b$ haplotype. Results from virus expression studies in the other two new crosses, AKR × DBA/1 and C57 × DBA/2 were roughly though not exactly analogous to those from the AKR × C57L cross.

Thus, these findings present us with the exciting possibility of identifying new lymphoma-regulating genes in the mouse. An interesting approach which may contribute to realizing this goal is provided by a recent finding of Bassin *et al.* (14), who showed that cells of origin from two different strains (NIH and DBA/2), which do not, however, appear to differ in *Fv-1* type, nevertheless show different mechanisms for restricting the infectivity of B-tropic, MuLV.

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THE ANOMALOUS ANTIBODY RESPONSE OF HYBRID MICE TO IMMUNIZATION WITH AN ABELSON VIRUS LYMPHOMA[1]

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ABSTRACT The humoral immune response of several hybrid mice to immunization with a parental Abelson virus lymphoma has been examined by serological and biochemical methods. Although all hybrid mice express the parental H-2 products of the immunizing tumor on their somatic cells, surprisingly they respond to the immunizing tumor with the production of cytotoxic and precipitating antibodies directed to those H-2 molecules. In all five positive responses, H-2K region products were recognized and in three cases H-2D region products were also recognized. Immunoprecipitation and gel electrophoresis of lactoperoxidase-radiolabelled cell surface molecules from normal spleen cells indicated the major reactivity was to molecules of approximate molecular weights 50,000 and 12,000 daltons, presumably the H-2K or D molecules plus beta-2-microglobulin. This "anti-self H-2" response of hybrid mice to immunization with this tumor may well reflect recognition of self in association with virus.

INTRODUCTION

The outcome of tissue transplantation in the mouse is governed by several loci, some products of which have been identified serologically and referred to as histocompatibility antigens (1, 2). The major histocompatibility locus of the mouse, H-2, has been implicated in susceptibility to leukemia induction by Gross (3), Friend (4), and more recently Abelson (5) viruses. Although the reason for the correlation of H-2 haplotype with sensitivity to leukemia induction by virus is not entirely clear, several workers have documented the topological and physical association of MuLV-related molecules with H-2 products (6, 7). In view of the accumulating evidence for the hypothesis that viral antigens including those of MuLV are recognized in associa-

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tion with self (8, 9) in the generation of cell-mediated immune responses, it seems likely the virus-H-2 association is a key factor in H-2 restriction.

During the course of immunization of hybrid mice with a parental Abelson virus lymphoma, we detected a high titer autoantibody directed to the $H\text{-}2^b$ products of the immunizing tumor. We suspect these autoantibodies may represent the converse situation to H-2 restriction, i.e. the recognition of self in association with virus.

METHODS

Immunizations. 8-12 week old female mice were immunized with 10^2 cells of the Abelson C57BL/6 (B6) lymphoma B6T1 (10). After regression, the immunizing dose, given in alternate weeks, was slowly increased to 10^8 cells per mouse, and this challenge was maintained. Mice were bled individually every 20-30 days.

Cytotoxicity Assays. Direct cytotoxic assays were performed as described (10) using rabbit serum prescreened for low toxicity and high complement activity as a complement source, at dilutions of 1/7 with tumor cells and 1/15 with normal spleen cells.

Biochemical Assays. Normal spleen cells were radiolabelled by the ^{125}I-lactoperoxidase technique as described (11), lysates prepared and immunoprecipitated (12). Immunoprecipitates were electrophoresed in polyacrylamide gels (13) with molecular weight markers.

RESULTS

Antiserum from one (C3H x B6)F_1 hybrid mouse hyperimmunized with the B6 Abelson lymphoma B6T1 was cytotoxic for the immunizing cell with a midpoint titer of 1/320. When antiserum was diluted 1/120 and absorbed with spleen cells from normal uninfected mice, B6 and C57BL/10 (B10) cells removed all cytotoxic activity, whereas spleen cells from congenic B6.C-$H\text{-}2^d$ or B10.BR ($H\text{-}2^k$) did not. This result indicated the presence of high titer antibody for $H\text{-}2^b$ controlled antigen(s). If this antiserum did contain anti-$H\text{-}2^b$ antibodies, then it would be expected to lyse normal cells from mice of the $H\text{-}2^b$ haplotype. In direct cytotoxicity tests this antiserum lysed 95% of spleen cells from B6, B10, 129 and (C3H x B6)F_1 mice with midpoint titers of 1/160 - 1/320, but did not appreciably lyse spleen cells

from C3H, B6.C-H-2^d nor B10.BR mice. Thus, a major antibody in the serum of the (C3H x B6)F_1 anti-B6T1 mouse was directed to antigens controlled by the H-2^b locus.

Several other hybrid mice have been hyperimmunized with B6T1, and their antisera examined for antibody cytotoxic for 95% of spleen cells from B6 but not cytotoxic for cells of the H-2 congenic strains B6.C-H-2^d or B10.BR. As the data in Table I indicate, 5/11 hybrid mice hyperimmune to B6T1 produced anti-H-2^b antibody.

Similar antibodies were not found in the sera of aged non-immune (C3H x B6)F_1 mice nor in the sera of aged non-immune (BALB x B6)F_1 mice. In addition 0 of 10 (C3H x B6)F_1 mice immunized with B6 bone marrow produced anti-H-2^b antibodies; and 0 of 15 B6 mice hyperimmune to B6T1 produced anti-H-2^b antibodies. The data in Table I emphasize the c t o ro c ng such mice, and for that reason the deleterious effects of this immune response are not yet clear, nor is the time course of its appearance.

TABLE I
ANTIBODY RESPONSE OF MICE

Mouse Strain	No. Immunized	No. H-2^b positive[a]/ No. Survivors
C3H x B6-old	---	0/11
BALB x B6-old	---	0/6
B6	113	0/15
C3H x B6	16	3/5
BALB/c x B6	15	1/2
C57BR x B6	11	1/3

[a]Positive mice produced antibody cytotoxic for 90% of B6 or B10 spleen cells at antisera dilutions of 1/80, and not cytotoxic for B6.C-H-2^d or B10.BR spleen cells (30% lysis). Antisera of non-responding mice showed or no cytotoxicity (30%) on B6 or B6 congenic spleen cells.

By testing the "anti-self H-2" antisera on spleen cells from H-2 recombinant mice, it has been possible to map the determinants recognized by each of the 5 "anti-self H-2" antisera. The conclusion of that experiment, for which the data is summarized in Table II, is that all sera recognized determinants controlled by the H-2K-IB regions and in addition some sera recognized determinants controlled by the H-2D subregion as well.

The nature of the molecules on normal spleen cells recognized by these antisera was determined by radiolabelling the cell surfaces by the lactoperoxidase technique, immunoprecipitating cell lysates with "anti-self H-2" sera, and then electrophoresing the lysates through SDS-polyacrylamide gels. The major proteins that appeared on fluorograms of the gels had approximate molecular weights of 50,000 and 12,000 daltons, and comigrated with the major protein species immunoprecipitated from these cells by the conventional anti $H\text{-}2^b$ antiserum $(A \times B10.D2)F_1$ anti B10.A-(5R). These proteins were the major species found in cells reactive in cytotoxic tests with the "anti-self H-2" antisera, e.g. B10 and B10.A (5R), and little or no reactivity was found by this method with any protein from B10.D2n lysates. The antisera that also reacted with B10.A(2R) cells in cytotoxic tests precipitated molecules of 50,000 and 12,000 daltons from these cells; although the intensity of the bands was lower, perhaps paralleling the lower cytotoxic titers. Thus, biochemical experiments confirmed the genetic mapping data, and indicated that the major reactivities of those anti-self antisera are for H-2K and sometimes H-2D molecules.

TABLE II
GENETIC MAPPING OF ANTI-SELF ANTIBODIES

Target:	B10	B10.A(5R)	B10.A(2R)	B10.D2n
Sera Haplotype:	K^bD^b	K^bD^d	K^kD^b	K^dD^d
S41#1	1/240[a]	1/160	1/60	-[b]
S41#2	1/160	1/80	-	-
S50	1/160	1/160	1/40	-
S64	1/640	1/160	1/160	-
S65	1/160	1/40	-	-

[a] The numbers indicate the midpoint titers (50% lysis). At lower antiserum dilutions cells were lysed to 90%.
[b] A negative(-) indicates 30% lysis at an antiserum dilution of 1/20.

DISCUSSION

The results presented here indicate that hybrid mice can with low efficiency be hyperimmunized to the syngeneic H-2^b Abelson lymphoma B6T1. In the process, they occasionally produce antibodies to the H-2 antigens present on that tumor; antibodies that also recognize H-2 antigens present on essentially all spleen cells of mice that carry the H-2^b haplotype. Questions about this phenomenon that remain to be answered include: 1) Is the elicitation of anti-self H-2 antibodies solely a property of B6T1 or can it be extended to other tumors of various etiologies and genetic backgrounds? 2) What genes must be heterozygous for the hybrid to respond in this fashion to B6T1? 3) Are the antibody-producing hybrids H-2^b immune in other arms of the immune system? 4) What are the pathological consequences of this response?

It should be noted that earlier workers have clearly demonstrated the immunity of hybrids to challenge with certain parental tumors (14, 15, 16) and bone marrow grafts (17), although none have reported serological correlates of those phenomena. Other workers have demonstrated the production of cytotoxic antibodies to various cell surface determinants by the hybrid mice immunized with parental tumors (18, 19, 20), although those cases do not include anti-self H-2 antibodies. In the case of hybrid resistance to parental bone marrow grafts, it has been proposed that homozygous cells express an antigen, linked to the H-2D region, that is lacking on heterozygous cells (17, 21). Clearly, this explanation is not applicable to the antibodies described here. It remains to be determined whether the antibody response documented here relates to hybrid histocompatibility and resistance.

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AKvr-1, A DOMINANT MURINE LEUKEMIA VIRUS RESTRICTION GENE, SEGREGATES IN LEUKEMIA-PRONE WILD MICE[1]

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ABSTRACT Leukemia-prone wild mice (Mus musculus domesticus) from a squab farm near Lake Casitas (LC) in southern California are polymorphic for a restriction gene, named Akvr-1, that suppresses AKR ecotropic virus. The restriction allele ($Akvr\text{-}1^R$) is dominant and exhibits 100% penetrance in prevention of viremia of AKR endogenous retrovirus and of virus-associated lymphoma in (LC^{RR} X AKR) F1 hybrids. Ecotropic and xenotropic AKR virus production is also suppressed in thymus, spleen and bone marrow of older resistant F1 hybrids. The restriction phenotype segregates as a single Mendelian locus in F2 and backcrosses to AKR mice. $Akvr\text{-}1^R$ likewise is effective in restriction of NB-tropic Moloney and Friend MuLVs in vivo but fails to restrict expression or pathogenesis of LC-derived amphotropic retrovirus. This gene apparently exerts a similar MuLV-suppressive effect in vitro upon various cell types. Akvr-1 does not map near to certain retroviral gene loci including Fv-1, Fv-2 and Akv-1. It may be related to the Fv-4 locus described in Japanese mice.

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INTRODUCTION

The high-MuLV, leukemia-prone Lake Casitas (LC) population of feral mice (Mus musculus domesticus) exhibits considerable diversity in retroviral genes and other complex gene families (1-3). We have reported (4, 5) that LC mice are monomorphic for Fv-1^N but polymorphic for a dominant strong MuLV restriction gene, named Akvr-1^R, that appears different from Fv-1 and other MuLV regulatory loci described in American laboratory mice. In this paper we describe the effect of Akvr-1^R *in vivo* and report that this gene apparently exerts a similar MuLV-restriction effect *in vitro* and does not map close to Fv-1 or certain other retroviral loci in the mouse genome.

METHODS

Infectious MuLV (ecotropic or amphotropic) was determined by the fluorescent antibody (FA) focus induction method (6) on SC-1 cells. The borderline sensitivity of this assay is $10^{1.2}$ fluorescent focus forming units/ml (FFFU/ml). In viremic F1 hybrids the virus titer was consistently $10^{2.0}$-$10^{3.0}$ FFFU/ml with slight variation. In Table 1, LC$^{(+)}$ indicates viremia with endogenous amphotropic MuLV; LC$^{(-)}$ indicates nonviremia. The SC-1 cells detect total infectious virus, whether amphotropic or ecotropic. Amphotropic virus (7) was detected by FA on the basis of growth for 6 days in rabbit cornea (SIRC) or human sarcoma (1080) cells; ecotropic virus was detected by the XC test from virus grown for 6 days on SC-1 cells. Only the ecotropic viruses registered in the XC test. The level of sensitivity for detection of either MuLV class was the same--$10^{1.5}$ FFFU/ml.

RESULTS

Evidence of a Dominant AKR Virus Restriction Gene Polymorphous in LC Wild Mice. A total of 18 LC wild mice were each bred to a different AKR mouse and three patterns of virus expression were observed in the F1 hybrid litters at weanling age: (i) nonviremic mice (RR); (ii) about 50% viremic mice (RS); and (iii) nearly 100% viremic mice (SS) (Table 1). These three phenotypes of the F1 hybrids suggested that a dominant AKR virus restriction gene (named Akvr-1^R) was segregating in LC mice. The recessive susceptible allele, previously designated r (5), is now called S. To confirm the dominant inheritance of this gene, the frequency of viremia was measured in F1 backcrosses to AKR and in F2

TABLE I

INFECTIOUS MuLV IN (AKR x LC) F1 HYBRIDS

Parental mice female X male	Akvr-1 genotype of LC parent (number of LC mice)	Number of litters	Viremia in F1 progeny/number tested at 2 months (percentage)	Number of lymphomas/ number of mice observed 9-12 months	13-24 months
AKR X $LC^{(+)}$	RR (1)	2	0/7	0/7	
AKR X $LC^{(-)}$	RR (4)	11	0/70	0/18	0/54
$LC^{(-)}$ X AKR	RR (1)	2	0/13	0/13	0/3
TOTAL	6	15	0/90 (0%)	0/38	0/57
AKR X $LC^{(+)}$	RS (1)	1	3/6		
AKR X $LC^{(-)}$	RS (2)	4	17/35		
$LC^{(-)}$ X AKR	RS (2)	3	5/12		
TOTAL	5	8	25/53 (47%)		
AKR X $LC^{(+)}$	SS (4)	8	36/36	0/21	
AKR X $LC^{(-)}$	SS (1)	3	22/23	0/9	0/5
$LC^{(-)}$ X AKR	SS (2)	4	13/15	0/13	
TOTAL	7	15	71/74 (96%)	0/43	0/5

mice using F1 parental mice of Akvr-1^{RS} genotype. Viremia was detected in 46% (37 of 81 mice in 10 litters) of the backcross mice with no difference related to sex of the AKR parent, and in 14% (6 of 43 mice in 5 litters) of the F2 mice. The differences between observed and expected numbers (50% and 25%, respectively) in a single dominant gene model in the backcross and F2 hybrids were not statistically significant. These results thus appeared consistent with segregation, in LC mice, of a single dominant gene for suppression of AKR virus.

Based upon the small sample of 18 mice, the allele frequency of Akvr-1^{R} was 0.47 in LC mice. The genotypes observed did not vary significantly (P=.05) from expectations of the Hardy-Weinberg equilibrium. Thus, the probable frequency of LC mice that contain at least one Akvr-1^{R} allele is 72%, whereas we observed 61% in our samples.

Evidence that the Restricted Virus is AKR Ecotropic Virus. In contrast to the ecotropic, XC positive AKR virus, the LC viruses are predominantly amphotropic and XC negative (7). Only amphotropic virus was detected in the sera of the parental viremic LC males used in this study. We previously showed that neither nonviremic LC mice nor viremic LC males transmitted infectious MuLV vertically (8). Therefore, the virus in their AKR F1 hybrids should be of AKR origin. Our results supported this contention. In crosses between AKR females and LC^{SS} or LC^{RS} males, regardless of whether or not the LC males were infected with endogenous amphotropic virus, the serum virus in each of 13 viremic F1^{SS} progeny grew only in SC-1 cells and gave large XC plaques typical of AKR ecotropic virus. In crosses between AKR males and uninfected LC^{SS} females, the virus in F1^{SS} progeny again grew only in SC-1 cells and gave large XC plaques. However, when viremic LC^{RS} females were mated to AKR males, the virus in some of the F1 progeny grew in both SC-1 and SIRC cells, and gave large XC plaques in SC-1 cells, thus indicating a probable mixture of LC amphotropic and AKR ecotropic virus. Progeny of viremic LC^{RR} females mated with AKR males yielded only amphotropic virus of LC origin. These results indicate that, except in crosses with viremic LC females, the virus present in the sera of two-month-old F1 hybrids inheriting the recessive Akvr-1^{S} allele from their LC parent is solely of AKR origin.

Duration of Virus Repression and Prevention of Lymphoma in F1 Hybrids. The F1 progeny of matings between AKR and LC^{RR} mice were retested for infectious virus at later ages

and observed for up to 22 months. Of 117 initially non-viremic $F1^{RS}$ mice, only 5 eventually became viremic between 6-12 months of age and only 1 of 30 $F1^{RS}$ mice became viremic in the second year of life. In each the virus was ecotropic and XC positive and, thus, of AKR origin. Similarly, only ecotropic, XC positive virus was recovered from the spleen, thymus and bone marrow of viremic $F1^{SS}$ hybrids. Virus (eco-, xeno- or amphotropic) was not isolated from serum, thymus, spleen or bone marrow of any of 5 $F1^{RS}$ hybrids from 9-18 months of age. None of 38 $F1^{RS}$ hybrids observed for 9-12 months or 57 other $F1^{RS}$ hybrids observed for 13-24 months developed lymphoma (Table 1). The only tumors found so far in these animals were one fibrosarcoma and one lung adenoma at 20 months of age. These F1 mice were remarkably vigorous, healthy and about twice as large as their wild parent. These findings indicate that the Akvr-1^{R} gene is strong and long-lasting in its suppression of competent AKR ecotropic (and xenotropic) virus in sera and tissues and is associated with the prevention of lymphoma. However, other genes in the LC mice also contribute to the prevention of lymphoma in these crosses, as evidenced by the absence of lymphoma in any of 48 $F1^{SS}$ viremic hybrids observed for >9 months, (Table 1).

By contrast, the same incidence (17%) and nonthymic type of lymphoma occurred in F1 progeny of viremic LC females (not typed for Akvr-1) and AKR males as occurred in parental viremic LC mice (1), suggesting that the Akvr-1^{S} allele present in AKR mice does not restrict LC amphotropic virus expression or pathogenesis.

Akvr-1^{R} is Restrictive for Moloney and Friend MuLVs but is not Restrictive for LC Amphotropic MuLV. Offspring of mice previously typed for their Akvr-1 genotype were challenged as newborns with NB-tropic Moloney MuLV. Homozygous Akvr-1^{SS} Swiss or 1^{RR} LC mice were susceptible (11 of 12) and homozygous Akvr-1^{RR} LC mice were resistant (0 of 10) to infection with Moloney MuLV (Table 2). AKR X LC heterozygotes, Akvr-1^{RS}, were, with one exception in five mice, resistant to Moloney MuLV. When additional LC X LC and AKR X LC crosses and backcrosses segregating for Akvr-1^{R} were challenged at 2-5 months of age with NB-tropic Friend MuLV (a mixture of spleen focus forming virus and helper virus), the ratio of resistant (spleen weight <0.5 gm) to susceptible (spleen weight >0.5 gm) mice was in close agreement with that expected on the basis of Mendelian segregation of a single dominant virus-restriction gene (Table 2). The hybrids that had previously been typed as Akvr-1^{SS} (i.e. ^{RS} viremic) were susceptible whereas the nonviremic Akvr-1^{RS}

TABLE 2

CONCORDANT RESISTANCE TO AKR ECOTROPIC VIRUS AND TO FRIEND AND MOLONEY NB-TROPIC VIRUSES

Cross		Akvr-1 genotype[a]	Resistance to MuLV	
Female	Male		Moloney[b]	Friend[b]
NIH Swiss	NIH Swiss	SS X SS	0/6	0/10
LC	LC	SS X SS	1/6	NT
LC	LC	RR X RR	10/10	NT
AKR	LC	SS X RR	4/5	NT
LC	LC	RS X RS	2/6	8/9
(AKR X LC) F1	(AKR X LC) F1	RS X RS	NT	13/15
AKR	(AKR X LC) F1	SS X RS	NT	23/30
(AKR X LC) F1	AKR	RS X SS	NT	10/25
LC	AKR	RS X SS	NT	2/6

[a]The Akvr-1 genotype of each LC parent was previously determined by the segregation of viremia in (AKR X LC) F1 hybrids. The LC mice were nonviremic with endogenous amphotropic virus.

[b]Newborn mice were challenged with 0.05 ml (intraperitoneally) of Moloney MuLV and viremia was determined at 2 months of age by the FA test on SC-1 cells. The Friend MuLV was a mixture of the spleen focus-forming virus and its helper virus serially passaged in NIH Swiss mice. At 2-5 months of age, mice were inoculated with 0.2 ml of 1:100 dilution of a 10% NIH Swiss splenic extract. Spleen weight was determined 9 days after inoculation.

hybrids were resistant. Thus, Akvr-1^{R} appeared to exert a strong restriction of Moloney and Friend MuLV proliferation *in vivo* but slight leakiness to the Moloney virus may exist.

Since about 90% of LC mice contain amphotropic virus (N-tropic for mouse cells) and about 70% of LC mice are expected to carry at least one Akvr-1^{R} allele, it is obvious that Akvr-1^{R} fails to suppress the highly prevalent amphotropic virus of its natural host. This conclusion is supported by the finding of high amphotropic virus titers in animals both homozygous and heterozygous for Akvr-1^{R} (Table 1). In addition, when an LC male of Akvr-1^{RR} genotype was mated with a viremic LC female, 100% (15 of 15 mice in 3 litters) of the weanling progeny were viremic. Furthermore, when 7 noninfected wild mice from another trapping area (BC) were mated to AKR females, 92% of F1 hybrids (47 of 51 mice in 8 litters) were viremic at weanling age (i.e. Akvr-1^{SS} genotype), indicating that the strong control of amphotropic virus noted in this other population of wild mice (9) could not be attributed to the Akvr-1^{R} allele. Thus, the target of Akvr-1^{R} found in endogenous AKR and the other laboratory ecotropic viruses is distinct from the target in the LC amphotropic virus.

Akvr-1^{R} Effect in Vitro. LC embryo fibroblasts, free of endogenous virus production and of known Akvr-1^{RR} genotype, were exposed in early subpassage on two separate occasions to various strains of MuLV and assayed for virus replication by the direct FA test. The cells were totally resistant to AKR, Friend and B-tropic BALB/c virus but were quite permissive to amphotropic virus and less permissive to Moloney virus and wild mouse ecotropic virus. Control SC-1 and NIH Swiss fibroblasts of Akvr-1^{SS} genotype were fully susceptible to all viruses except the B-tropic MuLV in Swiss cells. On the basis of reverse transcriptase assay, virus-free spleen, thymus and bone marrow cultures from 5 (AKR X LC) F1 adults of Akvr-1^{RS} genotype also appeared resistant to infection with AKR and Friend viruses while at least partially susceptible to amphotropic virus. Further *in vitro* studies are required, however, to confirm the virus-restrictive effect of Akvr-1^{R} in different cell types.

Mapping of Akvr-1. LC and AKR mice differ in color genes and electrophoretically at a number of allelic isozymes mapped to individual chromosomal loci (3). Thus far we have determined the recombinational frequency between the Akvr-1 locus and 8 such loci on different chromosomes of segregating (AKR X LC^{RR}) X AKR backcross progeny (Table 3).

TABLE 3

SEGREGATION OF Akvr-1^R IN (AKR X LC) X AKR BACKCROSS PROGENY

Locus[a]	Chromosome	Genotype		Number of mice	Percent recombination gene versus Akvr-1	χ^2[b]	Retrovirus loci on same chromosome
		AKR	LC				
Id-1	1	b	a, c	70	51.4	0.06	Xv-1; Akp-1
a	2	a	A	42	43.7	0.1	Rec-2
Gpd-1	4	b	a, b	68	51.5	0.06	Fv-1; end.MMTV
c	7	c	C	87	52.4	0.92	Akv-1; Fgv-1
Gr-1	8	a	a, b	52	48.1	0.08	Ram-1
Mod-1	9	b	a, b	48	60.4	2.1	FV-2
Es-3	11	c	a, c	70	57.1	1.42	Mtv-3
Got-1	19	a	a, b	42	57.1	0.86	-

[a] Isozymes were typed by electrophoresis and viremia by the FA test on SC-1 cells.

[b] χ^2 with one degree of freedom = 3.841 (P=.05).

No linkage was found, indicating that Akvr-1 is not situated near to certain retroviral structural and regulatory loci located on these chromosomes. Especially important is the evidence that Akvr-1 is not closely linked with the Fv-1 or Akv-1 loci.

DISCUSSION

Amplification and recombination of ecotropic and xenotropic proviral genes in the thymus are critical events in the pathogenesis of AKR lymphomas (10, 11). In hybrid AKR crosses the $Fv\text{-}1^b$ allele is of major importance in curtailing replication of ecotropic AKR virus and prevention of lymphoma (12, 13). Certain $Fv\text{-}1^n$ alleles also prevent lymphomas by conferring a restricted expression of ecotropic AKR virus specifically in F1 thymocytes (14, 15). Akvr-1 is clearly distinct from the Fv-1 locus and it also appears unrelated to other genetic loci (Fv-2, Fv-3, Rfv-3) (16-18) controlling resistance to Friend MuLV in American laboratory mice. H-2 linked Ir loci affect leukemogenesis in AKR (16) and other laboratory mouse strains (19), mainly by enhancement of the antiviral immune response. However, in some inbred strains, H-2 related genes also regulate early virologic events (20). Linkage of Akvr-1 to the H-2 region remains a possibility that would be supported if high titers of antibody to AKR virus are found in the resistant hybrids. $Akvr\text{-}1^R$ confers resistance *in vivo* and *in vitro* to N-tropic AKR virus and NB-tropic Friend MuLV but does not confer resistance to wild mouse amphotropic virus (also N-tropic). In these attributes $Akvr\text{-}1^R$ most closely resembles the Fv-4 locus described in the G inbred strain of Japanese mice (21, 22) and in some Japanese wild mice (*Mus molossinus*) (23). The mechanisms underlying this powerful and long-lasting virus restriction are yet to be explained at the molecular level.

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GENETIC CONTROL OF RESISTANCE OF MOUSE HEPATITIS VIRUS, STRAIN JHM, INDUCED ENCEPHALOMYELITIS[1]

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ABSTRACT Inbred mouse strains were crossed with the resistant SJL strain to detect strains carrying one of the two genes responsible for resistance to mouse hepatitis virus. Forty percent of the (SJL x BALB/c)F_1's were resistant. Backcrosses of F_1 on both the susceptible (BALB/c) and resistant (SJL) parent revealed the expected survival rates consistent with BALB/c genotype containing the Rhv-1 resistant, Rhv-2 susceptible genotype.

INTRODUCTION

Mouse hepatitis viruses (MHV) are generally commensal organisms existing as a latent infection of the mouse gastrointestinal tract (1). Stress can result in dissemination of the virus, generally resulting in fatal hepatitis (2). Among the many factors affecting the natural resistance to viral infections, the genetic makeup of the host appears to be one of the most important. Studies of the genetic basis of resistance to MHV induced hepatitis were first carried out by Bang and co-workers (3). Using strain 2 virus (MHV-2) they demonstrated a single recessive gene was responsible for resistance to acute hepatitis. Phenotypic expression was manifested in vitro by an inability of macrophages from a resistant animal to support viral replication (3).

MHV has recently come under intensive investigation, because it was found to induce at least two forms of neurological disease. MHV strain 3 (MHV-3) causes a spectrum of disease in its natural host from fatal hepatitis to an immunopathological chronic neurological disease resulting in hind limb paralysis (4). One H-2 linked gene is reported to control acquisition of the chronic neurological disease, while

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another non H-2 linked gene is responsible for resistance to acute hepatitis (5). Macrophages from animals exhibiting the 3 clinical-pathological types of MHV-3 disease exhibit parallel susceptibility to infection in vitro (6) although a T-cell component has also been implicated in resistance (7).

Another strain of MHV, the JHM strain, (MHV-JHM) also causes chronic neurological disease following recovery from acute encephalomyelitis (8,9). The chronic disease results from a persistent infection of the oligodendroglial cells, the cells that synthesize and maintain myelin in the central nervous system, resulting in chronic demyelination similar to human multiple sclerosis. In our earlier reported studies on the resistance of mice to MHV-JHM encephalomyelitis we found only SJL mice exhibited a marked resistance to intracerebral (i.c.) challenge (10). Analysis showed that two genes controlled resistance. One gene is dominant, designated Rhv-1, and the other recessive, designated Rhv-2.

MATERIALS AND METHODS

Mice B10.S, B10.M, A.SW, F1, and backcross mice were bred in the immunogenetics mouse colony, University of Southern California School of Medicine. All other mice were purchased from the Jackson Laboratory, Bar Harbor, Maine at 6-8 weeks of age. All animals were tested at 12 weeks of age. Males and females were used throughout, since we have previously reported no sex related difference in susceptibility (11).

Virus The seventh suckling mouse brain passage of MHV-JHM was used. The virus pool obtained from Dr. Leslie Weiner consisted of a 10% brain homogenate in phosphate buffered saline, pH 7.2. The lethal dose 50% (LD_{50}) was determined by the method of Reed and Munch using suckling Swiss-Webster mice obtained from Strong Laboratories, San Diego, California. Adult mice were infected intracerebrally (i.c.) with 0.032 ml at a dilution of virus calculated to contain 1000 suckling mouse LD_{50}. Following injection, the mice were observed daily for death. No deaths occurred later than 14 days post injection and mice surviving longer than 3 weeks were considered resistant.

RESULTS

We previously have reported that only the SJL strain and the SJL-Ig-1^a which differs from SJL only in immunoglobulin allotype were resistant to i.c. challenge with MHV-JHM virus (10). In addition to the strains previously tested, two

additional strains, A.SW and B10.M, have recently been found to be susceptible (Table 1). The susceptibility of A.SW confirms our previous finding, based on studies with B10.S, that resistance is not linked to the H-2^s allele at the major histocompatibility complex of the SJL mouse.

TABLE 1

MORTALITY OF VARIOUS STRAINS OF INBRED MICE FOLLOWING INTRACEREBRAL INOCULATION WITH 1000 SMB LD_{50} OF MHV-JHM

Strain	H-2 haplotype	Ig-1	Survivors/total	Percent resistance
A	a	e	0/10	0
C57BL/6	b	b	0/25	0
C57BL/10	b	b	0/10	0
BALB/c	d	a	0/50	0
NZB	d	e	0/10	0
B10.M	f	b	0/7	0
AKR	k	d	1/20	5
CBA	k	a	0/10	0
C3H/HeJ	k	a	0/20	0
DBA/1	q	c	0/10	0
RIII	r	c	0/10	0
B10.S	s	b	0/40	0
A.SW	s	e	0/40	0
SJL	s	b	80/100	80
SJL-Ig-1^a	s	a	2/2	100

Our earlier results suggested that multiple genes control the resistance in SJL vs. B10.S mice to i.c. challenge with MHV-JHM (10). Backcross analysis of (B10.S x SJL) F_1 x SJL suggested a single dominant gene segregating in that cross. We therefore began a systematic search of mouse strains for those strains which could contain the correct resistance gene, Rhv-1, but carried the susceptibility gene at Rhv-2. F_1's of this genotype would be predicted to be resistant but, the parental strain would be susceptible. Eight F_1's have thus far been tested by i.c. virus challenge (Table 2).

TABLE 2

MORTALITY OF F_1 MICE CROSSED WITH SJL AFTER INTRACEREBRAL INOCULATION WITH MHV-JHM

Strain	Rhv Rhv-1	Rhv-2	Genotypes of Inbred Mouse Strain % Survivors of Cross to SJL	
SJL	R	r	-	
BALB/cJ	S	r	41	(37/90)
A.SW	S	s	0	(0/30)
C57BL/10S_gS_n	S	s	0	(0/25)
C57BL/6J	S	s	0	(0/18)
C3H/HeJ	S(?)	s	20	(4/20)
DBA/2	S	s	2	(1/57)
RIII/J	S	s	11	(3/27)
NZB	S	s	0	(0/14)

(C3H/HeJ x SJL)F_1 showed some resistance (4/20) but insufficient animals have been tested to reach significance. Only (BALB/c x SJL)F_1's showed significant resistance. Unfortunately, resistance is not complete and the BALB/c x SJL F_1's showed only approximately 40% resistance. Whether this is a gene dosage effect, at Rhv-1 or the Rhv-2 alleles differ is not clear. In order to further test this finding, backcross were made between [BALB/c x SJL] F_1 x SJL, and [BALB/c x SJL] F_1 x BALB/c. As shown in Table 3, these backcrosses exhibited survival rates that are consistant with this model, this is, only the single dominant gene Rhv-1 (from SJL) is segregating in the BALB/c x SJL backcrosses.

DISCUSSION

The genetic control of resistance to MHV strains has been studied in a number of laboratories. Our analysis of resistance to encephalomyelitis following MHV-JHM virus infection suggested that resistance is controlled by at least two genes, one dominant and one recessive. Both genes are required for efficient resistance, and both function with nearly complete penetrance (10). Two other patterns of resistance to MHV have been reported. MHV-2 resistance is mediated by a single gene (3), while resistance to MHV-3 acute disease controlled by one gene and susceptibility to MHV-3

TABLE 3

BACKCROSS ANALYSIS OF RESISTANCE TO INTRACEREBRAL CHALLENGE WITH MHV-JHM

Cross	Postulated Rhv genotypes of Progeny	Phenotype	% Resistant[1]	% Survivors Observed	% Survivors expected
(BALB/c x SJL)F_1 x SJL	Rhv-1 R/R; Rhv-2 r/r	Resistant	(80%)	57% (8/14)	60%
	Rhv-1 R/s; Rhv-2 r/r	Resistant	(40%)		
(BALB/c x SJL)F_1 x BALB/c	Rhv-1 R/s; Rhv-2 r/r	Resistant	(40%)	19% (17/89)	20%
	Rhv-1 s/s; Rhv-2 r/r	susceptible	(0%)		

[1]Based on survival of these genotypes SJL 80%, (BALB/c x SJL)F_1 40%.

induced chronic neurological disease is controlled by a separate H-2 linked gene (5).

We have extended our previously reported data concerning the natural resistance of mice to MHV-JHM encephalomyelitis (10). Two additional strains of mice have been tested. One, A.SW confirms our previous conclusion that resistance is not due to the H-2^s major histocompatibility complex of the resistant SJL strain. The other, B10.M which has the H-2^f genotype controlling susceptibility to MHV-3 chronic neurological disease (5) was also susceptible to MHV-JHM encephalomyelitis.

When F_1 mice in SJL were tested for resistance, 40% of the (BALB/c x SJL)F_1's survived i.c. challenge with MHV-JHM, indicating that BALB/c carried the resistance gene, Rhv-2. Backcrosses of these progeny on their respective parents showed that segregation was as predicted, i.e., the expected percentage of resistance of the backcross on SJL was 60% which correlated well with the 57% observed resistance, while 20% was predicted for the resistance of the backcross on BALB/c and 19% was the actual value obtained. These results do not conclusively prove a two-gene hypothesis for resistance to MHV-JHM; however, the data is very suggestive that two genes are required and that BALB/c carries the recessive genes for resistance.

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LEARNING ABOUT THE REPLICATION OF RETROVIRUSES FROM A SINGLE CLONED PROVIRUS OF MOUSE MAMMARY TUMOR VIRUS

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ABSTRACT We have prepared molecular clones of an entire provirus of mouse mammary tumor virus (MMTV) and of the unoccupied integration site for this provirus in rat cell DNA in order to study the organization of interesting regions of viral DNA and the mechanisms of integration and transcriptional control. The provirus fortuitously lacks an apparently unclonable region of the MMTV genome as well as the single Eco RI site present in wild type proviruses; however, it includes both copies of a large direct repeat (ca.1300 nt) present at the ends of all MMTV proviruses, and it is expressed and regulated by glucocorticoid hormones in the infected rat cell. Sequencing studies of the relevant regions of the cloned DNA's show that the large units of directly repeated DNA include a short (6 nt) inverted repeat at their termini. The provirus is flanked by a 5 or 6 nt direct repeat apparently generated by duplication of a host sequence during integrative recombination, since this sequence is present only once in the unoccupied integration site. In addition, one or two nucleotide pairs predicted to be at the right hand end of viral DNA are missing from the provirus, implying loss at some stage subsequent to DNA synthesis. We have also identified proviral sequences within the large terminal repeats likely to serve as signals for synthesis and processing of viral RNA and other sequences adjacent to the repeat units involved in the priming of minus and plus strands of viral DNA.

Our findings, in concert with recent work in a number of laboratories, suggest that the replication of retroviruses involves the use of direct and inverted repetitions at several stages: the RNA genome is diploid, and each subunit bears short direct repeats; the linear double-stranded product of reverse transcription carries long direct terminal repeats composed of sequences unique to both the 3' and 5' ends of viral RNA; these terminal repeat units are concluded with short inverted repeats; closed circular DNA contains either one or two copies of the large direct repeats; integrated (proviral) DNA is co-linear with unintegrated linear DNA, including both

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copies of the large repeat unit; and the integration events duplicate a short sequence of host DNA which forms a direct repeat flanking the provirus. Although the functional implications of these designs are not fully understood, the provirus bears a striking structural relationship to transposable elements of bacteria and other organisms, and the repeated sequences seem likely to be instrumental in viral DNA synthesis, integration, and the control of transcription and processing of viral RNA.

INTRODUCTION: THE REPLICATION OF RETROVIRUSES

Ten years have now elapsed since the scientific world generally accepted Howard Temin's hypothesis, proposed nearly a decade earlier (1), that the replication of RNA tumor viruses proceeds via a DNA intermediate. Nurtured by the improvements in the biochemistry of nucleic acids---notably the DNA transfer procedure, recombinant DNA methods, and techniques for DNA sequencing---this simple notion, once a frail reed, has now blossomed, revealing unexpected and marvellous designs: nucleotide symmetries and redundancies, involved in all the major steps of the replication cycle.

A schematic representation of our current view of these designs is presented in Figure 1. Some older aspects of this scheme have been recently reviewed (2-5); new facets will be illustrated during a discussion of our studies of a provirus of mouse mammary tumor virus and are supported by unpublished work on a variety of retroviruses in several laboratories (personal communications from G. Vande Woude, D. Baltimore, H. Temin, I. Verma, G. Hager, R. Swanstrom, and S. Hughes).

Redundancy, a major theme, exists even at the most fundamental level, in that the viral RNA genome itself is diploid, composed of two single-stranded, identical subunits of ca.5-10 kb. For simplicity, only a single subunit is drawn, but it is possible that both subunits are normally used during the synthesis of viral DNA; diploidy may also contribute important structural features to the virion and facilitate genetic flexibility (e.g. heterozygosis and recombination).

The genomic RNA has features of eukaryotic mRNA's, being capped at the 5' end and polyadenylated at the 3' end; but these aspects of the RNA and the structural genes themselves are of less importance to the scheme under discussion than more unusual features indicated by the solid squares and rectangular boxes at the 3' and 5' termini. Each subunit ends with a short direct repeat (solid squares labeled "DR") used in an early phase of DNA synthesis to expedite a necessary transfer of reverse transcriptase between templates. The rectangular boxes are defined on one side by the ends of the

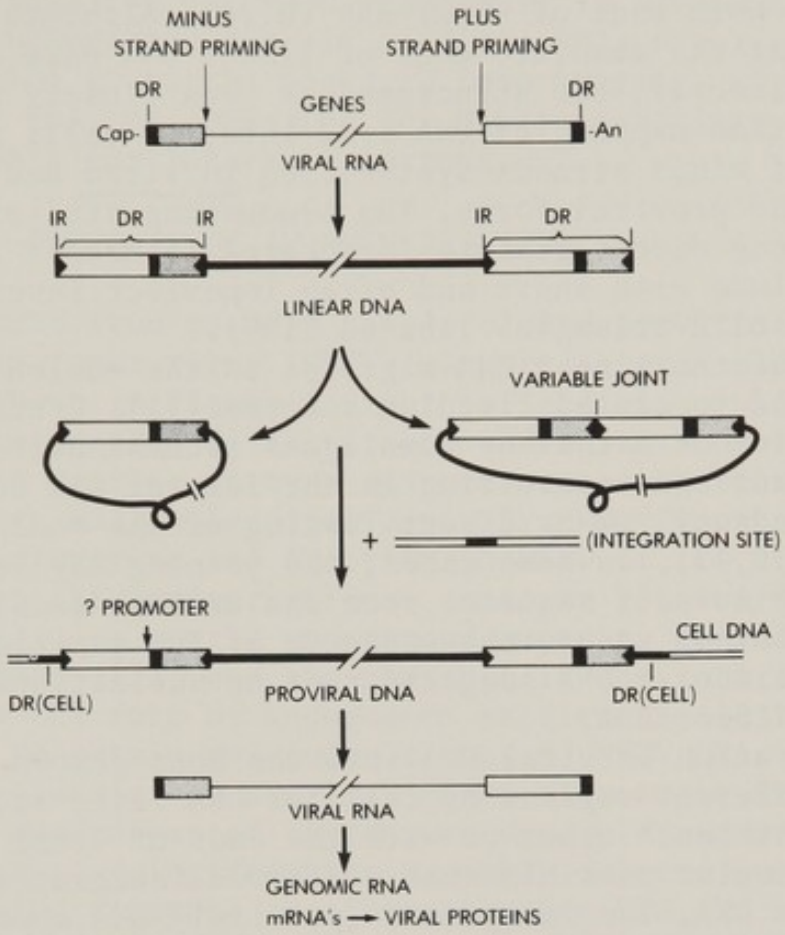

FIGURE 1. Replication of the genomes of retroviruses. A detailed explanation of the schema is provided in the text. DR = direct repeat; IR = inverted repeat. The 5' end of viral RNA is shown on the left, the 3' end on the right.

viral genome (the "cap" and the poly(A) sequences are added following RNA synthesis and are not virus-coded), and on the other side by the sites at which the first (minus) and second (plus) strands of viral DNA are initiated. Priming of the minus strand is performed by a cellular tRNA, the 3' end of which is hydrogen bonded to viral RNA 100-180 nucleotides from its 5' terminus (6). Priming of the plus strands may occur at several sites during synthesis of the DNA of some retroviruses, but all retroviruses appear to have at least one highly preferred site for initiation of plus strands (7,8). This site is located in a purine-rich region positioned 250 to 1200 bases from the poly(A) tract, but the nature of the primer is not known.

The completed product of DNA synthesis in the cytoplasm of infected cells is a linear duplex molecule; the linear DNA is slightly longer than a subunit of viral RNA and contains

long terminal redundancies composed of the boxed sequences present at both ends of viral RNA (9,10). Although nucleotide sequences at the absolute ends of linear DNA have not been determined directly, the structure has been closely approximated by restriction mapping of DNA from infected cells and by sequencing of minus strands synthesized in vitro and of cloned circular and proviral forms. The sequencing studies indicate that the long direct repeats (bracketed sequences labeled "DR") conclude with short and often imperfect inverted repeats (shown as solid triangles labeled "IR").

Some of the linear DNA migrates to the nucleus where some is converted to closed circular species (11). Circularization appears to occur either by homologous recombination between the redundant ends (resulting in the loss of one copy of the large redundancy) or by direct joining of the ends of linear DNA (9,10,12,13). In some cases, the joining may be accompanied by the loss of sequence from one or both ends, but in at least some other cases, the sequence of the junctional region of cloned circular DNA suggests that no nucleotides have been lost from linear DNA.

Integration of viral DNA into the host genome can occur in many different regions of cellular DNA (14), each lacking homology with each other or with the ends of viral DNA. However, it remains possible that preferred integration sites exist in host DNA. The integrated (proviral) DNA appears to be precisely colinear with the linear form of unintegrated DNA (14,15), proviruses thus ending with inverted repeats contained within the large direct repeats; however, the proximal precursor to the provirus has not been ascertained (hence the ambiguous arrow in Figure 1). The available sequence data from host-viral junctions reveal two additional, mechanistically significant attributes of proviruses: (i) the ends of proviruses appear to lack a few (generally two) nucleotide pairs present at the ends of linear DNA (and thus at the junction point in some of the larger circles); (ii) proviruses are flanked by direct repeats (DR) of a short sequence (4-6 nucleotide pairs) present once in unoccupied integration sites.

Proviruses normally harbor a complete copy of viral RNA, stretching from the point within the left hand terminal repeat corresponding to the cap site to a point within the other repeat corresponding to the poly(A) addition site. Moreover, there are additional sequences from the 3' terminus of viral RNA upstream (in a transcriptional sense) from the complete copy of viral RNA, and additional sequences from the 5' terminus of viral RNA downstream. Sequences likely to promote initiation of RNA synthesis at the cap site and to direct polyadenylation of viral RNA appear to be present at suitable locations within the redundancies. The primary transcript

initiated within the leftward redundancy is presumably equivalent to an unmodified subunit of viral RNA; after capping and polyadenylation it may be packaged into progeny virions, used as messenger RNA, or converted (by "splicing") into subgenomic messenger RNA's for internal cistrons.

PORTRAIT OF AN MMTV PROVIRUS

Our recent studies of a single cloned provirus of the mouse mammary tumor virus (MMTV) support several aspects of the scheme shown in Figure 1; in this section we will briefly review the rationale, difficulties, and results of that work.

Although similar in many respects to other retroviruses which are more malleable experimentally, MMTV invites study of proviral structure and function in view of its dramatic regulation at the transcriptional level by glucocorticoid hormones (16), its poorly understood capacity to induce mammary carcinomas (17), and its presence in the germ lines of many mice in the form of endogenous proviruses (18). However, our efforts to obtain molecular clones of MMTV DNA, starting either with circular or proviral forms, have been fraught with difficulty, regardless of whether we used plasmid or bacteriophage vectors. Judging from the variably deleted molecules isolated during attempts to clone circular DNA, it appears that a small region of the viral genome (less than 1 kb), near or in the gag gene, is relatively or absolutely unclonable in E. coli K12 host-vector systems. We refer to this sequence as the "poison sequence", and in Figure 2 we indicate its approximate position within a typical MMTV provirus using a traditional symbol.

Despite this obstacle, we have been able to clone an intact, steroid-responsive proviral unit from an MMTV-infected rat cell, because we have fortuitously isolated a cell containing a single provirus lacking the "poison sequence". The cell line was identified during a survey of proviruses in XC cells infected with MMTV produced by a cultured C3H mammary tumor line (19). Approximately two thirds of cells cloned nonselectively after infection at a low multiplicity contain MMTV proviruses, and about half of these have a single provirus. As expected from previous studies of MMTV DNA (20,21), the proviruses are located in different regions of host DNA and are generally colinear with the unintegrated linear species. However, the provirus in line E8 lacks most of the coding region for viral proteins (gag and pol) as well as the solitary Eco RI site present in the pol region of wild type MMTV DNA (Figure 2). The E8 provirus is transcribed into viral RNA, and the concentration of viral RNA is markedly stimulated by addition of dexamethasone to the culture medium. (The

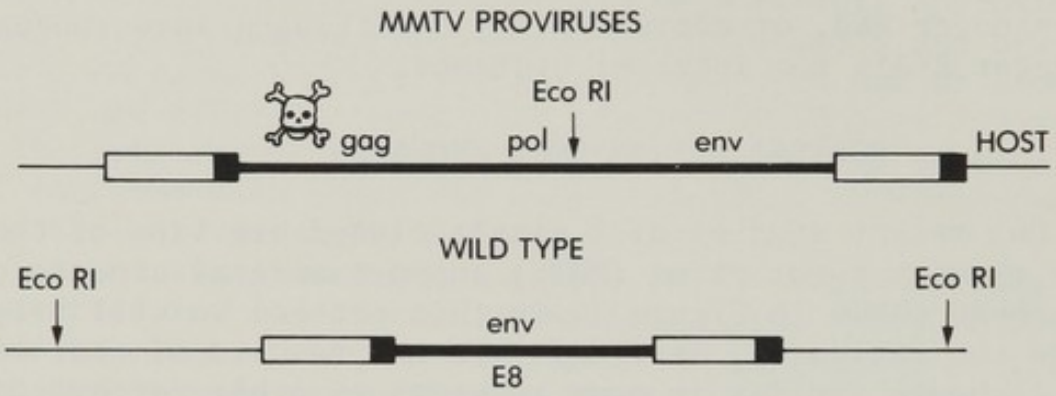

FIGURE 2. Proviruses of mouse mammary tumor virus. The relevant features of the typical MMTV provirus and the E8 provirus are discussed in the text. gag, pol, and env are structural genes common to replication-competent retroviruses (2).

presence of a complete env gene in the E8 provirus has been confirmed by the findings of normal env mRNA and env glycoprotein in E8 cells (unpublished results of D. Robertson and authors).)

The entire E8 provirus (ca.4.5 kb), accompanied by large regions of flanking host DNA, was cloned as an Eco RI fragment of 12 kb using Charon 4A lambda bacteriophage as vector and DP50supF as host. (Details of the cloning procedures and subsequent analyses will be described elsewhere.) DNA including the flanking regions was in turn used as hybridization probe to identify and clone an Eco RI fragment bearing the unoccupied integration site for the E8 provirus from another clone of XC cells. Analyses during these manipulations indicated that the flanking region is composed mostly if not completely of unique sequence DNA, and that no gross rearrangements or deletions had occurred in this region during the integration event.

The sequencing strategy was designed to explore primarily the host-viral junctions and possible regulatory signals encoded within the terminal redundancies. Precise definition of the ends of the provirus was facilitated by comparison of each junction with the corresponding internal region (the priming sites for plus and minus strands with left and right junctions, respectively; cf Figure 1). Identification of the

right end was also aided by independent sequencing of "strong stop" cDNA, primed by the native tRNA primer in an *in vitro* reaction. Alterations of the host sequence were determined by comparison of host sequence at the right and left ends of the E8 provirus with the corresponding sequence from the unoccupied integration site.

The following observation pertinent to the scheme drawn in Figure 1 were made during the sequencing studies of the E8 provirus:

(i) The large direct repeats (ca.1350 nt) at the ends of the provirus are concluded with a perfect inverted repeat of six nucleotide pairs $\binom{TGCCGC.....GCGGCA}{ACGGCG.....CGCCGT}$. (Here and below we follow the convention of showing the DNA plus strand above the minus strand, or only the plus strand, reading 5' to 3' from left to right.)

(ii). At least one and possibly two of the nucleotide pairs presumably present at the right end of linear DNA $\binom{(G)C}{(C)G}$ are missing from the right end of E8 proviral DNA.

(iii) A cellular sequence of 5 or 6 nucleotide pairs $\binom{(G)GTAAG}{(C)CATTC}$ present once in the unoccupied integration site is present as a direct repeat on both sides of the provirus. (The ambiguity about the size of the duplication---and about the number of bases missing from the viral sequence at the right end---arises because the G·C base pair at the virus-host junction could be contributed by either the host or the virus.

(iv) There is no evidence of homology between the ends of the provirus and the immediately surrounding cellular DNA. Moreover, there appear to be no similarities among the host sequences flanking E8 and those flanking the right ends of other MMTV proviruses from our infected cells or from a mouse mammary tumor line (Hager and Donehower, this volume).

(v) The site at which the critical plus strand is primed includes a purine-rich plus strand sequence (AAAAAGGGGGAAATG), the final 10 bases of which are identical to the functionally equivalent sequence in avian sarcoma virus (ASV) DNA (R. Swanstrom and P. Czernilofsky, unpublished). This finding is particularly striking since the genomes of ASV and MMTV are not homologous in conventional annealing tests, and their plus strand priming sites are different distances from the poly(A) sites.

(vi) The minus strand priming site is preceded by an 18 base sequence which would provide a completely matched binding site for $tRNA^{lys}_3$, recently identified as the minus strand primer for MMTV (G. Peters, personal communication). It is probably significant that the region of homology terminates

before the first modified base ($_m$1A) encountered when reading from the 3' end of the tRNA; the size of the binding site is thus probably determined by the extent to which reverse transcriptase is able to copy the tRNA into plus strand DNA (22, 23; R. Swanstrom, unpublished).

(vii) A sequence 24 to 32 nucleotides upstream from the cap site (TATAAAAGA) is similar to many other putative eukaryotic promoters and identical to a sequence similarly positioned upstream from the initiation site for the late RNAs of adenovirus 2 (24). A sequence of 10 nucleotides (CTTATGTAAA) is directly repeated in the region 40 to 60 nucleotides upstream from the cap site, but the significance of this redundancy is obscure.

(viii) The sequence AATAAA almost universally found just upstream from the poly(A) addition site (25) is not present in the expected region. (The precise site of addition of poly(A) addition is not known, since the size of the DR at the ends of MMTV RNA has not been determined.) The closest approximation of the signal for addition of poly(A) (AGTAAA) is located 4 to 10 nucleotides upstream from the cap site, and closely follows the sequence ATTTTTT.

IMPLICATIONS OF PROVIRAL STRUCTURE FOR MECHANISMS OF INTEGRATION AND TRANSCRIPTIONAL REGULATION

Although the available data are insufficient to determine the mechanism of integration---or even to decide which of the three well-defined species of unintegrated DNA is the proximal precursor to the provirus---several constraints must now be placed upon any proposals for mechanism. (i) Viral DNA must enter host DNA either randomly or by recognition of signals more subtle than simple homology of the type used for integration of lambda phage DNA. (ii) The provirus must be colinear with unintegrated linear DNA. This implies either that linear DNA itself is the substrate for integrative recombination (an attractive possibility in view of its free ends) or that host or viral enzymes with suitable specificity are available to open circular DNA at precisely defined sites. (iii) The absence of a deletion in host DNA at the site of integration suggests that the ends of viral DNA must be juxtaposed prior to integration, either as part of a circular intermediate or by some means for bringing the ends of the linear species together. (iv) A duplication of a short region of host DNA must occur during integration; this would be accomplished most simply by the production of a staggered cleavage of cellular DNA to create ends for ligation. Resulting single-stranded gaps on both sides of the provirus could then be filled by DNA polymerase, thereby completing the duplication. (v) It

appears that a small number of bases, generally two, is missing from at least one end of integrated viral DNA. A mechanism for eliminating this limited amount of viral DNA between synthesis and integration is required of any model for integrative events.

Whatever the mechanism of integration, it is apparent that the provirus is admirably suited to help regulate its own transcription and the processing of transcripts. In this light it seems probable to us that the response of MMTV gene expression to glucocorticoid hormones is controlled by a viral sequence (e.g. a binding site for activated hormone receptor) located within the ca.1200 nt upstream from the probable initiation site for RNA synthesis.

A virus-coded regulatory sequence would be the simplest answer to the problem of how MMTV proviruses at many different sites in several different cell types may be strongly influenced by glucocorticoid hormones (16). On the other hand, we do not mean to exclude the potential significance of flanking cellular DNA (or of chromatin conformation at the integration sites) as a determinant of the transcriptional activity of proviruses; the environs of a provirus could, for example, account for the complete absence of expression of certain MMTV proviruses (21). Use of the cloned E8 provirus and fragments of it as templates for transcription in vivo or in vitro should be helpful in defining these regulatory domains.

SIGNIFICANCE OF THE STRUCTURAL SIMILARITIES BETWEEN PROVIRUSES AND TRANSPOSABLE ELEMENTS

The structure of the E8 provirus (and others) has a striking similarity to the structure of certain transposable elements of bacteria (26), yeast (27),and Drosophila (28). The structural homology is illustrated dramatically by a comparison of a provirus such as E8 with the bacterial transposon it most resembles, Tn9 (Fig.3, ref.29). In both elements, domains encoding gene products are encompassed by large direct repeats; the direct repeats conclude with short inverted repeats; and the entire units are flanked by short duplications of host DNA generated during insertion. Despite the concordance of these physical symmetries, it would be premature to suggest that proviruses and transposons are functionally similar. For example, transposition in prokaryotes appears to depend upon the activity of transposases encoded by such elements (30), in conjunction with host enzymes used in DNA replication; movement of transposons is closely wedded to DNA synthesis, without the apparent generation of free forms, as discussed elsewhere in this volume by Starlinger. Proviruses can also be viewed as mobile genetic elements, but their

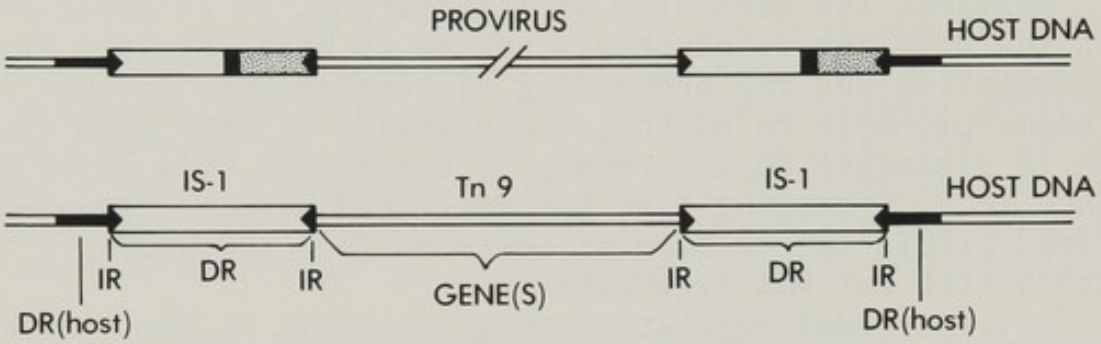

FIGURE 3. Comparison of provirus with Tn9. Symbols are used as in Figure 1. IS-1 is an insertion element present as a direct repeat at the termini of the transposon, Tn9 (ref.29). Comparative properties are discussed in the text.

transposition into new integration sites in new cells involves transcription into viral RNA by host RNA polymerase, transport of RNA in virus particles, DNA synthesis from RNA templates by virus-coded polymerase, and integration of free DNA into host chromosomes. It is entertaining in this context to consider reverse transcriptase as an analogue of transposases, but it is likely that the enzymatic functions are completely unrelated.

Although the mobility of transposons and proviruses probably differsmechanistically under usual circumstances, the strong structural relationship should prompt a concerted effort to seek unusual behavior of proviral DNA akin to the rare aberrancies exhibited by transposable elements (26). Strong selective pressures may well be required (as they are in procaryotes) to detect such rare events. In three separate contexts in which selective pressures exist, we and our colleagues have identified proviruses affected by deletions which involve the terminal redundancies:

(i) After selection for the rare descendents of an ASV-transformed rat cell in which the normal phenotype has been restored, we have occasionally encountered mutants bearing deletions in the left hand copy of the large terminal redundancy; such deletions eliminate the transcriptional activity of the single provirus in the parental cell and abort expression of the transforming (src) gene, (H.E.V. and N. Quintrell,

unpublished.)

(ii) Several proviruses endogenous to normal chicken cells exhibit deletions affecting the redundancy at the left end (31,32). Such proviruses are transcriptionally inert or incapable of producing virus particles; they are thus likely to be innocuous to their host, favoring their survival in the germ line.

(iii) Some lymphatic tumors induced in chickens by infection with Rous associated virus-2 bear single new proviruses which have sustained deletions in or near the redundancy at the left end (G. Payne, unpublished). If, as predicted, the structural genes of such proviruses are not expressed, the tumor cells may be likely to escape immune rejection by their hosts. (These observations also have interesting implications for the mechanism of tumorigenesis by this class of viruses.)

Although these examples are presently anecdotal and the mechanisms of deletion obscure, it may ultimately prove instructive to consider the possibility that the lesions in each case arose as a consequence of molecular behaviour also observable in structurally-related elements in lower forms of life.

ACKNOWLEDGEMENTS

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OBSERVATIONS ON THE DNA SEQUENCE OF THE EXTENDED TERMINAL REDUNDANCY AND ADJACENT HOST SEQUENCES FOR INTEGRATED MOUSE MAMMARY TUMOR VIRUS

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ABSTRACT Partial DNA sequences for the rightward extended terminal redundancies and adjacent cellular sequences of three integrated MMTV genomes have been determined. Viral-encoded sequences for the three independent molecules display perfect homology, whereas cell-specific sequences are non-homologous. A common DNA sequence that could serve as a sequence-specific integration site has not been found. Sequences that could serve as putative initiation and termination sites for viral transcription have been located within viral-encoded DNA.

INTRODUCTION

An important aspect of the regulation of retrovirus expression concerns the interaction, if any, between host cell and viral sequences. Current understanding of the mechanism for replication of retroviruses (1-3) and the organization of integrated proviral DNA (4-8) suggests a number of alternative models for the transcription of viral messenger RNA and genomic RNA from integrated viral DNA. These models are based on the unusual extended terminal redundant (E.T.R.) DNA sequence found at both ends of integrated proviral DNA. The critical feature of this integrated structure for the present discussion is the presence of information encoded at the 3' end of viral genomic RNA immediately proximal to sequences that serve as template for the 5' end of viral transcripts. The implications of this structure for viral expression are presented schematically in Fig. 1.

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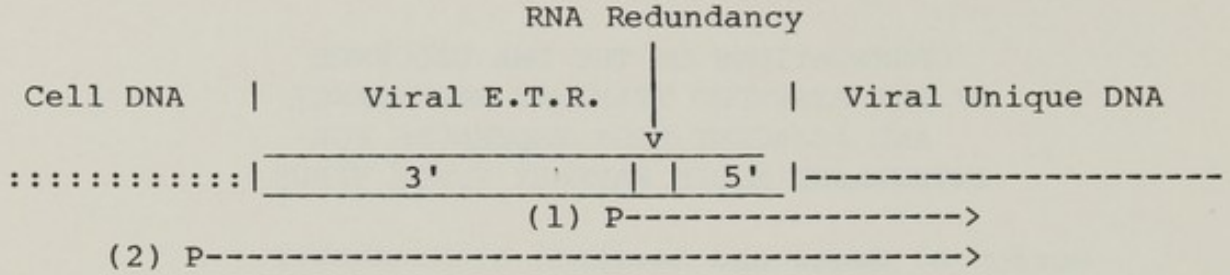

Figure 1. Models for initiation of viral transcription.

Two general models are immediately suggested by this structure. In the first case, viral transcripts would be initiated from sites encoded by viral DNA, presumably located in the region derived from the 3' end of the virus. Additional regulatory sites, if any, might also be located in this region. Under the second model, viral transcription would be promoted from cell-specific sequences, followed by processing to the mature viral RNA's. More elaborate models invoking regulatory contributions from both cell and viral DNA of course are possible.

In their most extreme forms, these models envisage, on the one hand, host cell DNA simply as a resident structure for the propagation of proviral sequences with the division of the cell, and on the other as providing critical and even necessary functions for the expression of viral information. A large body of evidence has accumulated (7,8,9), chiefly through the use of restriction endonuclease analysis, that exogenously-introduced retroviral genomes integrate at a wide variety of sites in the host cell genome. As has been previously discussed by others, this evidence indicates that a sequence-specific integration mechanism either is not operative in the case of retroviral insertion, or the putative sequence is too small to be detected by restriction endonuclease analysis.

Whether or not retroviral integration involves the recognition of sequence-specific sites is therefore of considerable relevance to the question of regulation of viral expression. We have accordingly undertaken the DNA sequence analysis of the 3' "joints" between Mouse Mammary Tumor Virus (MMTV) and host cellular sequences that are generated during the integration event. Three such joints have been analyzed. The data suggest that a "recognition sequence" for viral insertion probably does not exist, and that some other type of integration mechanism is utilized.

As discussed above for the first model (Fig. 1), promotion of viral RNA's from viral-specified DNA sequences implies that signals for the initiation of transcription should exist

in the extended terminal redundancy. In the simplest extension of this model, it would also be predicted that viral polyadenylation and termination signals should also be present in the E.T.R. (Fig. 2). Indeed, this proposed organization of

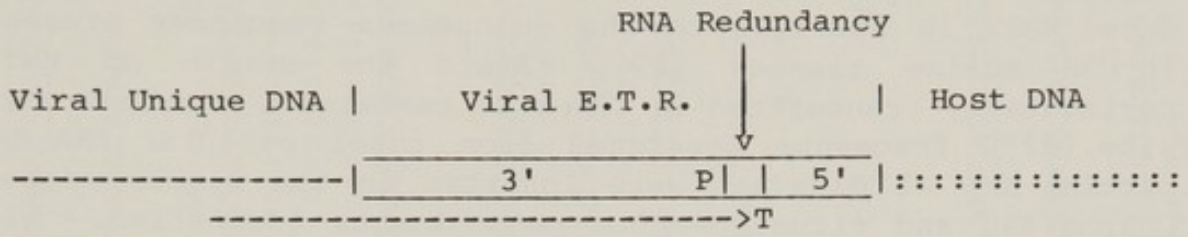

Figure 2. Rightward extended terminal redundancy. Predictions for transcription termination.

initiation and termination sites presents an interesting paradox. The sequence information in the leftward and rightward E.T.R.'s is identical. This postulate is strongly predicted by the fact that both sequences are derived from the same RNA information, and is supported by currently available restriction data. Viral transcripts initiated in the leftward E.T.R. (or in adjacent cellular DNA) must therefore "read through" the region where transcription presumably terminates in the rightward E.T.R.

Although our understanding of eucaroytic transcriptional signals is at a very primitive stage, the presence of suspected signal sequences has been noted in a number of eucaryotic systems (10-19). We have examined the E.T.R.-specific sequences from the integrated MMTV genomes for potential transcription signals, and have identified several potential sites.

METHODS

The approach we have used to address the questions raised in the introduction is to molecularly clone integrated copies of Mouse Mammary Tumor Virus representing several independent integration events and to determine the DNA sequence of specified regions of interest by the method of Maxam and Gilbert (20). The isolation and characterization of the molecular chimeras has already been described (21). Structural and sequence analysis of subgenomic regions will be detailed elsewhere (G. L. Hager and L. A. Donehower, manuscript submitted) and will not be further treated here.

RESULTS

We chose as the starting marterial for our investigation an MMTV-producing cloned cell line derived from a mammary carcinoma of a C3H mouse (21). This cell line contains a complement of 15-20 copies of exogenously introduced MMTV in addition to the endogenous sequences present in C3H murine tissues (29). Since the strain of MMTV horizontally transmitted in C3H mice contains a single Eco RI site (22), fragments generated from total cellular DNA by partial Eco RI cleavage were inserted in the lambda vector Charon 4A, and virus-specific recombinants identified. Although it was anticipated that some of the recombinants would contain complete integrated MMTV genomes, in reality only clones containing the 3' half of the genome and adjacent cellular sequences were identified (21). Numerous attempts to clone an accurate copy of the 5' half of MMTV have failed, both from unintegrated and integrated DNA. The left half of the genome apparantly contains a sequence that cannot be cloned in E. coli.

We pursued the analysis of the 3' clones, however, because the virus-cell joints at the right end of the genome represent the ideal candidates for initial study. A preliminary sequence of the MMTV "5' Strong Stop" was available (J. Lovinger and G. Schochetman, personal communication), and this sequence would precisely mark the right end of the viral genome (see Fig. 2). From a group of 86 independent recombinants, a group of 10 had been previously mapped (21). Fragments containing the virus-cell joint from three of these were transferred to the plasmid pBR322. The regions in the three clones corresponding to the cell-virus joints were then subjected to DNA sequence analysis. The results are presented in Fig. 3.

DISCUSSION

Integration Sites. The first issue to be addressed is the exact position of the virus-cell joint. As discussed above, this site should be approximately marked by the initial 5' strong stop sequence, although the mechanism of retroviral integration is insufficiently understood to permit an exact description. The incomplete determination of the 5' strong stop sequence is shown in Fig. 3. Although this sequence was very preliminary, it was of interest to search for its presence in the integrated sequences. An imprecise fit can be made between the initial strong stop and positions -1 to -28 in the integrated molecules. In itself, this correspondence is too ambiguous to clearly identify the beginning

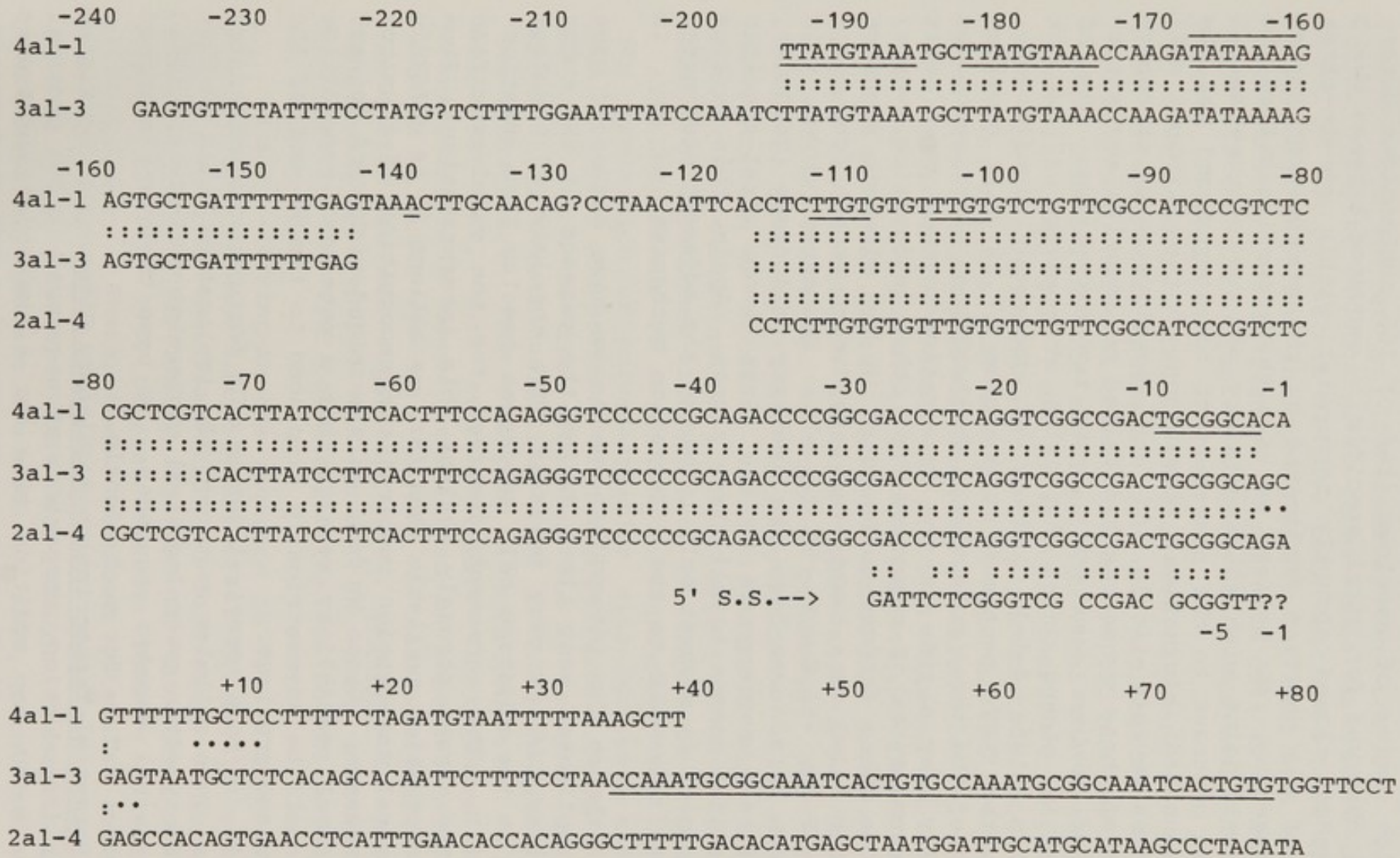

Figure 3. DNA sequence of three rightward virus-cell joints. Two dots (:) indicates homology between the three sequences, one dot (•) indicates homology between two.

of viral information. A more compelling observation is that the high degree of homology (166 of 168 nucleotides determined) observed for viral specific sequence between the 3 independent clones abruptly terminates within one-to-three bases of the position implicated by the preliminary strong stop sequence. We believe we have therefore identified the virus-cell joint with an uncertainty of one-to-three bases.

In contrast to the near-perfect homology found for the viral encoded information, comparison of the adjacent cell sequences reveals little, if any, homology. An extensive search of these sequences has failed to identify any structures of obvious similarity, with two exceptions. The AG doublet in clones 3a1-3 and 2a1-4 at position +2 is not found in clone 4a1-1, and is of uncertain significance. Again, the TGCTC pentamer at position +7 is only observed in two of the three molecules. Whether these short sequences actually play a role in the integration process must await the accumulation of sequences from other integration events.

One other feature to be noted in the adjacent sequence of clone 3a1-3 is a twenty-two base direct repeat from position +35 to +56, and +57 to +78. Contained within this sequence is a seven-base complimentary palindrome (TGCGGCA), that itself corresponds to the first seven viral-specific nucleotides common to all three clones. Again, this curious arrangement is present only in one of the adjacent sequences, and its relevance to the integration mechanism, if any, is unclear.

Although the integration events we have sequenced have been replicated many times during the passage of the tumor cell line, it is very unlikely that the lack of homology found in the adjacent cell sequences results from mutational drift. The agreement observed for the viral-specified sequence argues strongly against this interpretation. From the information available thus far, we believe it is unlikely that viral integration involves the recognition of a unique DNA sequence. Since we have only examined rightward joints, the formal possibility remains that a potential recognition site could be asymmetrically displaced to the 5' end of the integrated provirus.

If, in fact, proviral DNA is only fortuitously associated with a given cellular sequence, the implication would be that regulatory sites governing viral expression would be specified within viral encoded sequences. We have therefore examined the viral E.T.R. for such signals.

Potential Transcription Regulatory Sites. As mentioned in the introduction, comparison of sequences in DNA proximal to the m-RNA cap addition site for a number of eucaryotic genes reveals the frequent occurance of a common sequence, the so-called "TATAAA" or "Hogness" box 25-30 nucleotides upstream from the putative RNA ini

Although the exact sequence varies considerably in the several cases described, a fairly common feature is the presence of one or more TAT groups, followed by two-five A's. Although other examples of eucaryotic genes whose expression is mediated by RNA polymerase II have been described that lack the upstream TATAAA box analogue (23,24), it has been elegantly demonstrated that initiation *in vitro* in cell free transcription systems can occur at sites with a proximal TATAAA sequence (25,26).

The length of the 5' strong stop sequence for MMTV has been independently estimated to be 140 nucleotides (J. Taylor and H. Young, personal communication) to 150 nucleotides (27) long. Remembering the topology for viral DNA organization summarized in Fig.'s 1 and 2, it would therefore be predicted that the initiation site for MMTV transcription should occur between positions -140 and -150 in Fig. 3, and a transcription signal analagous to those at position -165 to -190. A heptanucleotide, TATAAAA, is in fact located at position -167, and would therefore fit the pattern described for many Pol II mediated genes. Another remarkable group of nucleotides occurs between positions -194 and -174. The nonanucleotide, TTATGTAAA, is repeated precisely. Whether these sequences have any functional significance will await the demonstration of specific viral initiation with these or similar DNA templates, and investigation of the functional impact of mutational alteration of the sequences in question.

A comparison of sequences in eucaryotic DNA distal to the termination or polyadenylation site for several mRNA's again reveals the presence of common features. In particular, an examination of the sequences distal to five different genes finds a tetranucleotide, TTTT or TTGT, precisely seven bases downstream from the poly A addition site. In all cases but one, this tetranucleotide is repeated further downstream, in a manner reminiscent of repeated termination signals in other systems. In the one case where this tetranucleotide was not repeated, the *X. borealis* 5S gene, the intriguing observation was made (19) that a single-base mutation of the TTTT tetranucleotide led to the abolishment of termination.

Again referring to the sequence in Fig. 3 and remembering that the termination site for viral RNA should be located downstream from the transcription initiation site by the number of bases terminally repeated in the viral RNA (Fig. 2), we find two TTGT tetranucleotides located at positions -112 and -104. These, then, represent potential candidates for viral RNA termination signals. Yamamoto *et al.* (28) have recently reported that two mRNA's transcribed from integrated ASV genomes are not, in fact, terminated within the viral E.T.R., but continue into cell DNA. This somewhat unexpected observation raises interesting questions about

the metabolism of viral mRNA's, but does not contradict the hypothesis that viral genomic RNA is terminated within the E.T.R.

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DERIVATION OF THREE MOUSE STRAINS CARRYING MOLONEY LEUKEMIA VIRUS IN THEIR GERM LINE AT DIFFERENT GENETIC LOCI

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ABSTRACT The exogenous Moloney leukemia virus (=M-MuLV) was integrated experimentally into the germ line of mice. Substrains of mice were derived carrying three new genetic loci which were designated as Mov-2, Mov-3 and Mov-4, and represent M-MuLV genes integrated into the mouse genome. Mice carrying the Mov-3 gene developed early viremia and died rapidly of leukemia, whereas animals transmitting the Mov-2 gene activated virus only occasionally late in life. The M-MuLV provirus integrated at the Mov-2 and Mov-3 locus was identical by restriction enzyme analysis and the virus activated from Mov-2, Mov-3 and BALB/Mo mice (=Mov-1) was identical by biological and biochemical criteria. The M-MuLV genome in Mov-4 mice was shown to have a partial deletion and no virus expression was observed in these animals. Together with previous results obtained in the BALB/Mo system, our data suggest that the chromosomal location of an endogenous virus influences its tissue-specific expression.

INTRODUCTION

Endogenous C type viruses are genetic elements in almost every vertebrate species and most probably entered the germ line by infection (1-5). Inbred strains of mice carry single or multiple copies of endogenous ecotropic virus. Genetic studies demonstrated that these virus genomes are transmitted as allelic or non-allelic genes in both closely and more distantly related mouse strains (6-10). Distinct phenotypes of tissue-specific virus expression have been observed in different mouse strains (11-13). Due to the presence of multiple copies of closely related endogenous viruses in every mouse strain, the genetic basis of the differences in expression of these genes is poorly understood. To avoid the complication of analyzing a multiple gene system, we have introduced the well defined exogenous Moloney leukemia virus (=M-MuLV) into the germ line of mice.

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A new substrain of mice, BALB/Mo, was developed which carries the exogenous M-MuLV as an endogenous gene (14,15). Hybridization probes specific for M-MuLV enabled us to study both the chromosomal location and tissue-specific expression of the integrated virus genome (13,16). The presence of this dominant gene, designated as Mov-1, was localized on chromosome No. 6 (17) and is associated with early viremia and high incidence of leukemia. Thus the BALB/Mo mouse is similar in phenotype to AKR mice which carry multiple ecotropic virus loci on different chromosomes and have a similar pattern of viremia and leukemogenesis (6). To investigate whether the distinct phenotypes of virus expression found in different mouse strains are associated with structural differences in the proviral genomes or with their chromosomal integration sites, new mouse strains carrying M-MuLV in their germ line were established. In the present communication we report the derivation of three new substrains of mice carrying each a single M-MuLV genome on different chromosomal integration sites. These new M-MuLV loci were characterized by restriction enzyme analysis, molecular hybridization and genetic segregation studies. Each locus was associated with a distinct phenotype of virus expression. Evidence is presented that defective viral genomes can be carried in the germ line of mice and that apparently identical viral genomes show differences in spontaneous virus activation.

MATERIALS AND METHODS

Mice. All mice used in the following experiments were bred in our colony at the Heinrich-Pette-Institut. The origin of BALB/Mo mice has been described previously (15, 22).

Infection of Preimplantation Mouse Embryos with M-MuLV. Four to eight cell embryos were isolated from pregnant mice, treated with pronase to remove the zona pellucida and cultivated in vitro as described previously (14,15). For infection with M-MuLV the embryos were cocultivated overnight with subconfluent Cl 1A cells (18). They developed into blastocysts and were subsequently transferred to the uterus of a pseudopregnant female.

Molecular Hybridization. Quantitative DNA-DNA hybridizations using M-MuLV-specific cDNA in excess and calculation of copy numbers have been described previously (22).

DNA-RNA Hybridizations. Virus-specific RNA was quanti-

tated by crt analysis as described previously (13).

Restriction Enzyme Analysis. Isolation of high molecular weight DNA from mouse tissues, separation of restricted DNA on agarose gels and detection of virus-specific fragments by specific probes were performed as published (16).

RESULTS

Preimplantation mouse embryos were infected in vitro with M-MuLV as described in Materials and Methods and were transplanted as blastocysts to the uterus of a pseudopregnant female. Approximately 30% of the transplanted embryos survived to birth. Twelve of 28 mice derived in this way were viremic when tested by RIA for p30 at the age of 4 weeks. For the experiments reported in this paper, one viremic male (No. 62) of ICR origin was selected and genetic and biochemical methods were employed to detect and analyze M-MuLV-specific information in the DNA of this animal.

Genetic transmission of virus was analyzed as follows: Male No. 62 was mated with normal females and the resulting offspring were tested for viremia as above. At 4 weeks of age 20 of 76 offspring analyzed were found to be viremic. This paternal transmission of virus suggests that M-MuLV was integrated in cells of the germ line of male No. 62. The fraction of viremic offspring observed (26%) deviates from the Mendelian expectations for transmission of a single dominant gene and suggests that only a fraction of the germ cells carried the virus. This is similar to our previous observation of "germ line mosaicism" in mice that were infected with M-MuLV at the preimplantation stage (15). Biochemical evidence (Fig. 1) indicates that male No. 62 was not only mosaic in the germ line but also in somatic tissues (see below).

When viremic sons of male No. 62 were bred with normal females, they transmitted viremia to approximately 50% of their offspring (Table 1). The pattern of Mendelian transmission observed suggests that they were heterozygous for a single viremia-inducing genetic locus and indicates that the exogenous M-MuLV had been converted to an endogenous virus in these mice.

Identification of Integrated M-MuLV Copies by Restriction Enzyme Analysis. In order to detect and to characterize biochemically the integrated M-MuLV copies, male No. 62 and 30 of his viremic and non-viremic offspring were partially hepatectomized (19), DNA was prepared and anal-

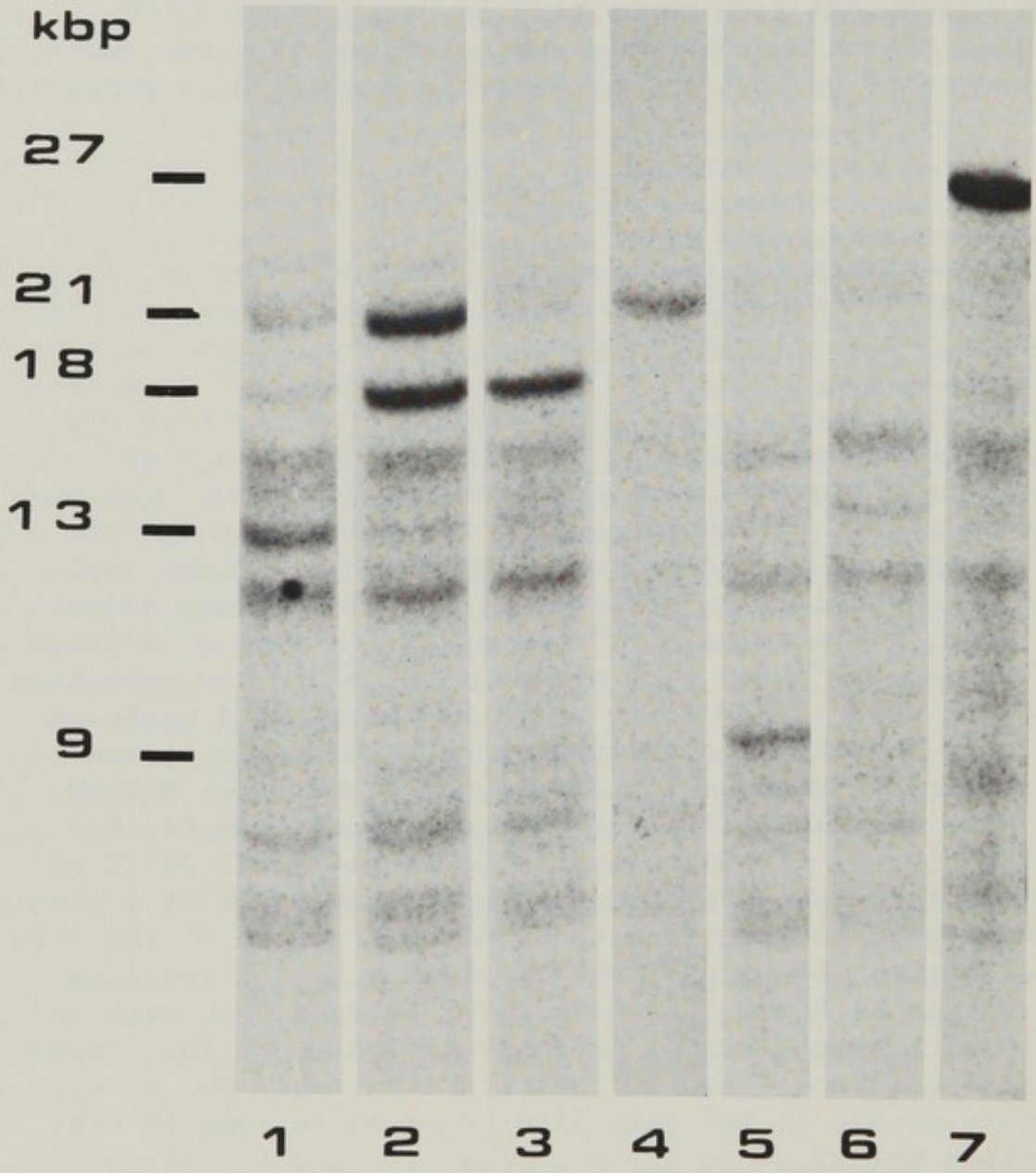

FIGURE 1. Restriction enzyme analysis of mouse liver DNA. High molecular weight DNA from liver biopsies was digested with EcoRI, separated on agarose gels, transferred to nitrocellulose filters and hybridized to M-MuLV-specific cDNA as described (16,37). EcoRI digested λ DNA was used as a size marker. Lane 1: DNA from No. 62; lanes 2-5: DNA from individual N-1 animals; lane 6: ICR control; lane 7: BALB/Mo DNA. The size and position of M-MuLV-specific fragments is indicated on the left.

TABLE 1
GENETIC TRANSMISSION OF M-MuLV

Male No.	Mode of infection	No. of viremic offspring (mated with normal ♀♀)	%
62	4-16 cell pre-implantation stage	20/75	26
Viremic males	N-1 from No. 62	85/162	52

Four to eight cell preimplantation ICR mouse embryos were exposed to M-MuLV in vitro and transplanted to the uterus of a pseudopregnant female as previously described (14). Animals were born and tested for viremia by RIA for the M-MuLV p30 protein (15) at 3 weeks of age. One viremic male (No. 62) was bred with normal females and animals of the N-1 generation were tested for viremia. Viremic N-1 males were further bred and the resulting N-2 mice were tested for viremia as above.

yzed by restriction enzyme analysis with EcoRI. The DNA was fractionated on agarose gels and transferred to nitrocellulose filters. Virus-specific sequences were identified by hybridization using a hybridization probe which detects preferentially M-MuLV-specific sequences as described previously (16). The use of a specific cDNA is crucial in order to detect M-MuLV sequences above the background of endogenous murine viruses. Confirming previous results, Fig. 1, lane 7, shows a single M-MuLV-specific fragment with a molecular weight of 27 Kbp in BALB/Mo liver DNA which was run as a control. This band represents the endogenous M-MuLV copy in BALB/Mo mice (16,20). Multiple bands with much lower relative intensities than the M-MuLV-specific bands are present in all mouse DNAs and are due to residual cross homology of our specific probe with endogenous murine viruses. Three new M-MuLV-specific fragments not present in the uninfected control (lane 6) with a molecular weight of 21, 18 and 9 Kbp, respectively, were detected in the offspring of male No. 62 (lanes 2,3,4,5). Since EcoRI does not cleave within the M-MuLV genome (21), every fragment represents a copy of M-MuLV sequences integrated at a different chromosomal site. In addition to the

three M-MuLV-specific fragments detected in his offspring, a fourth new band of 13 Kbp is seen in the liver DNA of male No. 62 (lane 1). The relative intensities of the 21, 18 and 9 Kbp fragments in the DNA of No. 62 are much weaker when compared to the respective fragments in the DNA of his offspring (compare lane 1 with lanes 2, 3 and 5). The 13 Kbp fragment, however, is not found in DNA of his offspring. This suggests that not all liver cells of male No. 62 carried the M-MuLV proviral copies so that individual fragments appear in submolar amounts. The quantitative hybridization using specific cDNA probes in excess (22) is in agreement with the "blot" analysis, detecting approximately 0, 1 or 2 copies per diploid genome in the respective mice carrying the different fragments (Table 2). Liver DNA of male No. 62, however, had only 2 copies by the quantitative hybridization, although a total of 4 acquired M-MuLV copies were identified by restriction enzyme analysis. Thus mosaicism was demonstrated biochemically in somatic tissues of male No. 62 complementing the genetic data on germ line mosaicism as discussed above (Table 1).

Thirty individual offspring of male No. 62 were partially hepatectomized and their genotype was characterized by restriction enzyme analysis. Five different genotypes were observed (Table 2). Each of the three M-MuLV-specific EcoRI fragments was found without the other two indicating independent segregation. The 21 and 18 Kbp fragments were transmitted separately or in combination indicating that these two genes are non-allelic. However, the 9 Kbp fragment was never seen in combination with any of the other fragments, and it cannot be excluded that this fragment was allelic to one of the other copies.

Segregation studies were performed in order to confirm that the M-MuLV-specific EcoRI fragments identified in Fig. 1 represent genetic loci. Males carrying the individual M-MuLV fragments were mated with normal females and the liver DNA was analyzed by quantitative hybridization using M-MuLV-specific cDNA (22). Table 3 shows that genetic transmission was observed for all of the fragments. The 21 Kbp and 18 Kbp fragments were transmitted at the expected Mendelian ratio of approximately 50% to the next generation, whereas only one out of the small number of five animals so far analyzed carried the 9 Kbp copy. Our results define three new genetic loci carrying M-MuLV-specific sequences. These genes will be designated Mov-2, Mov-3 and Mov-4 corresponding to the M-MuLV-specific EcoRI fragments of 21 Kbp, 18 Kbp and 9 Kbp length, respectively. The locus representing the M-MuLV gene in BALB/Mo mice has been designated as Mov-1 previously (17).

TABLE 2
CHARACTERIZATION OF GENETICALLY TRANSMITTED M-MuLV SEQUENCES AND PHENOTYPE OF MICE

Origin	No. of animals	M-MuLV in EcoRI fragment (size in Kbp)	No. of M-MuLV copies per diploid genome equiv.	Expression of viremia	Genetic locus
Male No. 62	1	21 18 13 9	2	+	-
N-1 of male No. 62	3	21	1-1.2	-	Mov-2
	5	18	.9-1.2	+	Mov-3
	8	21 18	2-2.4	+	Mov-2 Mov-3
	6	9	.9-1.1	-	Mov-4
	8	-	0	-	-
BALB/Mo	-	27	1	+	Mov-1

Backcross animals of the N-1 generation from male No. 62 were derived as described in Table 1. Viremic and non-viremic offspring were partially hepatectomized and the liver DNA was subjected to restriction enzyme analysis and to quantitative hybridization using specific M-MuLV cDNAs as hybridization probes (16,22). The size of M-MuLV-specific EcoRI fragments (see Fig. 1) and the number of M-MuLV-specific DNA copies per diploid mouse genome equivalent are given (22). Animals were tested for viremia at 3 weeks of age (see Table 1). Designation of genetic loci is explained in the text. Data on BALB/Mo mice are taken from previous publications (16,20).

The structure of the integrated M-MuLV genomes were analyzed after digestion of cellular DNA with the restriction enzyme Pvu II. This enzyme cleaves M-MuLV DNA internally and within the repeated sequences at both ends of the M-MuLV genome generating 4 internal M-MuLV fragments

TABLE 3
GENETIC TRANSMISSION OF M-MuLV SEQUENCES AND INCIDENCE OF VIREMIA IN OFFSPRING

M-MuLV-specific EcoRI fragment in father	Locus	M-MuLV-specific sequences present in DNA of N-1 mice/total tested	Viremic N-1 animals/total tested	%
18 Kbp	Mov-3	14/28	125/255[a]	49
21 Kbp	Mov-2	6/14	14/147[b]	9.5
9 Kbp	Mov-4	1/5	0/60	0

Males of genotype Mov-2, Mov-3 and Mov-4 were identified after partial hepatectomy as described in Table 2. They were bred with normal females and some of the offspring were again partially hepatectomized and the presence of M-MuLV-specific sequences in the liver DNA determined by quantitative hybridization (22). All animals were repeatedly tested for viremia using the RIA for p30 (15).

[a]Animals segregating the Mov-3 locus were viremic at the youngest age tested (3 weeks).

[b]Animals segregating the Mov-2 locus were non-viremic at 3 weeks of age; part of the mice developed viremia at later ages (between 2 and 4 months).

which correspond to 93% of the genome and two junction fragments containing viral DNA along with flanking cellular DNA sequences (23). No difference was observed between the internal M-MuLV fragments generated from Pvu II cleavage of DNA from mice of genotype Mov-1 (lane 2), Mov-2 (lane 3) or Mov-3 (lane 4). DNA from mice with genotype Mov-4, however, had lost the largest Pvu II fragment (lane 5). This suggests that the integrated M-MuLV in these mice contains a deletion in the 3' half of the viral genome. Further analysis with Sac I confirmed this conclusion (data not shown). Lane 1 in Fig. 2 shows that the B and D fragments are of very low intensity. The specific probe used for hybridization represents only 30% of the M-MuLV genome (20,22) and thus probably detects only weakly pol related sequences. The presence of the pol gene, however, was shown by digestion

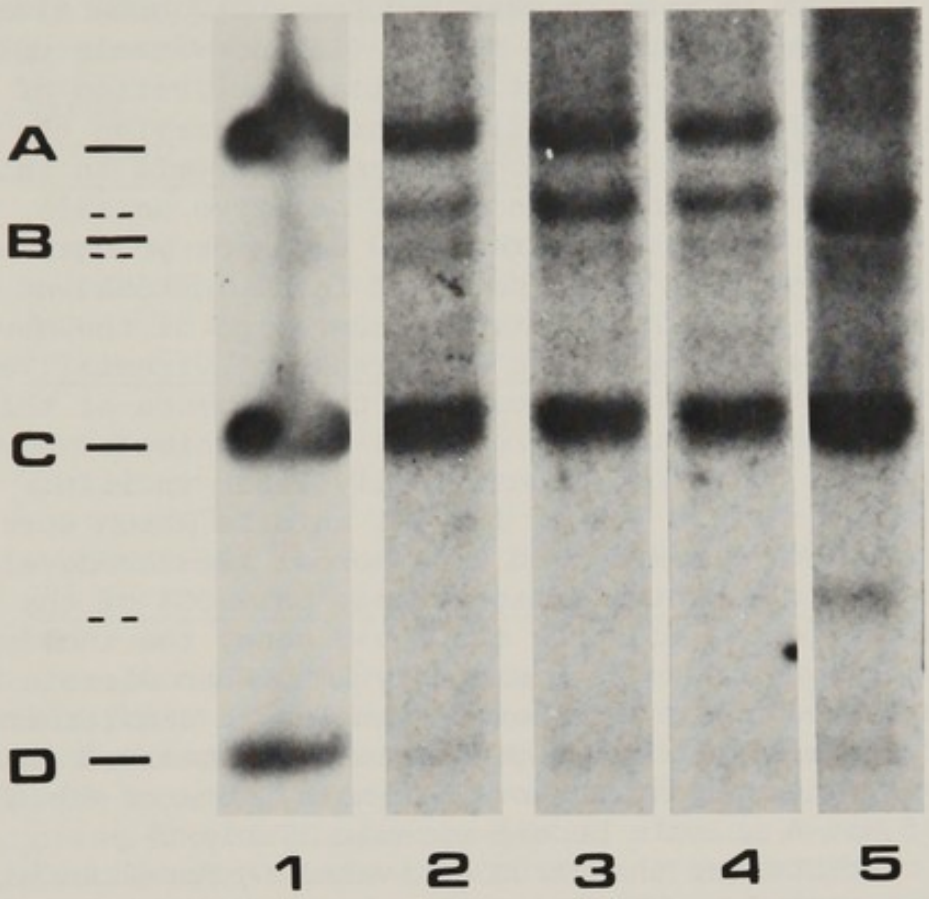

FIGURE 2. Digestion of liver DNA by Pvu II. DNA was analyzed as described in Fig. 1. Lane 1 shows a Hind III-Pvu II double digest of M-MuLV cloned in pBR 322 (provided by S. Goff); lane 2: BALB/Mo DNA (=Mov-1); lane 3: Mov-2 DNA; lane 4: Mov-3 DNA; lane 5: Mov-4 DNA. The letters A-D mark the positions of the M-MuLV fragments after Pvu II digestion as published (23).

with Sac I and Kpn I and these digestions did not reveal any differences in the cleavage patterns of DNA from mice with genotypes Mov-1, -2 and -3 (data not shown). Our results show that no major rearrangements in the M-MuLV genomes carried in the latter 3 substrains of mice have occurred and that partially deleted viral copies can be present in the germ line of mice.

Incidence of Viremia and Leukemia in Mice Carrying M-MuLV in the Germ Line. Table 2 correlates the genotype of the mice with the status of viremia. Three different phenotypes were observed among the five genotypes distin-

guished in the offspring of male No. 62: (i) early development of viremia in animals carrying the Mov-3 gene alone or Mov-3 in combination with Mov-2; (ii) no viremia until 3 weeks of age (Table 2), but occasional activation of M-MuLV later in life (Table 3) in animals carrying the Mov-2 gene alone; (iii) no occurrence of viremia in animals carrying the Mov-4 gene or in M-MuLV negative animals. Viremia in Mov-3 animals occurred at 3 weeks or younger and was followed by rapid development of fatal leukemia at 2 to 4 months of age. The genetic transmission of the Mov-3 gene correlates well with the occurrence of viremia (Table 3) and supports the conclusion that the presence of this gene induces early virus expression. Mov-2 animals, however, activate virus only occasionally later in life. Table 3 shows that 14 out of 150 N-1 animals which were derived from Mov-2 males bred with normal females developed viremia at 2 to 4 months of age. Since only 50% of the offspring are expected to carry the Mov-2 gene, the incidence of virus activation is approximately 20%. When viremic N-1 males were bred again with normal females, a similar incidence of viremia in the N-2 generation was observed (data not shown). In contrast to Mov-2 animals, none of 60 offspring of Mov-4 animals became viremic (Table 3).

To characterize the virus activated in Mov-2 animals, it was propagated on NIH 3T3 cells. Table 4 shows that this virus replicated equally well in NIH 3T3 and BALB/c 3T3 cells. The virus was cloned on either cell line and titrated again on NIH 3T3 and BALB/c 3T3 cells. The results shown in Table 4 indicate that the cloned virus grew equally well on both cell lines as is expected for NB tropic Moloney leukemia virus.

Mov-2 derived virus was characterized biochemically as follows: RNA was isolated from the infected NIH 3T3 cells and from the spleen of a viremic Mov-2 and Mov-3 animal and hybridized to M-MuLV cDNA. The M-MuLV probe hybridized equally well to the RNA from the tissue culture line and to RNA from both spleens (data not shown). Furthermore, when DNA from NIH 3T3 cells infected with virus activated from Mov-2 animals was digested with Pvu II, no differences in the cleavage pattern of integrated viral sequences was observed in comparison to Cl 1A virus, BALB/Mo virus or Mov-3 virus (data not shown). Our results suggest that Mov-2 animals activate NB tropic Moloney virus integrated into their germ line.

TABLE 4
TITRATION OF VIRUS FROM MOV-2 TYPE MICE

Virus (origin)	Titer on NIH 3T3	Titer on BALB 3T3
Mov-2 mice	1×10^5	7×10^4
M-MuLV Cl 1A	7×10^5	7×10^5
AKR	2×10^5	7×10^2
Mov-2 cloned on		
NIH 3T3	7×10^4	3×10^4
BALB 3T3	2×10^5	1×10^5

Virus was isolated from spleens of viremic Mov-2 type mice by cocultivation of spleen cells with NIH 3T3 cells. Virus stocks were titrated on NIH 3T3 and BALB 3T3 cells using the XC plaque assay (36). The virus was furthermore cloned on NIH 3T3 and BALB 3T3 cells and again titrated on both cell lines. Titrations with M-MuLV Cl 1A and with AKR virus are included as controls.

CONCLUSIONS

It is known that endogenous avian (2,3) and murine (7, 9,24,25) C type viruses are localized at several distinct chromosomal loci and some evidence suggests that the pattern of gene expression is different for individual proviruses (26-29). Our experiments are aimed to correlate the expression of endogenous viruses with the site of chromosomal integration. For this purpose, methods were developed which permit to introduce defined exogenous RNA tumor viruses into the germ line of mice with high efficiency. A high proportion of live mice obtained after transplantation of virus-infected preimplantation embryos to pseudopregnant foster mothers carry virus genetic information in many of their tissues including the germ line. Data presented here confirm our previous evidence (15) that these mice are mosaics in somatic and germ line tissues.

Animals heterozygous at several distinct virus loci were identified in the first backcross generation after mating of

a mosaic male to normal females. Expression of the M-MuLV genome introduced into different chromosomal sites was then correlated to the respective genetic locus of the virus. In our first attempt following this experimental approach, the BALB/Mo substrain of mice was derived carrying the M-MuLV genome at a genetic locus designated as Mov-1 (17). This genome has been shown to be transcribed specifically in lymphatic tissues during development of the animals (13). The present experiments define genetically and biochemically three new loci in the mouse designated as Mov-2, Mov-3 and Mov-4 which represent M-MuLV genomes integrated into the mouse genome.

The phenotype of these new substrains of mice was different. Every animal carrying the Mov-3 gene expressed regularly and early (3 weeks of age or younger) high titers of virus and died extremely rapidly from malignant lymphoma. In contrast to lymphatic tissue-specific expression of M-MuLV in BALB/Mo mice, preliminary experiments suggest that the virus genome is expressed also in other tissues (R. Jaenisch, unpublished). A different phenotype of virus expression is characteristic for Mov-2 mice which do not express their endogenous virus early in life. In 20% of these animals, however, infectious M-MuLV is activated after 2 to 4 months of age leading to virus-induced leukemia which is indistinguishable from the disease in BALB/Mo or Mov-3 type mice. The virus genome at the Mov-2 locus as well as the activated virus from the adult animal is indistinguishable from the M-MuLV used for the primary infection of the embryo (=male No. 62) or from the Mov-3 virus by all biological and biochemical criteria used. Virus negative Mov-4 mice were shown to carry a partially deleted M-MuLV provirus explaining their inability to activate infectious virus.

So far our data do not definitively exclude the possibility that the M-MuLV genome at the Mov-2 locus has a mutation and/or modification leading to less efficient expression in Mov-2 mice. Such an alteration occurring in integrated viral genomes was suggested in recent experiments with RAV O (30) and with AKR virus (31). In the latter experiments one of two apparently identical viruses replicated much less efficiently in tissue culture cells than the other. The virus we isolated from Mov-2 type mice appears to replicate as efficiently in tissue culture as the original M-MuLV used for infection of male No. 62. To definitively prove that no mutational alterations had occurred in the virus genome integrated at the Mov-2 locus, the integrated gene will have to be molecularly cloned and characterized by fine structure analysis.

Much evidence has accumulated suggesting that C type

virus expression is controlled at the transcriptional level (26), but it is not clear whether an integrated virus can be expressed independently from the surrounding cellular genes. Alternatively, virus genome expression may be under strict control of adjacent cellular regulatory elements.

Several considerations favor the notion that cis-acting cellular control elements are important for virus expression. (i) The efficiency of transfection with cellular DNA containing avian provirus is increased if the DNA is first sheared to genome length to remove adjacent sequences (32). Preliminary transfection experiments performed with liver DNA from Mov-1 (=BALB/Mo), from Mov-2 and Mov-3 mice indicated that XC plaques were induced only with Mov-3 DNA (H. Stuhlmann, preliminary results). This observation is compatible with a cis-acting control element determining the expression of the adjacent provirus. Alternatively, different levels of DNA methylation (30,33), which could also depend on the chromosomal locus, might determine the infectivity in the transfection assay and/or tissue-specific gene expression. (ii) Expression of the same endogenous virus in specific tissues and not in others, as has been observed in several systems (11-13), is most easily explained by the assumption of cellular control elements which activate adjacent cellular genes during cellular differentiation in specific tissues. This concept would attribute the tissue-specific expression of any endogenous virus to its chromosomal site of integration, as has been discussed in detail previously (34).

The experimental integration of viral genomes into different chromosomal locations in the germ line of mice offers access to study the control of gene expression by molecular and genetic means. In recent experiments we have derived nine additional mouse strains that carry M-MuLV at different chromosomal sites. Some of these strains show previously unobserved phenotypes, such as a characteristic hair color change as has been seen after manipulation of embryos in a different experimental approach (35). The availability of these defined mouse strains should facilitate investigations to understand the regulation of C type virus expression during cellular differentiation and its possible role in evolution.

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We thank Drs. S. Goff, E. Gilboa and D. Baltimore for providing us with the M-MuLV clone and Dr. J. Bilello and all members of the tumor virus laboratory for constructive criticism. The excellent technical assistance of E. Hoffmann is gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft and by Research

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A HUMAN HEPATOMA CELL LINE CONTAINS HEPATITIS B DNA AND RNA SEQUENCES[1]

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ABSTRACT. The genome of a human hepatoma cell line, the Alexander cell, contains at least six integrated copies of Hepatitis B viral DNA. Four of these integrated sequences appear to be full length viral genomes. Integration occurs at a specific region of the viral DNA, which is near the nick present in the native Hepatitis B genome. Alexander cells produce a major transcript of 2000 bases which hybridizes specifically to the viral surface antigen gene.

INTRODUCTION

There are at least three independent viral agents responsible for hepatitis. Hepatitis A virus is a small RNA containing virus (picornavirus) (1). Hepatitis B virus (HBV) is a DNA containing virus, visualized as a 42 nm spherical particle by electron microscopy (the Dane particle, 2). NonA-nonB hepatitis may be caused by two classes of viruses, one similar to Hepatitis A and the other similar to Hepatitis B, but with independent antigenic determinants (3).

Hepatitis B is perhaps the most serious of these viral infections. The response to HBV infection is different in different individuals (4). Approximately 90% of patients resolve the disease after several months without further complications. 5% of patients remain chronically infected, either with or without symptoms. An additional 5% of infected individuals develop liver cirrhosis, while 1% succumb to acute fulminant hepatitis early after infection. Analysis of the HBV genome may aid in understanding the various manifestations of this disease.

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There is a close correlation between HBV infection and primary liver carcinoma, the most common form of cancer in African and Asian males (5). The geographical areas of high HBV infection coincide with high rates of primary liver carcinoma, and individuals with liver carcinoma often have an antigenic history of HBV infection. Furthermore, a tissue culture cell line derived from a hepatoma of a Mozambican male was shown by Alexander et al. to produce HBV surface antigen when cultured in vitro (the "Alexander" cell) (6,17).

HBV contains the smallest mammalian viral DNA, a genome of 3200 base pairs (bp). This DNA is isolated from mature virus ("Dane" particles) as a partially double stranded circular molecule (Fig. 1) (8). The single stranded region, 1000-2000 bases in length, can be filled in by an endogenous DNA polymerase activity (9). The product of this reaction is a 3200 bp circular molecule containing nicks in both strands. These nicks are separated by ∿300 bp (10). Cloned HBV DNA has been entirely sequenced by our laboratory (accompanying article), and by Galibert et al. (11). In addition, Pasek et al. have sequenced ∿80% of the genome (12).

There are three antigens associated with HBV: the surface antigen (HBsAg, 25,000 mw) (13), the core antigen (HBcAg, 21,000 mw) (14), and the "e" antigen (HBeAg). The HBsAg gene has been located on the genome and the amino acid sequence has been identified (15,16). The HBcAg gene has also been localized on HBV DNA and the deduced amino acid sequence predicts that HBcAg is an avid DNA binding protein with protamine-like properties (15,17). We have tentatively located the DNA regions which may code for other genes (HBeAg, DNA polymerase?). There are no other regions on the genome capable of coding for proteins larger than 80 amino acids (neglecting splicing).

We report here an analysis of HBV in the Alexander human hepatoma cells using cloned HBV DNA sequences as probes. An important aspect of viral transformation is integration of the viral genome into the host genome. Such integration has been implicated in transformation caused by retroviruses (such as avian sarcoma virus, murine leukemia virus, and murine sarcoma virus) and by oncogenic DNA viruses. We have demonstrated that Alexander cell DNA contains six integrated copies of HBV DNA and no detectable free viral DNA.

METHODS

We have previously described the construction of cloned HBV DNA. This DNA was labeled in vitro by nick translation (18) and hybridized to DNA blots by the method of Southern (19). Alexander cells were provided by Drs. M. Hilleman, A.

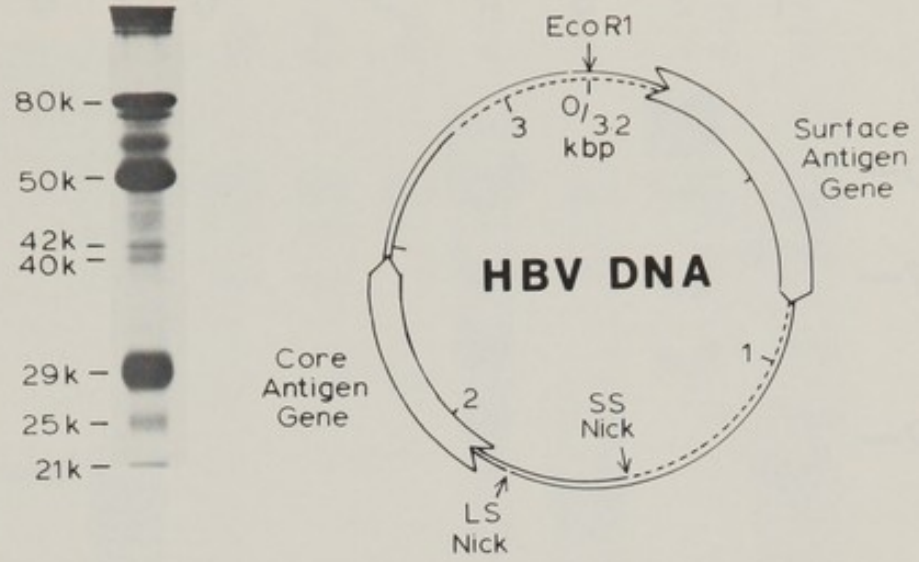

FIGURE 1. Map of HBV DNA (right) showing the coding regions for the HBsAg and HBcAg genes. The direction of transcription is indicated by the arrows. The single stranded region of native Dane DNA is represented by a dashed line. Positions of nicks in the long strand (LS) and short strand (SS) are indicated. The proteins of Dane particles are presented in the Coomassie blue stained polyacrylamide (12.5%) gel on the left. HBcAg has a size of 21,000 daltons, HBsAg is 25,000 daltons, and glycosylated HBsAg is 29,000 daltons. Other investigators have identified additional larger proteins (labeled) related to HBV. The band at 67,000 mw is the size of human serum albumins.

Tytell and W. McAleer of Merck Sharpe & Dohme laboratories. DNA was isolated from Alexander cells by proteinase K treatment followed by phenol/chloroform extraction and ethanol precipitation. RNA was isolated from Alexander cells by the method of Chirgwin et al. (20). PolyA enriched RNA was prepared by a single passage over oligodT-cellulose. RNA was electrophoresed through 1.0% agarose gels containing 4mM methyl mercuric hydroxide (21), transferred to diazotized cellulose paper, and hybridized to labeled, cloned HBV DNA by the "Northern" method of Alwine et al. (22).

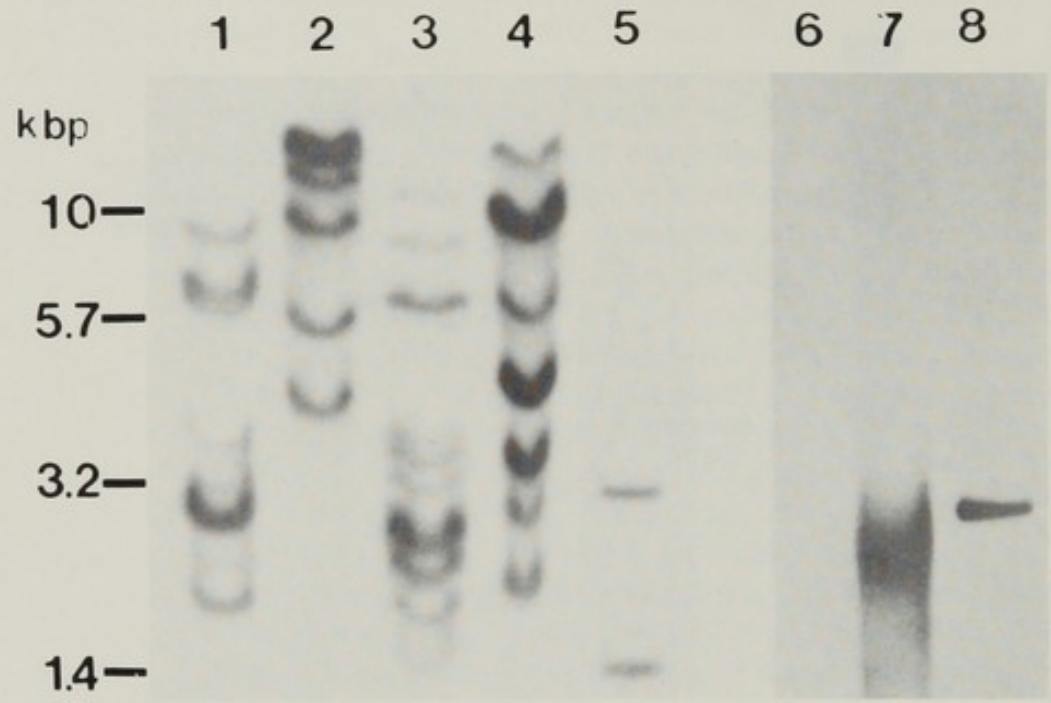

FIGURE 2. Autoradiogram of Southern hybridization of ^{32}P-cloned HBV DNA vs. restriction nuclease digested samples. Lane 1, EcoRI digested Alexander DNA. Lane 2, HindIII digested Alexander DNA. Lane 3, EcoRI + HindIII digested Alexander DNA. Lane 4, SacI digested Alexander DNA. Lane 5, EcoRI digested pHBV3200 and BamHI digested pHBV1400 (cloned HBV DNAs used as size markers, 12). Lane 6, EcoRI digested normal liver DNA. Lane 7, EcoRI digested chronic hepatitis liver DNA, slightly degraded. Lane 8, EcoRI digested Dane DNA.

RESULTS

HBV DNA is Integrated into Alexander Cells. In order to characterize the HBV DNA sequences in Alexander cells, we hybridized ^{32}P-labeled HBV DNA to Southern blots of Alexander DNA treated with various restriction nucleases. We chose the endonucleases HindIII and SacI, which fail to cleave free viral DNA, and EcoRI, which cleaves HBV DNA at a single site. HBV DNA hybridizes to six independent size classes of HindIII digested Alexander DNA, presented in Figure 2. Each band of hybridization is larger than 3200 bp, the length of HBV DNA. Thus, at least six copies of HBV DNA incorporated into different sites of the Alexander genome. No hybridization was observed to DNA of 3200 bp in size, suggesting that Alexander cells contain no free viral DNA.

HBV DNA hybridizes to seven independent size classes of SacI digested Alexander DNA, presented also in Figure 2. The SacI hybridization results support the HindIII hybridization

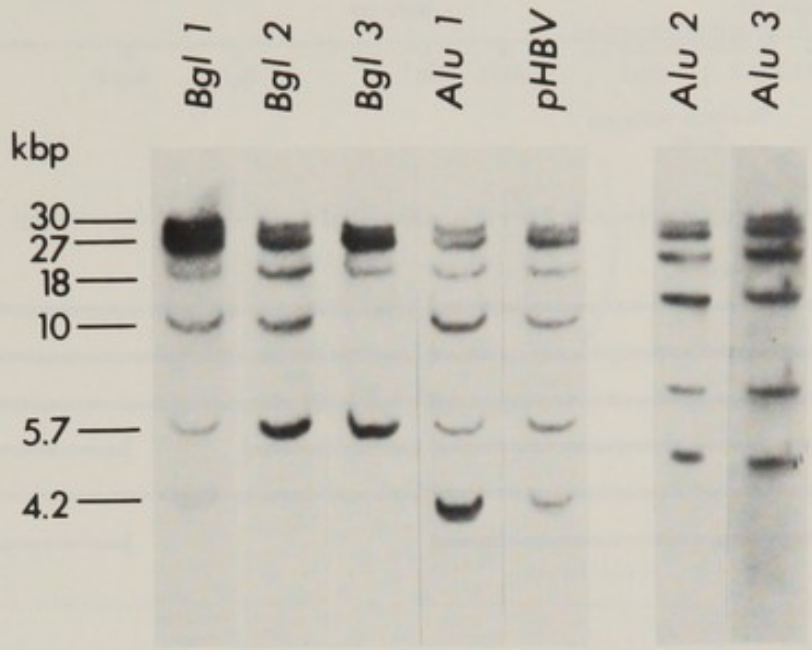

FIGURE 3. Autoradiograms of Southern hybridization of ^{32}P-cloned HBV DNA fragments vs. HindIII digested Alexander DNA. The origin of fragments is presented in Figure 4. "pHBV" represents total cloned HBV DNA.

results; there are at least six copies of HBV DNA integrated into high molecular weight Alexander DNA.

In contrast to Alexander cell DNA, HBV DNA does not hybridize to uninfected human liver DNA (Fig. 2). This suggests that there are no HBV related sequences in the normal human genome. Hybridization to EcoRI digested DNA isolated from liver of a chronically infected hepatitis patient (Fig. 2) shows a signal at 3200 bp, characteristic of free viral DNA. There is no indication of HBV integration into genomic sequences.

Ten hybridizing bands are observed in a Southern analysis of EcoRI digested Alexander DNA (Fig. 2). If there are six integrated copies, twelve bands would be expected in a hybridization pattern with an endonuclease which cleaves HBV DNA once, such as EcoRI. This discrepancy may be the result of multiple fragments with the same mobilities, which is suggested by the intense hybridization at 3000 bp (Fig. 2).

The fraction of HBV DNA in each of the integrated copies was determined by hybridization with fragments of HBV DNA prepared by restriction endonuclease digestion and electrophoretic separation. Six independent fragments (each representing approximately one-sixth of the HBV genome, presented in detail in Fig. 4) were hybridized to Southern blots of HindIII

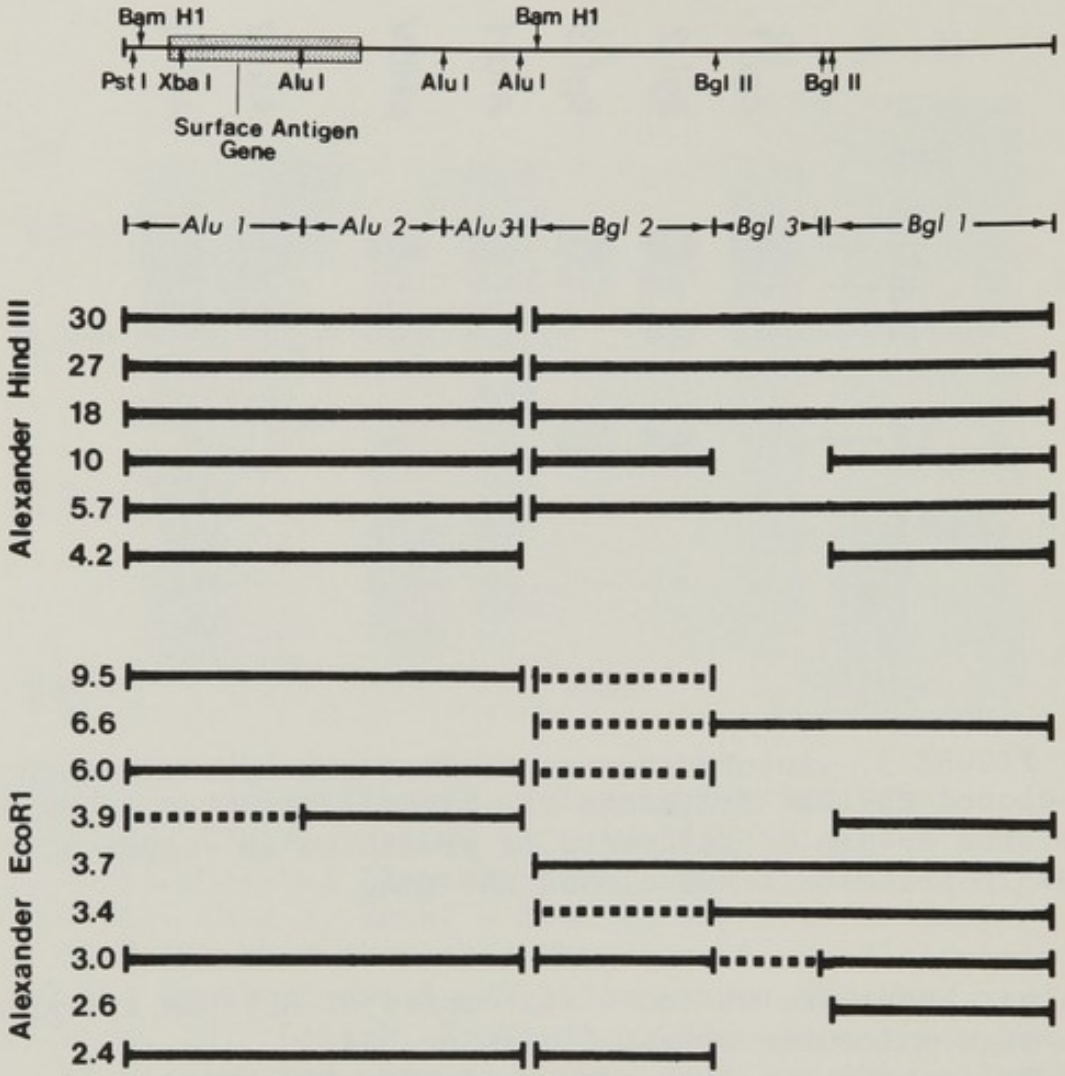

FIGURE 4. Maps of HBV DNA (top), probes used in hybridization analysis (directly below), and integrated HBV DNAs. Solid lines below each probe represent positive hybridization for that particular fragment. Dashed lines represent areas of weak hybridization. The gap between the Alu3 and Bgl2 probes represents an 80 bp region not tested. The SacI hybridizations are not shown, while the EcoRI hybridizations are presented in Figure 6.

and EcoRI digestions of Alexander DNA, presented in Figure 3. Four of the HindIII Alexander bands hybridize with all six fragments of HBV DNA. This suggests that with these four bands (30, 27, 18 and 5.7 kbp) contain substantially complete integrated copies of the HBV genome. However, the 10 kbp HindIII band fails to hybridize to two of the HBV fragments (Bgl2 and Bgl3), while the 4.2 kbp band does not hybridize with the Bgl3 HBV fragment. These two HindIII bands therefore appear to contain incomplete copies of HBV DNA.

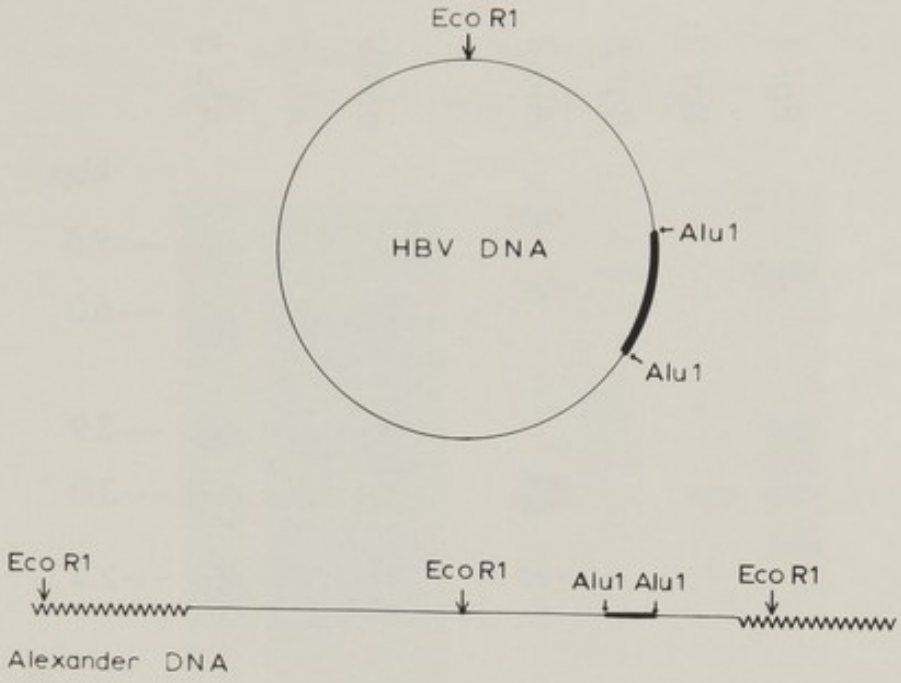

FIGURE 5. Analysis of integration of HBV DNA into Alexander DNA. A probe from one side of HBV DNA (Alu2 is represented by the heavy line) will hybridize only to the smaller EcoRI digested fragment of Alexander DNA.

Both lack DNA sequences specific for the HBcAg gene region. These results are summarized in Figure 4.

Integration site in HBV DNA. The orientation of HBV DNA integrated into Alexander DNA has been studied by hybridizing specific HBV fragments to EcoRI digested Alexander cell DNA. The single EcoRI site divides the integrated HBV genomes such that hybridization of a specific HBV fragment occurs to either side of the EcoRI site outlined in Figure 5; thus two patterns of hybridization are possible for different probes. The same HBV fragments outlined in Figure 4 were utilized as probes. Two patterns of hybridization were indeed observed, as shown in Figure 6. One of these patterns consists of approximately 1600 bp (not including Alexander DNA) on the HBsAg gene side of the EcoRI site. The other pattern consists of 1600 bp in the opposite direction, the HBcAg side of the EcoRI site. Consequently, the EcoRI site bisects the integrated HBV genomes approximately in half. Thus the site of integration is in the region of the nicks of native HBV DNA

There are three exceptions to these generalizations. The 3.9 kbp and the 3.0 kbp bands hybridize to HBV fragments from both sides of the EcoRI site. This may be a result of multiple EcoRI Alexander bands at these positions, perhaps due to

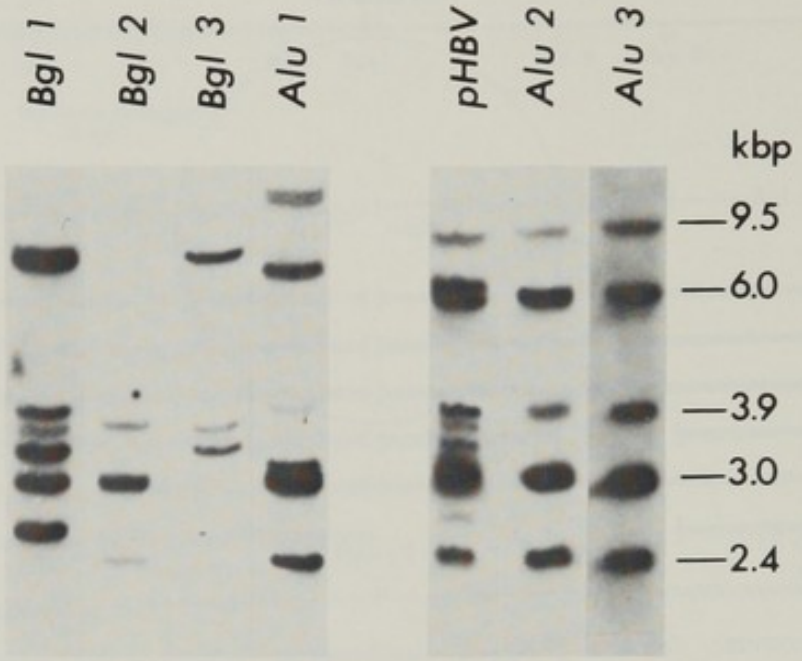

FIGURE 6. Autoradiograms of Southern hybridizations of ^{32}P-cloned HBV fragments vs. EcoRI digested Alexander DNA. The maps and origin of fragments are presented in Figure 4.

integration into Alexander DNA regions with similar EcoRI restriction site positions (such as repeated sequences or fortuitous EcoRI placement) or perhaps due to tandem integration of HBV genomes. The 2.6 kbp band hybridizes to only a small portion of the HBV genome, perhaps the result of integration of a partial HBV genome.

HBV Transcripts in Alexander Cells. Alexander RNA sequences complementary to HBV DNA in Alexander cells were analyzed by the "Northern" method. Alexander cell RNA was separated by electrophoresis in denaturing agarose gels, transferred to diazotized paper, and then hybridized with labeled HBV DNA. HBV DNA hybridizes with total Alexander RNA as shown in Figure 7. Hybridization with polyA enriched Alexander RNA is more extensive, suggesting that these transcripts are polyadenylated. An RNA approximately the size of 18S ribosomal RNA (2000 bases) is the predominant hybridization signal. HBV probes also hybridized to Alexander cell RNA both larger and smaller than the predominant transcript. This additional hybridization is HBV specific since negligible background is observed in the hybridization of HBV DNA to uninfected liver DNA (not shown). The HBV related transcripts larger than the predominant signal is obviously of considerable significance since it must either represent a precursor

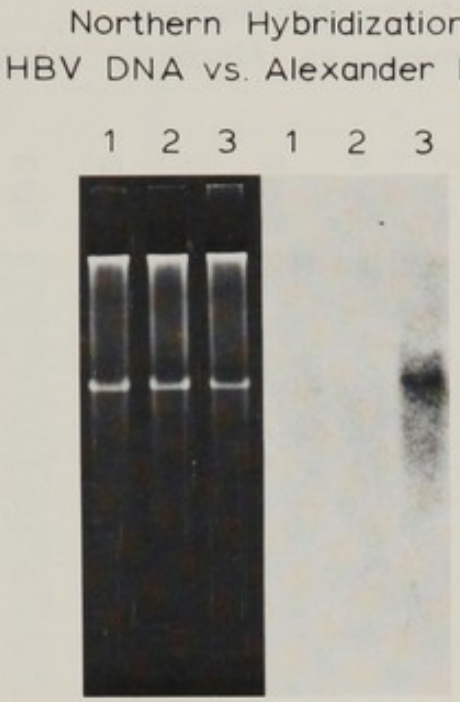

FIGURE 7. Northern Hybridization analysis of ^{32}P-cloned HBV DNA vs. Alexander RNA samples. The 28S and 18S ribosomal RNAs are apparent in the ethidium stained gel (left), while the autoradiogram indicates the presence of a single, diffuse signal (right).

to the approximately 2000 bp transcript or an independent transcript. The lower molecular weight material is more likely to represent processing or degradation of the predominant transcript as is seen in other systems.

This RNA has been mapped by hybridization to specific fragments of HBV DNA to Northern blots, similar to the Southern analysis described previously. The HBV-complementary RNA hybridizes to probes specific for the HBsAg gene, presented in Figure 8. This RNA also hybridizes to HBV fragments which are positioned 300 bp in the 5' direction of the HBsAg gene and 700 bp 3' to the gene. HBV DNA fragments outside of this colinear region of 1500 bases do not significantly hybridize to this major Alexander transcript.

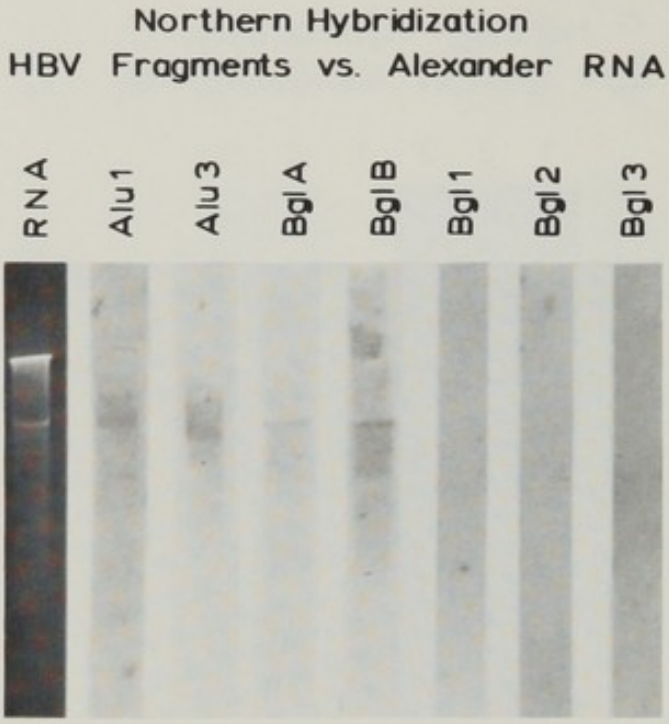

FIGURE 8. Northern hybridization analysis of ^{32}P-cloned HBV DNA fragments vs. polyA enriched Alexander cell RNA. BglA is a probe derived from Bgl1 (100 bp from the EcoRI site) while BglB is a probe derived from Bgl2 (100 bp from the BamHI site).

DISCUSSION

Our experimental results indicate HBV DNA is indeed integrated into the genome of a human hepatoma cell line, the Alexander cell. No unintegrated sequences were detected. In contrast, chronically infected liver contains free viral DNA but no integrated sequences. HBV DNA sequences are not present in uninfected liver. The Alexander genome contains at least four apparently full length, integrated copies of HBV DNA and two partial HBV copies.

We have considered the possibility of the HBV DNA integration pattern to be the result of a heterogeneous cell population with multiple integration patterns (e.g. six different cell types, each with a different integration pattern of HBV DNA). However, Southern hybridizations with Alexander cells cultured from a single clone are identical to the HindIII analysis shown in Figure 2. Thus, the HBV DNA integration pattern is a general and stable feature of the Alexander cell genome.

The major HBV-specific transcript of Alexander cells hybridizes with the HBsAg gene region, but not the HBcAg gene

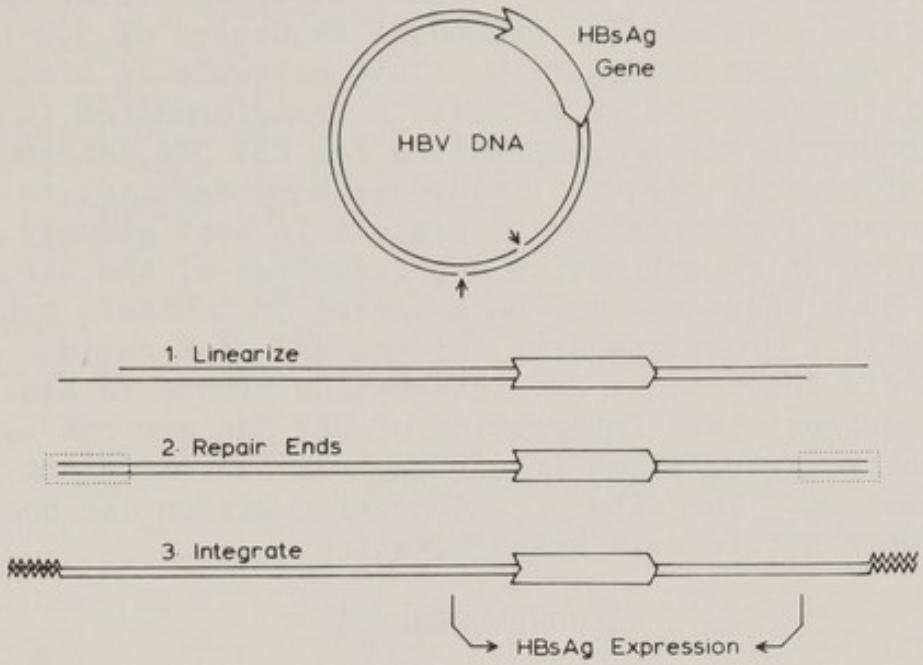

FIGURE 9. Possible events important to HBV integration into the human genome. The linearization and repair reactions have been demonstrated in vitro (7). The predominant transcript in Alexander cells hybridizes to the region delineated by the arrows, below.

region. This supports the observations that Alexander cells express HBsAg but not HBcAg. It is not known which integrated HBV sequences are productive in the formation of HBsAg mRNA. This may be the result of insertion of a partial HBV sequence within a transcriptionally active region of the Alexander cell genome. The absence of HBcAg expression may be a result of integration near the start of the gene; this may interfere with formation of a functional HBcAg mRNA.

The size of HBV DNA which hybridizes with the major transcript (1500 bases) appears significantly smaller than the observed size of the transcript as determined by electrophoretic mobility (2000 bases). The additional length may be contributed by transcription of Alexander genomic DNA and/or by polyA addition.

Viral transformation events are characterized by integration of the viral genome into host genomic DNA. The position of HBV integration is in the vicinity of the nicks of native Dane DNA. These nicks may be important to the process of integration. The distance between nicks is approximately 300 bp; partial denaturation of the HBV genome results in a linear structure with single stranded ends (7), which may

then be repaired into a fully double stranded linear molecule. The resulting molecule has redundant sequences of 300 bp at the ends, as shown in Figure 9. This molecule is similar to intermediates proposed in retrovirus transformation (23). Such a mechanism may be responsible for HBV DNA integration and transformation events. Sequences complementary to the HBV redundant sequences may be present in host genomic DNA and involved in recombination and insertion of the viral genome. Such sequences may be analyzed by Southern hybridizations with a probe specific for the HBV nick region.

The six independent HBV integration events in Alexander cell DNA suggest that integration of HBV DNA may not be rare. Consequently, integration itself may not be sufficient for transformation. The site of integration within the host DNA may be important in cell transformation.

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POLYOMA VIRUS HR-T GENE PRODUCTS[1]

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ABSTRACT Expression of the polyoma virus hr-t function leads to multiple alterations of the cell, affecting its structure and behavior in ways associated with malignant transformation. Mutants in this viral gene are unable to transform cells or induce tumors. Genetic and chemical methods have been used to localize and define the mutations in this class of mutants: all of them reside within a small segment of 315 base pairs in the early region of the viral genome. DNA sequencing of different hr-t mutant viral DNAs in this small segment shows three patterns of alterations: frameshift deletion, in-phase deletion, and triplet insertion plus single transition. Studies of the T antigen patterns induced by these different classes of hr-t mutants show that all produce normal large T antigen but are affected in different ways in both the small (22K) and middle (56K) T antigens. Based on these studies as well as others, it is clear that the 22K and 56K proteins are overlapping products encoded by the hr-t gene. *In vitro* protein kinase assays of T antigen immune precipitates show phosphorylation of the wild type but not of hr-t mutant middle T antigens. The amino acid sequence of these proteins in the region affected by hr-t mutants shows limited regions of homology with the pituitary glycoprotein hormones.

INTRODUCTION

Mutations in two parts of the early region of the polyoma viral genome can affect the ability of the virus to transform cells. Host range transformation-defective mutants (hr-t) map in the proximal part of the early region, and temperature-sensitive mutants of the "a" complementation group (ts-a) map in the distal part (1,2). The physiological

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properties of hr-t and ts-a mutants contrast strongly, indicating that different genetic functions are affected in the two classes of mutants. This supposition has been confirmed directly by complementation studies which formally define two viral functions in the cell transformation process (3,4).

The role of the ts-a gene in transformation seems to be a transient one, perhaps best understood as one which facilitates the integration of viral DNA into cellular DNA. The following observations are pertinent:

1. When cells are transformed at the permissive temperature by infection with ts-a mutants, and subsequently shifted to the non-permissive temperature, the cells remain transformed in most cases (5-9); [see, however, references (10,11) for indications that the ts-a gene may under some circumstances play a continuing role in transformation].
2. Ts-a mutants resemble wild type virus in inducing abortive transformation. That is, at the non-permissive temperature, ts-a mutants induce transient growth of cells in soft agar (12) as well as other cellular changes associated with transformation (13-16). However, in contrast to wild type viral infections in which stable transformants arise following the abortive transformation phase, few or no stable transformants arise following infections by ts-a mutants. Therefore, an event which stabilizes transformation and perpetuation of the viral genome in the cells is affected in these mutants which are otherwise fully capable of inducing the transformed state.
3. Cleavage of the viral DNA molecule in the ts-a gene does not impair the ability of the virus to induce tumors, and may indeed enhance that ability (17,18). Persistent expression of the ts-a function in transformed cells may lead to loss of the viral genome, perhaps through *in situ* replication of integrated viral DNA mediated by the ts-a gene product which is known to play an initiating role in viral DNA replication (see contributions of C. Basilico and J. Sambrook, this Symposium). These results suggest a role of ts-a in mediating both integration and loss of the viral genome and rule against its being essential for the maintenance of transformation.
4. The ts-a gene product is the 100K large T antigen (19-21) and is often lacking in polyoma transformed cells, as shown by immunoprecipitation experiments (19,22,23). The selection against persistent expression of the large T antigen in transformed cells is

greater the more permissive the host for replication (22). In some well studied cases, DNA sequences and RNA transcripts from the distal "ts-a" region are lacking in transformed cells (23,24).

The hr-t gene, in contrast to ts-a, plays a direct role n inducing the transformed phenotype. This has been shown in variety of experiments:

1. Hr-t mutants fail to induce abortive transformation, assayed as the ability to cause transient growth of cells in soft agar (9).
2. Hr-t mutants fail to induce cell surface changes assayed by lectin-mediated agglutination (25).
3. Hr-t mutants fail to induce morphological transformation accompanied by loss of stress fibers (16).
4. As will be discussed in detail below, the hr-t viral gene has two discrete products, the middle T and small T antigens. These viral products are uniformly present in polyoma transformed cells (18,19,22,23), suggesting an essential role in maintaining the transformed state. Conversely, cells carrying an integrated hr-t mutant viral genome and showing only the large T antigen are phenotypically normal (26).

RESULTS

There are three distinct early gene products of polyoma virus. These products are defined as T or tumor antigens detected by sera from polyoma tumor-bearing animals. The arrangement of sequences in the early region coding for the large, middle and small T antigens is shown in Figure 1. All three proteins share a common N-terminal domain. The small (22K) and middle (56K) T antigens share a common domain encoded by sequences which are removed from the large T antigen mRNA. The middle T antigen has a unique C-terminal domain specified by part of the same sequence which codes for the major C-terminal portion of the large T (100K), but read in a different frame. Figure 1 also indicates the location of hr-t and ts-a mutants as determined from "marker rescue" (1,2) and DNA sequencing experiments (32-34). Hr-t mutants map entirely within the region unique to the small and middle T antigens. The small T antigen which is found largely in the 'cytosol' fraction and the middle T antigen which is membrane-associated are therefore dual products of the hr-t gene. Ts-a mutants map in the distal part of the large T antigen and entirely downstream of the region of overlap with middle T. The large intranuclear T antigen is therefore the unique product of the ts-a gene.

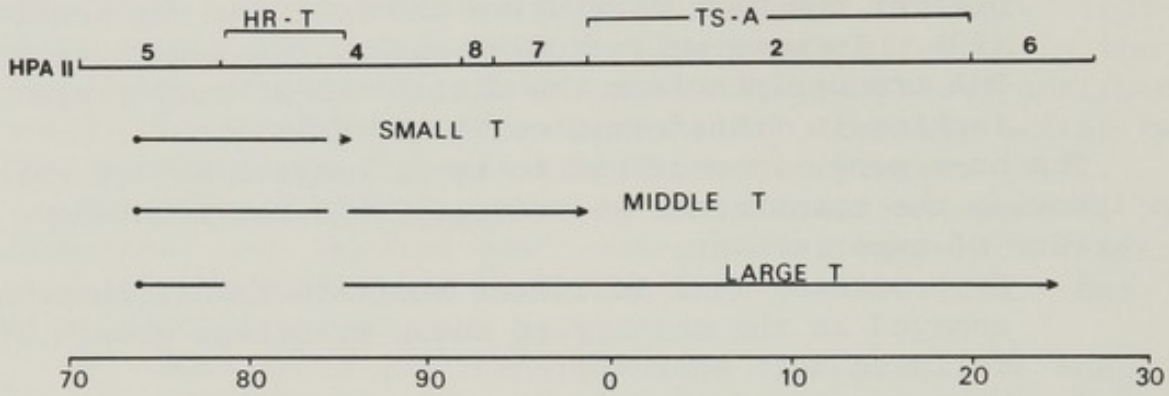

FIGURE 1. The early region of polyoma virus. The early region of polyoma extends from the unique DNA replication origin at about 71.5 map units to the 3' end of the early mRNA species at about 26 map units (27). The coding regions for the three primary gene products, the small (22K), middle (56K) and large (100K) T antigens, have been determined by a large body of data collected in several laboratories and including peptide mapping experiments (19,28,29), mRNA splicing studies (24) and DNA sequencing (30,31). All three T polypeptides are thought to share sequences at their amino termini. Different splices in their mRNAs lead to distal translation in different reading frames, generating unique carboxy-terminal domains.

The conclusion that both small and middle T antigens are direct products of the hr-t gene is based on a combination of data from DNA sequencing and immune precipitation experiments. Figure 2 shows the nucleotide sequence of wild type viral DNA in the hr-t region shown in Figure 1, along with the corresponding amino acid sequences which are both common and unique to the 22K and 56K proteins. Alterations in this sequence found in three different hr-t mutant DNAs are shown:

1. Mutant NG-18 has a deletion of 187 base pairs between nucleotides 526 and 713 (32,33). This frameshift deletion mutant is expected to make no 22K or 56K proteins, but should give rise instead to a truncated (8-9K) product.
2. Mutant SD-15 has a deletion of 141 base pairs between nucleotides 434 and 575 which does not alter the reading frame (34). This mutant should give rise to detectable middle and small T proteins reduced in size by 47 amino acids.
3. Mutant NG-59 was recognized as unusual among the hr-t mutants we have isolated in having no deletion in the hr-t region detectable by restriction mapping, (1,35). This mutant shows a triplet (ATA) insertion followed by a G to A transition at nucleotide position 721 in

the DNA (34). This alteration should add an 'extra' isoleucine and change the following amino acid from aspartic acid to asparagine. These changes in the 22K and 56K proteins should still allow these species to be detected by immune precipitation.

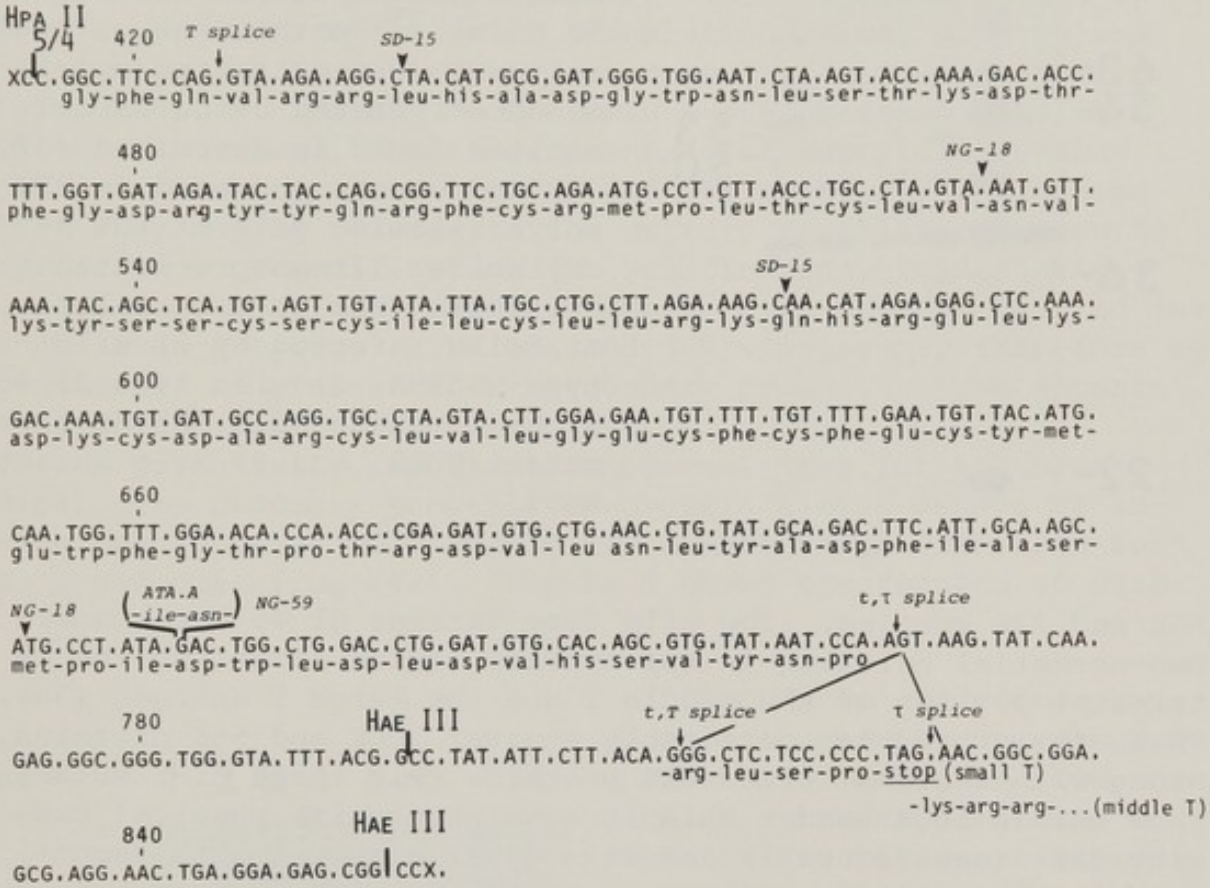

FIGURE 2. Fine structure of the hr-t region of polyoma virus. The sequence presented is that of the coding strand of the proximal half of Hpa II fragment 4 determined by us (32). The translated sequence below the DNA is presumed to be that of the domain common to both small and middle T antigens. All hr-t mutants isolated in our laboratory have lesions in this region. The boundaries of the deletions of hr-t mutants SD-15 and NG-18 are indicated by arrowheads. The postulated splicing junctions (24,30,31) for the viral proteins, small T (t), middle T (τ) and large T (T) are indicated by the small downward arrows.

The T antigen patterns induced by these three hr-t mutants and by two different wild type virus strains are shown in Figure 3. The standard wild type strain RA induces 100K,

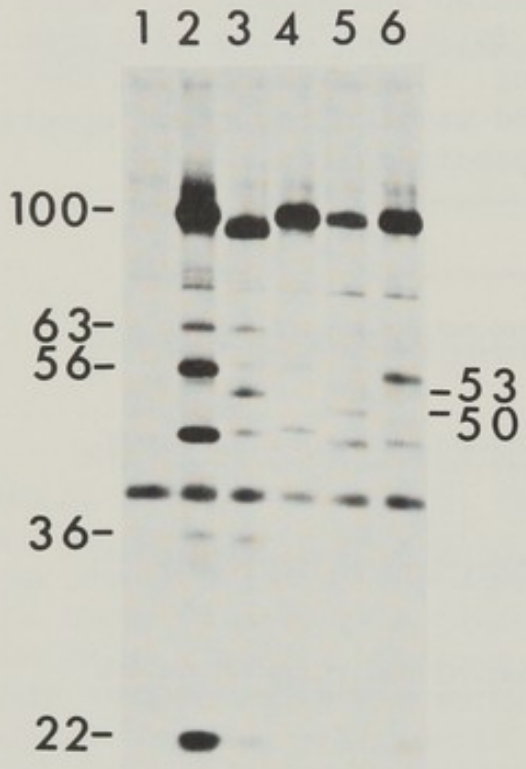

FIGURE 3. T antigens of wild type and hr-t mutant viruses. Infected or uninfected baby mouse kidney cells were pulse-labeled for 90 minutes at 24 hours after infection in Hank's salt solution containing 50 μc/ml ^{35}S-methionine. The T antigens were extracted and immunoprecipitated using anti-T ascites fluid as described (37, 38) and electrophoresed on SDS polyacrylamide gels of 10% acrylamide. Immunoprecipitates are shown from 1) mock cells, or cells infected by 2) wild type NG59RA, 3) wild type dl 45, 4) hr-t mutant NG18, 5) hr-t mutant SD15, and 6) hr-t mutant NG59.

56K and 22K species. The wild type variant dl 45 has a small non-essential deletion in the overlap region affecting the C-terminal portion of the middle T and the large T antigen (36). This strain produces detectably shorter 100K and 56K proteins, along with a normal sized 22K protein. All three hr-t mutants show normal 100K bands; this is consistent with physical mapping data (see Figure 1) and with genetic experiments demonstrating the ability of these mutants to complement ts-a mutants (3,4) which produce defective large T antigen (19-21). The expectations of alterations in small and middle T antigens in these mutants, based on the DNA sequence changes, are largely born out by the immune precipitation results. The out-of-phase deletion mutant NG-18 shows no detectable 22K or 56K species. Fragments smaller than 22K are sometimes seen with NG-18 and other similar mutants (21); such products may be degraded rapidly in the cell or be poorly reactive with antibody. The in-phase deletion mutant SD-15 shows a shorter middle T band of about 50K, consistent with the expected 47 amino acid deletion. Failure to detect a similarly shortened small T protein may again reflect rapid degradation or poor immunoreactivity. The insertion-substitution mutant NG-59 shows bands of the expected mobilities for 56K and 22K proteins. The middle T species appears to be made in normal amounts, while the small T is present at a reduced level compared to a wild type virus infection. In addition to altered immunoreactivity or rapid degradation, another possible basis for the

apparent underproduction of 22K in this mutant would be that the mutation may affect the splicing efficiency for small T mRNA. These data taken together confirm the assignment of the 22K/56K overlapping proteins as products of the hr-t gene.

Additional bands of 63K and 36K are also observed in immune precipitates from wild type infected and transformed cells (see Figure 3; 18,21,22,37). These species do not appear as products of *in vitro* translation primed with poly-A containing RNA from virus-infected cells (59; H. Lamfrom, G. Carmichael, R. Shaikh, T. Benjamin — unpublished results). On the other hand, the fingerprints of these proteins show partial identity with the 100K and 56K proteins, indicating that they may be related to the primary translation products by cleavage or modification (B. Schaffhausen, T. Benjamin — unpublished results). Interestingly, hr-t mutants fail to induce these proteins. The 63K and 36K species may therefore be considered at present to be secondary products whose appearance depends on the hr-t gene (21).

A potentially important functional test for the hr-t viral gene products consists of protein kinase assays of immune precipitates (38,40,41), in the manner first described for Rous sarcoma virus (42). Figure 4 shows the results of such

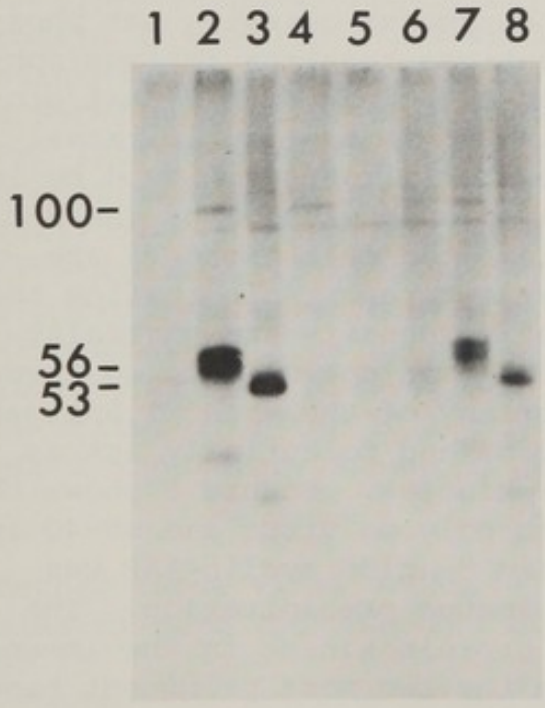

FIGURE 4. *In vitro* phosphorylation of polyoma viral T antigens. T antigens from unlabeled cultures identical to those of Figure 4 were extracted, immunoprecipitated and washed as described (38). In the mixing experiments equal volumes of extract from wild type and hr-t mutant infected cells were mixed prior to immunoprecipitation. The washed immunoprecipitates were incubated in 0.02 M Tris, 0.05 M $MgCl_2$, pH 7.5 containing 40 μc/sample γ-^{32}P-ATP for 15 minutes at 23°. The immunoprecipitates were then washed and electrophoresed on SDS gels of 10% acrylamide. 1) Mock-infected cells, 2) wild type NG59RA, 3) wild type dl 45, 4) hr-t mutant NG18, 5) hr-t mutant SD15, 6) hr-t mutant NG59, 7) mixture of wild type NG-59RA and SD15, 8) mixture of dl 45 and NG18.

assays. In the case of the wild type viruses RA and dl 45, there is strong phosphorylation of the middle T antigens. The phosphorylation of the 56K protein under these conditions appears to be at tyrosine residues (41), the same specificity as has been shown for the src kinase of avian sarcoma virus (43) and the kinase specific to Abelson leukemia virus (44). In the case of polyoma virus, this activity in the immune precipitates is not seen in any case involving hr-t mutants; this is true even for the two mutants, SD-15 and NG-59, which make altered but still detectable species of middle T antigen by S^{35}-methionine metabolic labelling (see Figure 3). The middle T-associated kinase reaction is specific and selective for middle T antigens from wild type viruses, as indicated by experiments in which extracts from different infected cells are mixed prior to immune precipitation and protein kinase assay. When assays are done on such mixed immune precipitates, bringing together deleted middle T of SD-15 with normal middle T of wild type RA, or conversely, the deleted middle T of wild type dl 45 with non-deleted middle T of NG-59, only the wild type products become phosphorylated. Similar results occur when assays are carried out with extracts of cells co-infected by these same pairs of mutants (38). At present, the data are consistent with the middle T itself being a protein kinase, but do not rule out either a role of small T antigen or of some cellular enzyme in the phosphorylation of the middle T antigen.

The possibility of cellular enzyme(s) carrying out phosphorylation of polyoma middle T antigen requires serious consideration, because experiments have shown directly that protein kinases are indeed present in the immune precipitates. Addition of histone H-1 to immune precipitates followed by incubation with ^{32}P-ATP results in phosphorylation of the H-1, even when pre-immune serum or uninfected cell extracts are used (38). In part because of earlier reports indicating that cyclic AMP could lower the saturation density and partially restore normal morphology to transformed cells (45), we investigated the possibility of whether any of the viral T antigens could be cyclic AMP binding proteins and potentially act as 'abnormal' regulators of cellular kinases. Figure 5 shows the results of an experiment with both polyoma virus and SV-40 in which photocoupling of P^{32}-labelled 8-azido cyclic AMP was carried out on extracts prior to immune precipitation. The presence of cyclic AMP-dependent protein kinase in the immune precipitates can be inferred from the two most prominent bands (right panel) which correspond to the two major classes of regulatory subunit for this enzyme. An unanticipated result from this experiment is the labelling of the large T antigens of both viruses with the cyclic AMP analogue. It is not known

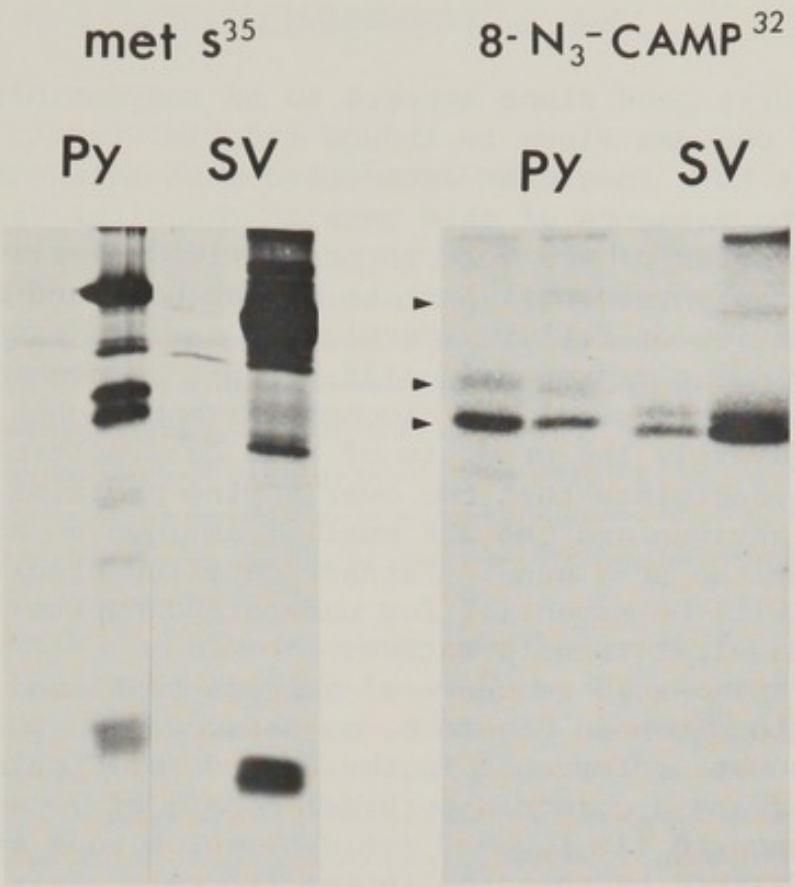

FIGURE 5. Labeling of SV-40 and polyoma large T antigen and types I and II regulatory subunits of cyclic AMP-dependent protein kinases by ^{32}P-8-azido-cyclic AMP. Left: met-S^{35}. T antigens were immunoprecipitated from extracts of SV-40-infected BSC-1 cells (SV) or polyoma-infected baby mouse kidney cells (Py) labeled with ^{35}S-methionine. They were electrophoresed on a 12.5% discontinuous buffer SDS gel. Pre-immune serum (left) or anti-T ascites (right) immunoprecipitates. Right: ^{32}P-8-N_3-cAMP. Extracts from unlabeled SV-40-infected BSC-1 or polyoma-infected baby mouse kidney cells were reacted with ^{32}P-8-azido-cAMP as described (38), immunoprecipitated, and electrophoresed. Pre-immune serum (left) or anti-T (right). The arrows from top to bottom: large T antigen, Type II and Type I regulatory subunits (46).

whether these viral products act as cyclic AMP binding proteins *in vivo* and what the significance of such binding might be.

DISCUSSION

The hr-t gene alone appears to be responsible for the ability of polyoma virus to induce the pleiotropic changes in cells that have long been associated with malignant transformation. The presence of this gene in the virus can be accounted for in terms of its role in productive infection, which is to alter the physiological state of the host and bring about the expression of cellular permissive factors essential for efficient virus reproduction (35,47,48). A combination of genetic, immunological and biochemical approaches have been used to identify the products of this gene. It can be stated with some certainty that two overlapping proteins — the 56K middle T antigen and the 22K small T antigen — are directly encoded by the hr-t gene. Further detailed studies of these proteins will be essential for understanding the initial steps in polyoma viral transformation.

The amino acid sequence of polyoma small and middle T antigens is given in Figure 6, as deduced from DNA sequence and other data referenced in the legend for Figure 1. The two proteins share a common N-terminal domain of 79 amino acids (also common to the large T antigen) which ends at the gln residue (marked by an arrow in the Figure). This sequence is somewhat hydrophobic (leu + val + ala + phe = 23) and has a slight preponderance of basic over acidic residues (arg + lys = 11, asp + glu = 8). The absence of any viable or conditionally viable mutants mapping in this region points to its essential nature, and at the same time hampers efforts to infer anything specific about its function.

The next 112 amino acids comprise the hr-t region *per se*. It represents the common sequence unique to the small and middle T antigens (see Figure 1). All hr-t mutants that have been studied map in this sequence. The region from the 5' end of the most proximal hr-t deletion to the 3' end of the most distal hr-t deletion includes 106 of the 112 amino acids, coming within 3 residues of either end of the common region (see brackets in Figure 6). A cysteine-rich region (8 of 34 amino acids) within this sequence contains two clusters with identical spacings of cys x cys x x cys. As first noted by Friedmann et al. (49), this sequence is invariably present in both the α and β subunits of all the pituitary glycoprotein hormones of the TSH, FSH, LH family, as well as being present in the small T antigens of SV-40 (50,51) and the human papova virus BK (52,53). This limited sequence homology between the hr-t gene products and the pituitary hormones may find some support at the functional level, considering the parallelism between the effects of the hr-t gene on altering microfilaments (stress fibers) and cell morphology (16) and those in-

duced by FSH in ovarian granulosa cells (54).

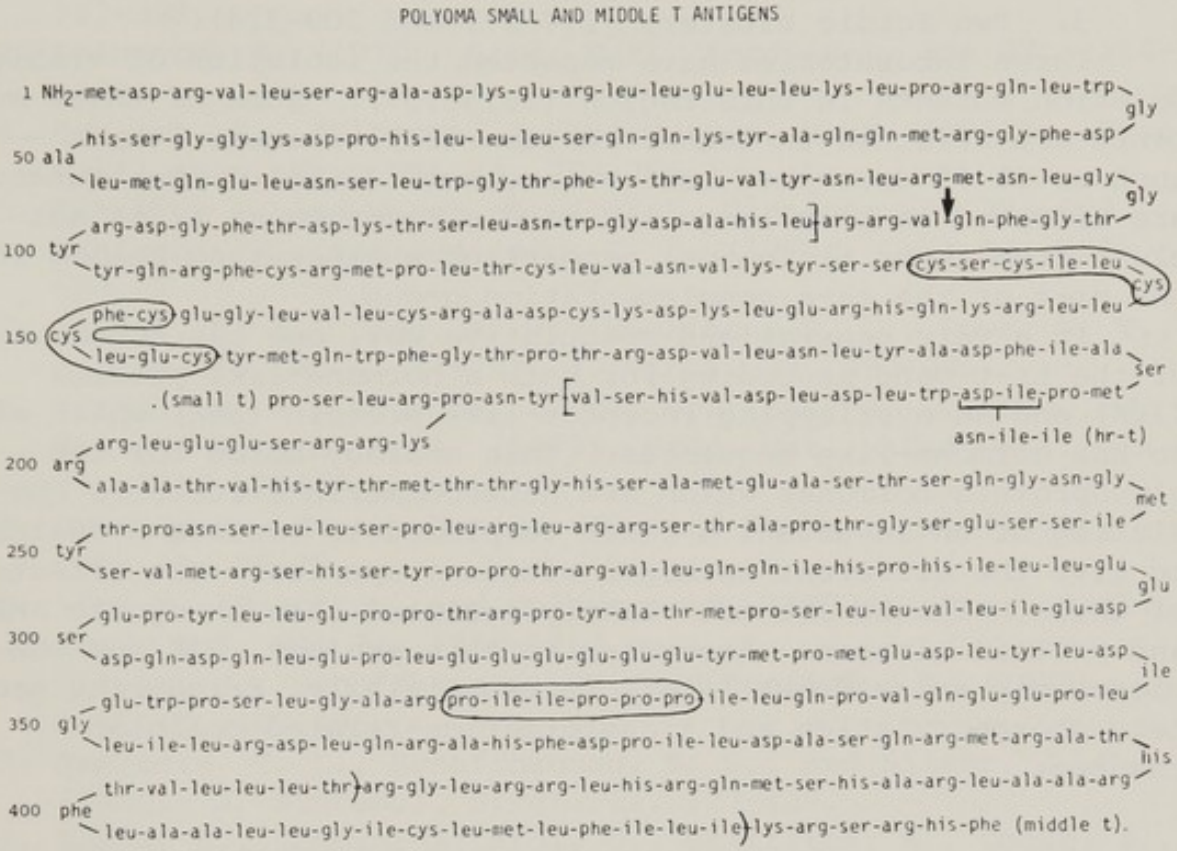

FIGURE 6. The hypothetical amino acid sequences of polyoma small and middle T antigens. The primary structure of these antigens is deduced from DNA sequencing, peptide mapping and RNA splicing data summarized in the legend to Figure 1. Circled sequences are regions of homology with hormones (see text). Hr-t mutations lie between the brackets in the small T/middle T common region, and the hydrophobic portion of middle T, near the C-terminus, is indicated by parentheses.

The introns for small and middle T antigens are thought to arise at precisely the same point in the RNA and to end at different positions in different reading frames (see Figure 2 and references 24,30,31). In the case of the small T, only four amino acids are acquired downstream of the splice, while the middle T gains an additional 230 amino acids. This latter domain, unique to the 56K protein, has several interesting features:

1. A pro-rich cluster (circled) homologous to a sequence near the carboxyl end of the β-subunit of human

chorionic gonadotropin (55);
2. a hydrophobic tail consisting of 22 residues (394-415) which most likely provides the 'membrane anchoring' site for the protein;
3. two acidic clusters (274-277 and 309-314).

Three laboratories have reported the isolation of viable deletion mutants in this region (36,56,57). Some of these mutants are affected in transformation (56), whereas others — such as dl 45 described here — are not (36,56). Such mutants are affected in both large and middle T antigens; it is not clear from their biological properties whether they belong in the hr-t or the ts-a complementation group.

In a very general and speculative way, one can conceive of the hr-t gene as coding for both a hormone-like protein (22K) and an overlapping receptor-like protein (56K) which also has hormone-like sequences. This analogy holds for the glycoprotein hormones which are heterodimeric structures consisting of an invariant α chain complexed to various β chains to give the different hormones within a species. The effects of these hormones are most likely mediated through cyclic AMP and protein kinases. According to this analogy, 22K/56K complexes may arise, most likely intracellularly, triggering protein phosphorylation and transformation-related cellular changes.

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COMPLEMENTATION STUDIES WITH TRANSFORMATION DEFECTIVE MUTANTS OF POLYOMA VIRUS[1]

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ABSTRACT Clones of transformed cells have been derived from the NRK line (normal rat kidney) by co-infections with hr-t and ts-a mutants of polyoma virus. Twenty-one such complementation transformed clones studied express the 56K (middle) T and 22K (small) T antigens, the dual products of the hr-t gene. In contrast, the expression of the 100K (large) T antigen is either greatly reduced (4 of 21 clones) or absent (17 of 21 clones). Upon fusion with permissive mouse cells, only four of the 21 clones were virogenic. One of these clones yielded only the ts-a mutant parent, the provider of the intact hr-t function. The overall results fit the view that the hr-t product(s) are required for the maintenance of the transformed phenotype, while the ts-a product is required only during the initial stages of the transformation process.

INTRODUCTION

The existence of two early genes of polyoma virus - hr-t and ts-a - defining two separate steps of viral transformation has been established (1,2). The hr-t gene is thought to provide the function which induces and maintains the cellular transformed state, while the ts-a gene is believed to be essential for the stabilization of the transformed phenotype, most likely facilitating the integration of viral DNA into cellular DNA (3). These conclusions are based upon experiments which demonstrate an absolute defect of hr-t

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mutants and a contrasting normal behavior of ts-a mutants in the viral pleiotropic induction of cellular changes referred to as abortive transformation (3-7). However, the results of analysis of clonal cell lines transformed by ts-a mutants do not yield to simple interpretations. When ts-a transformants, selected at permissive temperature, are shifted to non-permissive temperature, two categories of transformants are found: those whose transformed phenotype is insensitive to and those whose phenotype reverts with the temperature shift (3,8). The behavior displayed by members of the first category, in which one observes no effect of the ts-a mutation on the maintenance of transformation, is expected from the results of abortive transformation studies. In contrast, the existence of transformants with temperature-sensitive phenotypes is unexpected and suggests alternate or additional explanations, such as a role of the ts-a function on the maintenance of transformation under some circumstances, or an effect of the ts-a function on the expression of the hr-t function (3).

The analysis of transformation complementation clones, obtained by co-infections with hr-t and ts-a mutants described here provides an additional approach to studying the role of the hr-t and ts-a functions in the maintenance of transformation. Only genomes providing an intact maintenance function are expected to persist and be expressed in transformation complementation clones. In contrast, genomes providing the initiation function may be dispensed with once that function has been enacted, and thus may be neither expressed nor retained in the complementation transformed clones.

RESULTS

1. Derivation of Complementation Transformed Clones

Rat cells (NRK) were mixedly infected with hr-t mutant NG-18 and ts-a mutants ts-25D or ts-616, and transferred to agar at 39°C within 8 hr after infection as previously described (1,3). The results are shown in Table 1. The mixed infections give rise to 13-fold (NG-18 x ts-25D) or 23-fold (NG-18 x ts-616) increases over the background seen in the single infections. These results confirm previous reports of complementation for transformation between hr-t and ts-a mutants (1,2). Twenty-one transformed clones were picked, recloned and maintained in liquid cultures at 37°C.

TABLE I
COMPLEMENTATION FOR TRANSFORMATION[a]

Virus (total m.o.i.)	Number of transformants[b]
NG-18 (1-2)	0
ts-25D (5-10)	0
ts-616 (5-10)	1
NG-18 x ts-25D (6-12)	13
NG-18 x ts-616 (6-12)	23
wild type (5-10)	158

[a] NRK cells were infected at the multiplicity of infection (m.o.i.) shown as described previously (3) and transferred to soft agar at 39°C.

[b] Refers to the number of macroscopic clones per plate (i.e. per 10^4 infected cells).

2. Expression of Viral Proteins in the Complementation Transformed Clones

There are three distinct primary early gene products of polyoma virus: the 100K (large) T antigen, product of the ts-a gene; and the 56K (middle) T antigen and 22K (small) T antigen, dual products of the hr-t gene (See 10, this conference for a review and references). The expression of these proteins were studied in the complementation transformed clones. Virus specific proteins labeled with ^{35}S-methionine at 33°C were analyzed by means of immunoprecipitations with an anti-polyoma tumor serum, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (9). The results obtained for nine clones are shown in Figure 1 (lanes 3-11) where they are compared to the pattern obtained for a wild type lytic infection (lane 2). Both 56K and 22K bands are easily detectable in all nine clones. In addition, the presence of a 63K band previously described (9,11) is also clearly demonstrated in all clones. In contrast, the 100K band is either absent (in seven of the nine clones) or present at low levels (NG-18 x ts-25D #5 and NG-18 x ts-25D #11). This is in sharp contrast to the pattern observed with wild type lytic infection, in which the 100K is the most prominent band (lane 2). In two clones (NG-18 x ts-25D #8 shown in lane 5 and NG-18 x ts-616 #20 not shown) there is a unique band of approximately 45K and 60K respectively, which could be a fragment of the 100K protein.

Two wild type virus transformants as well as all twenty-one complementation transformed clones analyzed show a similar pattern with readily detectable amounts of 56K and 22K proteins and a strong reduction or absence of the 100K product.

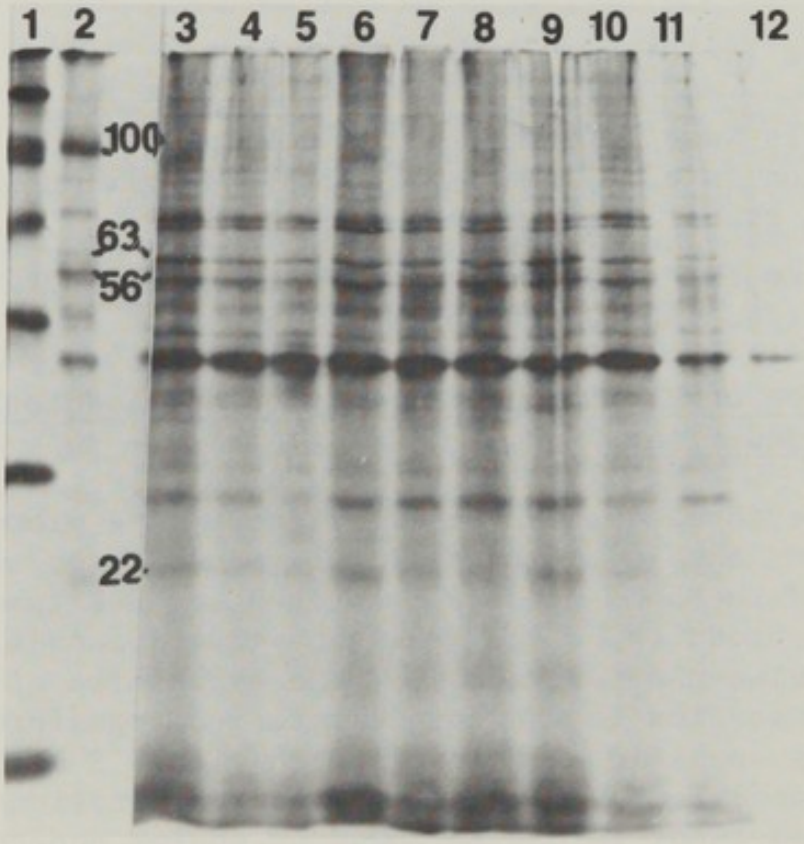

FIGURE 1. T antigen Patterns. T antigens were extracted and immunoprecipitated from cells labeled with ^{35}S-methionine. They were electrophoresed on an 11% discontinuous buffer SDS gel. The ^{14}C-labeled molecular weight protein markers (Amersham) displayed in lane 1 are: myosin (200K), phosphorylase-b (100K, 92.5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K) and lysozyme (14.3K). Lane 2 shows the pattern obtained in a wild type lytic infection; lane 3-11 show NRK clones NG-18 x ts-25D #5,6,8,11 and NG-18 x ts-616 #2,10,5,23,22 in this order. Immunoprecipitated extracts from NRK uninfected cells are shown in lane 12. The numbers shown refer large (100K), middle (56K) and small (22K) T antigens.

3. Analysis of Virus Rescued from the Complementation Transformed Clones

Attempts at rescuing virus from the complementation transformed clones were undertaken by fusing the transformed rat cells with permissive mouse UC1-B cells using polyethylene glycol (12). Fused cells were incubated for 7 days under conditions permitting the growth of both hr-t and ts-a mutants, i.e. using UC1-B cells (13) and low temperature. The fused cell lysates were assayed for hr-t and ts-a mutants as shown in Table II.

TABLE II
ASSAY CONDITIONS FOR THE DETERMINATION OF VIRAL GENOTYPES

	NIH-3T3		UC1-B	
Virus	33°C	39°C	33°C	37°C
ts-a	+	-	+	-
hr-t	-	-	+	+
wild type	+	+	+	+

Table III shows the results obtained for twenty-three clones analyzed. Most clones are non-virogenic upon fusion as is expected from the absence of large T antigen (14). Three virogenic clones (NG-18 x ts-25D # 2 and 4; NG-18 x ts-616 #17) yield both hr-t and ts-a mutants while one clone (NG-18 x ts-25D # 6) yields only the ts-a parent.

DISCUSSION

Twenty-one complementation transformed clones have been studied with respect to the viral T antigens expressed in these clones and the virus rescued by fusion with permissive cells. The overall results support the conclusion that the hr-t but not the ts-a gene product(s) are essential for the maintenance of the transformed phenotype. This is deduced from the fact that all clones express the 56K (middle) T and 22K (small) T antigens, the dual products of the hr-t gene, while the expression of the 100K (large) T antigen is strongly or totally suppressed. This situation has been observed previously in wild type transformants of rat and hamster cells (15-18).

TABLE III
ANALYSIS OF VIRUS RESCUED FROM TRANSFORMED CLONES[1]

Transforming virus	Clone number	Virus rescued by fusion
wild type	2	wild type
	3	-
NG-18 x ts-25D	1	-
	2	ts-25D, NG-18
	3	-
	4	ts-25D, NG-18
	5	-
	6	ts-25D
	7	-
	8	-
	11	-
	12	-
NG-18 x ts-616	2	-
	4	-
	5	-
	6	-
	10	-
	11	-
	17	ts-616, NG-18
	19	-
	20	-
	22	-
	23	-

[1]Transformed clones were fused with permissive mouse cells at 33°C and the fusion lysates were analyzed by plaque assays as described in Table II.

Most clones (17 of 21) fail to yield virus upon fusion with permissive cells. This is expected because of the known requirement for a functional 100K (large) T antigen in viral DNA replication (19) and in the viral excision process (14). One of the four virogenic clones yield only the ts-a parent genome, i.e. the genome providing the functional hr-t gene. This suggests that the hr-t mutant genome providing the functional ts-a gene can be lost while the cell remains transformed. This view is reinforced by the finding that two of four non-virogenic clones analyzed by Southern blotting fail to reveal the presence of genomes with the deletion characteristic of the NG-18 mutation (20). The loss of hr-t mutant genomes which provide the ts-a function strongly suggests that

the latter function has been fully enacted during the early steps of transformation when the stabilization of the viral genomes occurs in these transformed cells.

No wild type recombinants have been detected in the virogenic complementation transformed clones derived from NRK. This is in sharp contrast to the situation observed in complementation transformed cells derived from the F-111 line of Fisher rat cells (20).

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ANALYSIS OF ADENOVIRUS INDUCED CELLULAR DNA SYNTHESIS IN A ts MUTANT OF THE CELL CYCLE[1]

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ABSTRACT tsAF8 cells are temperature sensitive (ts) mutants of BHK cells that are arrested in G_1 at the non-permissive temperature. When made quiescent by serum restriction, they can be stimulated to enter S phase by 10% serum at 34°C but not at 40.6°C. Infection by Adenovirus 2 or 5 stimulates cellular DNA synthesis in tsAF8 at both 34°C and 40.6°C. Infection of these cells with deletion (Ad5dl312, Ad5dl313) and temperature sensitive (H5ts125, H5ts36) mutants of Adenovirus indicates that the expression of early regions 1a and 2 of the Adenovirus genome is needed to induce quiescent tsAF8 cells to enter S phase at the permissive temperature. To induce DNA synthesis at the non-permissive temperature, additional viral functions are required, such as the expression of early regions 1b and 5. In addition, we have shown that Adenovirus DNA intact or enzymatically digested is biologically active in inducing DNA synthesis when microinjected into the nucleus of tsAF8 cells.

INTRODUCTION

Our approach to the understanding of the regulation of G_0-S transition in eukaryotic cells has been to select, as an experimental model, the combined use of G_1 temperature sensitive mutants and DNA oncogenic viruses.

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G_1 ts mutants are operationally defined as growth mutants that are arrested at the non-permissive temperature in the G_1 phase of the cell cycle (1). Several of such mutants have been described including a cell line derived from BHK cells, tsAF8 cells (2), used in the experiments described in this paper.

When tsAF8 cells are made quiescent by serum deprivation and subsequently stimulated with 10% serum, they enter S phase at the permissive temperature, but they fail to do so at the non-permissive temperature (2-4).

A number of observations by several investigators has established that small DNA viruses, such as SV40, Polyoma and Adenoviruses, can induce the G_0-S transition in resting cells (5-8). The Adenovirus-infected cell is a particularly interesting system to investigate because the viral genome is small enough to be analyzed and, yet, its expression is regulated by different regions of the DNA (9,10).

Our initial studies using Adenovirus 2 (Ad2) infected tsAF8 cells have demonstrated the ability of Ad2 to induce cellular DNA synthesis in this mutant both at the permissive and non-permissive temperature (11).

This paper describes a further analysis of this feature in an attempt to localize the regions of the Adenovirus genome involved in the induction of cellular DNA synthesis in tsAF8 cells by the use of deletion and temperature sensitive mutants of the virus. In addition, our preliminary results on microinjection of Adenovirus DNA into the nucleus of tsAF8 cells open the possibility of a more precise localization of the viral genes required for the progression of cells from G_0 to S.

METHODS

Cell Culture. tsAF8, originally derived from BHK cells, were grown and made quiescent as previously described (3).

Viruses. Ad2, Ad5 and their ts mutants were grown in HeLa cell culture maintained in Dulbecco's modified Eagle Medium supplemented with 5% fetal calf serum. Ad5 deletion mutants were grown in 293 cell culture maintained in Dulbecco's modified Eagle Medium supplemented with heat-inactivated fetal calf serum. Virus was purified as described by Philipson et al. (12) with two cycles of CsCl density gradient centrifugation.

Purification and Digestion of Ad2 DNA. Adenovirus 2 DNA was purified as described by Petterson and Sambrook (13). The purified Ad2 DNA was digested to completion with

restriction endonucleases, Bam Hl, Hpa 1 and Hind III, under conditions suggested by the supplier (Bethesda Research Laboratories, Bethesda, Md.). After digestion the mixture was deproteinized with chloroform/isoamyl alcohol and ethanol precipitated at -20°C (14). DNA restriction fragments, when used, were resolved by electrophoresis in 1% agarose and visualized by long wavelength ultraviolet-induced fluorescence of ethidium bromide-stained gels, and DNA was isolated from cut gel slices by electroelution (15).

Microinjection. tsAF8 cells were plated on 22 x 22 mm glass slides and made quiescent by incubation at 34°C in 1% serum containing medium for 6 days. Purified, intact Ad2 DNA and Ad2 DNA fragments (see above) were dissolved in 10 mM Tris-HCl (pH 7.2) buffer at a concentration of 0.5 - 1.0 mg/ml and injected into nuclei of tsAF8 cells with the glass capillary microinjection technique of Graessmann (16). After microinjection the cells were incubated at 34°C in the same 1% serum-containing medium for 40 hours. For the last 24 hours of incubation [^{3}H]-thymidine was added to the medium (1 μCi/ml).

Immunofluorescence. tsAF8 cells were fixed for 15 min. with methanol (-20°C). Ad2 specific 72K protein was visualized in microinjected cells by indirect immunofluorescence using hamster anti 72K serum as the first antibody and FITC-labeled goat anti-hamster globulin as the second antibody.

Autoradiography. Incorporation of [^{3}H]-thymidine into tsAF8 was determined essentially as described by Baserga and Malamud (17). A minor modification of the procedure was used for autoradiography of microinjected cells. Methanol fixed tsAF8 cells were first stained by the immunofluorescence method for the Ad2 72K protein (see above) and then coated with NTB emulsion (Kodak Nuclear Track Emulsion diluted with H_2O, 1:1). The slides were developed after 3-4 days of exposure and mounted in Aqua-Mount (a non-fluorescing mounting medium from Lerner Laboratories, Stamford, Conn.)

RESULTS

Effect of Infection with Adenovirus 5 ts Mutants on the Induction of Cellular DNA Synthesis in Quiescent tsAF8 cells. After 48 hours of serum deprivation tsAF8 cells were stimulated by the addition of fresh medium containing 10% serum or infected with Ad5, H5ts125 or H5ts36 (M.O.I. = 500 - 1,000 pfu/cell). After one hour of virus absorption, conditioned medium was added to the cultures to be incubated

at the permissive temperature (32°C) and medium containing 10% serum, to the plates to be incubated at the non-permissive temperature (40.6°C). [^{3}H]-thymidine (0.01 μCi/ml) was added at the same time and the percentage of labeled cells was determined by autoradiography after 40 hours.

Table I shows the proportion of cells synthesizing DNA. As already reported (3), 10% serum stimulates quiescent tsAF8 cells to synthesize DNA at the permissive temperature but fails to do so at the non-permissive temperature. Ad5 is capable of stimulating tsAF8 cells to enter S phase either at 32°C or at 40.6°C as already reported for Ad2 (11).

H5ts125, in which the mutant gene product is coded by the Adenovirus DNA between 62.4 and 67.1 map units from the left end of the genome (18), i.e., in the early region 2, when tested on these cells was able to induce cellular DNA synthesis at the permissive temperature, but failed to do so at the non-permissive temperature. Similar results were obtained after infection with H5ts36 in which the ts function has been located on the Adenovirus genome between 18.5 and 22 map units (early region 5) (19).

Effect of Infection with Adenovirus 2 or Adenovirus 5 Deletion Mutants on the Induction of Cellular DNA Synthesis in Quiescent tsAF8 Cells. Quiescent tsAF8 cells were infected with Ad2d1305, Ad5d1312 or Ad5d1313. After virus absorption we followed the same procedure as described in the previous paragraph.

Table II shows the different behaviour of these deletion mutants in their ability to induce DNA synthesis in tsAF8 cells in relation to the wild types. The wild types (Ad2 and Ad5) as well as Ad2d1305, that has a deletion between 83 and 85 map units, i.e., in the early region 3, the non-essential region of the Adenovirus genome, were able to induce DNA synthesis in tsAF8 both at 32°C and at 40.6°C.

TABLE I

PERCENTAGE OF LABELED tsAF8

Treatment	32°C	40.6°C
Mock Infection	15.3	--
Serum Stimulation	90.0	20.0
wt Ad5 Infection	72.0	78.0
H5ts125 Infection (6214 - 67.1[a]:Reg. 2)	71.0	12.0
H5ts36 Infection (18.5 - 22[a]:Reg. 5)	80.0	11.0

[a] Map coordinates in which the ts function has been located.

TABLE II

DNA SYNTHESIS IN tsAF8 CELLS BY INFECTION WITH ADENOVIRUS DELETION MUTANTS

Treatment	% Labeled tsAF8 Cells 32°C	40.6°C
Mock Infection	15.0	--
Serum Stimulation	89.0	18.0
wtAd2 Infection	73.0	69.0
wtAd5 Infection	72.0	78.0
Ad2dl305 Infection (83-85[a]: Reg. 3)	73.0	77.0
Ad2dl312 Infection (1.5-4.5[a]: Reg. 1a)	18.0	8.0
Ad2dl313 Infection (3.5-10.5[a]: Reg. 1b)	76.0	10.0
Ad2+D1 Infection (64-74[a]: Reg. 2)	25.0	28.0
Ad2ND1-H71 Infection (81-86[a]: Reg. 3)	63.0	73.0

[a]Map coordinates in which the deletion has been located.

On the contrary Ad5dl312, that lacks the segment of the Adenovirus DNA located between 1.5 to 4.5 map units, is unable to stimulate cellular DNA synthesis both at the permissive and at the non-permissive temperature. Infection of tsAF8 with Ad5dl313 (deletion between 3.5 and 10.5 map units: early region 1b) resulted in a stimulation of DNA synthesis at the permissive temperature but not at the non-permissive temperature.

During these experiments we have tested two additional viruses, Ad2+D1 and Ad2ND1-H71. Ad2+D1 is a defective Adenovirus 2-SV40 hybrid virus (20) that includes in its DNA the entire early region of the SV40 genome, this SV40 insertion replacing the segment of the Adenovirus DNA mapping between 64 and 74 map units. In this regard, it is comparable to the H5ts125 since in both cases the deleted or mutated viral product is the 72K DNA binding protein.

As shown in Table II, Ad2+D1 fails to induce tsAF8 to enter S phase at both the permissive and the non-permissive temperature. We have also tested the non-defective Adenovirus 2-SV40 hybrid virus, Ad2ND1 in which the insertion of SV40 DNA is much smaller (28-11), and the deletion in the Adenovirus 2 genome is located in the non-essential region and maps between 81 and 86 map units. As expected Ad2ND1-H71

was able to stimulate cellular DNA synthesis at both 32°C and 40.6°C.

In order to determine whether the lack of induction of DNA synthesis at the non-permissive temperature observed in tsAF8 after infection with Ad5dl313, H5ts125 and H5ts36 was not simply due to the effect of the high temperature on the virus viability, we have tested these viruses on BHK cells, the parent cell line of tsAF8. Our results indicate that the addition of 10% serum to quiescent BHK cells, as well as infection with Ad5(wt), Ad5dl313 and H5ts36 increase the percentage of labeled BHK cells incubated either at 32°C or at 40.6°C, while H5ts125 infection of BHK cells results in induction of cellular DNA synthesis only at the permissive temperature, but not at the non-permissive temperature (data not shown).

Expression of the Adenovirus 2 Genome and Stimulation of Cellular DNA Synthesis in tsAF8 Cells Microinjected with Intact or Digested Ad2 DNA. tsAF8 cells were microinjected with either intact Ad2 DNA or with four types of Ad2 DNA fragments, i.e., unseparated complete digests of Bam Hl, Hpa 1 or Hind III endonucleases, and A+B fragments isolated from Bam Hl digest. After microinjection, the cells were incubated at 34°C for 40 hours ([^{3}H]-thymidine added for the last 24 hours) and then fixed, stained for the 72K protein and autoradiographed by the technique described in Methods. The cells were evaluated for immunofluorescence staining and autoradiographic grains by a Zeiss microscope using

TABLE III

DNA SYNTHESIS IN tsAF8 BY MICROINJECTION OF ADENOVIRUS 2 DNA

Injection	Staining for Ad2 72K Protein	% of labeled tsAF8 Cells
None	-	21.3
Intact Ad2 DNA	+	87.0[a]
Ad2 DNA-Bam Hl Digest	+	86.1[a]
Ad2 DNA-Hind III Digest	+	77.3[a]
Ad2 DNA-Hpa I Digest	+	79.2[a]
Ad2 DNA A and B Fragments of Bam Hl Digest	+	85.7[a]

[a]Percent of labeled cells in 72K positive cells.

alteranatively fluorescence excitation or transmitted light. The results are summarized in Table III. As can be seen, the cells injected with either intact or digested Ad2 DNA were positive for the presence of the Ad2 72K protein. In addition, 77.3 - 87% of the 72K positive cells were also labeled with [^{3}H]-thymidine. These values are more than three times higher than the percentage of labeled, non-microinjected cells (21.3%).

It can be concluded that Ad2 DNA, both in intact and fragmented form, retains its biologically activity upon microinjection into tsAF8 cells.

DISCUSSION

In a previous paper we have reported the ability of Adenovirus 2 to induce cellular DNA synthesis in tsAF8 cells (11), a cell cycle specific temperature-sensitive mutant derived from BHK cells (2), that arrest in G_1 at the non-permissive temperature.

The mutation of these cells has been shown to effect either the synthesis, the assembly or the stability of RNA polymerase II, since RNA polymerase II activity, as determined *in vitro* using isolated nuclei (3), as well as the number of α-amanitin binding subunits decrease at the non-permissive temperature with a halflife of 10-12 hours (3,21).

While at the permissive temperature Adenovirus DNA can be replicated, the cellular ts function inhibits viral replication (22) and, yet, a viral product(s) is able to initiate cellular DNA synthesis at the non-permissive temperature (11).

Although there is some information on the segment of Ad2 genome that is necessary for transformation (23), little is known about the early protein(s) coded by the Adenovirus that induce host DNA synthesis in resting cells. Our results indicate that, in our system, the expression of the early regions 1a and 2 of the Adenovirus genome is needed to induce tsAF8 cells to enter S phase at the permissive temperature.

Jones and Shenk (9) and Berk et al. (10) have reported that an Adenovirus early gene function located in the early region 1a is required for the production of cytoplasmic m-RNAs corresponding to early regions 2, 3 and 4. Our data have shown that a deletion in the Adenovirus genome corresponding to the early region 2, as well as a deletion in the region 1a, bring about the same results: a failure in the induction of cellular DNA synthesis. This conclusion is also supported by the results obtained after infection of tsAF8 with the H5ts125 mutant (region 2). However, the results obtained with the Ad2+D1 virus are complicated by the presence in the virus preparation of the helper virus (Ad2)

and by the insertion of the SV40 A gene, that could explain the slightly higher percentage of labeled cells in comparison to the mock-infected cultures.

Another interesting finding is that additional viral functions seem to be required at the non-permissive temperature for the induction of cellular DNA synthesis in tsAF8 cells, more precisely, the expression of region 1b and region 5 (also defined as minor region) (Williams, personal communication) as indicated by the results obtained with Ad5dl313 and with H5ts36. Finally, we cannot exclude the participation of the early region 4, since we have not tested mutant viruses of this region.

The results obtained with BHK cells rule out the possibility that the absence of DNA synthesis in tsAF8 at 40.6°C after infection with Ad5dl313 and the ts mutants of Ad5, was due to the effect of the high temperature on virus viability. They are in agreement with the findings by Williams indicating that H5ts36 is able to initiate DNA synthesis in rat embryo cells and in primary kidney cells both at 34°C and at 40°C (personal communication).

We have shown, in addition, that with the microinjection technique (16) it is possible to introduce in the nucleus of tsAF8 cells active Adenovirus 2 DNA molecules that can induce the synthesis of proteins recognizible by anti-72K-antiserum and are capable to stimulate host DNA synthesis. The activity shown by the Ad2 DNA, digested with restriction endonuclease, and by selected fragments, as Bam H1 A and B fragments, when microinjected in tsAF8 cells, opens the possibility to overcome certain limitation in the use of deletion and temperature sensitive mutants of the virus. This should give more precise information about the viral genes involved in the regulation of different steps in the transition from G_0 to S of the cell cycle.

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REGULATION OF ADENOVIRUS EARLY GENE EXPRESSION

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SUMMARY. Adenovirus early products are classified into four groups on the basis of studies with inhibitors of macromolecular synthesis and with virus mutants. (i) 'Immediate early' genes are resistant to stringent inhibition of protein synthesis and encode polypeptides of 13.5k and 52,55k. (ii) The 'pre-early' E1A gene block is sensitive to stringent protein synthesis inhibition, and is required for expression of the remaining early mRNAs. (iii) 'Delayed early' genes encode most of the conventionally recognized early proteins and are dependent on expression of the E1A region. (iv) 'Intermediate' genes are expressed in the absence of DNA replication but are more sensitive than the other early genes to protein synthesis inhibition; this group includes non-structural proteins as well as 'quasi-late' virion components. Mechanistic interpretations of this hierarchical organization are considered.

INTRODUCTION

The multiplication of adenovirus, a double stranded DNA containing virus which replicates in the nucleus of infected human cells, has been considered to take place in two stages delineated by the onset of viral DNA replication. During the early stage, before DNA replication takes place, the scene is set for DNA synthesis by the production of early messenger RNAs and proteins, some of which are involved directly in replication. Following DNA replication other gene banks are opened up and viral products are synthesized that are required for the production of progeny virus particles. This latter category of late products includes both the structural components of the virions and proteins involved in their assembly and maturation. Such a division of the lytic cycle into two phases is common to infections with many viruses, but the molecular basis of this division is generally poorly understood if it is understood at all. Several viruses, particularly those with larger and more

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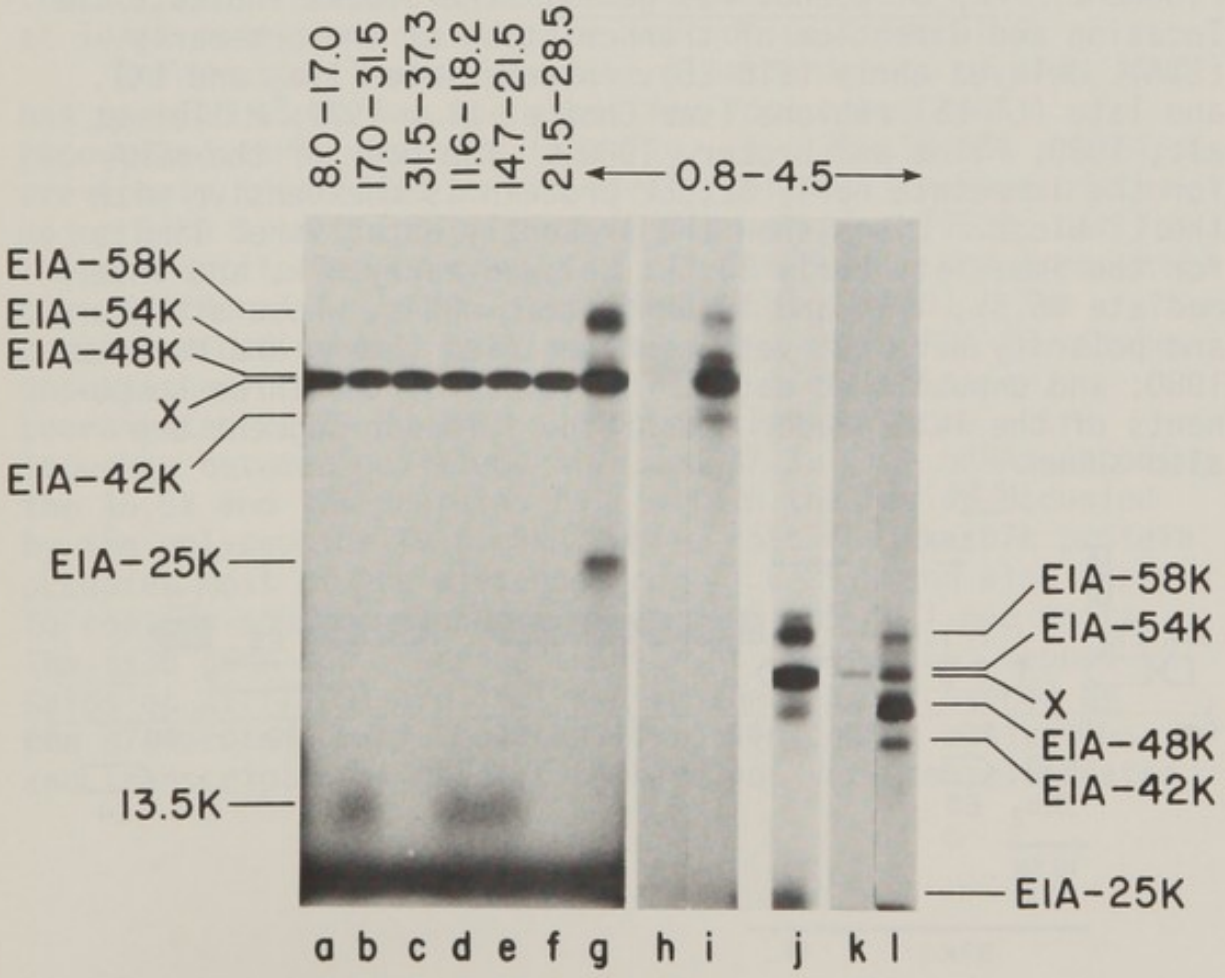
complex genomes, have a life cycle which can be divided more finely. One distinction which has been drawn is between those early viral products whose synthesis occurs in the infected cell without requirement for the prior expression of any viral proteins --these are termed the immediate early products--and, on the other hand, those early products whose production is contingent on the prior expression of the immediate early category--the delayed early products.

It would seem to be relatively simple to discriminate operationally among the immediate early, the delayed early, and the late classes of viral gene products by the simple device of including inhibitors of macromolecular synthesis in the medium in which the cells are cultured. Those early messenger RNAs which are produced in the presence of inhibitors of protein synthesis would then fall into the immediate early class; while those which are not present under these conditions, but which are produced in cells blocked for DNA replication, would be assigned to the delayed early category. The products which are not present under either of these conditions would be classified as late products. Distinctions drawn by the use of inhibitors are of course, beset by worries about the efficiency and specificity of the inhibitors used. They do, however, have the virtue that they can be related directly to mechanistic considerations. For example, one obvious hypothesis to explain the dependence on protein synthesis of expression of delayed early products is that formation of a viral protein is required to instigate their production.

In adenovirus, until recently the situation seemed to be relatively simple (see Flint and Broker, 1980, for a review). Both cytosine arabinoside (Ara C), an inhibitor of DNA replication, and cycloheximide, an inhibitor of protein synthesis, prevent the appearance of a large class of mRNAs encoding the late proteins. There is no obvious qualitative distinction between the early proteins made under either condition of inhibition, implying that the delayed early class does not exist in adenovirus. On the other hand, expression of certain early proteins lags behind other members of the class. Experiments with mutants of adenovirus that are incapable of growing in HeLa cells suggested that the products of one of the early genes known as early region E1A is required for the expression of the other viral early genes (Jones and Shenk, 1979; Berk et al., 1979), but left open the question of whether or not its products are immediate early as defined above. To investigate this question, we explored the use of very potent inhibitors of protein synthesis upon the expression of virus messenger RNA on the grounds that cycloheximide does not completely

obliterate all protein synthesis and the residual 2-5% might suffice to permit the production of a viral protein required in only small amounts. The inhibitor we have used is anisomycin, which at high concentration reduces protein synthesis to below one percent of the control level. The inhibition is reversible, allowing a measure of confidence that its use does not irretrievably hamper the cells' biosynthetic processes. Infections carried out in the presence of 100 μm anisomycin applied before infection yield greatly reduced amounts of the conventional early mRNAs, including those from region E1A which therefore does not fulfill the requirements for an immediate early gene. Other

FIGURE 1. Cell-free translation of viral mRNA. RNA was isolated 3 hr. after infection with Ad2 in the absence (tracks i, l) or presence of 100 μM anisomycin from 0.5 hr. before infection (tracks a-h, j, k). Adenovirus mRNAs were selected by hybridization to cloned DNA fragments with the map positions marked, and translated in vitro. The products were fractionated in an SDS-polyacrylamide gel which was exposed to fluorography for 1.5 hr. (tracks h, i, k, l) or 15 hr. (a-g, j).

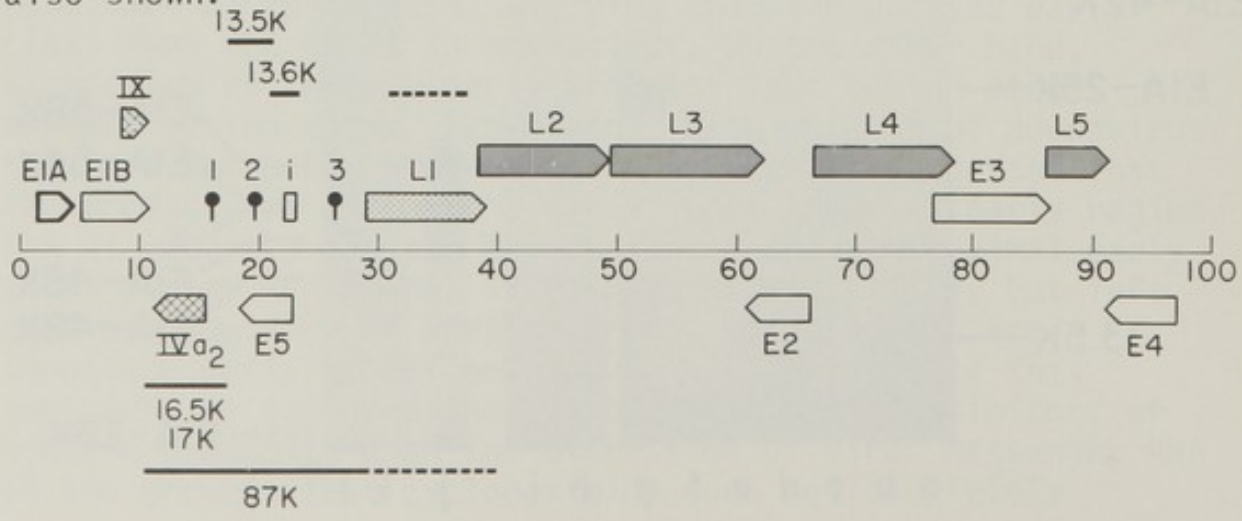


viral mRNAs are not depressed by this treatment, however, ruling out a non-specific interference of the drug with the course of the infection, and prompting a widescale survey of the mRNA species present in cells infected under a variety of conditions. The conclusions from this survey are discussed here.

RESULTS

We have employed a translation-based assay to analyze the viral mRNAs present in HeLa cells infected with wild-type or mutant adenoviruses. Cytoplasmic RNA isolated at various times post-infection from cells maintained under assorted drug regimes is selected by hybridization to viral DNA or its subfragments, generally cloned in pBR322, immobilized on nitrocellulose (Ricciardi et al., 1979). The eluted viral mRNA is translated in the messenger-dependent reticulocyte lysate (Pelham and Jackson, 1976) and the products identified by SDS-polyacrylamide gel electrophoresis and fluorography. In this way, individual viral mRNAs can be mapped on the genome and their approximate quantitative

FIGURE 2. Map of adenovirus genes. The blocks indicate the location and direction of transcription of the pre-early (E1A), delayed early (E1B-E5), intermediate (IVa_2 and IX), and late (L1-L5) regions (see Chow et al., 1979; Miller et al., 1980; Flint and Broker, 1980). The body of the mRNA for the immediate early 52,55k protein is coextensive with the L1 block. Lines show the presently established limits for the immediate early 13.5k, delayed early 87k, and intermediate 16.5k, 17k, and 13.6k protein mRNAs, whose structures and polarity have not yet been specified (Lewis and Mathews, 1980; and unpublished data). Positions of the three components of the late leader and of the i leader segment are also shown.



representation in the population can be assessed.

An example of such an analysis is shown in Fig. 1, which displays the results obtained with RNA isolated at 3 hr. post infection with Ad2. Selection with DNA specific for the E1A region (tracks i and L) results in the appearance of the four characteristic polypeptides, E1A-58k, 54k, 48k and 42k, with a heavy predominance of the 48k species. Using RNA from a parallel infection carried out in the presence of 100 μM anisomycin to block protein synthesis, the amount of E1A proteins synthesized is drastically reduced (tracks h and k); a longer exposure (tracks g and j) reveals that among the residual mRNAs, that for the 58k species is preeminent. Also clearly visible is a 25k polypeptide that is not a major product of RNA from unblocked cells. The only other viral mRNA detectable in the RNA from anisomycin treated cells, apart form a trace of E4-21k mRNA (not shown), encodes the recently described 13.5k protein (Lewis and Mathews, 1980). The mapping of this species is illustrated in the remaining portion of Fig. 1, which shows that the 13.5k mRNA hybridizes to DNA fragments with coordinates 17-31.5, 11.6-18.2 and 14.7-21.5 (tracks b, d and e) but not to DNA from 8-17, 31.5-37.3 and 21.5-28.5 (tracks a, c and f). This data indicates that the mRNA is transcribed from DNA between coordinates 17 and 21.5, with at least some sequences to the left of position 18.2.

Information drawn from several experiments of this kind has enabled us to map the genes for four other newly recognized early proteins (see Fig. 2). The 52,55k proteins, previously identified as the first (leftmost) member of the major late transcription unit (Miller et al., 1980), carries the tripartite leader common to all late mRNAs and frequently the additional i leader also (Chow et al., 1979). The species encoding the 13.6k protein is a short RNA (H. Esche, unpublished) mapping in the vicinity of the i leader, coordinates 22-23.2, with the possibility of some extra sequence between positions 31.5 and 37.3. The mRNAs encoding the 16.5k and 17k proteins fall within the region occupied by the polypeptide IVa_2 mRNA, while that for the 87k protein occupies most of the distance from 11.6-29.4 and also appears to contain some sequences between 31.5 and 40.1 map units. The ts36 gene and l-strand transcript (region E5) mapped by Galos et al (1979) are included in the region covered by the 87k protein mRNA, but their protein coding activities and transcriptional regulation have not yet been elucidated.

TABLE I
PATTERNS OF ADENOVIRUS EARLY GENE EXPRESSION

HeLa cells in suspension culture were infected with wild type Ad2 (or Ad5), or with mutant hr1 and dl312, and harvested at the times shown (hours post infection). RNA was isolated, selected by hybridization to specific viral DNA fragments and translated in a reticulocyte lysate cell-free system. Proteins synthesized were identified and estimated by SDS-polyacrylamide gel electrophoresis followed by fluorography. Inhibitors were added at the times shown (hours post infection) as indicated: cytosine arabinoside (AraC), 25 µg/ml; anisomycin (Aniso., An), 10 µM or 100 µM; emetine (Emet.), 50 µg/ml; puromycin (Puro.), 100 µM. The symbols in the body of the table have the following significance: —, not detectable; (+) to ++++, present in trace amounts to abundance; ND, not determined.

a) Protein spectrum shown is restricted to low multiplicity infection; at higher multiplicity pattern resembles wild type.
b) Cannot be reliably estimated in presence of hr1 26k protein or of large amounts of 42-58k E1A proteins, because of partial translation products.
c) Replaced by 26k fragment in hr1 infection.
d) In some gels the 100 µM anisomycin-resistant 58k protein seemed to migrate slightly slower than the usual E1A-58k.
e) Symbols refer to major species (E4-21k, E3-13k, 15.5k); the levels of the minor species follow coordinately.
f) A very small amount of Ad5 E4-17k is detected.
g) Additional minor band visible at 60k.

	Wild type Ad2											hr1[a]		d1312	
Protein	Unblocked		AraC	Aniso. 10uM			Aniso. 100μM			Emet.	Puro.	AraC	An10	AraC	An10
Harvest time	3	20	16	6	6	6	3	6	6	6	6	6	6	6	6
Inhib. addition			+1	-0.5	+1	+3	-0.5	-0.5	+1	-0.5	-0.5	+1	+1	+1	+1
52,55k	—	+++	++	+	+	+	ND	+	+	ND	++	+	+	—	—
13.5K	—	++	++	++	++	++	++	++	++	ND	++	—	++	—	+
E1A-25K[b]		—					+	+	++					—	—
58K[c]	+++	++	+++	+++	+++	+++	+	+[d]	++	+++	+++	—	—	—	—
54K, 42K	+++	++	+++	+++	+++	+++	(+)	(+)	++	+++	+++	+++	+++	—	—
48K[c]	++++	++	+++	++++	++++	++++	(+)	(+)	++	++++	++++	—	—	—	—
28K[b]		++					—	—	—					—	—
E4[e]	++	ND	++	++++	++++	++++	(+)	(+)	+	++	++	—	++	—	(+)[f]
E3[e]	ND	+	+++	+++	+++	+++	—	—	ND	+	ND	+	ND	—	—
E1B-57K	+	+	++	+	+	+[g]	—	—	ND	—	ND	—	ND	—	—
18K	—	—	ND	+	+	+	—	—	ND	—	ND	—	ND	—	—
15K	+	++++	++++	+++	++++	++++	—	+	ND	++	ND	+	ND	—	—
22K	—	+	—	—	—	—	—	—	ND	—	ND	—	ND	—	—
E2-72K	+	++++	++++	+++	+++	++++	—	—	—	+	+	+	+	—	—
87K	—	—	—	+	++	++	—	—	—	ND	—	—	—	—	—
16.5K, 17K	—	+	++	—	—	—	—	—	—	ND	ND	+	+	—	—
13.6K	—	++	++	—	—	—	—	—	ND	ND	—	—	—	—	—
IVa_2	—	++++	++	+	+	+	—	—	—	ND	ND	ND	+	—	—
IX	ND	++++	++	+	+	+	—	—	ND	ND	ND	ND	ND	—	—

DISCUSSION

Results from a large number of experiments similar to that of Fig. 1 are collated in Table I. The early genes of adenovirus can be divided into four categories reflecting the requirements for their expression as cytoplasmic mRNA: this classification is summarized in Table II.

Immediate Early Genes. Two messenger RNAs are found in adenovirus infected cells under conditions of stringent protein synthesis inhibition (100 μM anisomycin added one half hour before infection): these encode the 13.5k protein and 52,55k pair of proteins. Their map positions allow the possibility that both are transcribed from the major late promoter, which Weil et al. (1979) have shown to be active in uninfected cell extracts. Under even more stringent conditions (500 μM anisomycin added before infection), only the 52,55k mRNA is detected, suggesting that the immediate early phase may perhaps be divided into two stages. Later in the infection increased quantities of the 52,55k mRNA are found, as expected for a product of the late region, but levels of the 13.5k mRNA remain steady. Both mRNAs are seen in extracts of cells infected with the E1A mutant hr1, but only the 13.5k mRNA is detectable in cells infected with the "tighter" dl312 mutant, and then just in trace amounts. Perhaps E1A proteins boost the production of the immediate early RNAs, as well as of the delayed early RNAs (see below), and the lack of 52,55k mRNA in dl312 infections may be attributed to a combination of this circumstance with the observation that its mRNA is always less abundant than that for the 13.5k polypeptide. Detection of minor species at early times also often requires moderate inhibition of protein synthesis, presumably to stabilize the mRNA as suggested by Wilson et al. (1979).

Although the dependence on protein synthesis of E1A expression is capable of other interpretations, we consider it likely that the immediate early genes govern the generation or stabilization of E1A mRNAs. There are few clues as to the nature of this involvement, but it is a striking observation that blockade of protein synthesis not only suppresses the total quantity of E1A mRNA, but distorts the ratio of the individual species in the resistant residue. Whereas the 48k protein is usually the most abundant, the 58k protein predominates when protein synthesis is stringently inhibited. Moreover, peptide analysis of the 25k protein synthesized from mRNA extracted from stringently blocked cells suggests that it may represent the product of an unspliced RNA. Thus it is possible that the immediate

TABLE II
CLASSIFICATION OF ADENOVIRUS EARLY GENES

Class	Protein	Factors affecting mRNA appearance	Persistence of mRNA in late phase
Immediate early	13.5k 52,55k	Present in cells under stringent protein synthesis inhibition.	Possibly present, but not ↑ ↑↑
Pre-early	E1A-58k, 48k 54k, 42k 28k	Accumulation substantially blocked by stringent inhibition of protein synthesis.	Present, but in decreased amounts. Persists.
Delayed early (shut-off)	E4-11k, 13k, 17k, 19k, 21k, 24k24k	Requires presence of E1A protein(s) at least in small amounts.	Undetectable. Prob. shut-off early (particularly subject to enhancement).
	E3-13k, 14k, 15.5k, 20k	" " "	Maintained, but not ↑ .
	E1B-57k, 18k	" " "	Poss. present, but ↓ .
(continuously accumulated)	E1B-15k	" " "	↑↑
	E2-72k	Requires E1A product(s) but slightly more sensitive to protein synthesis inhibition.	↑↑
(shut-off)	87k	Requires E1A product(s) but rather more sensitive to protein synthesis inhibition.	Undetectable.
Intermediate	16.5k, 17k,	Present at early times, but only if protein synthesis has not been inhibited, even partially.	↓ or none.
	13.6k		↑
(quasi-late)	IVa_2 IX	Small amounts present at early times, E1A - dependent.	↑↑ ↑↑

early proteins play a role in controlling the splicing reaction, or the stability of variously-spliced species.

Pre-early Genes. The term 'pre-early' was coined to describe the E1A genes, which studies with the mutants dl312 and hr1 have shown to be required for expression of mRNAs from regions E1B-E4 (Berk et al., 1979, Jones and Shenk, 1979). In our hands, no viral mRNA could be detected in dl312 infected cells apart from that encoding the immediate early 13.5k protein, and, in trace amounts, the E4-17k protein of Ad5 (corresponding to E4-21k in Ad2). Modest amounts of the latter and of some other early mRNAs were found in hr1 infections, either because of the measurable "leakiness" of this mutant or because of partial activity of the residual E1A proteins and protein fragments for which it codes. Stringent inhibition of protein synthesis (>99%) reduces expression of the E1A genes by 95-98% and of the genes which depend on E1A products by 99% or more. Milder inhibition of protein synthesis (95-98%) does not alter the level of the E1A mRNAs, making it seem that relatively small amounts of the proteins that govern E1A expression can suffice. Quite possibly for the same reason, it has not been feasible to obtain with protein synthesis inhibitors the same interference with delayed early gene function that is seen in the mutant infected cells. Anisomycin at 10 μM gives the enhanced pattern of expression of the conventional early genes that was first observed with cycloheximide (Craig and Raskas, 1974) and is probably attributable to messenger stabilization as mentioned above, or to suppression of a negative effector (Carter and Blanton, 1978; Eggerding and Raskas, 1978). At 30 μM, the drug reduces the amounts of mRNA from regions E1A and E4 and obliterates E2 mRNA (the results for other regions were not decided). Emetine and puromycin give an intermediate picture: E1A mRNA is present at the enhanced level but expression of the delayed early genes is blocked to a large degree. Interpretation of these findings is complicated by the toxicity of the drugs, which are not readily reversible, and the fact that some of the delayed early products appear later in the early phase than do others (for example, E4 precedes E2 and E3).

The studies of the E1A mutants make it clear that the E1A products are involved in the production or stabilization of the delayed early species. Whether the same or different factors are responsible for the fluctuating ratios of the individual E1A mRNAs remains to be seen.

Delayed Early Genes. The other conventionally recognized early genes are delayed early, in that their expression is

predicated on the presence of one or more E1A products. This group includes E1B-E4 together with the gene for the 87k protein. The delayed early genes are inhomogeneous in other regards, however: E4 products appear sooner after infection than their counterparts from E2 and E3; some mRNAs are largely confined to the early phase (E4), others presist throughout the infectious cycle (E3 and perhaps E1B-57K and 18K), and yet others accumulate in greater amounts in the late phase (E2, E1B-15k); the mRNA for the 87k protein is particularly sensitive to inhibition of protein synthesis whereas those from E4 are substantially more resistant than most delayed early RNAs; and the enhancement by moderate protein synthesis inhibition is greatest for E4 and least for E3 RNAs. The E2-72k protein appears to exert a negative control over the delayed early genes (Carter and Blanton, 1978), and may be responsible, in part at least, for the enhancement by moderate inhibition of protein synthesis, but it is clear that regulation of this group of genes must involve multiple factors.

Intermediate Genes. This category contains a motley grouping of proteins united by the common feature that their mRNAs are detectable in the presence of AraC, but are absent (16.5k, 17k, 13.6k) or present in only trace amounts (polypeptides IVa_2 and IX) when protein synthesis is blocked. All are lacking in dl312 infections and under stringent protein synthesis blockade, so presumably depend directly or indirectly on E1A expression. The two mRNAs which map in the IVa_2 region (16.5k, 17k) are present in reduced amounts at late times, whereas those for the 13.6k protein and especially for the 'quasi-late' polypeptides IVa_2 and IX increase in concentration possibly because of expansion of the transcriptional template pool brought about by DNA replication.

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METHYLATION AND EXPRESSION OF ADENOVIRAL DNA IN INFECTED AND TRANSFORMED CELLS[1]

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ABSTRACT It has been shown previously that the DNAs from purified adenovirus type 2 (Ad2) and type 12 (Ad12) are not methylated. The isoschizomer pair of restriction endonucleases HpaII and MspI was used to investigate the extent of adenoviral DNA methylation in infected and transformed cells. In human cells productively infected with Ad2, parental and newly synthesized viral DNA were not found to be methylated nor was the integrated form of Ad2 DNA. Hamster cells (BHK21) and Muntiacus muntjak cells are abortively infected by Ad12. In both systems Ad12 DNA remained unmethylated. On the other hand, Ad12 DNA integrated into the genomes of four different Ad12-transformed hamster cell lines was found to be extensively methylated. The segments of integrated Ad12 DNA comprising early genes, which were expressed in two of the lines, were strikingly less methylated in comparison to unexpressed late viral genes which were extensively methylated. Similar results were obtained with viral DNA in Ad2-transformed hamster cells. In contrast, in two lines of Ad12-induced rat brain tumor cells some of the late viral genes were expressed as m-RNA. These late regions of the Ad12 genome were undermethylated in comparison to the high level of methylation in the same regions in Ad12-transformed hamster cells. Thus, a striking inverse correlation exists between the levels

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of methylation in specific segments of integrated viral DNA and the levels of expression of these segments. This correlation may provide a clue to the analysis of gene regulation in mammalian cells.

INTRODUCTION

The regulation of gene expression in eukaryotes poses one of the fascinating problems in molecular biology. Several years ago, it has been reported that DNA methylation in developing tissues is at a lower level than in cells that are completely differentiated (1,2). In adenovirus type 12 (Ad12) transformed hamster cells an inverse correlation was reported between the extent of DNA methylation in specific genes of integrated viral DNA and the level of expression of these genes (3,4). The DNA of herpesvirus saimiri, which is thought to persist in a nonintegrated state in lymphoid tumor cells, is extensively methylated in non-producer lines, whereas viral DNA sequences in virus-producing lymphoid lines are undermethylated (5). Similarly, it was observed in a number of eukaryotic systems that DNA sequences in specific genes, e.g. the globin gene in rabbits (6) and in chickens (7), the ribosomal genes in Xenopus laevis (8,9) a variety of genes in Echinus esculentus (10), and the conalbumin and ovomucoid genes in chickens (11) are strongly methylated when these genes are not expressed. In the active state the DNA in the same genes is undermethylated.

DNA methylation in eukaryotes is found almost exclusively in 5-methylcytosine (12), and the bulk of the modified cytosine residues is found in 5'-CCGG-3' doublets (13). Waalwijk and Flavell (14) have discovered that the restriction endonucleases HpaII and MspI are isoschizomers cleaving at 5'-CCGG-3' sequences. HpaII cannot cleave at the sequence 5'-C^{m}CGG, whereas the sequence 5'-^{m}CCGG-3' seems to be cut (15). MspI is able to cleave at methylated and unmethylated sequences.

In the present report we demonstrate that in human KB cells productively infected with Ad2, neither the parental nor the newly synthesized viral DNA was found to be methylated at any time after infection. It appeared that the integrated form of Ad2 DNA in productively infected cells (16) was not

methylated either. In BHK21 cells (17) or Muntiacus muntjak cells (18) abortively infected with Ad12, the viral DNA remained unmethylated.

The levels of DNA methylation of specific integrated Ad2 and Ad12 DNA segments are correlated with the extents of their expression. In particular, in Ad12-transformed hamster cells the late regions of the integrated viral genomes, which are not expressed as mRNA, are strongly methylated. The same regions are at least partly expressed in Ad12-induced rat brain tumor cells, and the same segments are strikingly undermethylated.

MATERIALS AND METHODS

Cells. All virus-cell-systems investigated in this study were previously described. Human KB cells and human embryonic kidney cells were productively infected with Ad2 (16,17). Baby hamster kidney cells (subline B3) were infected with Ad12 at multiplicities between 1500 and 2000 plaque forming units (PFU) per cell (17,19). The abortive Ad12-Muntiacus muntjak cell system was also described elsewhere (18). Multiplicities of 500 PFU/cell were used. The Ad12-transformed hamster cell lines T637 and HA12/7 (22,15), and the Ad12-induced rat brain tumor lines RBT12/3 and RBT12/6 (30) were described elsewhere. We have also analyzed the DNA from five hamster cell lines (Ad2 HE_{1-5}) transformed with UV-inactivated Ad2 (20,21). Primary cultures of LSH hamster embryos had been used for transformation experiments.

Adenovirus and viral DNA. Ad2 and Ad12 were propagated in suspension cultures of human KB cells (17). The virus was purified and the viral DNA was extracted as previously reported (17,19).

Preparation of viral DNA and viral DNA fragments. Nick translation of Ad2 and Ad12 DNAs. Ad2 DNA was cleaved with the EcoRI restriction endonuclease, and the fragments were separated preparatively on cylindrical 0.5% agarose gels as described elsewhere (22,25). Adenovirus DNA and specific viral DNA fragments were ^{32}P-labeled by nick translation (23) as outlined earlier (24).

Isolation of cellular DNA. Nuclear DNA from

adenovirus-infected or transformed cells was extracted and purified as described elsewhere (25). The high molecular weight forms of DNA from human cells productively infected with Ad2 were purified as detailed before (16).

Analysis of the total intranuclear DNA from infected or transformed cells. Viral DNA sequences in adenovirus-infected or transformed cells were analyzed by cleavage with the HpaII or MspI restriction endonuclease. The DNA fragments were subsequently separated by gel electrophoresis on horizontal 1.5% agarose slab gels. Viral DNA fragments were identified by Southern blotting (26), DNA-DNA hybridization (27) using ^{32}P-labeled adenovirus DNA or viral DNA fragments followed by autoradiography. All methods employed were previously described in detail (3,4,22,24,25).

RESULTS

Absence of Methylation in Ad2 Virion DNA and in Intracellular Viral DNA from Productively Infected Cells. We have previously reported that Ad2 and Ad12 DNAs are not methylated to any significant extent (28). These results were confirmed by an enzymatic analysis of adenovirus DNA using the HpaII or MspI restriction endonuclease, followed by Southern blotting and DNA-DNA hybridization. Both enzymes yielded the same cleavage patterns with Ad2 DNA (Fig. 1,4). HpaII and MspI also cut Ad12 DNA in the same way (Fig. 2). The intranuclear Ad2 DNA isolated from productively infected human cells was analyzed by the same methods. The total intranuclear DNA was extracted between 1 h and 20 h postinfection (p.i.) of KB cells or between 1 h and 10 h p.i. of HEK cells with Ad2. Multiplicities of infection in these experiments ranged from 5 to 100 PFU/cell. Upon cleavage with the HpaII or MspI restriction endonuclease, the cleavage patterns of nuclear Ad2 DNA extracted from KB cells (Fig. 1) or HEK cells under a variety of conditions were identical. The same results were obtained with DNA extracted as late as 20 h p.i. It was important to analyze the intranuclear DNA in these experiments or else the DNA from virions unspecifically attached to the cytoplasma membrane might have been included in the analysis and would have obscured the cleavage

patterns. Furthermore, low multiplicities of infection were used in some of the experiments to avoid too high a concentration of intracellular viral DNA at early times after infection. We conclude that Ad2 DNA is not methylated at 5'-CCGG-3' sites in human cells at any time after productive infection.

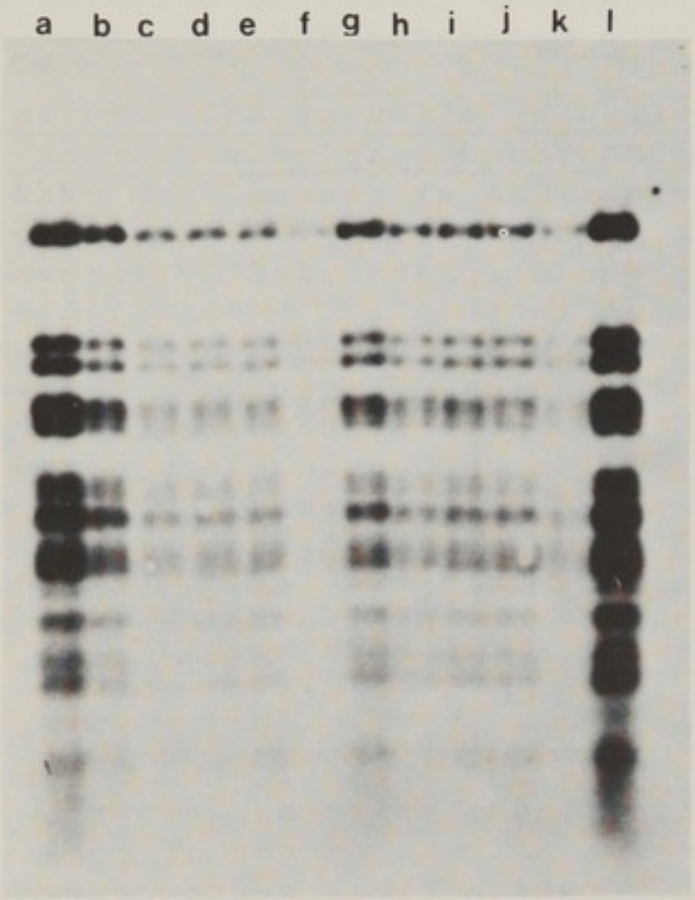

FIGURE 1. Absence of methylation in Ad2 DNA extracted from purified virions or from productively infected human cells.

The DNA was cleaved with the HpaII (a-f) or the MspI (g-l) restriction endonuclease and analyzed as described (3,4). The DNA was isolated from Ad2 virions (a,l) or from the nuclei of Ad2-infected KB cells at 1 h p.i. (f,k), 2 h p.i. (e,j), 3 h p.i. (d,i), 4 h p.i. (c,h) or 5 h p.i. (b,g). KB cells were infected with 100 PFU/cell of Ad2.

It was of particular interest to investigate whether the high molecular weight forms of Ad2 DNA

in KB cells became methylated. Previously, we have demonstrated that this form of Ad2 DNA is covalently linked to cellular DNA (16). High molecular weight forms of Ad2 DNA were prepared as described (16) and analyzed by Southern blotting after cleavage with the MspI or HpaII restriction endonuclease. The data (not shown) demonstrated that the integrated Ad2 DNA sequences from productively infected cells were not methylated extensively. This finding would suggest that the Ad2 sequences linked to cellular DNA might be expressed into messenger RNA.

Absence of Methylation of Ad12 DNA in Abortively Infected Hamster and Muntiacus Muntjak Cells. The total intranuclear DNA extracted from BHK21 (17) or from Muntiacus muntjak cells (18) abortively infected with Ad12, was also analyzed by cleavage with the HpaII or MspI restriction endonuclease followed by Southern blotting and DNA-DNA hybridization. The data (not shown) indicated that Ad12 DNA was not methylated in these cell systems, since the HpaII and MspI restriction endonucleases cleaved Ad12 DNA both in BHK21 and Muntiacus muntjak cells equally efficiently. It was concluded that extensive methylation could not explain the failure of Ad12 DNA to replicate in hamster or Muntiacus muntjak cells, since Ad12 DNA was not methylated in these systems.

Differential Methylation of Integrated Adenovirus DNA Sequences. We investigated the possibility that correlations might exist between the levels of methylation in specific sequences of eukaryotic DNA and the extent to which these sequences were expressed. Adenovirus-transformed cells are a suitable system for such studies, because the entire viral genome persists in the transformed cells, but not all of the viral sequences are expressed as mRNA (19,22,25). In Ad12-transformed hamster cells, only the early regions E_1-E_4 or parts of them are expressed as mRNA, as has been shown for lines HA12/7 and T637 (19). It is apparent from the transcriptional map of Ad12 DNA (Fig. 2 and ref. 29) that the early regions of the Ad12 genome are located mainly in the EcoRI fragments A and C. The EcoRI fragment B on the other hand, contains exclusively late viral DNA sequences (Fig. 2) which are not expressed in the Ad12-transformed hamster lines HA12/7 and T637 (19).

We therefore analyzed the levels of methylation

in early and in late Ad12 DNA segments persisting in the integrated state in transformed hamster cells. The DNAs of the Ad12-transformed cell lines T637 and HA12/7 were cleaved with the isoschizomer enzyme pair MspI and HpaII. The DNA was blotted and then hybridized to ^{32}P-labeled EcoRI fragment A,B, or C. In the hamster lines T637 (Fig. 2) and HA12/7 (data not shown), practically all of the 5'CCGG-3' sequences located in EcoRI fragment B, which comprised late genes of Ad12 DNA, were methylated.

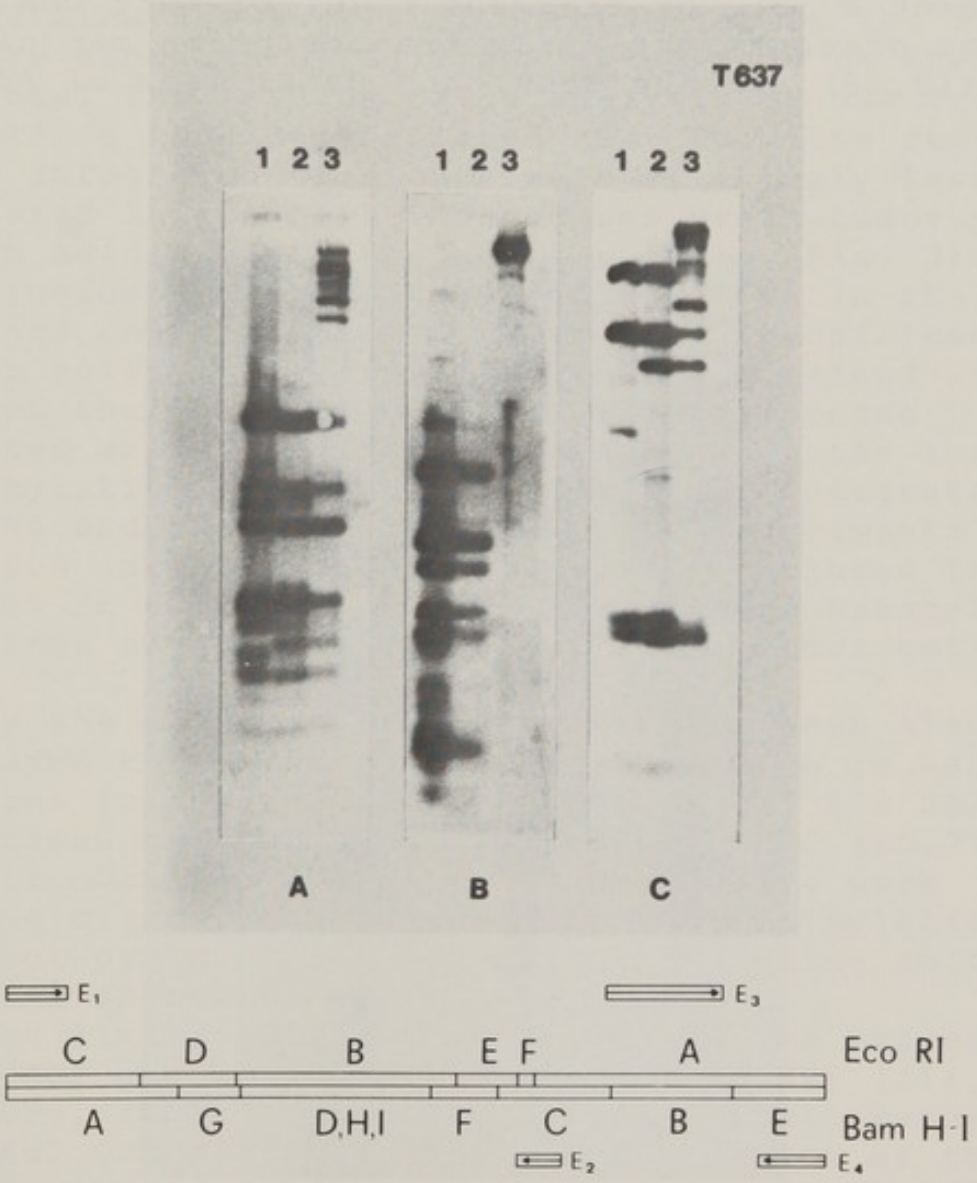

FIGURE 2. Autoradiograms of Southern blots of T637 hamster cell DNA digested with MspI (lane 2) or HpaII (lane 3). Lane 1 is Ad12 marker DNA cleaved with MspI; identical patterns were obtained when

The integrated viral DNA sequences located in the EcoRI fragment B were not cleaved by HpaII, whereas MspI cut all 5'-CCGG-3' sequences in this fragment. In the EcoRI fragments A and C, which contained early genes of Ad12 DNA, most of the 5'-CCGG-3'

(Fig. 2, contd.) Ad12 DNA was cut with HpaII. The fragments were separated by electrophoresis on 1.5% agarose slab gels and blotted onto nitrocellulose filters (26). Three such filters were prepared for hybridization with ^{32}P-labeled EcoRI fragment A (A), fragment B (B), or fragment C (C) of Ad12 DNA. The scheme presents the early transcription map of Ad12 DNA (19,29) and the EcoRI and BamHI maps of Ad12 DNA.

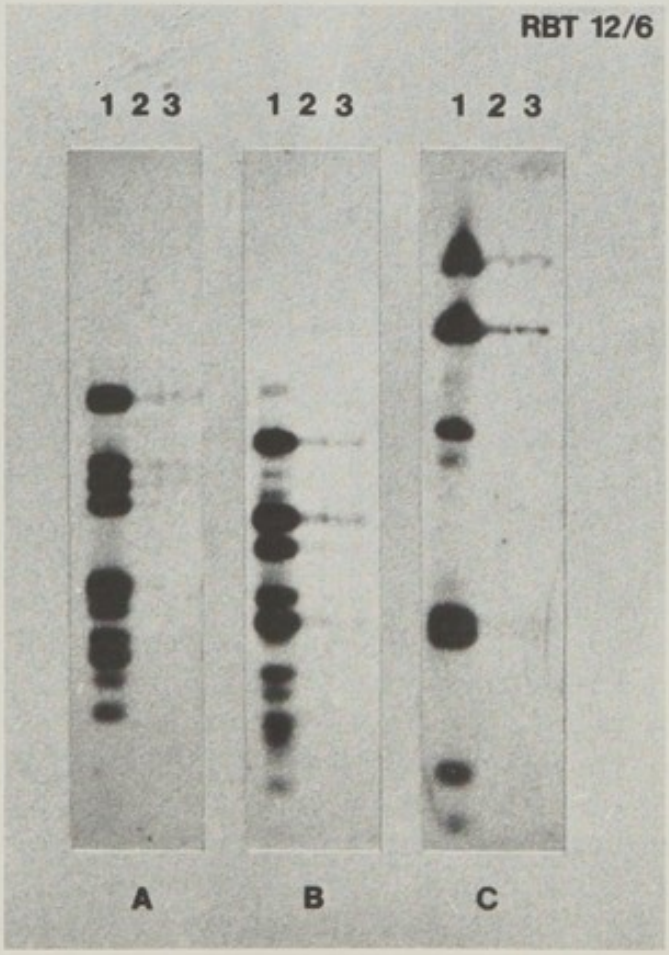

FIGURE 3. Autoradiograms of Southern blots of RBT12/6 rat cell DNA digested with MspI or HpaII. Experimental conditions and the arrangements of the autoradiograms are the same as in Fig. 2.

sites were not methylated in the two hamster lines studied, as revealed by a comparison of the HpaII and MspI cleavage patterns. It should be noted that only 60-70% of the EcoRI fragment C constituted early sequences (19), a fact that could explain the finding that some of the 5'-CCGG-3' sequences in this fragment were methylated.

It has recently been shown that in the Ad12-induced rat brain tumor lines RBT12/3 and RBT12/6, late sequences located in the EcoRI fragment B of the integrated Ad12 DNA are at least partly expressed as mRNA (30). It was therefore interesting to determine the levels of methylation in the early and late segments of Ad12 DNA integrated in these rat lines. The results of an analysis of DNA from the RBT12/6 line demonstrated that the late regions of the integrated viral DNA were strikingly less methylated in the Ad12-induced rat brain tumor cells than in Ad12-transformed hamster cells (Fig. 3). The early regions of the integrated Ad12 DNA in the rat line were undermethylated as in Ad12-transformed hamster cells. Similar findings were obtained with DNA from the RBT12/3 line. The data presented suggested an inverse correlation between the levels of methylation in specific segments of integrated Ad12 DNA and the extent to which these segments were expressed into mRNA and presumably translated into proteins in a number of Ad12-transformed hamster cell lines and Ad12-induced rat brain tumor cell lines.

By the methods outlined above, we have also investigated the extents of DNA methylation in Ad2 DNA sequences integrated into the genomes of the Ad2-transformed hamster cell lines HE_1 to HE_5 (20,21). The data obtained demonstrated that there were striking differences in the extent of methylation in different parts of the Ad2 genome depending on the cell line investigated.

Too little is known as yet about the expression of the Ad2 genome in cell lines HE_1 to HE_5 (21). Therefore, functional correlations between methylated Ad2 DNA sequences and the expression of these sequences could be drawn only in a few instances. The scheme in Fig. 4 presents the functional map of Ad2 DNA in a simplified form. It is apparent that the DNA-binding protein of Ad2, the 72K protein, is encoded in the EcoRI fragment B of Ad2 DNA. The results on the expression of viral functions in the

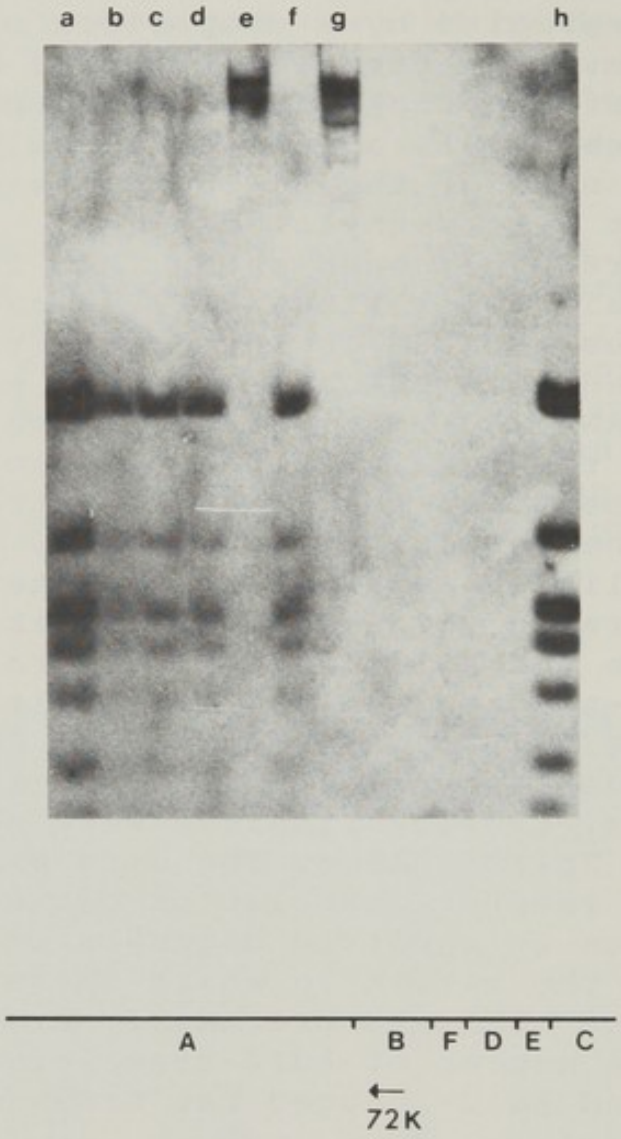

FIGURE 4. Autoradiogram of Southern blots of HE1, HE2, and HE3 hamster cell DNA digested woth the restriction endonucleases HpaII (a,c,e,g) or MspI (b,d,f,h). DNA preparations were extracted from Ad2 virion DNA (ah) and from the Ad-transformed hamster lines HE1 (b,c), HE2 (d,e), and HE3 (f,g). The DNA was hybridized with the 32p -labeled EcoRI fragment B of Ad12 DNA. The scheme indicated the EcoRI map of Ad2 DNA and the location of the gene for the 72K protein, the DNA-binding protein of Ad2.

HE_1 to HE_3 cell lines published by Johansson et al. (21) suggested that the DNA-binding protein of Ad2 was synthesized in line HE_1 and not in the four other Ad2-transformed hamster cell lines. It was therefore of particular interest to investigate the extent of DNA methylation in EcoRI fragment B in line HE_1 in comparison to that in lines HE_2 and HE_3 (Fig. 4).

The restriction endonucleases HpaII and MspI cleaved the DNA in EcoRI fragment B of line HE_1 equally well, whereas the same DNA regions from lines HE_2 and HE_3 were not cleaved at all by the restriction endonuclease HpaII. This finding demonstrated that the DNA in EcoRI fragment B was not methylated in line HE_1, where it was expressed. In lines HE_2 and HE_3 the same segment of viral DNA was completely methylated, and there was no evidence that the DNA-binding protein was synthesized in these lines (21). Preliminary results indicate that in vitro translation experiments using messenger RNA from cell line HE_1 yield 72K protein, whereas similar experiments using mRNAs from lines HE_2 and HE_3 do not (H. Esche, unpublished results).

DISCUSSION

In Ad2- and Ad12-transformed hamster cells, as well as in Ad12-induced rat brain tumor lines (3,4), a very striking inverse correlation has been found between the degree of DNA methylation in specific segments of the viral genome and the level of expression of the same viral DNA segments into messenger RNA. This correlation appears to apply to integrated viral genomes (3,4) or to viral genomes stably anchored within the cell in an episomal state (5). Much further work will be required to elucidate the possibly quite complicated mechanisms relating modification of specific DNA sequences and their availability for expression. The results presented raise tantalizing questions as to the role of DNA methylation in the regulation of gene expression in transformed cells and perhaps in mammalian cells in general. Of coures, it is conceivable that DNA methylation or demethylation is the consequence rather than the cause of different levels of genetic activity in specific regions of the genome. DNA methylation may somehow constitute a signal for long-term inactivation of genes. In preliminary experi-

ments we have shown that treatment of T637 cells with dexamethasone (10^{-5}M) does not lead to a decrease of methylation in the genetically inactive EcoRI fragment B of the integrated Ad12 genomes.

In contrast, viral DNA in productively or abortively infected cells or virion DNA (28,31) remain unmethylated, even at very early times postinfection when late viral functions are not yet expressed. This finding has recently been confirmed by the results of hybridization experiments in which cloned fragments of Ad2 DNA from different parts of the Ad2 genome have been used as probes. If DNA methylation was in fact the cause or the consequence of genetic inactivation of specific DNA sequences, it would not be advantageous for viral replication to become subject to cellular regulatory mechanisms. It is, however, completely enigmatic, how viral DNA can escape modification by cellular methyl-transferases.

It is worth noting that the high molecular weight form of Ad2 DNA in productively infected cells, which represents viral DNA covalently linked to cellular DNA (16), does not become methylated either. It remains to be determined whether the integrated viral DNA sequences in productively infected cells are expressed as messenger RNA. Absence of methylation in integrated viral DNA might suggest that the integrated Ad2 DNA sequences in productively infected cells are genetically active.

In BHK21 cells abortively infected with Ad12, only the early Ad12 genes are expressed as messenger RNA (19). The entire viral genome, however, remains unmethylated as late as 24 h postinfection. These data indicate again that free viral DNA is not modified. We have not separately analyzed the integrated form of Ad12 DNA described in this system earlier (32).

ACKNOWLEDGMENTS

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SYNTHESIS OF ADENOVIRUS 2 RNA *in vitro*: PROPERTIES OF THE MAJOR LATER TRANSCRIPT AND ITS PROMOTER

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ABSTRACT *We have developed a new* in vitro *transcription system which we feel will be useful for the synthesis of mRNA in mammalian cells. The system consists of a crude whole-cell extract, exogenously added DNA and small molecules and salts needed for transcription. If the added DNA includes sequences which encode the 5' end of the adenovirus 2 (Ad 2) major late transcript, as well as 5' flanking sequences, easily detectable amounts of transcripts with the same 5' ends as* in vivo *RNAs are formed. Transcription continues until the polymerase reaches the end of the particular DNA fragment used as template, and we have observed transcripts ranging in size from 175 to 4,360 nucleotides. We can also detect an RNA species which is the size expected for a molecule which has undergone the first splice in the pathway to mRNA production. By using a recombinant plasmid containing appropriate fragments of Ad2 DNA, we found supercoiled DNA to be a much poorer template for* in vitro *transcription than is the linearized DNA. Finally, by using a recombinant plasmid containing a very small fragment of Ad2 DNA, as well as templates truncated by digestion with the appropriate restriction endonucleases, we show that all the sequence information needed for accurate initiation of transcription by RNA polymerase II at the major late promoter site is contained within a region from -52 to +33 nucleotides from the RNA cap site.*

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INTRODUCTION

Regulation of gene expression occurs at the level of transcription, and a large number of examples exist which show that control of transcription initiation is of primary importance. In order to understand how such regulation is mediated, it is first of all required that we know what sequences on the DNA are necessary to bring about accurate initiation of transcription; i.e., what is a promoter? In bacteria, examination of a number of DNA sequences around transcription start-sites have detected certain sequences which are highly conserved (for review, ref. 1). Specifically, a heptanucleotide sequence centered 10 bp upstream from the mRNA startpoint is highly conserved (2). Another conserved sequence is located at about -35 bp (3). These sequences, appear to constitute the bacterial promoter region. This deduction is supported by experimental evidence which shows that a large number of point mutations which affect promoter function are located within these conserved regions. In addition, DNA protection experiments show that RNA polymerase actually covers this region of DNA during initiation.

Elucidation of the structure of promoters in eukaryotic organisms has been much more difficult. There are several reasons for this. First, until the advent of recombinant DNA technology, the ability to analyze eucaryotic genes did not exist with the exception of some viral genes. Second, the relative lack of genetics in higher organisms has made it difficult to obtain mutants with which to define presumptive promoter sequences. However, techniques for selectively mutagenizing DNA *in vitro* are rapidly becoming available. Finally, *in vitro* transcription systems for eucaryotic cells have not been available, making difficult the analysis of any presumptive promoter mutations.

Still another complexity in defining promoter sequences in eucaryotes is the existence of three separate classes of RNA polymerase (RNAP, see ref. 4). RNAP I transcribes ribosomal RNA genes; RNAP II transcribes mRNA genes and RNAP III transcribes 5S, tRNA and several other genes which encode small RNAs. To date the most progress in understanding eucaryotic promoter structure has been made for the genes transcribed by RNAP III. To a large degree this has been due to the existence of *in vitro* DNA-dependent transcription systems which accurately transcribe class III genes (5,6). Using such a system, coupled with recombinant DNA and *in vitro* mutagenesis techniques, a promoter, or control region, for transcription of *Xenopus* 5S RNA genes has been defined (7,8). Surprisingly, this region does not lie in the 5' flanking sequences, but rather within the gene itself.

Thus, the only sequences needed to bring about accurate transcription initiation are contained in a region between +50 and +83 nucleotides from the RNA start-site.

Much less is known about the sequence needed for initiation by RNAP II. Comparison of the 5' flanking regions of a number of eucaryotic mRNA genes has revealed a conserved hexanucleotide sequence centered approximately 27 nucleotides from the apparent transcription start-site (9). However, no evidence exists which indicates that this sequence is of any functional importance. One reason for this has been the lack of *in vitro* transcription systems that will accurately transcribe class II genes. Recently, though two such systems have been developed (10,11). Here we present further characterization of the system which we described previously, and show it can be used to define RNAP II promoter sites.

MATERIALS AND METHODS

Preparation of the HeLa whole-cell extracts and recombinant DNAs containing the Bal I E fragment of Ad2 DNA (14.7-21.4 m.u.) inserted into the Bam H I site of pBR 322 (pBalE), were carried out as described previously (11, 12). Briefly, HeLa cells were lysed by hypotonic swelling and homogenization, afrer which the nuclei were broken by addition of $(NH_4)_2SO_4$ to 10% of saturation. The lysate was centrifuged at 170,000 x g for 3 hr, and the supernatant concentrated by addition of $(NH_4)_2SO_4$ to 80% of saturation. The precipitate was collected, redissolved and dialyzed against a buffer which contained the appropriate ions, etc. for transcription. Construction of the recombinant plasmid p 4, P which contains Ad2 sequences from 15.3-16.5 m.u. inserted between the EcoRI and Hind III sites of pBR322 will be described elsewhere (Hu and Manley, manuscript in preparation). *In vitro* transcription reactions were performed as described (11). Extraction and analysis of RNAs was done as described previously (11, 13, 14).

RESULTS AND DISCUSSION

Analysis of In Vitro Synthesized RNA by S1 Nuclease Mapping. The mRNAs encoded by the human adenoviruses have been extensively studied over the last several years (15, 16). Not only do they undergo all the processing reactions that cellular mRNAs do but in many cases these reactions were first detected in the adenovirus system. Specifically, the major late transcription unit has been quite well characterized, and has served as a model for several studies dealing with transcription initiation. Fig. 1 diagrams the first 5,000 nucleotides of the long (25kb) primary transcript. The

capped 5' end is encoded at position 16.5 (9). The three segments of the 228 nucleotide tripartite leader are complementary to DNA sequences located at coordinates; 16.5, 19.5 and 26.5 (17, 18).

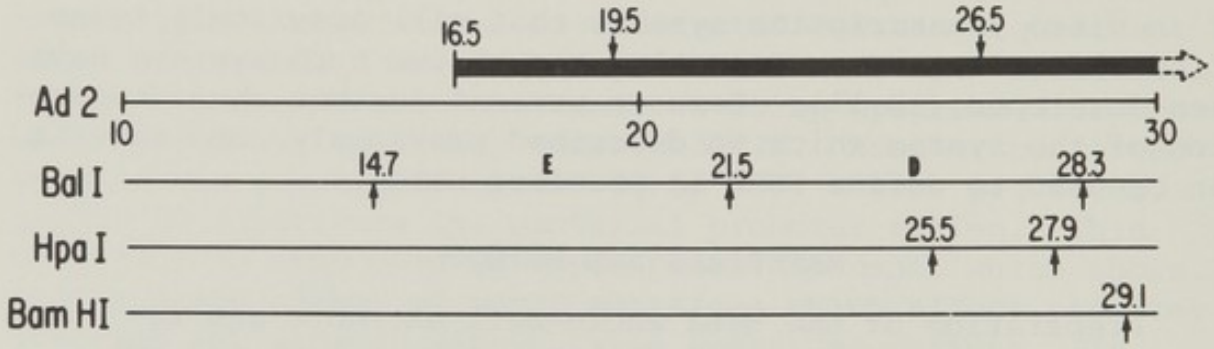

Fig. 1. The 5' end of the Ad2 late transcription unit. Transcription begins at approximately map coordinate 16.5 and continues rightward to almost the end of the Ad2 genome (coordinate 100). Since one map unit is roughly 350 bp, the length of the entire transcription unit is over 29,000 bp. Although at least 13 mRNAs are encoded in this transcription unit, none are located within the region shown in the figure. However, the approximately 200 nucleotides which ultimately form the leader segment common to all mRNAs from this transcription unit are located here. They are encoded in the genome in three segments, which are ultimately joined by RNA splicing. The coordinates of these segments are indicated by the numbers above the solid arrow. The sites at which the restriction endonuclease Bal I, HpaI and Bam HI cleave this region of the Ad2 genome are indicated.

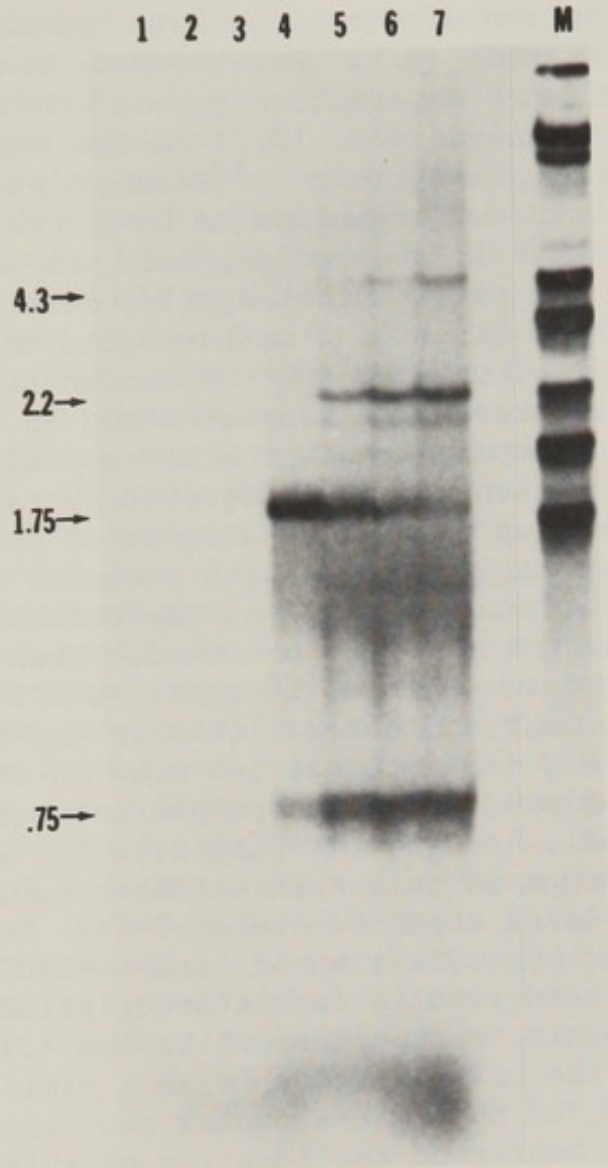

Fig. 2. Hybridization-S1 nuclease analysis of transcription products complementary to pBal E. Standard reaction mixtures contained either pBal E_{Bam} (lanes 2-7) or no exogenously added DNA (lane 1) 12.5 g/ml; lane 4, 50 g/ml; lane 5, 75 g/ml; lane 5, 100 g/ml; and lane 7, 125 g/ml. After in vitro synthesis and purification of the RNA, 25% of each sample was hybridized to 0.5 g pBal E_{Bam} DNA in 15 l hybridization mixtures (14). After S1 nuclease treatment, RNA-DNA duplexes were resolved by electrophoresis in a 1.4% agarose gel and bands visualized by autoradiography of the dried gel. The sizes indicated are expressed in kilobases, and were determined by comparison with an Eco RI digest of ^{32}P-labeled Ad2 DNA (M).

We have previously shown that RNAP II will accurately initiate transcription of the major late transcription unit in a whole cell extract (11). Here we have analyzed the products of *in vitro* transcription by the hybridization-nuclease S1-gel technique (14, 19). In the variation of the method used in these experiments, ^{32}P-labeled RNA, synthesized *in vitro*, was hybridized to unlabeled DNA, under conditions of DNA excess. Unhybridized nucleic acid was digested with S1 nuclease, and the S1-resistant hybrids resolved by agarose gel electrophoresis. In Fig. 2 we have used this method to examine the effect of varying the DNA concentration in *in vitro* reaction mixtures. The samples shown in lanes 1-7 were derived from reaction mixtures which had contained varying amounts of pBAl E which had been digested by Bam HI (pBal E_{Bam}). Following purification of RNA, an aliquot of each sample was hybridized to pBal E_{Bam}, treated with nuclease S1, and electrophoresed through an agarose gel. At DNA concentrations of 25 µg/ml or less, no bands were observed. When the concentration of DNA was increased to 50 µg/ml, a 1750 nucleotide RNA-DNA duplex could be detected. This is the result expected if RNA synthesis had initiated at 165 m.u. on the Bal E fragment, and continued until the polymerase reached the end of the DNA fragment. As the DNA concentration was further increased, the amount of this species decreased, and several other RNA-DNA duplexes appeared (lanes 5-7). The two larger species arose from hybridization of "end-to-end" transcripts, i.e., the 2.2 kb band results from transcription from one end of the Bal E molecule to the other while the 4.3 kb species is transcribed from the pBR 322 molecule in a similar manner. A large amount of 750 bp RNA-DNA duplex can also be detected. While we have not confirmed the identity of this species, it is interesting to note that this is the size expected of a molecule which had undergone the splicing reaction which joins the first leader segment (42 nucleotides) to the second, encoded at position 19.5. Direct nucleotide sequence analysis of this transcript will be required to confirm its identity. If this is indeed a spliced molecule, it is of interest to ask why its production is enhanced at high DNA concentrations. One possibility is that some sequence(s) in the DNA can interact with the RNA primary transcript to stabilize some intermediate in the splicing reaction. A similar role in splicing has been proposed for small nuclear RNAs (20), and it is not inconceivable that, under *in vitro* conditions, a DNA molecule might substitute in this role. We have also detected this species under different conditions, which will be discussed below.

Time course of Appearance of 1750 Nucleotide Transcript. The 1750 nucleotide transcript accumulates at an approximately linear rate for at least 60 min of *in vitro* incubation. This is shown in Figure 3, where we have analyzed the products of reaction mixtures incubated *in vitro* for different lengths of time. RNA was extracted from reaction mixtures, denatured with glyoxal, resolved by electrophoresis through a 1.4% agarose gel. Lanes 1-4 show that the 1750 transcript and also the 750 nucleotide species, increased in concentration in a nearly linear manner for 60 min. Reaction mixtures incubated for longer times (up to 3 hours) showed no further increase in the concentration of these species. However, no significant decrease was detected indicating non-specific degradation did not occur during *in vitro* incubation. A diffuse band can be observed across the bottom of the gel. This species, which was also present in the zero time sample, increased in concentration during the first 20 min of incubation then decreased. We believe this resulted from end-labeling of endogenous small RNAs (tRNA or 5S RNA). It is not clear why the concentration of this species decreased during *in vitro* incubation. However, the predominance of this species at early times made monitoring of time course reactions by TCA-precipitations impossible, because nearly equal amounts of radioactivity became acid precipitable at all the times we tested. These results also show that no appreciable lag occured in the appearance of the 1750 nucleotide transcripts, in contrast with the synthesis of 5S RNA in similar RNAP III *in vitro* systems (6).

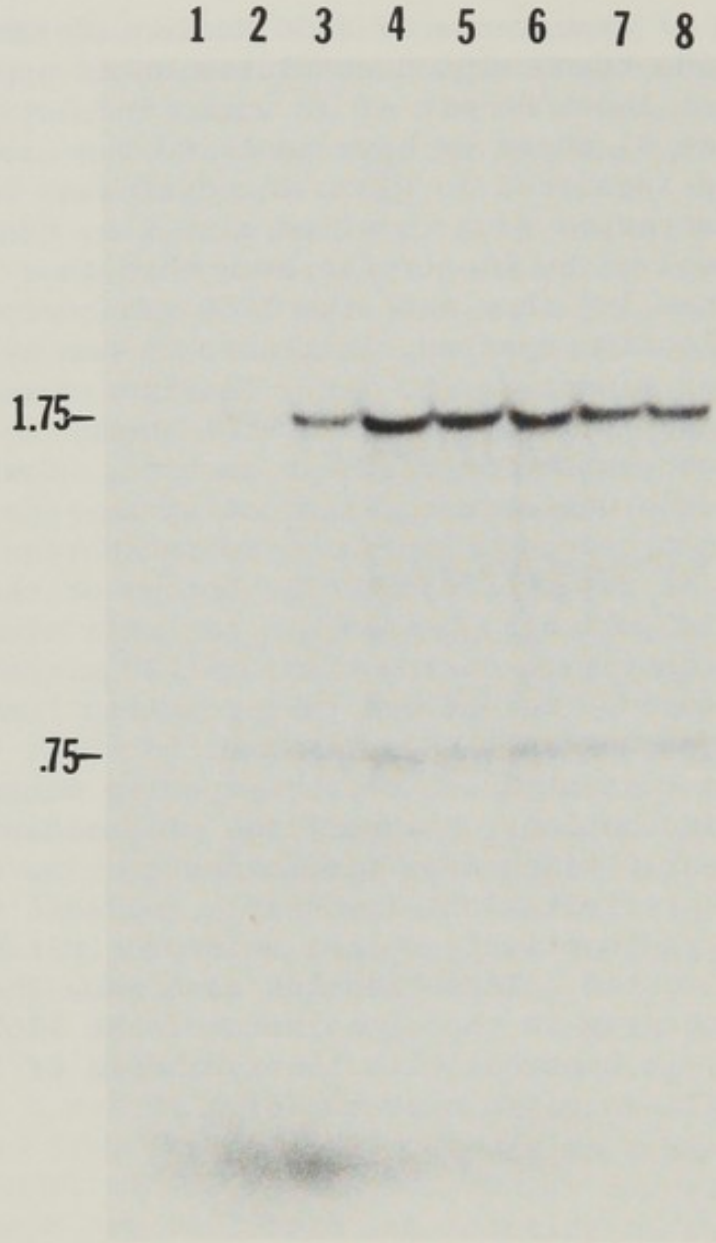

Fig. 3. Time course of appearance of 1750 nucleotide transcript. Standard reaction mixtures were incubated for 0 (Lane 1), 10 (Lane 2), 30 (Lane 3), 60 (Lane 4), 90 (Lane 5), 120 (Lane 6), 150 (Lane 7) or 180 min (Lane 8). RNA was extracted denatured by treatment with glyoxal, and resolved by agarose gel electrophoresis (11). The sizes indicated are expressed in kilobases.

Transcription Initiation From Other Forms of DNA Containing The Major Late Prompter. In the experiments described above, the template for transcription was the recombinant plasmid pBalE which had been linearized by digestion with the restriction endonuclease BamH I. Figure 4, lanes 1 and 2, compares the template efficiencies of supercoiled pBalE DNA with linearized (Bam H I digested) pBal E DNA. Since the supercoiled DNA contains neither a termination

signal nor any ends, we did not expect synthesis of discrete RNA species. Therefore, transcription products obtained from reaction mixtures containing either of these DNAs were hybridized to pBal E_{Bam} DNA, and the resultant RNA-DNA duplexes analyzed by the S1-nuclease technique. Although the amount of radioactivity incorporated into TCA precipitable material was approximately 50% greater when supercoiled DNA was used as template, the amount of specific synthesis, i.e. of the 1750 transcript, was much less. However, the concentration of the 750 nucleotide transcript, here present as an apparanet doublet, of two bands approximately 750 and 700 nucleotides in length, was actually higher when supercoiled DNA was used as template. A possible explanation of this result is discussed below.

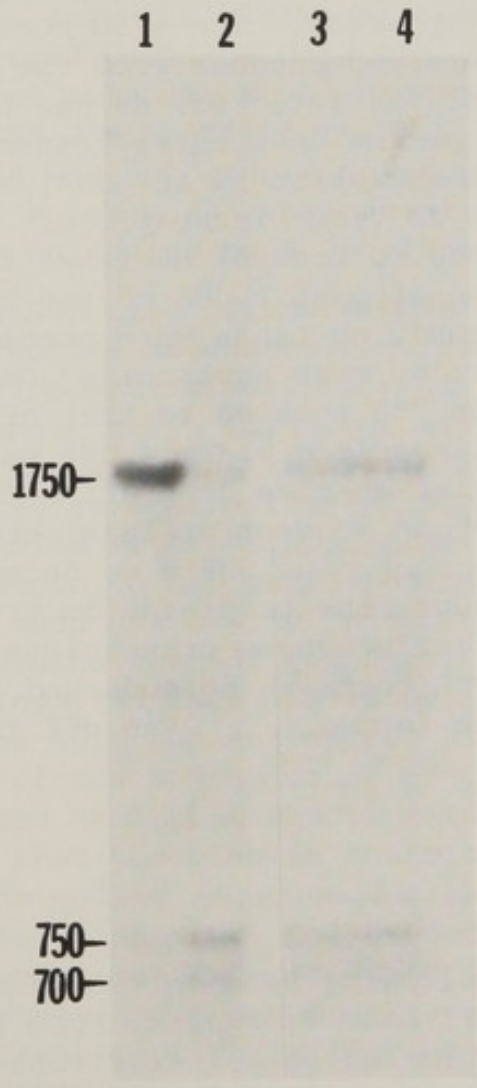

Fig. 4. Nuclease S1 analysis of in vitro *transcripts. RNA was extracted from standard reaction mixtures which had*

We showed previously that Ad2 DNA which had been extracted from virions can function as a template for initiation of transcription from the Ad2 major late promoter (11). This conclusion was based on analysis of "run-off" transcripts produced in reaction mixtures which had contained Ad2 DNA with the restriction endonucleases Bal I, Hpa I or Bam H I (see Fig. 1). However, the pattern of transcripts obtained in each case was complex and in order to determine which RNA species are encoded by the region of the genome proximal to the late promoter site, we hybridized RNA from two such reaction mixtures to pBal E_{Bam} DNA, and analyzed the products by the nuclease S1 technique. Figure 4, line 3, shows the results obtained with RNA extracted form a reaction mixture which had contained Ad2 DNA restricted by Hpa I as template, Figure 4, lane 4, from a reaction mixture in which the Ad2 DNA had been previously restricted by Bam H I. In both samples, which gave virtually identical results, only a small number of discrete transcripts are apparent. The 1750 nucleotide transcript, which is the expected product from the initiation at the major late promoter was detected, as was the 700-750 doublet. This result proves that these transcripts are indeed products of transcription of Ad2 DNA, since no pBR22 DNA was present in the *in vitro* incubations. Furthermore, protection of these species from S1 nuclease digestion was dependent upon addition of pBal E DNA to the hybridization - if pBR322 was substituted, no bands were protected (results not shown). Note that, as with supercoiled DNA the ratio of the intensity of the 750 species to that of the 1750 species is greater than when linearized plasmid DNA was used as template. If the smaller species does arise from splicing of the larger, than we can suggest at least one explanation for these results. In each case where we detected higher levels of the 750 product, the template for transcription was such that significantly longer transcripts could be formed. Supercoiled DNA contains no ends and in theory very long transcripts can be formed. Run off transcripts

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(Continued Fig. 4)

contained one of the following DNAs as template: Lane 1, pBal E digested with Bam H I; Lane 2, pBal E, Form I (greater than 90% supercoiled, as estimated by gel electrophoresis): Lane 3, Ad2 digested with HpaI; Lane 4 Ad2 digested with Bam H I. An aliquot of each RNA sample was hybridized to pBal E_{Bam} DNA treated with S1 nuclease, and the resistant hybrids resolved by gel electrophoresis. The sizes indicated are expressed in base pairs.

beginning at the late promoter and continuing to the end of Hpa I cut Ad2 DNA will be 3200 nucleotides in length, while a similar transcript with Bam H I cut DNA will be 4400 nucleotides long. Two explanations come to mind which explain how this might effect the levels of RNA splicing. First, for unknown reasons longer RNAs may be better substrates for splicing. Another possibility is that the actual substrate for splicing are nascent RNA chains. Thus, since longer RNAs would be nascent chains for a longer period of time these transcripts might be spliced more efficiently. We have in fact presented evidence which suggests that this splice occurs on nascent chain (14). The presumptive spliced molecule may exist as a doublet for either of two reasons. Recall that the segments of RNA which would be spliced together are 42 and about 700 nucleotides long (700 is the length of RNA encoded by the Bal E fragment). If the splicing reaction didn't always go to completion (i.e., only the cleavages took place) a doublet of the size observed would be detected. Alternatively, a completely spliced molecule could have given rise to a doublet if the RNA-DNA hybrid formed by the 42 nucleotide RNA and Bal E DNA was not stable under the high formamide conditions used, which would then result in degradation of this part of the molecules by S1 nuclease.

Mapping the Ad2 Late Promoter. We have begun to analyze the nucleotide sequences required to bring about accurate initiation of transcription of the major late transcript. We have taken advantage of the known nucleotide sequence around the apparent transcription start-site (9) and have generated several different DNA templates by cleaving recombinant plasmids with the appropriate restriction endonucleases. To begin to map the 3' boundary of the promoter region, we have made use of the recombinant plasmid pϕ4. This plasmid contains the Ad2 sequences from 15.3 to 16.5 m.u. which had been inserted into pBR322 by attachment of a Hind III linker at the 16.5 end and an Eco RI linker at the 15.3 end. A portion of the DNA sequence of this plasmid has been determined (Hu and Manley, manuscript in preparation). This sequence showed that the plasmid contains the 5' flanking sequences and the coding sequence for the first 33, nucleotides of the late transcript, followed by the Hind III linker and pBR322 sequences reading clockwise from the Hind III site. To determine whether these sequences are sufficient to bring about accurate transcription initiation, we have digested aliquots of pϕ4 with restriction endonucleases which cleave at known sites in pBR322 (21), and used these DNA's as templates for *in vitro* transcription. If pϕ4 contains

sufficient information so that initiation of the late transcript can occur, then we should be able to detect discrete sized chimeric RNA molecules containing 33, nucleotides of Ad2 sequences joined to pBR322 encoded sequences, the length of which would be determined by the restriction enzyme which was used to cleave the DNA. Figure 5, lanes 1-4, show an example of the results we obtained which prove that the Ad2 sequences contained in pϕ4 are indeed sufficient to bring about accurate initiation of the late transcript. Specifically, when aliquots of pϕ4 DNA which had been restricted with either when AvaI or Hinc II were used as templates, transcripts of 1400 or 650 nucleotides were detected following RNA extraction and gel electrophoresis (lanes 1 and 2). These are precisely the sizes expected. We have confirmed this result by using templates produced by cleavage with a number of other restriction endonucleases (unpublished results). Also these transcripts contain the same 5' cap structure as found on *in vivo* RNA (unpublished results). The RNAs obtained from reaction mixtures which had contained pBal E DNA digested with either Bam H I (a 1750 nucleotide transcript) or Sma I (a 530 nucleotide transcript) are shown in lanes 3 and 4. A comparison shows that not only is the late promoter contained on pϕ4 active, but it is just as efficient as the promoter contained on pBal E. We conclude that no Ad2 sequences further downstream than +33 nucleotides from the presumptive RNA start-site are required for initiation of the Ad2 late transcript by RNAP II. Furthermore, we have recently shown that nucleotides downstream from +3 can also be deleted without adversely affecting transcription (Hu and Manley, manuscript in preparation). This contrasts with results obtained recently which show that the promoter, or control region, for transcription of *Xenopus* 5S genes by RNAP III, is located between 50 and 83 nucleotides downstream from the transcription start-site (7,8). Thus, the mechanisms by which these two classes of polymerases initiate transcription may be quite different.

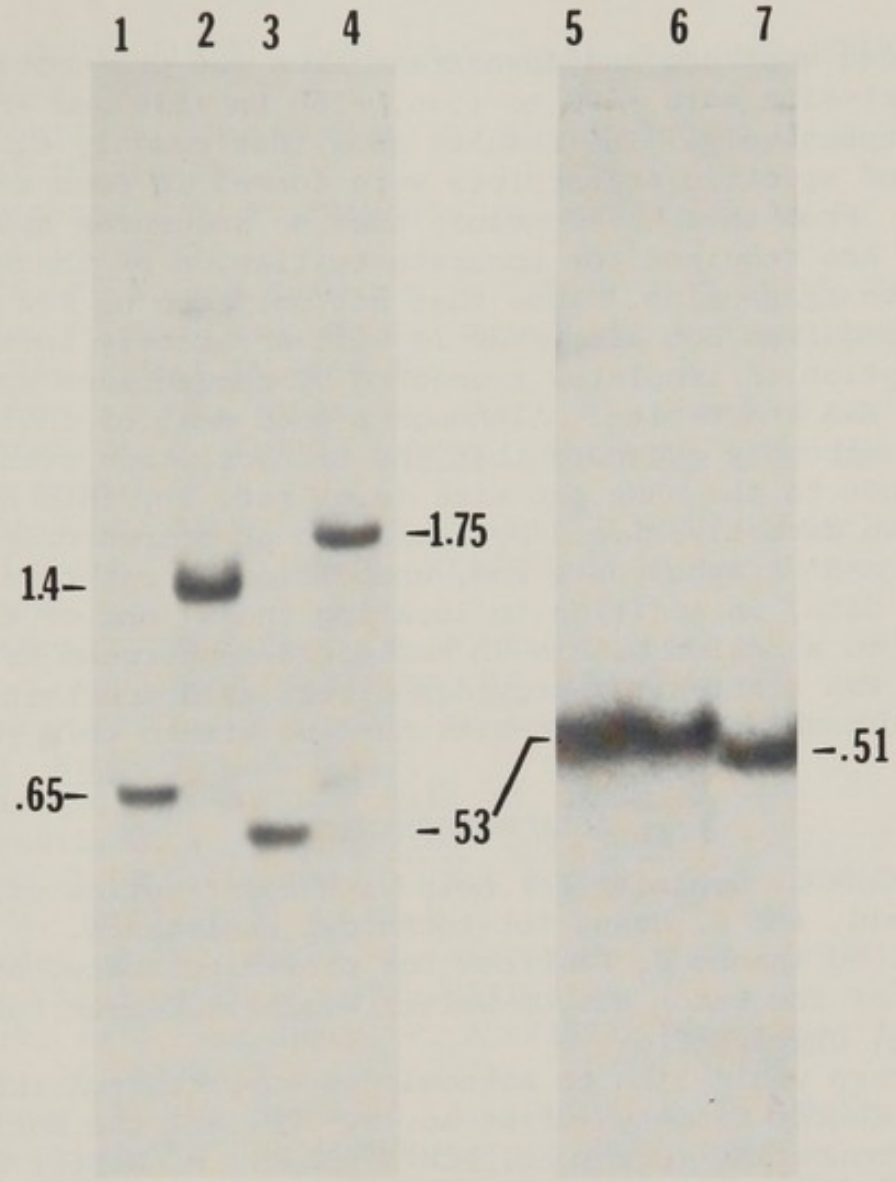

Fig. 5. Localization of the Ad2 late promoter by in vitro *transcription. RNA was synthesized in standard reaction mixtures which contained the following DNA templates: Lane 1, pϕ4$_{Hinc\ II}$) Lane 2, pϕ4$_{AvaI}$, Lane 3, pBal E$_{Sma\ I}$, Lane 4, pBal E$_{Bam\ H\ I}$, Lane 5, pBal E$_{Bam\ H\ I\ +\ Sma\ I}$, Lane 6, pBal E$_{Xho\ I\ +\ Xma\ I}$, Lane 7, pBal E$_{Hpa\ II}$. Following extraction RNA was denatured with glyoxal and resolved by electrophoresis in a 1.4% agarose gel. Sizes are expressed in kilobases.*

To map the 5' boundary of the Ad2 late promoter, we have used aliquots pBal E DNA which had been digested by different restriction endonucleases as templates for *in vitro* transcription reactions. Specifically, in the experiments shown here we used pBal E restricted by Bam HI + Sma I (Fig. 5, lane 5); Xho I + Sma I (Fig. 5, lane 6); or Hpa II (Fig. 5, lane 7). The sizes of the promoter containing fragment produced, expressed in number of

nucleotides upstream and downstream from the presumptive mRNA start-site were -690 to +536, -260 to +536 and -52 to +507, respectively. The results show that roughly equal amounts of specific transcripts were formed in each reaction mixture. From this, we conclude that no sequences upstream from -52 are required for accurate initiation of the Ad2 major late transcript. Note that not only are no Ad2 sequences required but also RNAP II will accurately initiate transcription on templates truncated 52 nucleotides upstream from the RNA start-site. Although a good deal of indirect evidence strongly suggests that the transcription start-site corresponds to the mRNA cap site (e.g. ref. 9), this has not been shown directly; i.e., by isolation of a transcript which contains a 5' triphosphate end, unmodified by cap formation. Thus our data, in addition to locating the 5' end of the promoter to a region within 52 nucleotides upstream from the *apparent* RNA start-site, provides direct evidence that the *actual* transcription start-site must be within this region.

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A GENE FUNCTION OF HERPES SIMPLEX VIRUS REQUIRED FOR EXPRESSION OF ALL EARLY VIRAL GENES[1]

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ABSTRACT The herpes simplex virus mutant HSV-1 (HFEM) tsB7 fails to express any detectable viral polypeptides and fails to inhibit host cell protein synthesis in infected cells maintained at the non-permissive temperature. The mutant can complement the growth of other viruses and therefore can enter doubly infected cells. The mutation maps in the middle of the L component in or near genes coding for virion structural proteins and near a previously mapped function regulating inhibition of host cell protein synthesis. Abundant transcripts and mRNA from this region accumulate only after DNA synthesis and thus the gene appears to code for a late protein. All of our data are consistent with the lesion being a defect in a virion structural protein required for expression of all early viral gene products and possibly for inhibition of host cell protein synthesis. This mutation defines a new early stage of infection prior to expression of any viral gene products at which the replication of herpes simplex virus can be regulated.

INTRODUCTION

Herpes simplex virus (HSV) rapidly inhibits the expression of host cell proteins early after infection. At least a portion of this inhibition is due to a virion component in that irradiated virus can inhibit protein synthesis in enucleated cells (1) demonstrating that viral nucleic acid synthesis is not necessary for host cell inhibition. The extremely rapid host inhibition due to HSV-2 has been mapped by analysis of HSV-1 X HSV-2 intertypic recombinants to the map positions 0.52 to 0.59 in the L component of the viral DNA (2).

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HSV expresses at least three alpha or immediate early proteins immediately following infection. The genes for these proteins are thought to be transcribed by the host cell RNA polymerase II and this process requires no prior viral protein synthesis.

In this report we present an initial characterization of a mutant HSV-1 (HFEM) tsB7 that is defective for both early viral gene expression and inhibition of host cell protein synthesis. Our data suggest that the temperature-sensitive lesion is within a gene coding for a virion structural protein that functions early in infection to promote the expression of early viral genes.

RESULTS AND DISCUSSION

Polypeptide Expression by tsB7. To examine the nature of the block in replication at the non-permissive temperature, 39°C, we examined the proteins synthesized in cells infected with tsB7 at 39°C and 34°C. As shown in Figure 1, tsB7 expressed the same profile of polypeptides as its wild type parent, HFEM ts^+, at 34°. The time scale for progression of the tsB7 infection was always slower, consistent with the slow growth of tsB7 at 34°. HFEM also expressed an equivalent pattern of polypeptides at 39°. However, tsB7 expressed no viral polypeptides at 39°. The pattern of polypeptides labelled in these cells was equivalent if not identical to that of the mock-infected cells at 39°. Thus, there appeared to be no inhibition of host cell protein synthesis in cells infected by tsB7 at 39°.

Complementation of Other Mutants by tsB7. Schaffer et al (3) previously reported that tsB7 could complement other ts mutants but in light of our observations concerning the leakiness of tsB7, we reexamined this question using a strict temperature control. Table 1 shows the result of a representative complementation with an early mutant, ts502. This virus forms syncytial (syn) plaques while tsB7 forms non-syncytial plaques (syn^+). The growth of ts502 was enhanced 300-fold by co-infection while the growth of tsB7 was enhanced 2.7-fold. The progeny were almost entirely still temperature-sensitive. Therefore tsB7 can complement the growth of a co-infecting virus and thus can enter doubly infected cells. Also, it appears that the defect in tsB7 can be complemented in trans by the co-infecting virus.

Marker Rescue Mapping of tsB7 with HSV-1 DNA. The genome location of the lesion in tsB7 was determined by the co-transfection method of marker rescue (4). Table 2 summarizes the results of rescue by co-transfection of mutant DNA with each of the individual fragments of HSV-1 (MP) DNA generated by

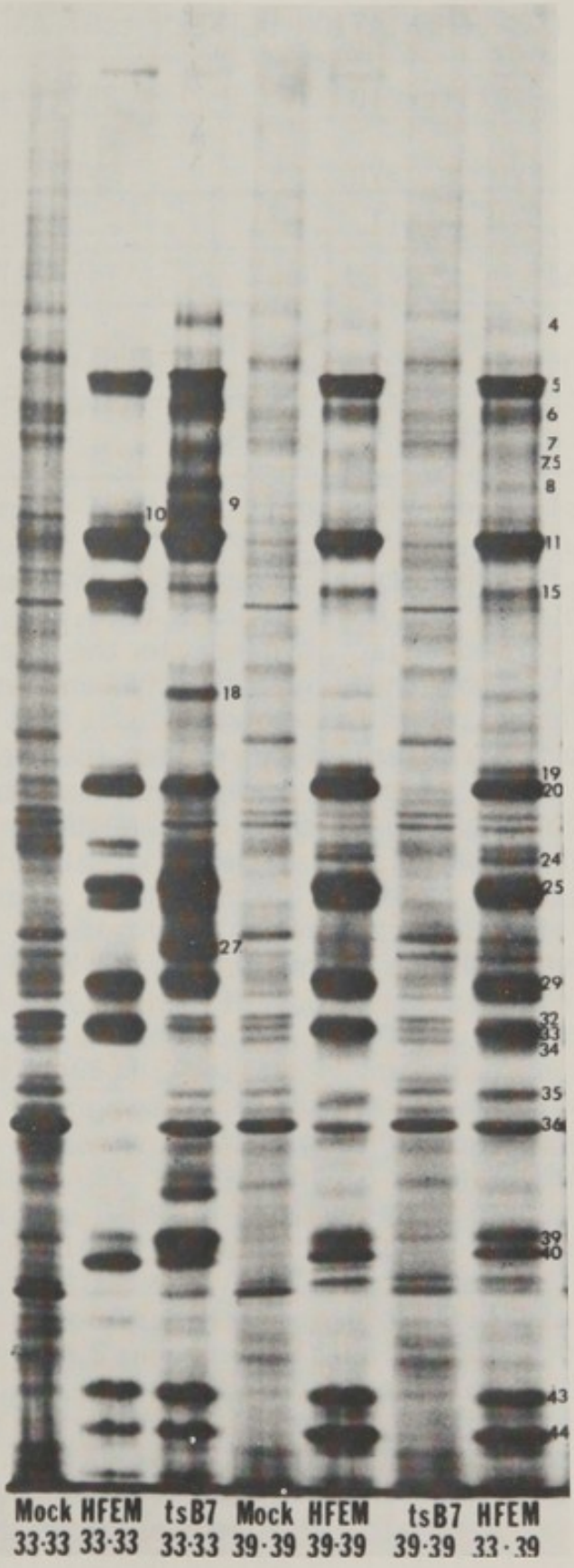

FIGURE 1. Autoradiogram of a polyacrylamide gel containing infected cell polypeptides. Numbers at bottom represent temperatures of absorption and infection, respectively.

TABLE 1. COMPLEMENTATION BETWEEN tsB7 AND ts502[a]

	34° titer x 10^5		39° titer X 10^5	
	syn	syn$^+$	syn	syn$^+$
tsB7 syn$^+$	-	11.7	-	0.01
ts502 syn	0.64	-	Titer<10^2	-
ts 502 + tsB7	204	32	0.19	1.85

[a]Performed as described in ref. 3.

simultaneous cleavage with Bg1II and HsuI (Fig. 2). Two DNA fragments, HsuI/Bg1II-F and HsuI/Bg1II-E, were capable of rescuing tsB7 to ts$^+$ phenotype. These two fragments map at positions 0.42 to 0.53 and 0.70 to 0.83 (Fig.2). Although these two fragments formed adjacent bands in the agarose gel, our results indicate that these rescuing DNA sequences are probably non-identical.

To further characterize the rescue by the two regions of HSV-1 DNA, we isolated ts$^+$ recombinants from the progeny of the transfections by four cycles of cloning under agarose overlay medium at 39°. Table 3 summarizes the properties of

TABLE 2. Marker Rescue Mapping of HSV-1 (HFEM)tsB7 with Restriction Endonuclease Fragments of HSV-1(MP) DNA

Transfection Mixture	Ratio of Titers (39° /33°) x 10^3
A. Mapping with Hsu I/Bg1 II DNA Fragments	
tsB7 Alone	0.01
+E	21
+F	43
+G	2.4
+H	0.2
+I	0.4
+J	0.4
+K, L	0.3
+M	9.8
+N	1.0
+O	0.05
+P	0.01
+Q	0.03
+R	0.04
+S	0.08

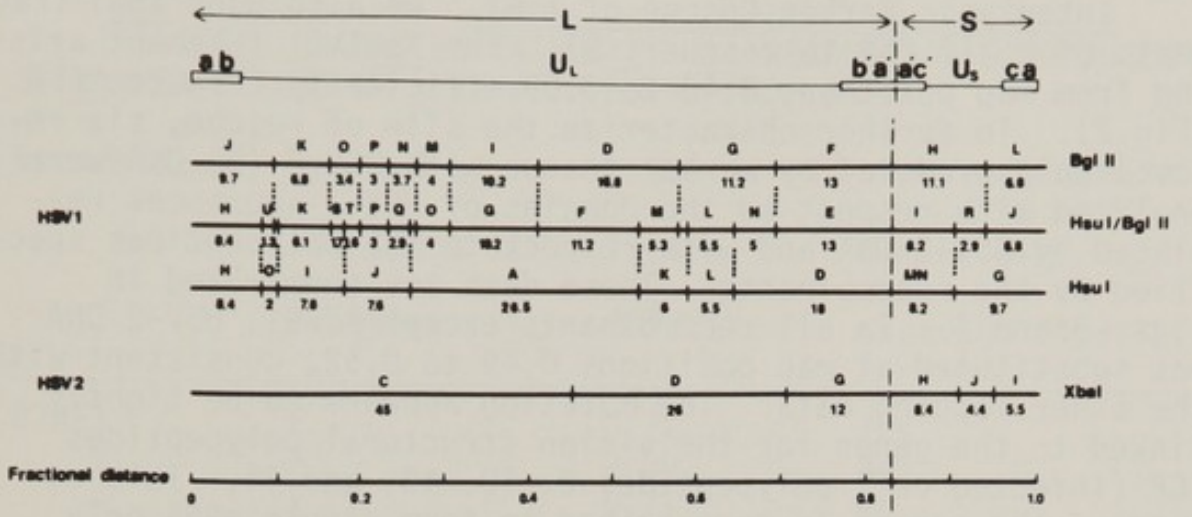

FIGURE 2. Restriction endonuclease cleavage maps of the DNAs used for the marker rescue of tsB7.

two classes of recombinants produced by the marker rescue tests. Class 1 constitutes recombinants produced by rescue of tsB7 with the HsuI/Bg1II-F fragment. Most of the recombinants in this class had an efficiency of plating ($39^{o}/33^{o}$) greater than 0.6 (mean =0.68)and in addition produced large plaques that were characteristic of ts^{+} virus. Analyses of the DNAs of the recombinants showed that 4 of the 6 recombinants in this class lost the cleavage site between the BamHI fragments D and H. (Fig.4). This cleavage site is present in tsB7 DNA but absent in MP DNA. The cleavage site between the BamHI-D and H fragments maps at approximately 0.516 map units and is contained within the HsuI/Bg1II-F fragment. These results are consistent with the hypothesis that the sequence rescuing the tsB7 mutant was contained within the F fragment and that the region of homology was sufficient to permit replacement of sequences beyond the BamHI H-D cleavage site.

Class 2 recombinants constitute those produced by marker rescue of tsB7 with the HsuI/Bg1II-E fragment. Most of the recombinants in this class had a ratio of titers (39/33) less than 0.3 (mean = 0.3) and in addition produced very small plaques at 39^{o}. However, at 33^{o} the plaques were comparable in size to those of the class 1 clones. These class 2 clones were therefore still partially temperature sensitive. Analyses of the DNAs of these recombinants showed that 9 of the 10 recombinants retained the BamHI D-H cleavage site. These results suggest that rescue by the HsuI/Bg1II-E fragment occurs at a different site from the rescue by the HsuI/Bg1II-F fragment or that the homology is not sufficient to allow replacement of the D-H cleavage site by the HsuI/Bg1II-E fragment. Data not shown here support the latter hypothesis.

Intertypic Marker Rescue of tsB7. We also used XbaI fragments of HSV-2 DNA to rescue tsB7. The XbaI-D fragment arising from map positions 0.43 to 0.69 efficiently rescued tsB7 (Fig.2). To further characterize the site of rescue, six recombinants produced by marker rescue with HSV-2 (G) DNA were analyzed with respect to the domains of HSV-1 sequences replaced by HSV-2 DNA and with respect to the polypeptides specified by the recombinants. These data are summarized in Figs. 3 and 4. In all recombinants except B7RG1, HSV-2 DNA was substituted at map positions 0.49 to 0.52, consistent with the other mapping data. The mutation appears to be tightly linked to the genes for the virion structural polypeptides ICP (infected cell polypeptide) 2, 10, 43, and 44. This region of the genome is transcribed to form stable mRNA only after viral DNA synthesis (5) and therefore the mutation appears to be in a late gene.

Comparison with Other Viral Mutants. This type of mutation differs from the other two classes of viral mutants that show a complete lack of viral gene expression under non-permissive conditions, the tsD mutants of SV40 and polyoma (6,7). The SV40 and polyoma mutants map in the late region of the genome (8,9) and appear to be defective in uncoating of the viral DNA. Therefore the DNA of these mutants cannot be transcribed to yield early mRNA. These mutants are unable to complement other co-infecting mutants and thus this uncoating function is a cis-acting function (7).

The group I host range mutants of adenovirus map in the region I of the genome and only the mRNAs for that specific region is expressed from the mutant genome in non-permissive cells (10,11). This appears to be a "pre-early" gene function whose synthesis is needed for expression of the early genes. The mutation of tsB7 thus differs from these because 1) it can be complemented by a co-infecting virus and 2) the existing evidence strongly argues that the alpha gene products require no prior viral protein synthesis (5, 12). Several different inhibitors of protein synthesis have been used to allow accumulation of alpha mRNA. There is no evidence to suggest that a "pre-alpha" protein gene product must be synthesized prior to expression of alpha genes.

Nature of the Defective Gene Product. The current evidence suggests that the defective gene product in tsB7 is a virion component that can be complemented by another co-infecting virus. This gene product could act upon the tsB7 capsid to allow it to express the alpha genes. Alternatively, it could alter the host cell RNA polymerase so that it can trans-

TABLE 3. CHARACTERIZATION OF $\underline{ts}^+$ RECOMBINANTS PRODUCED BY MARKER RESCUE OF $\underline{ts}$B7 WITH HSV-1 (MP) DNA.

Recombinant	Rescued by Hsu I/Bgl II Fragment	Ratio of Titers $39^o/33^o$	Presence of Bam HI D-H Cleavage Site
B7RF1	F	0.72	Yes
2	F	0.59	No
4	F	0.71	No
6	F	0.85	Yes
9	F	0.32	No
11	F	0.79	No
B7RE1	E	0.17	Yes
2	E	0.30	Yes
3	E	0.22	Yes
4	E	0.58	Yes
5	E	0.55	Yes
6	E	0.54	Yes
7	E	0.13	Yes
8	E	0.42	Yes
9	E	0.02	Yes
10	E	0.76	No

scribe the viral genome efficiently or it could alter the host cell in other ways to promote transcription of the viral genome. These functions would be required only for infections initiated by virions because it is known that rigorously deproteinized HSV DNA is infectious, albeit at lower efficiencies than with virions.

These studies show that the herpesvirus lytic cycle can be modulated at a stage that may not be lethal to the host cell. This may mimic the situation found in a cell that is latently infected with HSV.

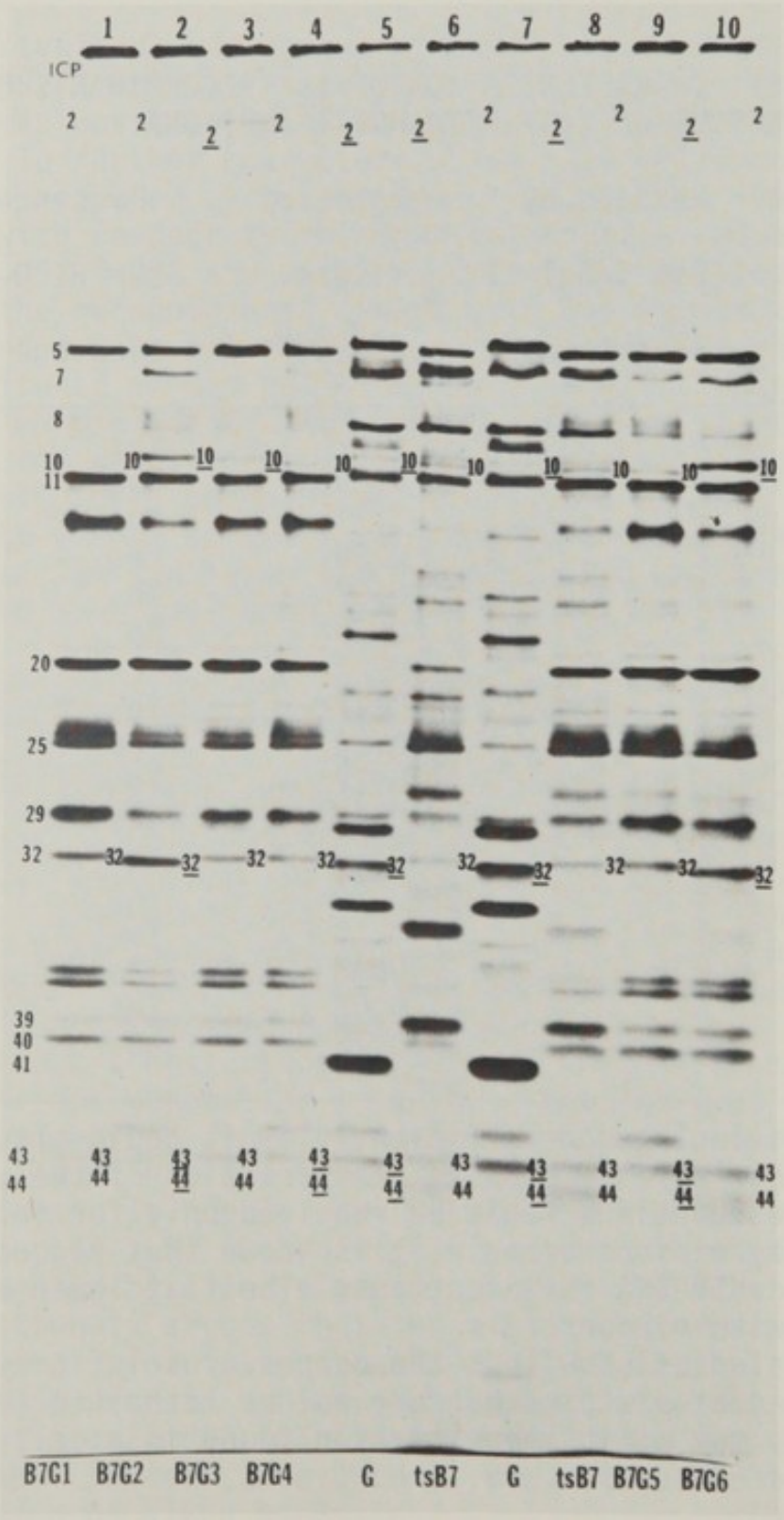

FIGURE 3. Autoradiogram of a polyacrylamide gel containing electrophoretically separated polypeptides from cells infected with intertypic recombinants.

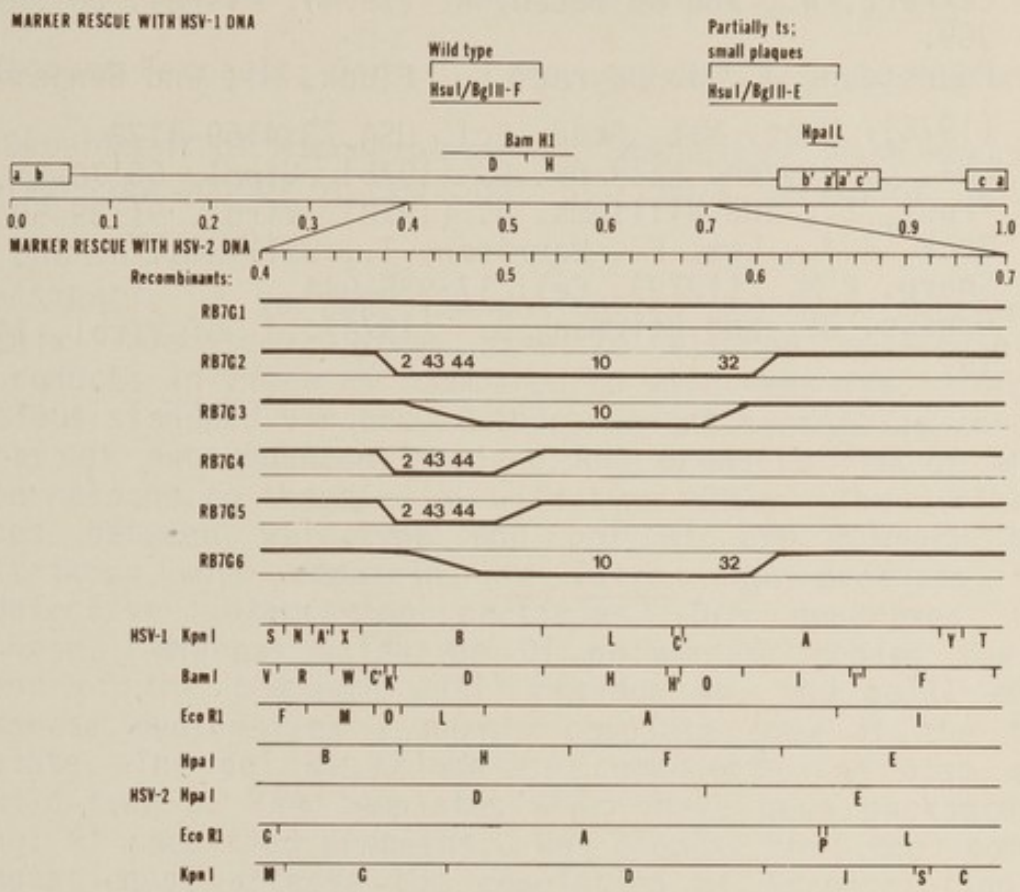

FIGURE 4. SUMMARY OF THE DNA STRUCTURE AND POLYPEPTIDES SPECIFIED BY INTERTYPIC RECOMBINANTS PRODUCED BY RESCUE OF tsB7 WITH HSV-2 DNA.

ACKNOWLEDGEMENTS

We sould like to thank Cathy Nosal and Mayer Stein for their expert performance of some of these experiments.

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A VARIANT VSV GENERATES DEFECTIVE INTERFERING PARTICLES WITH REPLICASE-LIKE ACTIVITY *IN VITRO*[1]

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ABSTRACT. A variant VSV designated pol R1, isolated in this laboratory, gives rise to altered transcription products *in vitro* as compared to wild-type VSV. Infectious standard virions of this variant synthesize an array of heterogeneously-sized RNA products few of which correspond to the size of wild-type mRNAs. The differences between wild-type and pol R1 are however most striking when comparing *in vitro* RNA products from defective interfering particles (DI) generated from these. Whereas wild-type DI polymerase copies the 3' end of the template until residue 46, the polR1 polymerase synthesizes a nearly complete copy of the template. The pol R1 polymerase components can also copy wild-type DI RNA templates which have been packaged by pol R1 specified proteins. We propose that the pol R1 mutation(s) affects the regulation of transcription vs replication functions of the VSV-coded polymerase.

INTRODUCTION

The transcription of eucaryotic negative-stranded RNA viruses, exemplified by vesicular stomatitis virus (VSV), can be readily studied *in vitro* since the virus particle core, comprised of only three virus-coded proteins (N, NS, and L) carries out this function faithfully including capping, methylation, and polyadenylation of RNA chains (for review see ref. 1). The mechanism responsible for the initiation and sequential appearance of the five VSV mRNAs in the order found on the genome, i.e., 3'-N-NS-M-G-L-5', is not yet clear. A model involving a single initiation site at the 3' end of the (-) strand genome followed by cleavage of a growing nascent chain has been proposed (2) but other more recent evidence favors independent initiation of each mRNA (3,4,5).

Several lines of evidence suggest a close coupling between the VSV transcription and replication functions but

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little is known regarding the latter (1). At least three laboratories have described conditions for the synthesis of at least some template-size (+) strands in vitro (5,6,7) and in one case this synthesis was achieved solely with purified virion cores under conditions suggesting that phosphorylation of a regulatory protein might be involved (5).

The synthesis of (-) strand virion RNA has not been observed in vitro so far. However, an initiation event analogous to this genome (-) strand synthesis takes place in vitro with purified VSV DI (for review see ref. 8). VSV DI contain deleted (-) strand genomes, but a stretch of ~50 bases at the 3' end of most of these DI RNAs is identical to that of the intracellular full-size (+) strand of the standard virus genome (9, see also Fig. 5). The 3' end of these DI template is copied into a 46-base-long (-) strand RNA which is the only major RNA species synthesized in vitro by these DI (10,11). This small-size product is thus analogous to a standard genome (-) strand initiation on a (+) strand template. For unknown reasons this synthesis terminates abruptly at residue 46.

We report here a preliminary characterization of a VSV variant (obtained following multiple cycles of heat inactivation and growth of survivors) which unlike wild-type VSV gives rise to nearly full-size copies of the deleted DI templates in vitro. We discuss the implications of this mutational alteration, which also affects the nature of the products made by infectious virus, in terms of regulation of VSV transcriptive vs replicative functions.

RESULTS

Growth, labeling, and purification of standard virus stocks and DI derived from the VSV Indiana serotype (standard Mudd-Summers strain) were described previously (12). In some experiments the heat-resistant HR VSV (Toronto strain) standard virus was also used. According to a recent suggestion concerning the nomenclature of DI particles (Reichmann, M. E., et al., submitted for publication), the three DI particles employed here are designated:

VSI ts^+ ATCC DI 0.22 (5', 20%) (13)
VSI pol R1 ATCC DI 0.21 (5', 13%)
VSI pol R1 ATCC DI 0.29 (5')

The pol R1 DI stocks were generated after three successive undiluted passages in BHK_{21} cells.

Enhanced RNA Synthesis by Pol R1 DI. Fig. 1 shows the time course of ^{3}H-UTP incorporation by purified wild-type vs pol R1 virus particles in a standard in vitro polymerase

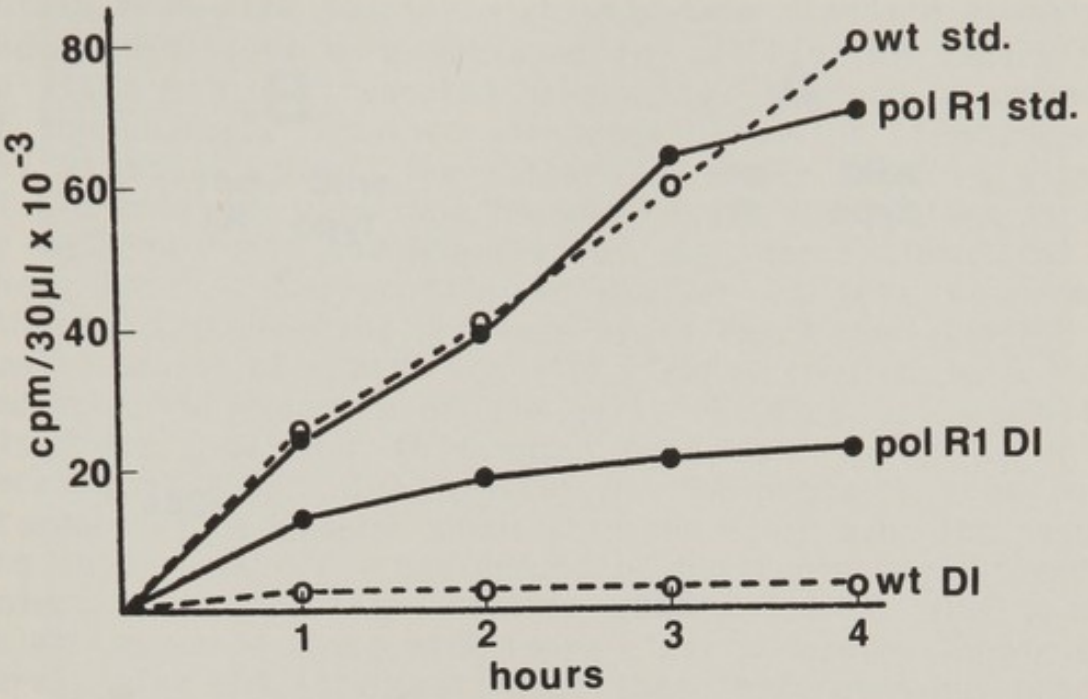

FIGURE 1. Time course of ^{3}H-UTP incorporation by purified standard and DI particles. Reaction mixtures contained 50 mM Tris-Cl pH 8.2, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% NP-40, 5 mM dithiothreitol, 1 mM each of ATP, GTP, and CTP, 0.1 mM UTP, 50 µCi/ml ^{3}H-UTP, and 100 µg/ml virus protein. Incorporation was measured as described previously (13).

reaction. No significant differences were observed between the two types of standard virions but polR1 DI 0.21 consistently showed a 5 to 10-fold higher level of activity as compared to wild-type DI 0.22. Pol R1 DI polymerase activity is similar however to wild-type DI in showing a leveling off of incorporation after about 2 hr of reaction time, as opposed to continued linear incorporation in standard virions for at least 4 hr. At the end of this particular 4 hr period the standard virion reactions accumulated ~10-fold excess of product over template by weight, the pol R1 DI ~3.7-fold, and the wild-type DI ~0.4-fold. Varying the concentrations of various components of the transcriptase reaction mixture did not reveal any major differences in polymerase assay requirements between wild-type and pol R1 particles.

Size Distribution of Wild-Type and Pol R1 Products. We next examined the size of *in vitro* synthesized RNA products by analysis on agarose gels *after* denaturation of the RNA with glyoxal. In agreement with the results of Breindl and Holland (6) we found that substitution of 300 mM potassium acetate instead of 100 mM sodium chloride was necessary to

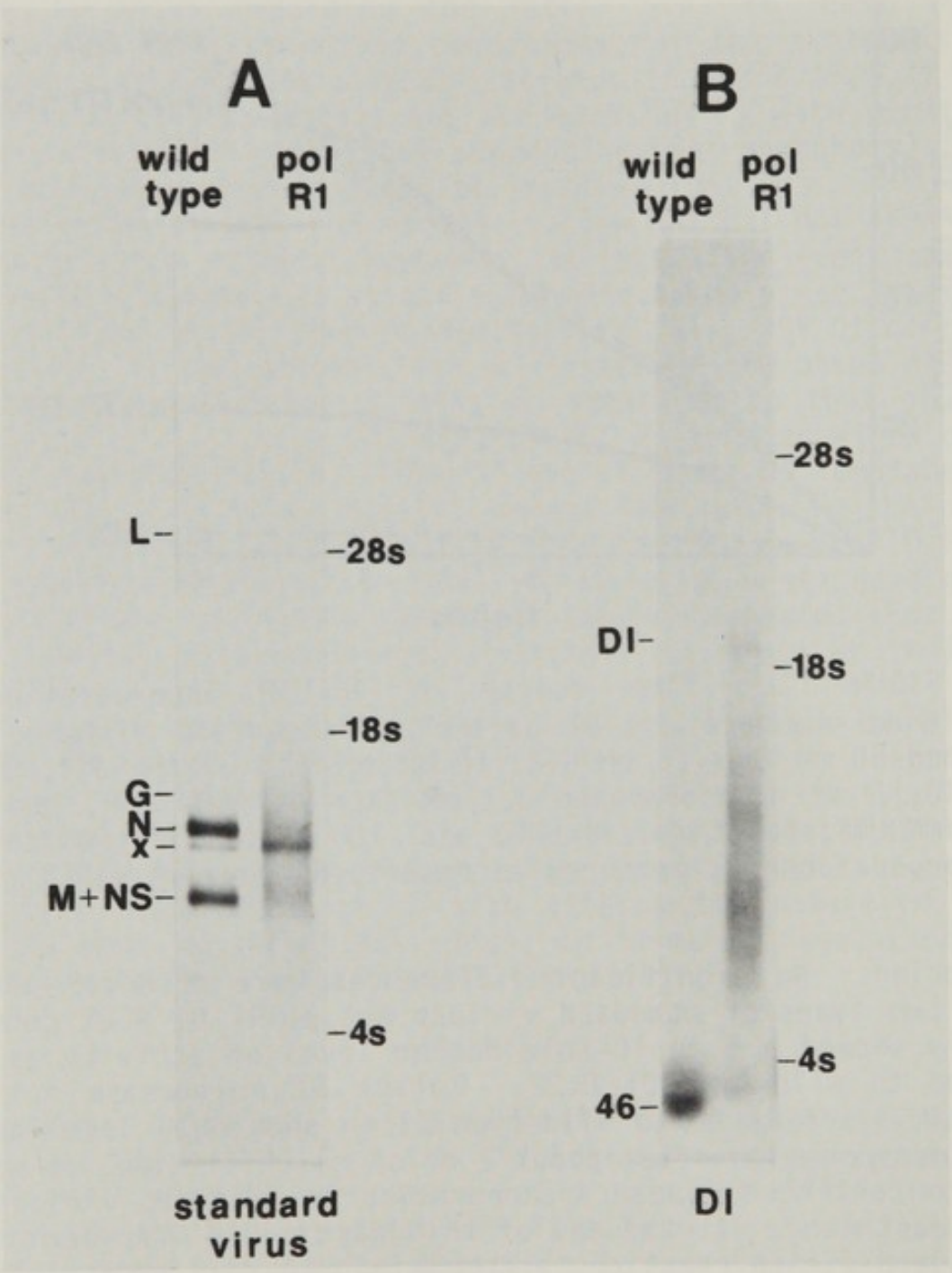

FIGURE 2. Size distribution of ^{3}H-uridine-labeled polymerase products synthesized in vitro from wild-type and pol R1 virus. All reactions contained 300 mM potassium acetate instead of 100 mM NaCl and standard virus reactions contained purified virus cores (equivalent to 2.5 mg/ml whole virus) and 1 mM UTP (see text). The DI products were purified by proteinase K digestion and phenol-chloroform extraction (12) followed by Sephadex G-50 chromatography. Standard virus products were purified by the CsCl method (6). RNAs were denatured with glyoxal and analyzed in 1.1% agarose gel as described before (14).

obtain mRNA-size species with wild-type standard virions and these conditions were employed for all further analysis of in vitro products reported here. Fig. 2A shows a comparison of the products obtained with core particles of standard HR VSV vs pol R1 VSV. The pattern of mRNAs observed with the HR (Toronto) or wild-type (Mudd-Summers) VSV Indiana strains, or between cores and whole virus, was nearly identical (not shown here). The profile is similar to that observed by others except for the presence of an extra band labeled X (a small amount of L mRNA was also clearly seen on longer autoradiographic exposure of the gel). Studies to be published elsewhere show that this extra band corresponds to N mRNA lacking poly(A). The profile of RNA products from pol R1 standard virus however shows only one clear band (corresponding to X) against a background of heterogeneously-sized RNA molecules ranging from smaller than M and NS mRNA to molecules as large as L mRNA (more clearly seen on longer exposure). The characterization of these heterogeneous products is the subject of a separate communication to be published elsewhere (see Discussion below).

Fig. 2B shows the profile of products synthesized by wild type DI 0.22 vs pol R1 DI 0.21. As expected from our previous studies the wild-type DI synthesized a small product migrating slightly faster than the 4S size t-RNA marker RNA. As shown previously almost all of this represents a 46 base-long species complementary to the 3' end of the template (10). In contrast, the pol R1 DI synthesized an array of products approximately 500 to 1600 bases long for the most part with a distinct band at ~2300 bases. As shown below, this latter band appears to be very near the size of the whole template.

Hybrydization of Pol R1 DI Template and Products. The appearance of a distinct large size product in the pol R1 DI reaction suggested to us that these particles might indeed be capable of synthesizing a complete (+) strand copy of the template. We therefore examined the hybridization properties of the pol R1 DI template and products to verify this hypothesis. Table 1 shows that only ~8% of the sequences in the pol R1 DI template formed a double-stranded hybrid structure immediately following denaturation. This level of "snapback" is consistent with the presence of short inverted terminal complementary sequences found in most VSV DI RNAs (14,15). Exhaustive self-annealing of the pol R1 DI RNA template preparation showed only a small (~2.5%) level of unlinked complementary (+) strand templates. The pol R1 DI template thus appears to be similar to several other genome 5'-end-derived DIs obtained from wild-type and ts mutant virus (12,15).

Labeled pol R1 DI product RNA was RNase sensitive after

TABLE I
HYBRIDIZATION OF POL R1 DI 0.21 TEMPLATE AND PRODUCTS[a]

^{3}H-Uridine labeled RNA	Unlabeled RNA	Hybridization time (hr)	% RNAse resistance
Template (~1 ug/ml)	--	Melted and quick cooled only	8
Template (~1 ug/ml)	--	72	13
Products (~2 ug/ml)	Template (100 ug/ml)	3	102
Template (~1 ug/ml)	Products (50 ug/ml)	2	98

[a] Hybridization conditions and RNase resistance assay were described previously (14).

melting and quick-cooling as expected (not shown here) and was essentially totally protected by an excess of unlabeled pol R1 DI template indicating that essentially all of it is complementary. When labeled pol R1 DI template was annealed to an excess of unlabeled pol R1 DI products again almost all of the template was protected indicating that the majority of the template was copied by the polymerase (Table 1).

Synthesis of a Nearly Complete and Contiguous Copy of the Pol R1 DI Template. The above results strongly suggest that the large distinct pol R1 DI product, ~2300 bases long, might indeed represent a faithful copy of the template. We examined this question more critically by determining the size of the labeled template fragment protected from RNase digestion after hybridization with excess unlabeled product. As shown in Fig. 3, this protected template fragment obtained after glyoxal denaturation of the duplex, is only slightly smaller than the pol R1 DI 0.21 template (~50 to 100 bases shorter). Since any mismatch or discontinuity of complementarity between the product and the template would lead to sites sensitive to RNase in the duplex structure (14) we conclude that a contiguous segment representing ~96% of the template is faithfully copied into a complementary product.

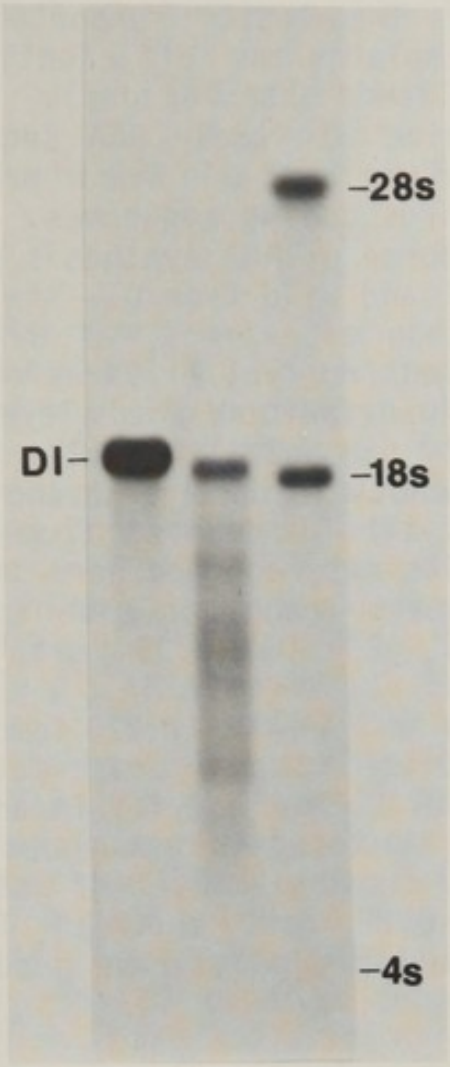

FIGURE 3. Size comparison of pol R1 DI 0.21 template RNA and fragment protected from RNase digestion after annealing with excess in vitro synthesized polymerase products. Left lane, ^{3}H-uridine pol R1 DI 0.21 template labeled in vivo: middle lane, labeled template after annealing for 35 min with a 40-fold excess of unlabeled DI 0.21 product RNA (100 µg/ml) followed by RNase digestion as described previously (14): right lane, ^{3}H-uridine labeled marker HeLa cell RNAs. Samples were analyzed as in Figure 2.

Mutational Alteration of a Polymerase Protein Component in Pol R1. The nature of the large distinct pol R1 DI polymerase product suggests that the enzyme complex in this variant either, 1) "reads-through" the termination site 46 bases from the 3' end of the template found in wild-type DI, or 2) initiates synthesis at a different site within the first 50 to 100 bases at the 3' end of the template. A priori this result could be achieved by 1) a critical change in the sequence of the template responsible for initiation and for termination in the wild-type, and/or 2) a change in one or more components of the polymerase complex rendering it unresponsive to the wild-type template signal.

In order to distinguish between a template vs a polymerase component alteration in pol R1 we constructed in vivo a "mixed" DI particle consisting of wild-type DI 0.22 template RNA packaged in pol R1 specified protein. This can easily be done since purified wild-type DI such as 0.22 do not give rise to translatable transcripts in vivo (8). Wild-type DI (free of their helper wild-type standard virus)

were employed in co-infection experiments with pol R1 standard virus (free of pol R1 DI). The latter supported the replication of wild-type DI templates as efficiently as homologous wild-type virus (unpublished observations). The resulting "mixed" DI progeny derive all their RNA sequence from the wild-type input DI template and all their protein from the helper standard pol R1 virus coding sequences.

Fig. 4 illustrates the time course of RNA synthesis mediated by these "mixed" DI vs pol R1 and wild type DI. The larger pol R1 DI 0.29 employed in this experiment, as well as several others independently derived from pol R1 virus (unpublished observations), clearly show the enhanced level of polymerase activity seen previously for pol R1 DI 0.21. This result demonstrates that the phenotypic change associated with pol R1 virus is common to all DI derived from this strain so far. Furthermore, results not reported here indicate that a large size product nearly equal in size to the template is synthesized in pol R1 DI 0.29 catalysed reactions.

The "mixed" DI containing wild-type DI 0.22 template also clearly shows the same enhancement of polymerase activity as compared to pure wild-type DI 0.22 (Fig. 4). In addition, "mixed" DI containing pol R1 template sequences and wild-type virus derived proteins display a level of activity comparable to wild-type DI 0.22 (not shown). These results suggest strongly that the altered polymerase products

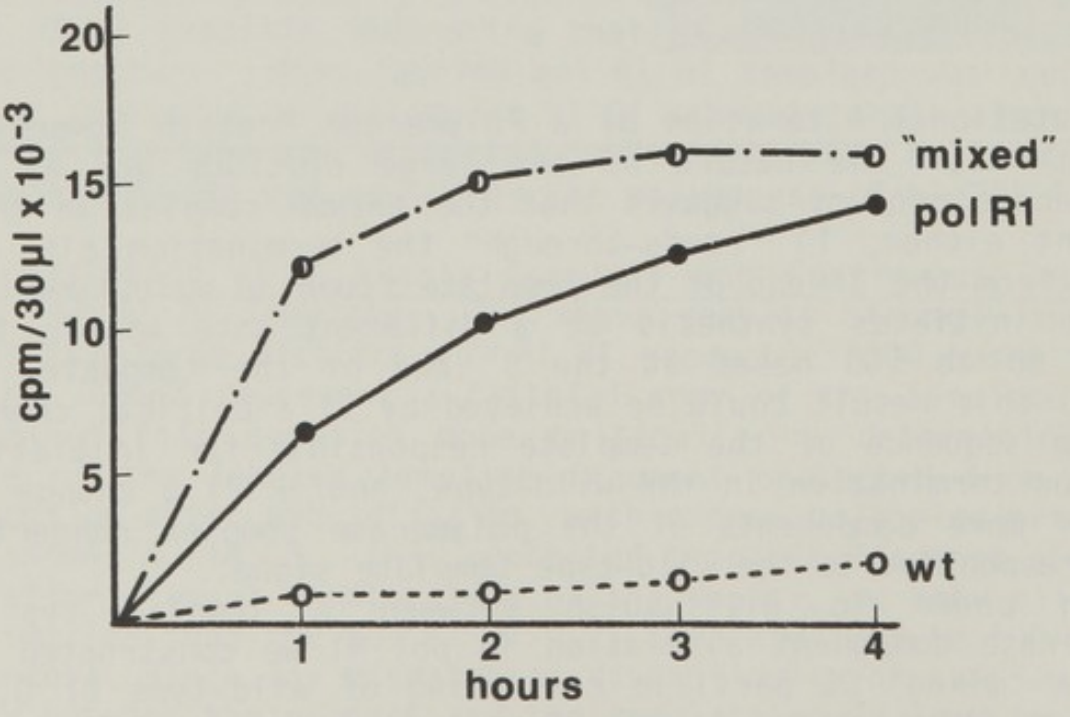

FIGURE 4. Time course of ^{3}H-UTP incorporation by pol R1 0.29, wild-type, and "mixed" DI. Conditions were as described in Figure 1.

seen in pol R1 DI are due to a mutational alteration in one or more critical polymerase components, i.e., N, NS, or L proteins. This conclusion is supported by other preliminary data which indicate that the annealing properties of the "mixed" DI RNA products correlate with the nature of the proteins and not the templates.

DISCUSSION

We report here the preliminary characterization of a VSV variant, pol R1, which displays unusual polymerase properties *in vitro*. It is not yet clear why the particular heat selection procedure employed (details to be described elsewhere) generated this variant. Surprisingly pol R1 virus infectivity and transcriptase activity are not significantly more heat resistant than wild-type virus (unpublished results). Although this variant could have originated by chance alone it is likely to have had a selective advantage over wild-type at least at some point during the selection procedure. It has since remained stable on continued passage.

The present communication focuses on the *in vitro* polymerase activity of pol R1 DI particles. Our observations can be summarized as follows: 1) as opposed to the 46 base-long product synthesized by wild-type DI, pol R1 DI synthesize much larger products heterogeneous in size; 2) a small but significant subset of these products corresponds to molecules only ~50-100 bases shorter than the template DI RNA molecules; 3) this large RNA product represents a faithful, contiguous (+) strand copy of almost all of the template; 4) other DI derived from pol R1 virus, as well as "mixed" DI containing wild-type templates and pol R1 proteins, also show these properties; and 5) the pol R1 phenotype is due to one or more mutationally altered protein component(s).

The properties of the pol R1 DI polymerase activity are of special interest for the following reason. The template structure of VSV DI differs from that of infectious virus in a critical way as illustrated in Fig. 5. Except for the rare class of internal deletions in VSV DI (obtained from the HR VSV strain) which map at the 3' end of the standard genome, all VSV DI map at the 5' end of the genome (8,14). In addition all the 5' end DI examined so far contain inverted complementary terminal sequences 40 to 65 bases in length (14, 15,16). The 5' end of these DI RNAs corresponds to the 5' end of the standard virus (-) strand. The 3' end of the DI RNA however corresponds to the 3' end of the standard virus (+) strand (see Fig. 5). We have shown previously that wild-type DI polymerase copies the 3' end of its template *in vitro* until residue 46 and then abruptly stops (10). Some

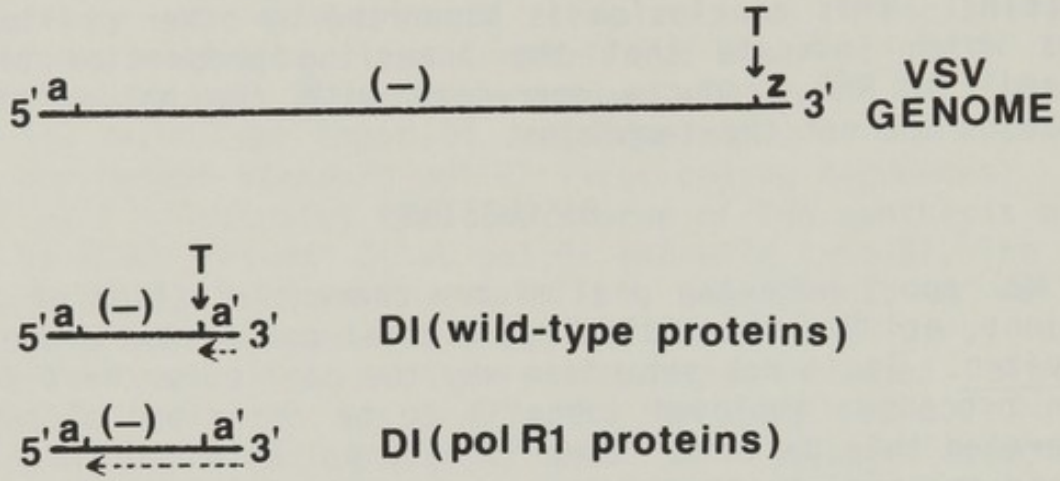

FIGURE 5. Structural relationship between standard and DI genome RNAs and proposed DI template interaction with wild-type vs pol R1 polymerase components. The letters a and a' denote inverted terminal complementary sequences (roughly 50 bases long). The letter z denotes that the 3' terminal sequence of the standard genome differs completely from that of DI RNAs beyond 20 bases from the 3' end (16). The letter T denotes a polymerase termination site 47 to 48 bases from the 3'terminus of the standard genome and 46 bases from that of the DI. The dotted line indicates the proposed pol R1 versus wild-type DI polymerase products synthesized *in vitro* from 5'-end derived DI templates.

termination at this same site also seems to occur *in vivo* (17,18). This synthesis can be viewed as an "abortive" (−) strand synthesis since the template promoter sequence is identical to that of genome (−) strand synthesis. We propose that the synthesis of near template size RNA by pol R1 DI *in vitro* represents an event analogous to synthesis of standard genome (−) strand synthesis *in vivo*. A corollary to this proposal is that pol R1 virion proteins alone can carry out this process (at least under some conditions and to the extent observed here *in vitro*) without the simultaneous need for additional factors present only in the infected cells.

Standard pol R1 virus also gives rise to products which differ drastically in size from wild-type standard virus (Fig. 2A). We will show elsewhere that this size heterogeneity is due to giant poly(A) tails at the 3' ends

of apparently otherwise normal VSV mRNAs. This observations is intriguing since a similar result was obtained by others when *in vitro* transcription of standard VSV was carried out in the presence of the methylation inhibitor, S-adenosyl-homocysteine (19). Preliminary results indicate that pol R1 standard virus also synthesize greatly reduced amounts of the 48 base-long (+) strand leader RNA *in vitro*. At present the probable relationship, between the changes observed in the standard pol R1 transcriptase products and those seen in pol R1 DI *in vitro* is intriguing. So far we have seen few but significant differences between wild-type and pol R1 RNA products *in vivo*. It is abundantly clear however, that the mutational alteration(s) in pol R1, and other such variants if they can be found, will provide us with a new approach to elucidating the controlling events in VSV transcription and replication.

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SEPARATION OF FULL LENGTH TRANSCRIPTS AND GENOME RNA PLUS AND MINUS STRANDS FROM CYTOPLASMIC POLYHEDROSIS VIRUS OF BOMBYX MORI

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ABSTRACT Under optimal conditions, CPV transcription in vitro was maintained for more than 24 hr, resulting in large quantities of full-size mRNAs. The mRNAs were resolved into nine discrete bands by agarose gel electrophoresis in the presence of 7 M urea. The genome dsRNA segments coding for each mRNA were identified by hybridizing the separated, ^{32}P-labeled mRNA to a mixture of genome RNAs. Each of the genome segments appears to be transcribed at the same rate, producing equal quantities by weight but different molar amounts of the separated individual mRNA species. These results imply that each of the genome segments is transcribed repeatedly by an equal number of template-associated RNA polymerases at the same rate of chain elongation. The genome RNAs of CPV and reovirus, labeled at the 3'-termini with ^{32}pCp by RNA ligase, were separated by agarose gel electrophoresis into the plus and minus strands. The RNA strands of plus polarity were found to migrate faster in the gel than those of minus polarity for all CPV dsRNA genome segments, whereas the opposite was seen for most of the reovirus genome segments. The basis of this separation is due to retention of secondary structure during the electrophoresis even in the presence of 7 M urea.

INTRODUCTION

Cytoplasmic polyhedrosis virus (CPV) infects the silkworm Bombyx mori and causes a disease which results in the formation of virus inclusion bodies, the polyhedra, accompanied by diarrheal symptoms and eventual death. The virus, like human reovirus, is a member of Reoviridae and contains a ten-segmented, double-stranded RNA genome (1) and a virus-associated RNA polymerase (2). CPV purified from infected silkworms directs mRNA synthesis in vitro (3). The CPV transcription

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system is unique in that S-adenosylmethionine (AdoMet), a methyl donor, markedly stimulates mRNA synthesis (∿70-fold) (4). The stimulatory effect of AdoMet apparently occurs at the initiation step (5,6) and the CPV mRNAs synthesized in the presence of AdoMet were the first shown to contain a methylated cap structure, $m^7GpppA^m_pG$, at the 5'-terminus (7). mRNA-methyltransferase associated with the virus particles appears to be involved in the initiation of the transcription (8). With a goal to assigning coding properties of the genome segments and synthesizing active viral enzymes in a mRNA-dependent cell-free translation system, we have established condition of transcription in vitro which resulted in the formation of capped, full-size viral mRNAs (9). During these studies we discovered that agarose gel electrophoresis which separates a mixture of ten mRNAs can also resolve complementary strands of CPV and reovirus genome dsRNAs (10).

MATERIALS AND METHODS

Isolation and Purification of CPV. CPV was isolated from infected silkworm (Bombyx mori) midguts as previously described (11).

In vitro Transcription of CPV. Transcription reaction mixtures (100 µl) contained 70 mM Tris-OAc (pH 8), 10 mM $Mg(OAc)_2$, 100 mM NaOAc, 4 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP containing 7.3 µCi [α-^{32}P]UTP (specific activity 20 Ci/mmole), 1 mM AdoMet, 40 µg bentonite, 20 µg proteinase K and 30 µg CPV. The reaction was carried out by incubation at 31°C. The incorporation of ^{32}P-label into TCA-precipitable RNA was monitored by extracting reaction aliquots of 12 µl at 0, 2, 4, 8, 17 and 26 hr for Fig. 1.

Purification of mRNA product was performed as described previously (12).

Separation of RNAs by Agarose Gel Electrophoresis. RNAs were separated by a method similar to the one described by Rosen et al. (13) and Smith and Furuichi (9). Samples containing mRNA or double-stranded RNA were denatured by boiling for 2 min in 4.5 M urea or by incubation in 90% DMSO at 37°C for 30 min. The samples were then cooled and applied to the 1.75% agarose gel containing 25 mM sodium citrate buffer (pH 3.5) and 7 M urea. Electrophoresis was performed at 4°C for 30 hr at 25 mA unless otherwise noted.

Labeling of the 5' and 3'-Termini of CPV and Reovirus Genome RNAs. 3'-Termini of dsRNAs were labeled with [5'-^{32}P]

pCp by T4-induced RNA ligase and 5'-termini of the (-) strands of dsRNA were labeled with [γ-^{32}P]ATP and polynucleotide kinase according to the methods of England and Uhlenbeck (14), and Miura et al. (15), respectively.

Treatment of mRNAs and genome dsRNAs of CPV and Reovirus by Glyoxal. The reaction mixtures (20 µl) contained about 0.1 µg of ^{32}P-labeled mRNA or genome dsRNA, 25 mM Na citrate buffer (pH 3.5), 50% DMSO (N_2 flushed) and 1 M glyoxal (deionized) (16). The reaction mixtures were incubated for 1 hr at 50°C. The RNAs were then subjected to electrophoresis on 1.75% agarose gel (10).

RESULTS

Efficient Synthesis of Full-Size mRNAs by CPV-Associated RNA Polymerases. Synthesis of CPV mRNA continues for many hours under the optimized condition which includes NaOAc (100 mM), high concentration of rNTPs (10 mM in total) and proteinase K (Fig. 1, left panel). In a 26 hr incubation, the amount of synthesized CPV mRNA reached a level of about 6-fold greater than the genome templates present in the virus particles. mRNAs that accumulated in the reaction mixture maintained their full size (Fig. 1, right panel) and were active in messenger-dependent cell-free translation systems from rabbit reticulocyte and wheat germ.

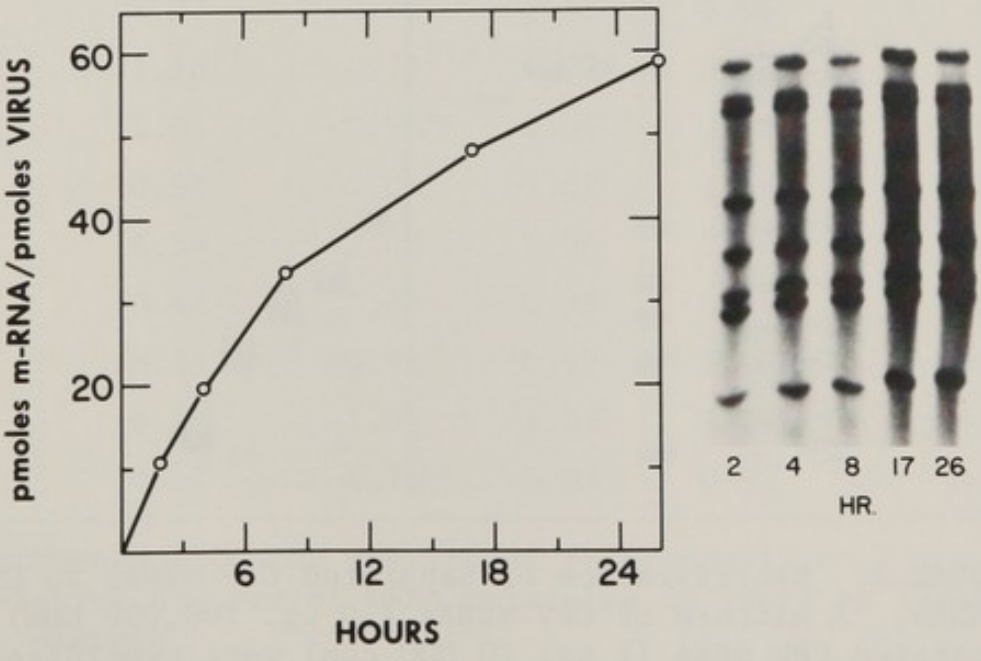

FIGURE 1. In vitro synthesis of CPV mRNA. Conditions of the synthesis and the separation of CPV mRNAs are described in Materials and Methods.

Apparently, the relatively high ionic strength of NaOAc and high rNTP concentration help CPV RNA polymerase to complete chain elongation, and the presence of proteinase K prevents product RNA nicking.

Relationship between Viral Genome Segments and mRNAs. To determine the relationship between genome segments and the corresponding transcripts, CPV mRNAs were synthesized in the presence of [α-^{32}P]UTP under the improved reaction conditions described above. The mRNA species were separated by gel electrophoresis, extracted from the gel and freed from agarose as described (9). Each of the isolated mRNAs was then hybridized to the mixture of denatured CPV genome dsRNAs. The annealed ^{32}P-labeled dsRNA (whose plus strands had been replaced by the corresponding ^{32}P-labeled RNA made *in vitro*) were separated by 4% polyacrylamide gel electrophoresis and compared with CPV genome RNAs (Fig. 2).

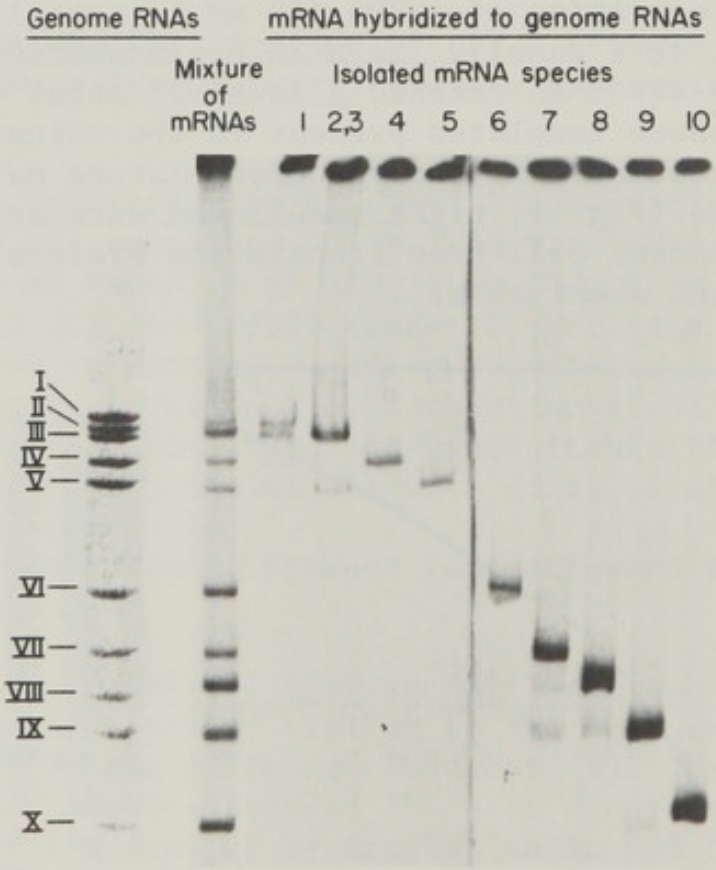

FIGURE 2. Hybridization of Separated CPV mRNAs to CPV genome RNAs. A mixture of CPV mRNAs (10 µg, 100,000 cpm) and each separated CPV mRNA (1 µg, 10,000 cpm) were hybridized respectively to 10 µg of a mixture of CPV dsRNAs as described previously (4). Electrophoresis of the RNA on 4% polyacrylamide gel was performed for 12 hr at 300 v.

As Fig. 2 clearly demonstrates, each mRNA separated by agarose gel electrophoresis hybridized with a specific genome RNA segment. mRNA-10, for example, hybridizes to genome RNA-X. Similar specific hybridization occurred between mRNA-1 through 9 and genome RNA segments I through IX, respectively (Fig. 2). These results demonstrated that mRNA-10 is the transcript of genome RNA-X. The remaining mRNAs have a similar relationship to the genome RNAs as follows: 9-IX, 8-VIII, 7-VII, 6-VI, 5-V, 4-IV, 2 and 3 -II and -III, and 1-I, respectively.

Relative Efficiency of Transcription of the Genome Segments of CPV. CPV contains segments of dsRNA ranging in m.w. from 0.35×10^{-6} to 2.6×10^{6} daltons (17). Synthesis of full-size mRNAs, an efficient separation of the mixture of CPV mRNAs by agarose gel electrophoresis and the assigned relationships between genomes and mRNAs permit a detailed analysis of the transcription rates of the individual genome segments. Nine of the ten mRNA species (mRNA-2 to 10) are synthesized in almost the same amount as determined from the incorporation of

TABLE I

DISTRIBUTION OF CPM mRNA SPECIES
PRODUCED IN VITRO TRANSCRIPTION REACTION

mRNA	Molecular weight ($\times 10^{-6}$ dalton)*	Ratio of the intensity Incubation time 1 hr	2 hr	4 hr	Relative numbers of mRNA molecule**
1	1.27	0.76	0.58	0.56	4.4
2&3†	1.18††	0.79†††	0.78	0.65	5.5
4	1.01	0.98	0.99	0.93	9.2
5	0.91	1.01	1.09	1.05	11.5
6	0.56	1.00	1.00	1.00	10.0
7	0.42	0.89	0.85	0.96	22.8
8	0.31	0.96	0.99	1.00	32.3
9	0.28	1.05	1.03	1.14	40.7
10	0.17	0.76	0.85	0.92	54.1

*Estimated from the results of reference 17.
**Relative numbers of mRNA molecules in the 4 hr incubation mixture.
†Mixture of mRNA-2 and mRNA-3.
††Averaged value for mRNA-2 and mRNA-3.
†††Averaged value per band.

nucleotides including [α-^{32}P]UMP into RNA (Table I). These results show that each genome segment is transcribed at the same rate. The apparent small differences in mRNA-2 to 10 are probably due to minor differences in base composition or trailing of the mRNAs during gel electrophoresis. The one exception is mRNA-1 which is synthesized in an amount of about 40% less than the other mRNAs. Probably because of its large molecular weight, premature termination of mRNA-1 may occur even under the improved conditions of synthesis.

Since all mRNAs are synthesized in similar amounts, their rates of transcription on a molar basis depend upon the size of their respective genome templates. Random binding of RNA polymerase to the promoter sites of each genome RNA cannot account for these observations nor can continuous transcription of an entire set of linked genome segments by a single polymerase. Instead, an equal number of RNA polymerase molecule may associate with each genome template and transcribe each dsRNA segment repeatedly. The RNA polymerase would be conserved on the same template for initiation, chain elongation and reinitiation in a "recycling" mode of transcription.

Separation of the Complementary Strand of Genome dsRNA of CPV and Human Reovirus. We have found that agarose gel electrophoresis which separates CPV mRNAs can also resolve the plus and minus strands of virus genome dsRNA. The two 3'-termini of each of the genome segments were labeled with [5'-^{32}P]pCp by T_4-induced RNA ligase. When the ^{32}P end-labeled CPV dsRNA mixture was heat-denatured in 4.5 M urea or in 90% DMSO and subsequently subjected to agarose gel electrophoresis in citrate buffer (pH 3.5) containing 7 M urea, it was resolved into as many as 18 discrete bands (Fig. 3a, lanes B and E). This result clearly demonstrated that the (+) and (-) strands of genome dsRNAs are separated because at least 9 bands comigrated with CPV mRNAs synthesized *in vitro* (Fig. 3a, lane A). The rest of the bands comigrated with the (-) strands of CPV genome RNA which were specifically ^{32}P-labeled at the 5'-termini with [γ-^{32}P]ATP and polynucleotide kinase (Fig. 3a, lane C). Most of the bands in lane B were distinctly identified as either (+) strand or (-) strand as shown in lane E. Several faint bands shown by astericks apparently resulted from incomplete strand separation of duplex RNA or reannealing with contaminated dsRNA (lanes A and B), since these bands disappeared or faded under the stricter condition of strand separation by 90% DMSO (lane E). In addition to the separation of the (+) and (-) strands of each dsRNA segment, it should be noted that the (+) strands of CPV genome always migrated faster than the corresponding (-) strands.

Reovirus dsRNA labeled with ^{32}P-pCp were resolved into 13 bands (Fig. 3b, lane B). The apparent incomplete separation

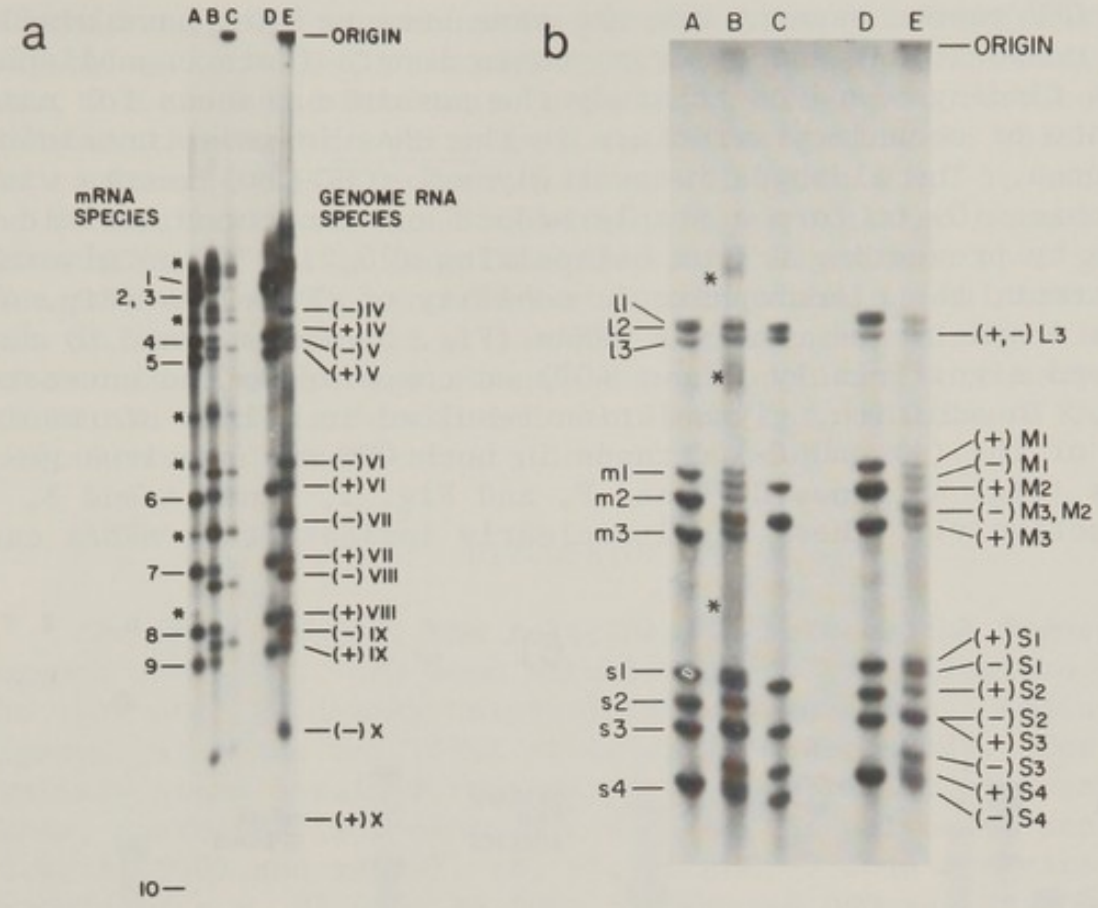

FIGURE 3. Separation of the complementary strands of CPV and human reovirus genome RNAs. a: CPV; b: reovirus. Genome dsRNAs ^{32}P-p*Cp-end-labeled were denatured either by heating (lanes B and C) or incubation in DMSO (lanes D and E) and separated by electrophoresis on a 1.75% agarose gel in the presence of 25 mM Na citrate buffer (pH 3.5) and 7 M urea. Lane A, mRNAs of CPV or reovirus synthesized in vitro.

is due to comigration of several ssRNAs of similar or identical m.w. from unrelated dsRNA species. Nevertheless, it is likely that each of the dsRNA segments is separated into its component two strands (Fig. 3b, lane E). Interestingly, most of the (-) strands of reovirus genomes, contrary to CPV genomes, migrated faster than the corresponding (+) strands.

Guanine-Mediated RNA Secondary Structure Affects Relative Mobility of the Complementary Strands. Electrophoretic mobility of RNAs in a gel depends on both their m.w. and conformation. Removing secondary and tertiary structure should make the electrophoretic mobility a simple function of molecular weight. Gels containing denaturing agents such as formamide (18) and 7 M urea (19) have been used for m.w. determinations based on these considerations.

The molecular weights of two complementary strands of

each CPV genome segment are the same because they have similar base compositions and the same chain length (data unpublished). These findings lead us to study the possible reasons for maintenance of secondary structure in the RNAs in gels containing 7 M urea. The aldehyde reagent glyoxal (OHC-CHO) reacts with guanosine (G) to form a stable adduct and denatures nucleic acids by preventing G from basepairing (20,21). Upon glyoxal treatment, the electrophoretic mobility of CPV mRNAs (Fig. 4, lanes A and B) and reovirus mRNAs (Fig. 5, lanes C and D) decreased significantly (about 40%) as compared to the untreated RNAs. In addition, glyoxalation resulted in a loss of resolution of the (+) and (-) strands in both CPV and reovirus genomes (Fig. 4, lanes C, D and F, and Fig. 5, lanes A and B, respectively). These results clearly indicate that mRNAs can

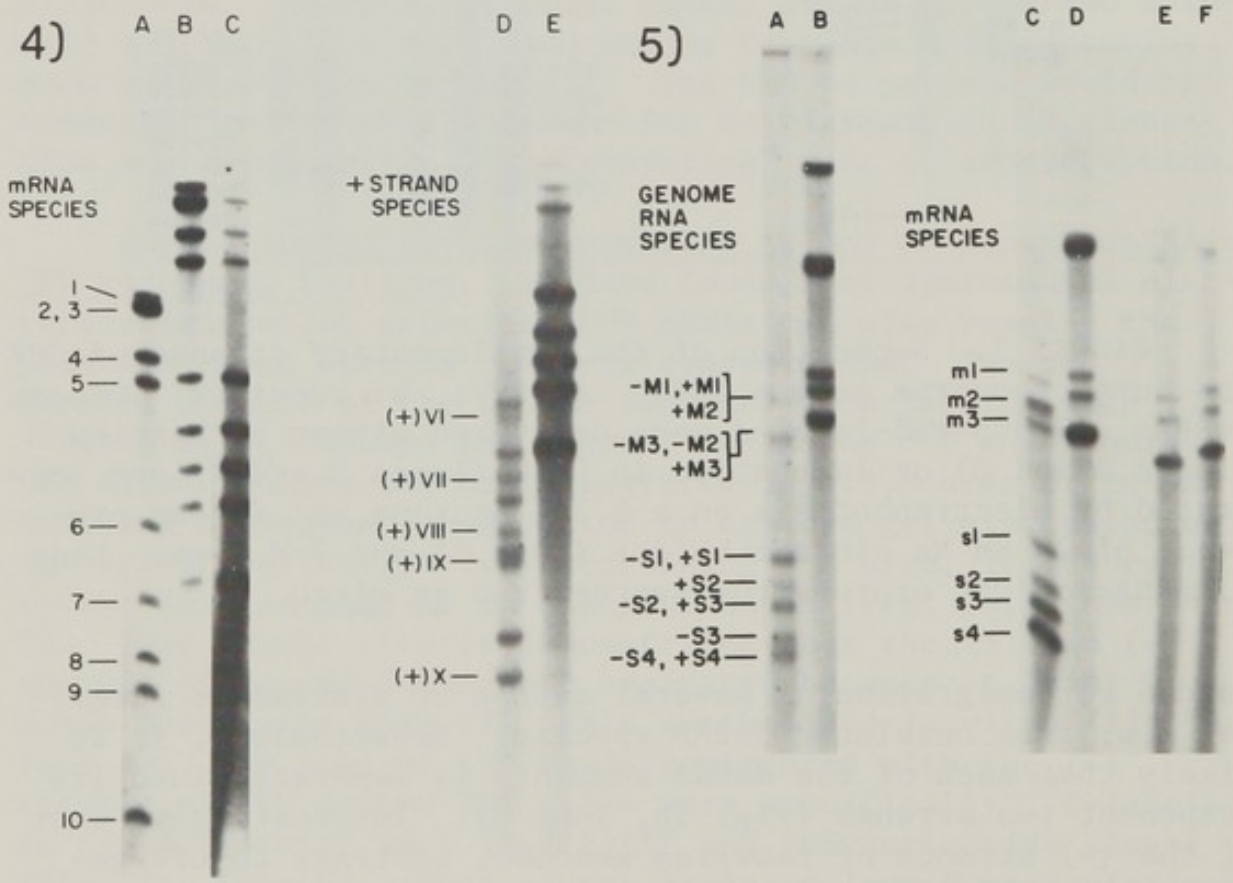

FIGURE 4 (left) Effect of glyoxalation on the electrophoretic mobility of CPV mRNAs and the plus and minus strands of the genomes. A, CPV mRNA; B, glyoxalated CPV mRNA; C and E, glyoxalated CPV genome RNA; D, denatured CPV genome dsRNA.

FIGURE 5 (right) Effect of glyoxalation on the electrophoretic mobility of genome RNAs, mRNAs and ITP-mRNAs of reovirus. A, denatured genome RNA; B, glyoxalated genome RNA; C, glyoxalated mRNA; D, reovirus ITP-mRNA; E, glyoxalated reovirus ITP-mRNA. Reovirus ITP-mRNAs were prepared under the same condition as described previously (9) except for the replacement of GTP (2 mM) by ITP (4 mM).

maintain a folded structure by partial base-pairing (presumably by weakened G-C pairing) even under denaturing conditions, i.e., 7 M urea and low ionic strength. Moreover, reovirus mRNAs synthesized *in vitro* in a reaction mixture in which GTP was replaced by ITP (referred to as ITP-mRNA, Fig. 5, lanes E and F) similarly migrated as slowly as glyoxalated normal reovirus mRNAs (Fig. 5, lane D). Thus, it is evident that guanosine is involved in secondary structure which is maintained even in the presence of 7 M urea. Differences in migration between the (+) and (-) strands depend upon the secondary structure which is formed inherently in the individual RNA molecules upon strand-separation of the duplex.

DISCUSSION

CPV gene mapping, the relationship between the segmented genomes and mRNAs, has been established by hybridization of the separated full-size transcripts to genome RNA. Coding assignment of mRNAs for viral proteins is under study. Our preliminary study obtained from *in vitro* translation of separated mRNAs, indicated that mRNA-10 codes for the polyhedra peptide (m.w. 31,500) and mRNA-9, -8, -7, -6 and -5 code for viral protein of m.w. 37,000, 54,000, 60,000, 66,000 and 83,000 daltons (22), respectively.

An unique transcription by a recycling RNA polymerase is proposed for each of the segmented genomes of CPV. "Circular" or "Pan Handle" structures are predicted for each of the genome segments for efficient transcription which yields equal weights of mRNAs of various chain length.

Reoviridae genome dsRNAs are separated into the complementary strands by 7 M urea-agarose gel electrophoresis. The basis for this separation is due to differences in the conformation of ssRNA which is formed presumably by G-C base pairing. Specific secondary structures are suggested for the (+) strands of CPV and (-) strands of reovirus genome RNA which migrate faster than the corresponding strands of counter polarity. Whether or not these structures are involved in the important functions of these two RNA species, translation for the (+) strand and RNA polymerase binding site for the (-) strand, remains to be clarified.

In any case, the technique for separating complementary strands should prove useful for more general studies on RNA and DNA structure.

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DIFFERENTIAL METHYLATION OF ENDOGENOUS AND ACQUIRED MOUSE MAMMARY TUMOR VIRUS-SPECIFIC DNA[1]

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ABSTRACT Using the restriction endonucleases MspI and HpaII, isoschizomers which distinquish methylated from non-methylated sites (CCGG), and the DNA blotting technique, DNAs from uninfected and mouse mammary tumor virus (MMTV) infected tissues of BALB/c inbred mice were analyzed to determine the 5-methylcytosine content of proviral DNA. Endogenous proviral sequences were found to be extensively methylated while acquired proviruses were not. This hypomethylation was found in infected normal and transformed cells. In addition to the specific hypomethylation of virus-specific sequences, significant hypomethylation of cellular sequences was observed in mammary tumors.

INTRODUCTION

Mouse mammary tumor virus (MMTV), a retrovirus, is transmitted among inbred strains of mice as either a milk-borne (horizontal transmission) or genetic (vertical transmission) agent (1,2). From studies characterizing the virus-specific DNA found in tumors from infected mice (3), it was demonstrated that MMTV-specific proviral DNA can recombine with many sites on the mouse genome and that the orientation of resulting proviral sequences is colinear with that observed with viral RNA. Insertion of proviral DNA at new sites in host cell DNA is a common feature of all mammary tumors arising in virus-infected, inbred mice with the single exception to date of a GR strain mammary tumor in which no proviruses in addition to those transmitted genetically could be found (Cohen and Varmus, in preparation). Recent data revealing that the genetically

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transmitted MMTV proviruses appear to be structurally related to those acquired by milk-borne infection (4) and that MMTV strains commonly transmitted horizontally may also exist as vertically transmitted viruses in some inbred mouse strains (5) indicates that integration of new MMTV proviral DNA greatly enhances its carcinogenic effect. Since tumor incidence among inbred mice lacking the milk-borne virus is usually much less than that of the infected animal (1,2), mammary tumor induction by genetically transmitted MMTV-specific sequences is normally repressed in the mouse. Thus, reinfection and integration are required in most cases for expression of the oncogenic potential of this virus.

The variable tumorigenicity of MMTV proviral sequences could be related to either the normal repression of vertically transmitted proviruses or to the requirement for insertion of the proviral DNA into specific site(s) on the host genome for tumor induction. This latter possibility would suggest that genetically transmitted viruses represent those rare infection events of the germ line that occur in cells resistant to MMTV-induced transforming proteins or which involve insertion of proviruses into regions of the mouse genome not participating in mammary carcinogenesis. At the present time, since mutants of MMTV can not be obtained, it is not possible to determine whether a single virus-specific protein is essential for mammary tumorigenesis. However, the apparent heterogeneity of sites for MMTV integration (3) would argue for the former hypothesis and suggest that an important event in MMTV-induced transformation would be the alteration or exclusion from normal repressor functions of the eucaryotic cell by virus-specific sequences.

In most eucaryotic cells the only modified base is 5-methylcytosine (m^5C which occurs predominantly in the dinucleotide CpG (6,7,8,9,10). Although this modified base in addition to 6-methyladenosine is present in procaryotes and is thought to function in host restriction (11,12), the significance to the eucaryotic cell has yet to be determined. Several investigators (13,14,15) have suggested that methylation could play a role in selective silencing of genes during cellular differentiation. Data supporting this concept was obtained by recent studies demonstrating organ-specific hypomethyl- of avian cells (16,17). Furthermore, determination of m^5C content in rat liver malignancies revealed a significant decrease over that of normal tissue indicating that hypomethylation of cellular sequences was occurring in this tumor (18).

In this study the level of methylation of MMTV proviral sequences acquired by either genetic or milk-borne transmission was evaluated by digestion of DNA extracted from uninfected and infected tissues with restriction endonucleases shown

to be inhibited by m^5C occurring at the cleavage site (19,20). Of these enzymes the isoschisomers HpaII and MspI are the most useful, since the former is inhibited by m^5C while the latter is resistant (20). Thus, by comparison of parallel digestions of these two enzymes one can determine directly the level of methylation of cellular DNAs at the specific cleavage site (5'-CCGG). These restriction endonucleases were used to digest DNA extracted from infected and uninfected tissues of the BALB/c mouse strain. Previous studies have shown this strain to be particularly useful when infected by foster-nursing newborns on virus producing C3H females. In this infected BALB/c substrain, BALB/cfC3H, the liver remains uninfected while the mammary gland and tumors which produce virus have demonstrable new proviruses which may be differentiated by specific restriction fragments (1,2,3). Biologically the BALB/c strain exhibits a very low tumor incidence while the BALB/cfC3H substrain has a tumor incidence approaching 100% by 12 months. Thus, in this relatively simple model the milk-borne proviruses have a definitive role in mammary carcinogenesis and the genetically transmitted proviruses are suppressed.

RESULTS

To determine the extent of methylation of MMTV proviral sequences, DNA extracted from either uninfected normal (BALB/c liver and lactating mammary gland) or infected normal (BALB/cfC3H lactating mammary gland) or transformed (BALB/cfC3H mammary tumors) was digested with either MspI or HpaII (20). After electrophoresis on agarose gels, the digested DNA was transferred to nitrocellulose filters by a modification of the method of Southern (21) as described previously (3). Virus-specific fragments were detected by hybridization with ^{32}P labeled cDNArep synthesized using AMV reverse transcriptase and calf thymus primers followed by autoradiography with intensifying screens (22).

As shown in Fig. 1, DNA from uninfected BALB/c liver (lane A) digested with MspI yielded a series of 10 virus-specific fragments ranging in size from 0.5-7.0kb indicating the presence of multiple sites for cleavage occurring in the endogenous proviruses. However, upon treatment with the isoschizomer HpaII, which is inhibited by methylation at the cleavage site, a single high-molecular weight, virus-specific fraction is observed (lane B). Thus, all sites for MspI-HpaII digestion must be methylated. Similar data was obtained with other mouse strains having different MMTV genotypes (5).

When DNA from BALB/cfC3H infected mammary tumors was

digested with MspI and HpaII (lanes E-H) a result strikingly different from that of uninfected BALB/c liver DNA was obtained. The digestion with the methyl insensitive enzyme, MspI,

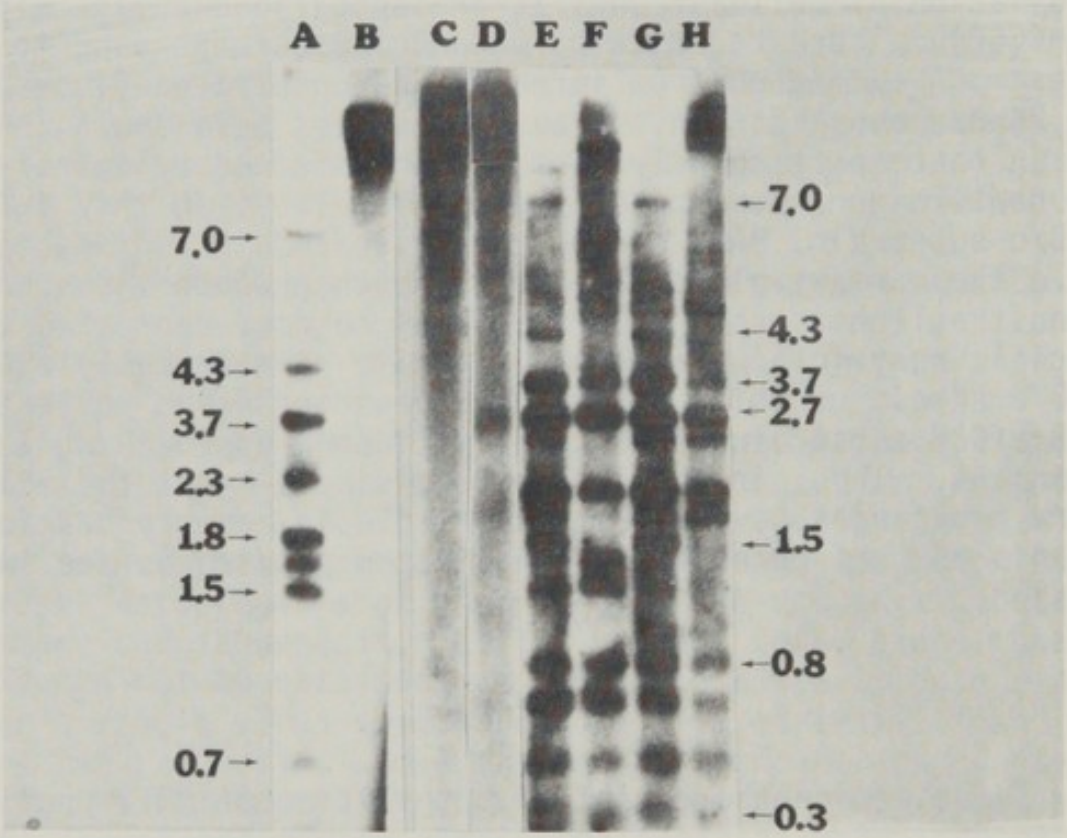

FIGURE 1. Methylation of MMTV proviral DNA. DNA extracted from either BALB/c liver (A and B) and lactating mammary gland (C) or BALB/cfC3H lactating mammary gland (D) or individual mammary tumors (E and F, G and H) was digested with either MspI (A, E, and G) or HpaII (B, C, D, F, and H), analyzed on 1.0% agarose gels and DNA blotted as described previously (3). Sizes (kb) of selected virus-specific fragments are indicated on the left for lanes A and B and on the right for lanes C-H.

produced several virus-specific fragments varying in size from 0.3-0.7kb. Of theses fragments the 2.7, 0.8, and 0.3kb fragments occur in both MspI and HpaII digestions of independently derived mammary tumors and are not present in the digestions of BALB/c liver DNA. Therefore, these three cleavage products must be derived from the milk-borne proviral sequences. Digestion with HpaII (F and H), unlike data obtained with BALB/c liver, produced a series of fragments indicating the presence of unmethylated sites in MMTV proviruses. Of particular interest is the fate of the endogenous versus the

exogenous proviral fragments. Those fragments derived exclusively from acquired proviral sequences (2.7, 0.8, and 0.3) are always present in HpaII digestions. However,fragments common to both MspI digests of DNA from uninfected and infected tissues and thus derived either from endogenous proviruses or shared by both endogenous and acquired proviruses are upon HpaII treatment of tumor DNA either not present (e.g. 7.0, 4.3, and 1.5kb); present in one but not both (e.g. 2.3kb, lane H) or occur in reduced molar concentration (e.g. 3.7, 0.8, 0.7, and 0.5kb). Thus, all virus-specific fragments possibly originating from endogenous proviruses are altered in some way in the HpaII digests of infected mammary tumor DNA. These data are interpreted as indicating that the acquired proviruses are hypomethylated while the endogenous sequences remain methylated in the transformed cell. In support of this conclusion the high molecular weight fraction of HpaII-resistant, MMTV-specific sequences seen with BALB/c liver DNA (B) is also present in digests of BALB/cfC3H mammary tumor DNA with HpaII (F and H).

The specific hypomethylation of exogenous sequences could represent either a functional alteration of methylating activities in the transformed cell or the inability of the cell to normally modify DNA sequences exogenously acquired. To evaluate this question, DNA from uninfected BALB/c (C) and infected BALB/cfC3H (D) lactating mammary gland (tumor-free) was analyzed with HpaII (these lanes overexposed to detect minor bands). As can be seen in Fig. 1, the digests of DNA from BALB/c lactating mammary gland resembled those obtained with BALB/c liver, while DNA from infected BALB/cfC3H mammary gland revealed the presence of the 2.7kb fragment characteristic of the milkborne MMTV proviral DNA. Thus, the specific hypomethylation of acquired viral DNA also is found in a random population of infected, normal cells and must reflect the inability of the cell to methylate acquired proviral sequences.

From the data presented in Fig. 1, one can not distinguish endogenous from acquired proviruses by MspI-HpaII digestion since identical fragments are derived from both. Therefore, the extent of differential methylation can not be evaluated using these endonucleases alone. However, restriction endonuclease mapping studies of MMTV proviruses endogenous to the BALB/c mouse strain (4) have demonstrated the presence of multiple sites of cleavage by the enzyme HpaI, which does not cleave DNA of the milkborne, C3H strain of MMTV. HpaI digestion of DNA from infected BALB/c tissues would effectively distinguish the endogenous proviruses (major HpaI fragments at 7.3, 6.7, and 0.8kb) from the acquired proviruses (minimum size equivalent to genome length of

approximately 10kb), as shown in the HpaI digestions of BALB/cfC3H mammary tumor DNA presented in Fig. 2 (lanes D and (G).

Digestion of DNA from BALB/c liver with MspI in addition to HpaI (B) results in the loss of the major HpaI fragments at 7.3 and 6.7kb (a minor MspI fragment at 7.0kb is present) and produces many of the MspI derived fragments previously seen (Fig. 1, A). HpaI and HpaII double digestion (C),as expected,does not alter the major HpaI virus-specific fragments of the endogenous, MMTV proviruses, although a decrease is noted in the intensity of these bands in this and all double

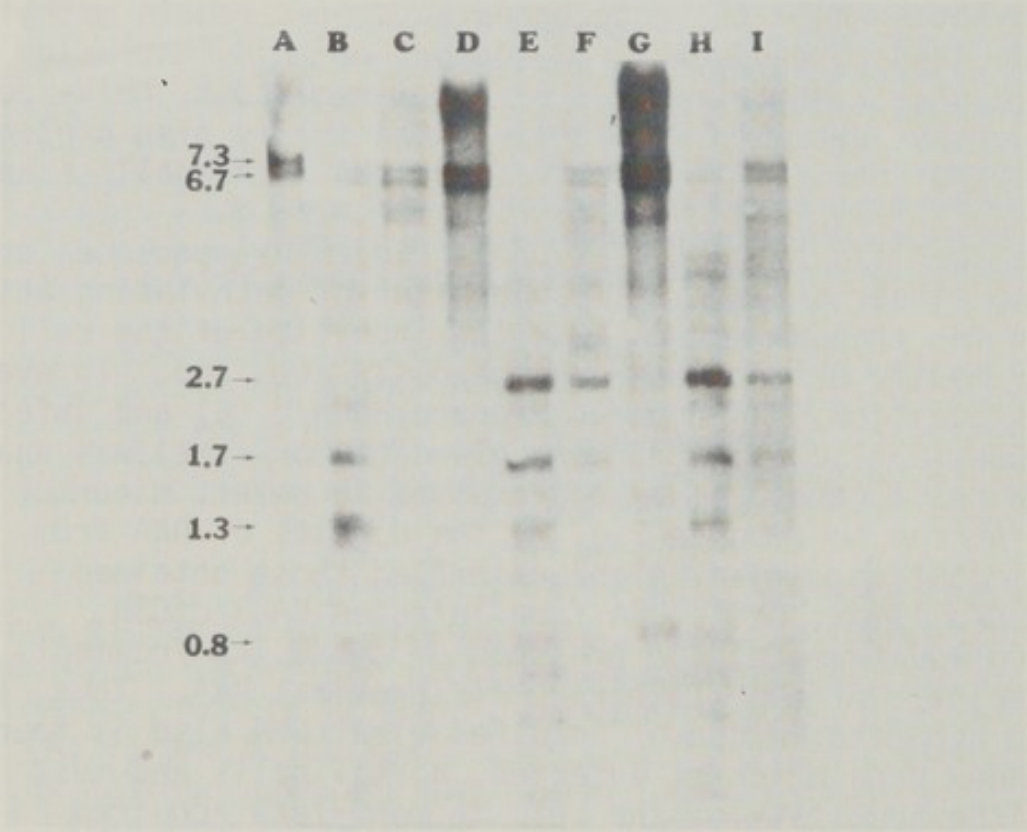

FIGURE 2. Specific hypomethylation of acquired MMTV proviruses. DNA from either BALB/c liver (A-C) or two BALB/cfC3H mammary tumors (D-F and G-I) was digested with either HpaI (A, D, and G) or sequentially with HpaI and MspI (B, E, and H) or HpaI and HpaII (C, F, and I). DNAs were analyzed as described previously (3) and sizes of virus-specific fragments are indicated on the left in kb.

digestions possibly due to contaminating exonuclease activity in the restriction endonuclease preparations. Therefore, all sites for HpaII cleavage occurring in the endogenous proviral sequences defined by HpaI digestion are methylated.

HpaI digests of DNA from BALB/cfC3H mammary tumors (D and G) produced the expected heterogenous set of virus-specific fragments in the region of the gel representing sizes greater than 10kb. In addition the three HpaI fragments of the endogenous proviruses are produced. HpaI and MspI double digests (E and H) resulted in both the MspI fragments characteristic of the acquired proviruses (i.e. 2.7 and 0.3kb) and those derived from endogenous sequences (i.e. 1.3kb). Treatment of tumor DNA with HpaII in addition to HpaI (F and I) as with BALB/c liver DNA did not significantly affect the HpaI fragments of the genetically transmitted proviruses, but did result in the complete digestion of all virus-specific sequences migrating at greater than 10kb). In addition, the 1.3kb fragment seen in double digestion of uninfected BALB/c liver (B) is completely absent from the HpaI, HpaII digested mammary tumor DNA. These results are consistent with the conclusion that endogenous, MMTV-specific proviruses are extensively methylated while the acquired proviruses are not methylated in the infected cell.

TABLE I

m^5C CONTENT OF NORMAL AND TRANSFORMED BALB/c TISSUES[a]

Liver	Lactating Mammary gland	Mammary tumor[b] C3H	Mammary tumor[b] C3Hf
0.94	0.91	0.83	0.82
0.93		0.78	0.73
0.90		0.82	0.80
0.90		0.85	0.80
0.86		0.76	
0.95		0.86	
0.91±0.03[c]		0.82±0.04	0.79±0.04

[a] mole percent

[b] mammary tumors were obtained independently from BALB/c mice infected with MMTV strain from either C3H or C3Hf mice.

[c] Mean±relative standard deviation.

During analysis of MMTV methylation with MspI and HpaII, it was noted that the ethidium bromide fluoresence of electropherograms from mammary tumor DNA showed significantly more digestion with HpaII than those from liver DNA. This suggested that a substantial fraction of tumor DNA sequences were less methylated than normal DNA sequences. To analyze directly the relative content of m^5C in DNA from various mouse mammary tumors and uninfected normal tissues (liver and lactating mammary gland of BALB/c mice), the DNAs were hydrolyzed to deoxymononucleosides with DNaseI, nuclease P1, and E. coli alkaline phosphatase and the digests characterized with a very sensitive, reversed phase, high performance liquid chromatography system (Gehrke and Ehrlich, unpublished results). DNA

samples isolated from 10 different mammary tumors, one lactating mammary gland, and six livers produced no significant difference in the mole% deoxycytidine, deoxyguanosine, deoxythymidine, or deoxyadenosine, since the relative standard deviation for each of these was 1-2%, which was the limit of reproducibility for a given sample. In contrast, there was a small but significant difference ($p < 0.01$) between the content of m^5C of DNA from tumors and that from either normal liver or lactating mammary gland (Table 1). The liver and lactating mammary gland DNAs had a higher percentage of m^5C than all but one of the tumor DNAs. The average m^5C content of tumor DNAs was approximately 10% less than that of liver DNAs.

DISCUSSION

The specific hypomethylation of acquired, MMTV proviruses in the presence of extensive methylation of endogenous sequence was demonstrated in infected normal and transformed tissues of the BALB/c mouse strain. In addition, significant hypomethylation of cellular sequences was found in transformed cells (Table 1). These observations are of possible importance both to the essential replicative steps of the virus and to the transformation event leading to mammary carcinogenesis.

The transcriptional activity of endogenous MMTV sequences of the BALB/c and other inbred strains has been shown to be repressed (24,25). However, upon infection by milkborne virus the synthesis of viral RNA is increased up to 1000 fold. From the work presented here there is an inverse correlation between the level of methylation of MMTV DNA sequences and rate of transcription seen in the mouse cell. A similar relationship has been demonstrated in both adenovirus and herpesvirus infected cells (26,27).

The general hypomethylation of cellular sequences in the transformed cell could represent either an essential step in tumorigenesis or a phenomenon occurring as a consequence of the altered physiological state of the cell. If hypomethylation is an integral part of virus-induced mammary carcinogenesis, then the finding that undermethylation correlates with increased transcriptional activity suggest that derepression of normally repressed cellular genes might occur. If such cellular genes included those abrogating growth control, their activation could be responsible for the transformed phenotype of the cell. This model would imply that quantitative or qualitative alterations of DNA methylase activity or of the accessibility of certain DNA sequences to methylation could be a primary insult in mammary tumorigenesis.

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SFFV SPECIFIC GENE EXPRESSION IN INFECTED AND ERYTHROLEUKEMIA CELLS

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ABSTRACT *Virus from a Friend erythroleukemia cell line producing an excess of the defective spleen focus forming virus (SFFV) was used to isolate SFFV non-producer cell lines. These cell lines together with various Friend erythroleukemia cell lines were analysed for SFFV specific gene expression. The major molecular species precipitating with anti-envelope glycoprotein (gp 70) sera in both erythroid and non-erythroid cell lines is a glycoprotein of approximately 55,000 molecular weight (gp 55). gp 55 type proteins are present in Friend cells transformed by either the anaemia (FV-A) or polycythaemia (FV-P) inducing Friend virus but are not detectable in cells infected solely with the helper virus. A Rauscher virus transformed erythroleukemia cell line (RA-1) and the Friend cell line T3C12 express a related glycoprotein of 52,000 molecular weight (gp 52). The series of SFFV-non producer cell lines obtained in this study are negative for the gag-related gene expression. A correlation was established between the presence of SFFV specific RNA and gp 55 expression. All SFFV non-producer cell lines express genomic 32S RNA whereas only certain non-producers express a sub-genomic RNA of 21S. Cells expressing 21S RNA constitutively synthesize gp 55.*

INTRODUCTION

The Friend virus complex contains a replication defective component, the spleen focus forming virus (SFFV) which induces a rapid proliferative disease resulting in the

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appearance of macroscopic spleen foci and splenomegaly (erythroblastosis) in adult mice (1, 2). Transformed erythropoietic cell lines can be isolated from the spleens of such mice. These cell lines are arrested in maturation but can be induced to differentiate (3, 4, 5). Racevkis and Koch (6) first reported that a 55,000 molecular weight glycoprotein (gp 55) was the major intracellular species precipitable by anti-gp 70 serum in several Friend polycythaemia virus transformed erythroleukemia cell lines. SFFV specific gene expression in cells infected with SFFV in the absence of helper virus, has been studied in several laboratories with somewhat conflicting results (7-12). In an effort to clarify these studies of SFFV gene expression, viral RNA and protein synthesis has been analysed in a series of SFFV non-producer cells derived from direct cloning of Friend virus from an erythroleukemia cell line producing SFFV in excess.

METHODS

Cell culture, labelling and immunoprecipitation. The cell lines used were donated by the investigators involved in their isolation (3, 5, 7, 13-15). Propagation of cells, labelling of cells, immunoprecipitation and polyacrylamide electrophoresis were all performed as described previously (10). The SFFV non-producer clones were derived by infection of SC-1 cells with virus released by Friend cells (10, 16). Table I indicates the biological properties of these SFFV non-producer clones compared with those obtained in other laboratories.

Synthesis of viral cDNAs. Synthesis of cDNAs to the Friend virus (FV) complex and to the SFFV specific sequences has been described previously (16). The SFFV specific cDNA hybridizes to 80% with excess template FV RNA, to 10% with LLV 70S RNA in 100-fold RNA excess and contains sequences related to xenotropic virus.

RNA isolation and hybridisation. Cytoplasmic RNA was mixed with SFFV specific or FV cDNA at a ratio of 1:20,000, hybridised at 60°C for varying periods of time and the degree of hybridisation determined as described previously (10, 16).

Agarose gel electrophoresis and transfer of RNA to DBM paper. Cytoplasmic RNAs were denatured by incubation at 50°C in the presence of 50% DMSO, 1*M* glyoxal and subjected to electrophoresis in 1% agarose. The RNA was then

TABLE I

EXPRESSION OF VIRAL PROTEINS IN SFFV

NON-PRODUCER CELLS

SFFV type	$SFFV^{+}$ LLV^{-} cell line	SFFV titre following rescue	p15	p12	p30	gp55
FV-P (SL)[a]	NRK clone 1	high	yes	yes	no	yes
FV-P (A)[b]	NIH 3T3 NP 9	high	yes	no	no	not reported
	NRK NP 502	high	yes	no	no	
	NIH 3T3 NP 215	high	no	no	no	no
FV-P (M)[c]	6S26	moderate - high	no	no	no	yes
	SC 204	low	no	no	no	no
	SC 204 on rescue	low	yes	yes	yes	yes

[a] FV-P (SL) lines derived from FV-P obtained from Dr. R. A. Steeves
[b] FV-P (A) cell lines kindly supplied by Dr. A. Bernstein (Toronto)
[c] Using FV-P obtained from Dr. A. Axelrad (Toronto)

partially degraded, and transferred from the gel to activated diazobenzyloxymethyl (DBM) paper (17). Pre-hybridisation, hybridisation with a (^{32}P-) FV cDNA and conditions for washing and autoradiography have been described elsewhere (10, 17 and 18).

RESULTS

Expression of a modified glycoprotein in Friend erythroleukemia and SFFV non-producer cell lines. Previous studies of virus specific protein synthesis in Friend cells have shown that a 55K glycoprotein is the major species precipitable by anti-gp 70 serum (6, 8 and 19). Figure 1

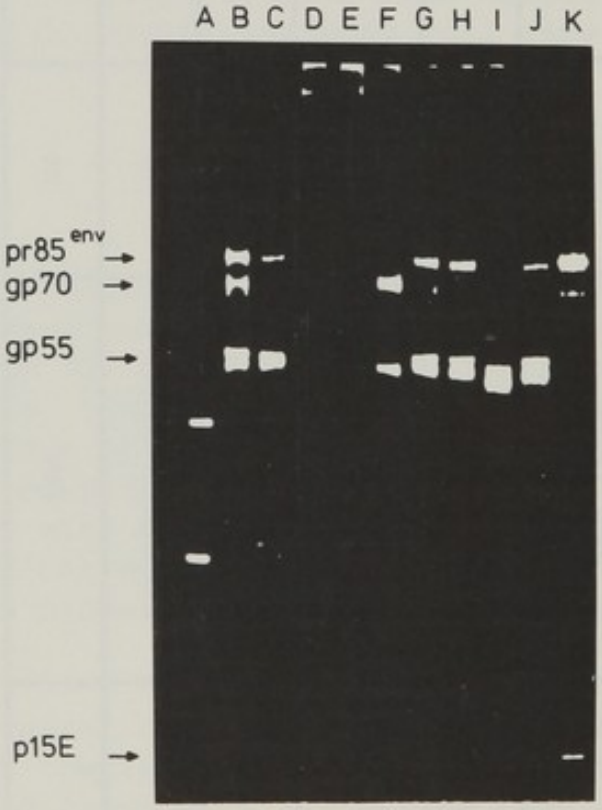

Fig. 1. Immunoprecipitation analysis of erythroleukemia cell extracts. ^{35}S-menthionine labelled cell extracts were immuno-precipitated with anti-Friend virus gp 70 sera as described in the Methods. Lane A, vesicular stomatitis virus (VSV) structural proteins markers of 195K, 66K, 48K, 29K in descending order. Erythroleukemia cell extracts pulse labelled for 30', lane B, F4-6; C, 6D4A1; D, D5A1; E, RED-2; F, RA-1; G, F4N; H, 745a; I, T3C12; J, G1; K, G2.

extends this observation to a number of other erythroleukemia cell lines transformed by the Friend anaemia virus (FV-A), Friend polycythaemia virus (FV-P), or by Rauscher virus.

All Friend and Rauscher cell lines tested were found to contain a gp70 related 52-55K protein whereas the rat erythroleukemia cell line RED-2 and its azaguanine-resistant derivative clone D5A1 lack all murine virus related proteins including gp 55 (Figure 1). Examination of (^{35}S-) methionine labelled SFFV non-producer extracts precipitated with monospecific antisera to Friend or Balb/c xenotropic virus gp70 demonstrated that several such SFFV non-producer clones constitutively expressed gp 55 in the absence of pr 85^{env} or gp 70 expression (Figure 2). No gp 55 expression was observed in uninfected SC-1 cells. Certain SFFV non-

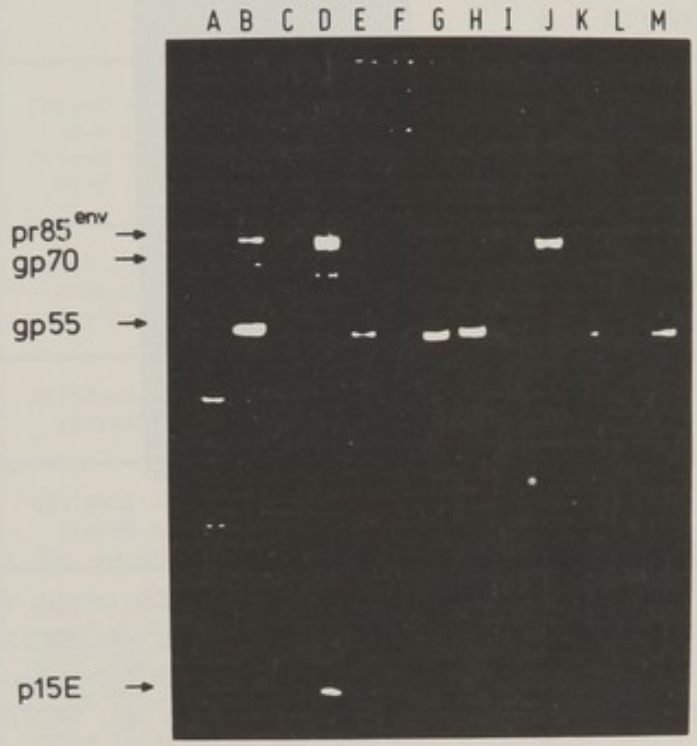

Fig. 2. Anti-gp70 immunoprecipitates of SFFV non-producer cell extracts. Cell extracts from control and non-producer cells were immunoprecipitated with antisera against either Friend gp70 (B-G) or Balb/e xenotropic virus gp70 (H-M). Labelled VSV proteins lane A. Cell extracts: lanes (B,H), F4-6 cells; (C,I) SC-1 cells; (D,J) 643/22; (E,K) 6S21; (F,L) 6S23; (G,M) 6S26.

producer cell lines do not express gp 55, e.g. SC204 (Figure 3). Infection of this cell line with either Gross or Moloney helper viruses or induction with BUdR, however, results in the induction of gp 55 synthesis (Figure 3, Lanes B and C). No gp 55 was observed in virus from

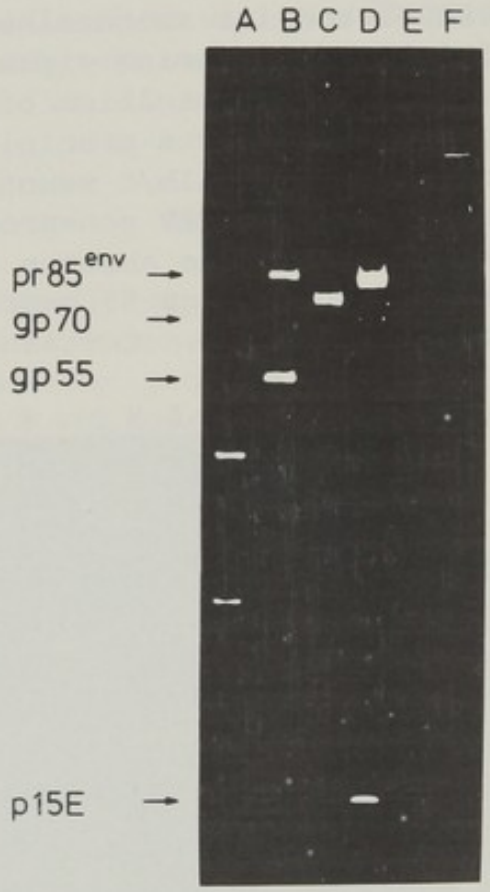

Fig. 3. Expression of gp55 during rescue of SC204 cells. ^{35}S-methionine labeled cell extracts were immunoprecipitated with anti-Friend virus gp70 as described. Lane A, VSV proteins. Cell extracts: B, SC204 superinfected with G-MuLV; C, SC204 + Mo-MuLV; D, 643/22; E, SC204; F, SC1.

uninduced or induced Friend cells, from rescued SFFV non-producers, nor in preparations of Gross, Moloney, Rauscher or MCF virus. Our SC-1 series of SFFV non-producer lines was completely negative for *gag* gene related expression as assayed by either radioimmunoprecipitation or radioimmunoassay (data not shown).

SFFV specific RNA sequences in Friend erythroleukemia cell lines. The levels of cytoplasmic RNA hybridising to either FV cDNA or specifically to SFFV cDNA were determined in various Friend and rat erythroleukemia cell lines (Table II). Irrespective of whether transformed by FV-A (Gl, FM2) or FV-P (F4-6, T3Cl2) all Friend virus transformed cell lines express similar levels of viral RNA and RNA hybridising to a Friend SFFV specific probe (Figure 4).

TABLE II

EXPRESSION OF FRIEND VIRUS AND SFFV SPECIFIC SEQUENCES IN ERYTHROLEUKEMIA CELLS AND IN SFFV$^+$ LLV$^-$ CELL LINES

Cell line	Origin (reference)	SFFU/ml	% of total cytoplasmic RNA hybridizable to	
			FV cDNA[a]	SFFV cDNA[a]
F4-6	Friend erythroleukemia FV-P induced	1.2×10^4	0.056 (1.0)	0.052 (0.52)
T3C12	Friend erythroleukemia FV-P induced	0	0.041	0.052
C-1	Fetal liver cell transformed by FV-A erythroid	0	0.87	0.052
SC1	Uninfected mouse fibroblast	0	0	0
643/22	SC1 cell infected with cloned LLV	0	0.56	0
6S21	SFFV$^+$ LLV$^-$ SC1	3×10^3[b]	0.009	0.005
6S26	SFFV$^+$ LLV$^-$ SC1	1.5×10^3	0.01 (0.051)	0.0096
6S23	SFFV$^+$ LLV$^-$ SC1	3×10^2	0	0
6S9	SFFV$^+$ LLV$^-$ SC1	5×10^2	0	NT
SC204	SFFV$^+$ LLV$^-$ SC1	9×10^2	0 (0.0035)	0
SC204+M-MuLV	SC204 infected with Moloney MuLV	2×10^2	NT	0.0011
NP215	SFFV$^+$·LLV$^-$ NIH-3T3	NT	0.005	NT
D5A1	Rat erythroleukemia chemically induced	0	0	0

[a] Figures in parentheses correspond to percentage of respective cDNA probes hybridising to polysomal poly A(+) RNA for the cells indicated.
[b] The SFFV titres indicated are that of parallel non-producer cultures superinfected with virus from 643/22 cells.

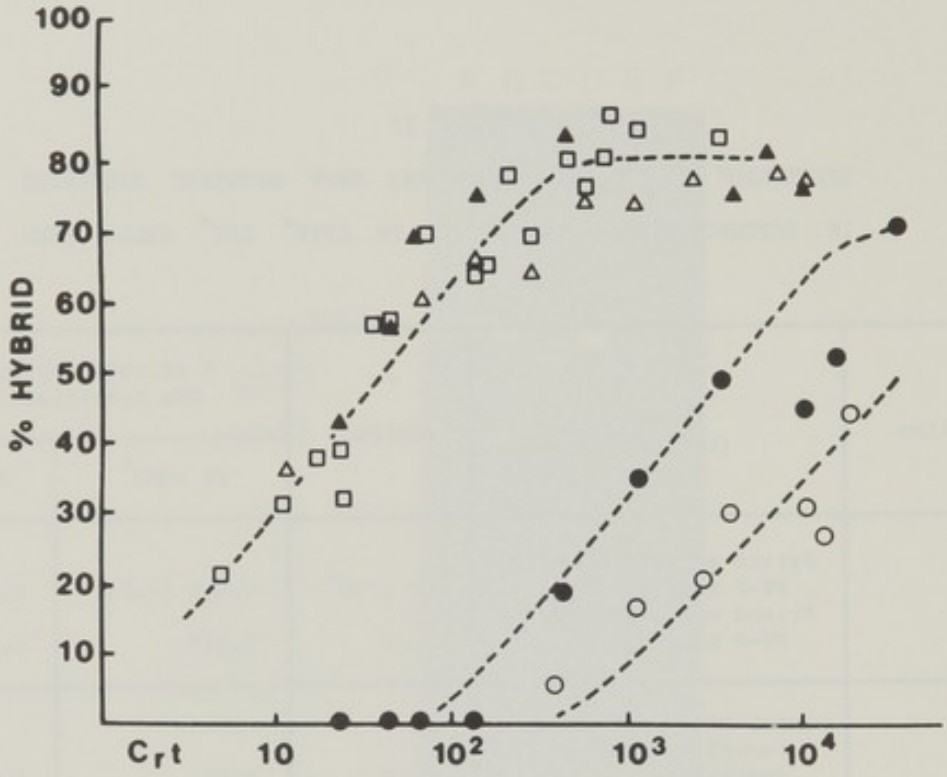

Fig. 4. Levels of SFFV specific RNA in cytoplasmic RNA from erythroleukemia and SFFV$^+$ LLV$^-$ non-producer cells. SFFV specific cDNA was prepared and hybridised to polysomal poly A$^+$ RNA from the following cell lines: F4-6 ( ); G1 (Δ); T3C12 (Δ); SC204 (O); SC204 + MoMuLV (O).

Expression of SFFV specific sequences in SFFV non-producer cell lines. The SFFV non-producer cell lines were examined also for levels of cytoplasmic RNA related to both the Friend virus complex and specifically to the SFFV component of this complex. Closely similar levels of Friend virus related and of SFFV specific sequences were found in each non-producer cell line examined (Table II), suggesting the absence of helper virus genomes in these cell lines. The SFFV non-producer lines varied with respect to the levels of SFFV specific RNA expressed as follows:

(a) Low or non-expressor clones such as 6S23, SC204 or NP215 and
(b) High producers such as 6S26.

gp 55 was only detected by radioimmunoprecipitation in those cell lines which expressed high levels of SFFV specific RNA (Table I, Figure 5). Rescue of one of the low level

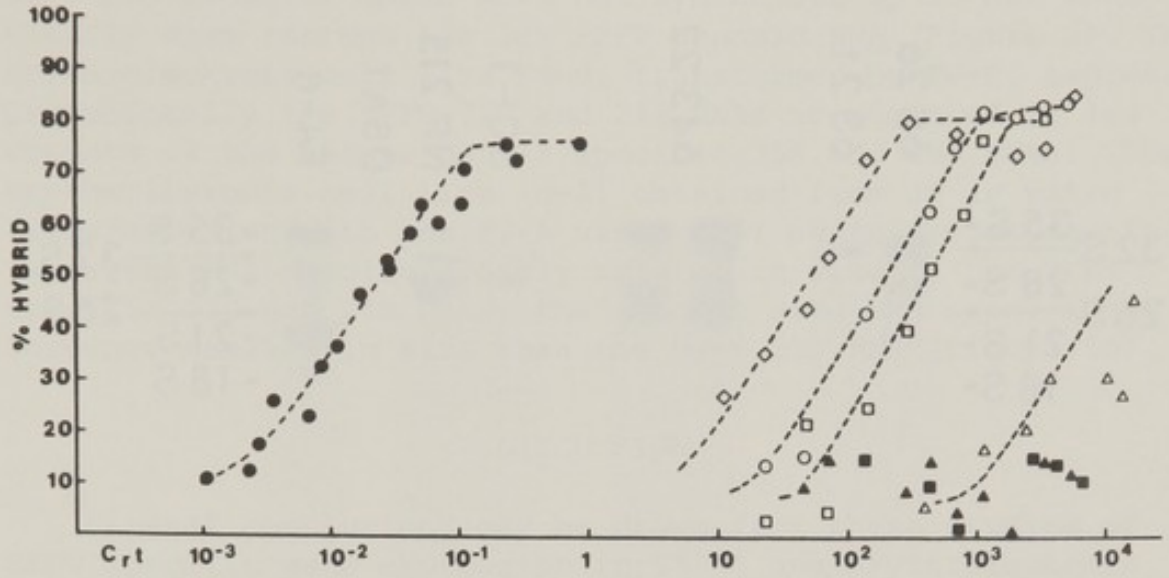

Fig. 5. Levels of SFFV specific RNA in cytoplasmic RNA from control and SFFV$^+$ LLV$^-$ non-producer cells. SFFV specific cDNA was prepared and hybridised to 70S viral RNA (●) and to polysomal poly A$^+$ RNA from the following cell lines: SC-1 (▪); 643/22 (▲); 6S21 (▫); 6S26 (O); 6D5A1 (◇); SC204 (Δ).

SFFV RNA cell lines (SC204) with Moloney LLV helper virus led to an increase in SFFV specific cytoplasmic RNA sequences and identification of gp 55 in immunoprecipitates (Figure 4 and Table II).

Size characterisation of LLV-F and SFFV specific viral RNAs in SFFV non-producers and Friend erythroleukemia cell lines. Two major species of virus specific RNA were detected in a Friend helper virus (LLV-F) infected SC-1 cell line 643/22 (Figure 6) when glyoxalated cytoplasmic RNA was

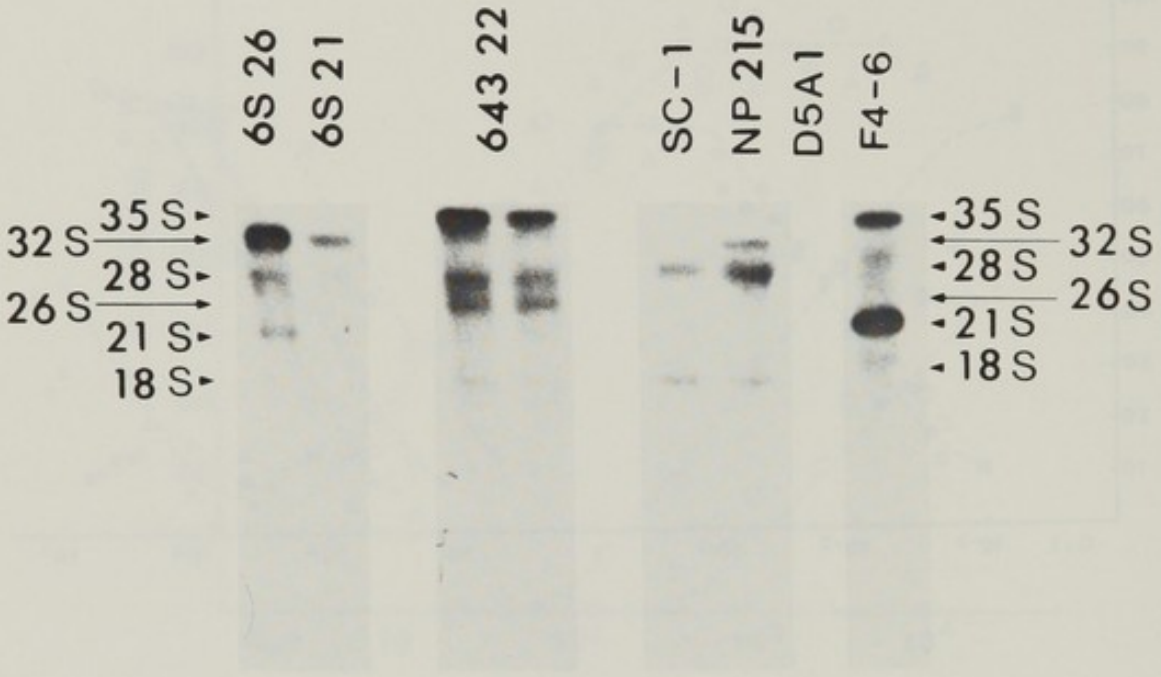

Fig. 6. Analysis of cytoplasmic RNA of Friend virus infected cells. Autoradiograph of Friend virus related cytoplasmic RNA separated in agarose gels. 5 µg of cytoplasmic RNA from the indicated cell lines were transferred to diazobenzyloxymethyl paper and hybridised with ^{32}P-FV cDNA. 6S21, 6S26 and NP215[11] are SFFV-non-producer cell lines, 643/22 is a cloned line releasing LLV-F. SC-1 cells are the uninfected fibroblasts used to produce the 6S series of SFFV non-producers. F4-6 is an FV-P erythroleukemia line[7] and D5A1 a rat erythroleukemia cell line[13]. The autoradiograph panel containing RNA from the SC-1 control cells and the NP215 cell line has been exposed for a four-fold longer time period to demonstrate the low amount of 32S RNA present in the NP215 line.

fractionated on 1% agarose gels and viral molecules identified by hybridisation after transfer to activated DBM paper. 643/22 contained 35S RNA and a subgenomic species of approximately 26S which, by analogy with the 23S RNA found in Moloney and Rauscher MuLV infected cells (20, 21), is a candidate for the subgenomic message for the *env* precursor of MuLV-F (gp 85). In spite of extensive purification of the 30-35S viral RNA template, some minor ribosomal RNA contamination is nevertheless transcribed in cDNA, resulting in the detection of 28S and 18S RNA species by our FV cDNA. The SFFV$^+$ LLV$^-$ non-producer cell lines 6S21 and 6S26, which express high levels of gp 55, synthesize correspondingly large amounts of SFFV specific RNA. This SFFV-RNA is present in two major size classes of RNA,

32S genomic size RNA and a subgenomic RNA species of 21-22S. This 21S SFFV-RNA is not found in NP-215 cell line, a $SFFV^+$ LLV^- non-producer which does not synthesize gp 55 but which clearly does express the 32S SFFV genomic RNA (Figure 6). The erythroleukemia cell line F4-6, transformed by FV-P, expresses predominantly the SFFV 32S and 21S RNAs as compared to low amounts of the helper MuLV-F specific 35S and 26S RNAs. The erythroleukemia cell line (G-1) obtained from an *in vitro* transformation with the FV-A virus (15) again predominantly expresses RNA species closely related in size to the SFFV specific 32S and 21S RNAs; the genomic size RNA appearing somewhat smaller in size than the F4-6 32S RNA (Figure 10).

DISCUSSION

Several conclusions may be drawn from these studies of SFFV specific gene expression in S^+ L^- and erythroleukemia cell lines. The genomic RNA of SFFV is 32S and is expressed in all Friend and Rauscher erythroleukemia cells and also in all SFFV S^+ L^- non-producer cell lines tested. The SFFV provirus, however, also expresses a major sub-genomic RNA of 21S which is present in all erythroleukemias tested but in only some S^+ L^- SFFV non-producers. All Friend and Rauscher cell lines examined express an envelope related gp 52-55 glycoprotein as a major cellular protein. The expression of gp 52-55 is linked to the presence of the SFFV specific sub-genomic 21S RNA species as certain SFFV S^+ L^- non-producer cell lines contain only the SFFV sub-genomic 21S RNA but still express gp 55. Furthermore, cell lines which do not express 21S RNA do not synthesise detectable amounts of gp 55. Also the relative levels of SFFV specific RNA and in particular 21S RNA in different non-producer cell lines appear to correlate with approximately equivalent levels of gp 55 expression. The 32S RNA found in cytoplasmic extracts of the $SFFV^+$ LLV^- non-producers and Friend erythroleukemias is indistinguishable in size from the sub-unit genomic SFFV RNA identified in virions released from DMSO induced Friend cells (11, 12, 13); furthermore such RNA does not appear to direct the synthesis of gp 55 in *in vitro* translation systems (Bilello, unpublished). Taken together these observations suggest that gp 55 is encoded for by the 21S sub-genomic SFFV RNA. The provirus of the MuLV-F helper also expresses two major size classes of RNA, a genomic size RNA of 35S and a sub-genomic RNA of approximately 26S.

Erythroleukemia cells transformed either by FV-P, FV-A or Rauscher virus all express higher levels of the SFFV-related RNA species than of RNA molecules related to the helper MuLV-F RNA. The clear correlation of expression of

SFFV RNA and a gp 55-type *env* related gene product in these erythroleukemias and not in untransformed or chemically-transformed erythroleukemias such as RED-2 and D5A1, strongly suggests an involvement of this gene product in the maintenance of the "transformed" state of the cells that gave rise to these cell lines. Although FV-A, FV-P and Rauscher virus all initiate a distinctly different disease pattern in susceptible mice, this is not reflected in any obvious differences in levels or type of SFFV or LLV specific gene expression in cells transformed by these viruses. Presumably the specificity resides in the particular modification of the gp 52-55 molecule present in cells transformed by these different viruses.

Detailed studies of the SFFV specific RNAs, their modified *env* gene products and the sites of action of these different moieties in the target cell are obviously required to further elucidate the role of these viral gene products in the induction of proliferation and transformation.

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MOLECULAR GENETICS AND CELL CULTURE ASSAYS FOR HELPER-INDEPENDENT AND REPLICATION-DEFECTIVE COMPONENTS OF THE FRIEND VIRUS COMPLEX

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ABSTRACT From the Friend virus complex, the helper-independent component, F-MuLV, and the replication-defective component, SFFV, have been biologically cloned, and subsequently molecularly cloned in bacterial vectors. The molecularly cloned forms of each virus are infectious in gene transfer studies. F-MuLV induces an erythropoietic proliferative disease with anemia in newborn Swiss mice, while SFFV determines a polycythemic erythroblastosis in adult mice. By molecular cloning techniques, we have identified that the 3' 3.1 Kb of F-MuLV carries the disease-inducing gene(s) of F-MuLV. Cell culture conditions have been developed which allow long-term erythropoiesis in Dexter bone-marrow cultures, and effects of F-MuLV and of SFFV have been observed in this culture system. The results suggest for the first time a cell culture assay for the growth promoting effects on hematopoietic cells of helper-independent type-C leukemia viruses.

INTRODUCTION

The Friend murine leukemia virus, discovered in 1957 by Dr. Charlotte Friend, has been widely used to study both mechanisms of viral-induced leukemia and mechanisms of erythroid differentiation. Several cell lines have been isolated either from diseased tissues of mice infected with the Friend virus complex, or from infections of hematopoietic cells in cell culture with Friend virus complex. A variety of different cell lines have been isolated from such infections:

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(1) tumor cells with erythroid potential which can be induced in cell culture to differentiate along the erythroid pathway (1); (2) nonmalignant cells with granulocyte potential with the properites of normal CFU-C; (3) nonmalignant cells with the characteristics of pluripotent stem cells, CFU_S; and (4) malignant myeloid cells which appear to produce a myeloid leukemia when transplanted to susceptible mice (2,3). Although much useful information has been gained from these studies, certain problems have existed which have hampered a more detailed understanding of the mechanisms of diseases induced by the Friend virus complex. First, the Friend virus complex was passaged in mice for several years to preserve the leukemogenicity of viral stocks, and such stocks contained complex mixtures of both helper-independent and replication-defective viruses. Thus it was difficult to be certain which component(s) of the complex were responsible for the observed biological effects in vivo or in vitro.

Over the past four years, we have been studying the virology and molecular biology of the Friend virus complex. We first biologically cloned individual viral components present in several independently passaged stocks of Friend virus, and tested the biological activity in mice of each component (4, 5). We analyzed the molecular structure of a replication-defective virus, spleen focus forming virus (SFFV), found in all mouse-passaged Friend virus stocks (6). More recently, we have molecularly cloned two viruses with differing disease-inducing properties and have begun to analyze which viral gene(s) are responsible for the disease syndromes (7,8). We report, herein, studies which have begun to define for SFFV and F-MuLV, a helper independent virus present in all Friend virus stocks, which gene(s) are responsible for their disease-inducing properties. In addition, we report the development of an in vitro cell culture system which can be used as an assay for the growth-promoting properties of both SFFV and F-MuLV.

RESULTS

A sumary of the viruses found in several different stocks of Friend virus is shown in Table 1. There are three major ways to classify the viruses biologically cloned from these different virus stocks. (1) Ability to replicate: There are replication-defective viruses which can replicate only with the aid of a helper virus. These are called spleen focus forming viruses or SFFV. There are helper-independent viruses called F-MuLV, Friend murine leukemia virus. There are also envelope gene recombinant viruses (9) Friend MCF viruses (5).

TABLE 1
BIOLOGICAL PROPERTIES OF FRIEND VIRUS COMPONENTS

Virus	Properties		
	Replication	Pathogenicity	Hematocrit
Anemia-inducing			
F-MuLV	Competent	Newborn Swiss or BALB/c	<25
$SFFV_{FVA}$	Defective	Adult Swiss or DBA/2	38-45
Polycythemia inducing			
SFFV Lilly-Steeves	Defective	Adult Swiss or DBA/2	>55
SFFV Mirand	Defective	Adult Swiss or DBA/2	>55
SFFV Axelrad	Defective	Adult Swiss or DBA/2	>55

TABLE 1. The viruses were biologically cloned by methods detailed (5,10). The source of different Friend virus stocks is also given in those manuscripts.

(2) Ability to induce disease in certain strains of newborn or adult mice: The replication-defective viruses can be pseudotyped with a non-Friend helper virus such as Moloney virus and will cause a rapid erythroblastosis when this complex is injected into newborn or adult mice. The helper-independent virus, F-MuLV, will induce rapid disease (3-4 weeks) only in certain strains of newborn mice (Swiss or BALB/c) but not in adult mice. In addition certain mouse strains such as DBA/2 are resistant even as newborns to rapid disease induction by F-MuLV but are susceptible to disease induction by SFFV as adults or newborns. (3) Kind of disease induced: The helper-independent virus causes an erythroproliferative disease in newborn Swiss mice characterized by normal lymph nodes and thymus, enlarged livers and spleens, and profound anemia. The replication-defective SFFV determines either an erythroblastosis and polycythemia, or an erythroblastosis with normal

to slightly low hematocrits. In addition, all the SFFV viruses produce characteristic splenic foci on the surface of the spleens of susceptible mice injected with SFFV and an appropriate helper virus. The target cell for SFFV seems to be a late-stage BFU_E (11) cell, whereas the target cell for F-MuLV has not been determined.

Although both the helper-independent virus, F-MuLV, and the replication-defective virus, SFFV, cause rapid proliferation of hematopoietic precursor cells, neither virus has any growth-promoting effect on undifferentiated cells in cell culture. Thus both components of the Friend complex, although they cause rapid disease, differ from other retroviruses which can be classified as rapidly oncogenic. The biological differences as well as the molecular differences between different retroviruses which rapidly induce cellular proliferation are summarized in Table 2. Class one is currently composed of two viruses, Rous sarcoma virus and Moloney sarcoma virus. Both viruses have their transforming gene, src, located at the 3' end of the virus. The gene is a unique sequence cellular gene acquired by recombination, and the protein product of that src gene is synthesized from a subgenomic mRNA. Class two is a broader group of viruses. These viruses apparently have their onc genes located at the 5' end of their genomes. Their onc genes are again unique sequence cellular genes, acquired by recombination. As far as is known, the onc protein in each case is made from an mRNA, which is essentially the size of the viral genomic RNA. The wild type strains of these viruses can transform fibroblasts in cell culture as well as hematopoietic precursors in susceptible hosts. There are differences between the viruses, such as the precise location of the onc genes at the 5' end of their genomes and the way in which the onc genes are fused to the helper viral genes. There may be other differences which will emerge in further study of these viruses. However, at this point in time, they seem to share many properties. The last class of virus is the spleen focus forming virus, SFFV. In studies on the molecular biology of the SFFV genome, no evidence has been attained for the existence of a unique sequence cellular gene in SFFV (6,12). The candidate onc protein of SFFV is a env gene recombinant glycoprotein, gp52 (13,14) (Table 3). The gp52 is synthesized from a gene located at the 3' end of the SFFV genome, from a subgenomic mRNA. All strains of SFFV code for a gp52-like molecule related to the gp70s of a novel class of murine retroviruses, called mink cell focus inducing viruses, or MCF viruses (9). No phosphotein candidate onc gene product has been detected in SFFV infected cells. Thus SFFV seems to be a unique retrovirus. It determines a rapid

TABLE 2
CLASSES OF RAPIDLY ONCOGENIC TYPE-C RETROVIRUSES

Virus	Properties	
	Biological	Molecular
C Region Recombinants		
RSV	Helper-Independent	src 3' location subgenomic mRNA
Mo-SV	Replication-Defective	src3' location subgenomic mRNA variable 5' gag products src unique sequence gene
Gag Region Recombinants		
Abelson, MC29, AEV Fe-SV, Ra-SV	Replication-Defective Transform certain differentiated cells and fibroblasts	Putative onc 5' location only genomic size mRNA known gag-polyproteins
Ki-SV, Ha-SV		onc 5' location not gag linked onc unique sequence gene
Env Gene Recombinants		
SFFV	Replication-Defective Transformed only certain hematopoietic target cells Not fibroblasts	Subgenomic mRNA constancy - 3' end (gp52) variable 5' gag proteins no known unique sequence gene

erythroproliferative disease with no effect on fibroblasts and no apparent unique sequence gene. Perhaps SFFV does not induce malignant proliferation of cells in the same way that group one or group two viruses do. Clearly, future studies will be necessary to determine if SFFV remains as a unique class of retrovirus, or if SFFV belongs in group two viruses when its onc gene is more precisely defined.

TABLE 3
PROPERTIES OF SFFV GP52

1. Specific relationship to MCF viral gp70 molecules
   a) Distinct from known xenotropic virus gp70 molecules
   b) Tryptic peptides and serological analysis
2. Deleted at 3' end of the env gene
   a) Lacks p15E in env precursor
   b) Lacks group specific and interspecies determinants present on gp70
3. Not released from infected cells
   a) Not secreted
   b) Not in pseudotype virions
   c) IS on cell surface
4. Common to all SFFV strains: Polycythemic or Anemic SFFV Lilly-Steeves; Mirand; Axelrad; Friend, present in all Friend cell lines
5. Made from subgenomic mRNA

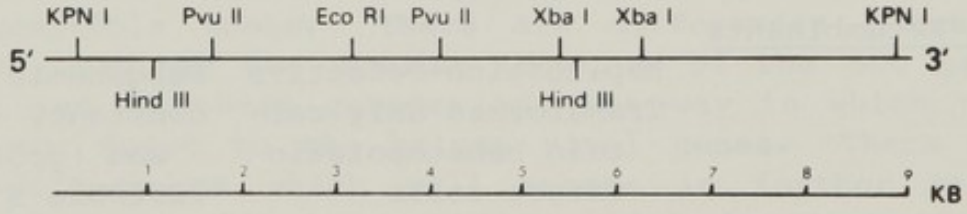

FIGURE 1. Restriction map of F-MuLV clone 201 linear DNA. Schematic representation of the restriction enzyme map of the F-MuLV clone 201 in vivo linear DNA molecules using EcoRI, KpnI, XbaI, HindIII and PvuII. Relative distances between enzyme sites were obtained from the analysis of restriction fragment sizes. The 3'-5' orientation is deduced from the autoradiograph of the in vivo linear DNA after digestion with EcoRI-KpnI, HindIII-KpnI, and XbaI-KpnI, and subsequent hybridization to the 3' F-MuLV probe.

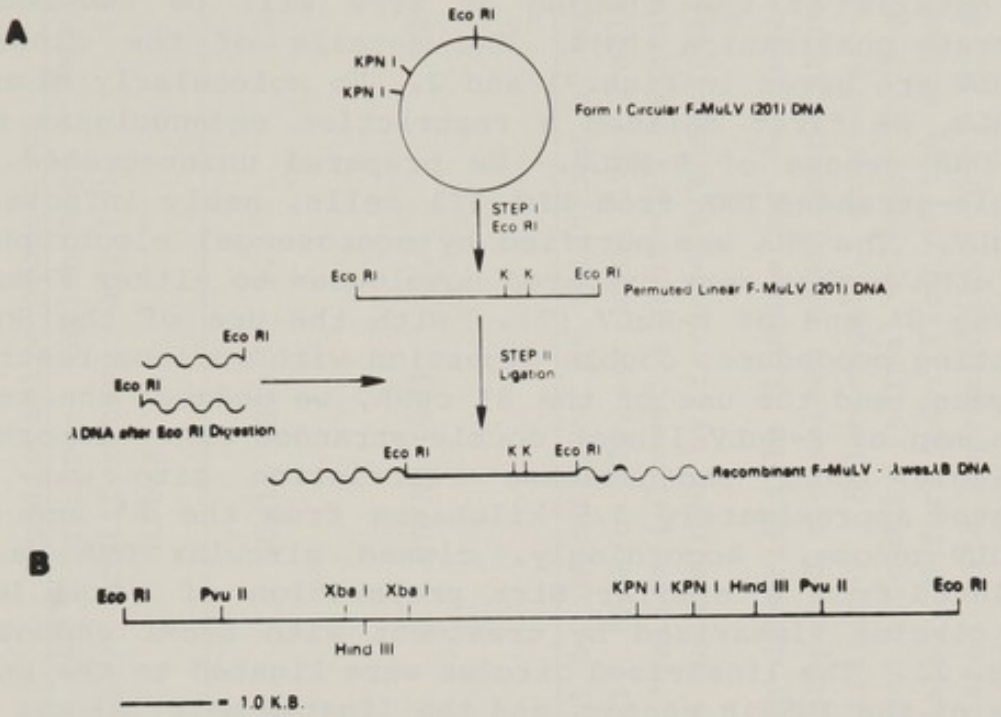

FIGURE 2. Molecular cloning procedure. (A) The schematic model for the molecular cloning of F-MuLV clone 201 DNA begins with the isolation of form I circular DNA corresponding to the linear DNA from the low-molecular-weight Hirt DNA. These molecules have a single EcoRI restriction enzyme site. Step I involves the cleavage of these circular molecules with EcoRI enzyme, which then generates a permuted linear F-MuLV clone 201 DNA molecule with EcoRI sequences at both ends of the molecule. These permuted linear DNA molecules are then mixed with the EcoRI arms of λgtWES•λB DNA, which also have EcoRI-specific sequences at one end of each molecule. In step II, the permuted F-MuLV DNA and the EcoRI-digested λgtWES•λB DNA molecules are ligated together at their EcoRI-specific ends, generating the λ-F-MuLV recombinant DNA. (B) The restriction enzyme map of the permuted linear F-MuLV clone 201 DNA molecule is shown with EcoRI, XbaI, HindIII, PvuII, and KpnI as predicted from the actual restriction enzyme map of the in vivo linear DNA molecules of F-MuLV clone 201. The permuted map was generated from the in vivo map by joining the two ends of the in vivo linear map, forming a circular map, and then opening this circularized map at the EcoRI site, forming a new (permuted) linear map.

In order to further understand the F-MuLV and SFFV induced diseases, we have molecularly cloned each virus (7,8). The details of the cloning of SFFV will be covered in a separate publication (8). The details of the cloning of F-MuLV are given in Figs. 1 and 2. To molecularly clone F-MuLV, we first defined a restriction endonuclease map on the DNA genome of F-MuLV. We prepared unintegrated linear double-stranded DNA from NIH 3T3 cells, newly infected with F-MuLV. The DNA was purified by agarose gel electrophoresis and cDNA probes were prepared homologous to either F-MuLV, or to the 3' end of F-MuLV (7). With the use of the Southern blotting procedure, double digestion with various restriction enzymes, and the use of the 3' cDNA, we deduced the restriction map of F-MuLV linear double-stranded DNA. Importantly, a single EcoRI endonuclease restriction site was found, located approximately 3.5 kilobases from the 5' end of the F-MuLV genome. Accordingly, closed circular DNA was then isolated from a similar Hirt preparation of viral DNA and the circles linearized by treatment with EcoRI endonuclease (Fig. 2). The linearized circles were ligated to the purified arms of the λWESλB vector, and the ligated material was transfected using *in vitro* packaging methods into a certified host. Molecular clones were detected by the Davis filter hybridization procedure, and the positive colonies amplified and analyzed. Eight molecular clones were identified; one of them, a clone 8.5 kb long, was found to be infectious when the viral DNA was released from the vector and transfected onto NIH 3T3 fibroblasts (7). Since the virus, F-MuLV clone 57, recovered from the transfection had been molecularly cloned, we tested it for its disease-inducing properties in newborn Swiss mice. The results are shown in Table 4. F-MuLV clone 57 behaved in an identical manner to the starting virus, F-MuLV clone 201. In 3-4 weeks, infected newborn Swiss mice developed anemia with enlarged spleens and livers with no enlarged lymph nodes or thymus. Morphological analysis of the spleens revealed abundant early hematopoietic precursors which could be classified as blasts or erythroblasts, and many maturing normoblasts. Despite this partial erythroid maturation, the mice became severely anemic, and the virus thus seems to cause an erythroproliferative disease with a defect in the terminal stages of erythroid differentiation. Further characterization of the disease is in progress. However, the effects of F-MuLV *in vivo* suggest that the differentiation block seen in Friend leukemia cells *in vitro* may be caused by F-MuLV present in the cells rather than the SFFV which is also present.

In order to identify which portions of F-MuLV (and SFFV) were responsible for inducing the erythroproliferative

TABLE 4

COMPARISON OF THE BIOLOGICAL PROPERTIES OF VIRAL CLONE F-MULV (201) VERSUS F-MULV (57)

Viral clone	Host range titer (PFU/ml)[a]			Proliferative properties			
	NIH 3T3 cells	BALB/c 3T3 cells	Mink lung cells	Latency period (wk)	Erythroproliferative disease	Splenomegaly (g)	Hematocrit (%)
F-MuLV 201 biological isolate	$10^{6.0}$	$10^{6.2}$	$<10^{0.3}$	3-4	10/12 (83)[b]	1.2	22-34
F-MuLV 57 molecularly cloned isolate	$10^{6.1}$	$10^{5.9}$	$<10^{0.3}$	3-4	28/30 (94)	1.4	14-36

[a] Host range of each viral isolate was ascertained by inoculation of dilutions of each virus into 60-mm culture dishes containing 10^5 NIH 3T3, BALB/c 3T3, or mink lung cells. Mouse cells were assayed for virus by the XC test. Titration followed single-hit kinetics on both mouse cell lines. Titers for mink cells were determined by reverse transcriptase assay.

[b] The percentage of inoculated animals developing identifiable disease by 4-5 weeks postinoculation, when the animals were sacrificed and autopsied and spleen touch preparations were made to assess for hematopoietic cellular infiltration. Splenomegaly, expressed in grams, is the average weight of the spleens of the diseased mice only (normal spleen = 0.09 g). Hematocrits were obtained by ophthalmic puncture immediately before sacrifice of the animals (normal hematocrit = 42 to 46%). All mice were inoculated with approximately $10^{5.0}$ XC PFU of each virus preparation. Inoculations were untraperitoneally into newborn mice less than 24 hours old.

diseases, we devised a genetic scheme shown in Table 5. For F-MuLV, we chose to employ the technique of marker rescue. The mouse type-c retrovirus, amphotropic virus 1504A, was chosen as one parent in the scheme. This virus grows on NIH 3T3 cells but does not cause XC cells to fuse and does not cross-interfere with mouse ecotropic or xenotropic type-c retroviruses (15). Importantly, when 1504A is injected into newborn Swiss mice, the virus replicates in the spleen and marrow but no disease is detected for up to eight months after the mice are infected with 1504A. We used this virus to infect NIH 3T3 cells and simultaneously transfected the cells with a subgenomic piece of F-MuLV DNA shown in Fig. 2B. A restriction fragment bounded by two HindIII restriction sites was cloned in PBR322 from the permuted DNA of F-MuLV clone 57. This H-H piece has 3.1 kb of the 3' end of F-MuLV, one terminal repeat, and approximately 400 bases of the 5' end of F-MuLV. The total length of the piece is approximately 4.0 kb. The results of this infection-transfection are shown in Fig. 3. NIH cells either transfected alone or infected with 1504A (Fig. 3A and 3B) do not fuse XC cells. The mixed infection-transfection yields several XC-positive progeny (Fig. 3C). Two XC-positive progeny have been biologically cloned from such an experiment and both cause the rapid erythroproliferative syndrome in newborn Swiss mice. Detailed analyses of these recombinant viruses are underway to determine which parts of the H-H piece have recombined with 1504A. Also we are attempting to molecularly clone 1504A so as to be able to make more precise recombinants with the H-H piece or smaller fragments of the H-H piece. Such studies have localized the disease-inducing portion of F-MuLV essentially to the 3' end of the genome. This approach should allow us to define precisely which portion of F-MuLV is required to induce this erythroproliferative disease.

For SFFV, results will be reported by Linemeyer et al. in a separate publication (8). The schema, similar to that employed for F-MuLV, is shown in Table 5. We wish to note only in passing the cotransfection assay shown in Table 5 with SFFV DNA and other infectious helper viral DNA, an assay which should allow an unambiguous definition of the "leuk" gene of SFFV.

Cell Culture Assays for Viruses. To understand the mechanisms by which F-MuLV or SFFV induce hematopoietic disease, we have explored the long term bone marrow culture system of mouse bone marrow cells developed by Dexter (16). In this culture system, the contents of a single femur of an adult mouse are flushed into 10.0 ml of Fisher's medium supplemented

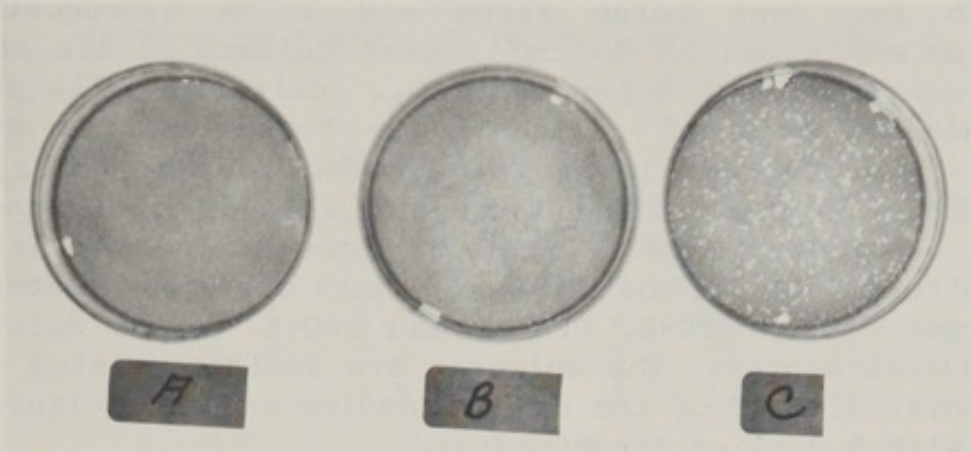

FIGURE 3. Marker rescue of XC function of F-MuLV.
Plate A: Transfected with 2.0 μg of HindIII piece of F-MuLV DNA.
B. Infected with amphotropic virus 1504A.
C. Infected and transfected.
Plates were tested for XC plaques 14 days after infection.

TABLE 5
SCHEMA FOR GENETIC DEFINITION OF ONC OF SFFV AND F-MuLV

1. Molecularly clone each virus from closed circular unintegrated form
SFFV in pBR322 at HindIII site
F-MuLV in λWES at EcoRI site
Each is permuted in molecular clone: 3'-5' joint attached

2. For SFFV: Transfect NIH 3T3 cells with SFFV DNA
A. No prior digestion with restriction enzymes
B. Prior digestion to release from plasmid
C. Prior digestion within SFFV genome
Coinfect with helper virus OR cotransfect with helper viral DNA
Test progeny in mice for SFFV disease

3. For F-MuLV: Infect NIH 3T3 cells 1504A amphotropic wild mouse virus
A. XC negative
B. Diesase negative
Cotransfect NIH 3T3 cells: Non-infectious F-MuLV DNA
a) not restricted from vector
b) subgenomic piece
Test: XC positive progeny
F-MuLV disease

with 25 per cent horse serum and 10^{-6}M hydrocortisone. Cultures are grown in 35 cm^2 closed culture flasks at 33°C in an atmosphere of 5 per cent CO_2. Cultured cells produce an adherent layer of cells on the bottom of the culture flask. The adherent layer is composed of fat cells, epithelioid cells, and fibroblasts and islands of hemapoietic cells. Hemapoietic cells are also present in suspension in the culture fluid in the flask. In such cultures hematopietic precursor cells, CFU-S, CFU-C, and BFU-E can be maintained for several months. The cultures are semidepopulated weekly by removal of half of the culture medium and the cultures are refed with 5.0 ml of fresh medium.

We have infected such cultures with F-MuLV clone 57. After 3 weeks in culture, the control and infected cultures were placed at 37°C and allowed to shake gently on a rocker platform in order to minimize contact between the adherent cells and the hematopoietic cells. The total number of cells and the morphology of the cells in the control and infected cultures was examined over the next three weeks. In the cultures which were both infected and placed on the rocker platform, a dramatic change in the morphology of the cells became apparent. A striking increase in the number of blast cells was observed; after 21 days over 50 per cent of the suspension cells were blasts as shown in Table 6. The complete morphological analysis of the cultures is also shown in Table 6. Control cultures or infected cultures had only 3-5 per cent blasts in the culture fluid, and late granulocytes or macrophages predominated. The exact requirements for these differences have not been defined. However, the results suggest that the Dexter culture system can be developed as an assay for the growth-promoting properties of helper-independent murine leukemia viruses. Such a cell culture assay has not existed for this class of oncogenic retroviruses. Future studies will be directed at defining the exact culture conditions and virus requirements to induce the observed effects, and to determine if the same gene(s) in F-MuLV that are responsible for the *in vivo* disease are also responsible for the effects in the Dexter culture system.

We have also utilized this culture system to study the effects of SFFV. Cultures were infected with a polycythemia-inducing strain of SFFV pseudotyped with a Moloney helper virus. After two weeks in culture, the media was changed to Fisher's medium with 25 per cent fetal calf serum and 10^{-6} M hydrocortisone. In earlier studies, fetal calf serum was found to be more favorable for erythropoiesis in the long-term culture system (17). After two more weeks, cultures were fed with fresh medium, or fresh medium containing 1% (v/v) or 10% (v/v) sera from profoundly anemic BDF_1 mice

TABLE 6
EFFECTS OF F-MULV IN DEXTER CELL CULTURES

Group	Days of Agitation	Cells/ml $\times 10^5$	Morphology (%) BL	EG	LG	Mon
Control	7	-	3	17	76	5
	14	3.2	4	22	70	3
	18	2.4	3	13	73	10
	21	1.9	3	12	85	--
Infected	7	-	4	15	75	7
	14	5.1	9	35	53	3
	18	4.7	22	22	53	3
	21	2.9	52	9	35	3

TABLE 6. Long term bone marrow cultues were initiated from Swiss mice by procedures previously described (3,16). Cultures were infected at the time of initiation by adding 1.0 ml of F-MuLV clone 57 produced in NIH 3T3 cells to the culture flasks. Approximately 10^6 infectious units of the virus were added to 10^7 nucleated marrow cells. The marrow cultures were placed on the rocker platform for mechanical agitation 3 weeks after culture. At that time, the cultures were producing approximately $10^{5.0}$ XC PFU per ml of F-MuLV. The suspension cells in each culture were examined at the indicated times for total cell counts, and morphology examined after staining with Giemsa and May-Grunwald stains (3). Classification criteria for cells has been previously described (3).

(anemic mouse serum - AMS). The results are shown in Tables 7 and 8. Over the next four weeks (8 weeks after initiating cultures and infection) a profound effect on erythropoiesis was observed. Cultures, fed with 10% AMS, at 37°C, with no mechanical agitation, continuously produced large numbers of erythroblasts, and benzidine positive erythroid cells; 20-40 per cent of the suspension cells in such cultures were mature, nonnucleated erythrocytes. The results suggest that the AMS can be an easily prepared reagent to induce erythropoiesis in long-term marrow cultures. It is not known what ingredients in the AMS are required for induction of the erythropoiesis, and whether erythropoietin alone, or epo plus other factors are required. However such cultures are an easy source of populations of cells highly enriched for erythroid

TABLE 7
ERYTHROPOIESIS IN LONG TERM CULTURES INDUCED BY ANEMIC MOUSE SERUM

Supplements to FSS (Days in culture)		Cells/ml	Morphology (%) BL	EG	LG	MO	Erythroid Cells (%) EB	BZ+	ME
(7)									
HS		--	--	--	--	--	--	--	--
(14)									
FCS		$3x10^5$	3	15	68	10	0	0	0
(21)									
FCS		$5x10^5$	6	19	63	12	0	0	0
(28)									
A FCS	AMS	$1.3x10^5$	3	24	44	29	0	0	0
B FCS		$2.4x10^5$	3	8	88	9	0	0	0
(35)									
A FCS	AMS	$3x10^5$	4	22	73	4	1	0	0
B FCS		$3.3x10^5$	4	16	68	12			
(39)									
A FCS		$5x10^5$	3	4	49	0	10	18	16
B FCS		$6x10^5$	3	10	82	5	0	0	0
(46)									
A FCS		$5x10^5$	0	5	14	0	14	27	40
B FCS		$4.3x10^5$	1	16	83	0	0	0	0

TABLE 7. Erythropoiesis in marrow cultures. Long term bone marrow cultures from 6-8 week old Swiss mice were established in Fisher's medium (FSS) at 33°C containing 25 per cent horse serum (HS) and 10^{-6} M hydrocortisone (HC). Each time the cultures were fed as indicated, one half of the media was replaced with fresh media. Where indicated, the HS was replaced by 25 per cent fetal calf serum. On day 28, serum from anemic BDF_1 female mice (AMS) was added to a final concentration of 10 per cent v/v to the culture indicated, A. All cultures were then incubated at 37°C. On day 35, fresh AMS was added as indicated. AMS as prepared as follows: 6-8 week old BDF_1 mice were given 600 rads of x-irradiation and the next day injected sc with 60 mg/kg phenylhydrazine. Eight days later mice were exsanguinated. The serum was filtered through through an 0.45 mμ millipore filter prior to use. EG = early granulocyte; LG = late granulocytes; MO = monocytes; EB = erythroblasts; BZ^+ = benzidine positive cells; ME = mature erythrocytes. Anemic mouse serum had approximately 20 units per ml of erythropoietin. All cultures contained 10^{-6} M hydrocortisone.

TABLE 8
SFFV INDUCED ERYTHROPOIESIS IN LONG TERM CULTURES

Group (Days in culture)		Supplements to FSS	Cells/ml	Erythroid Morphology (%) Erythroblasts	BZ+	ME
Control						
(28)	A	none	--	0	0	0
	B	1% AMS	--	0	0	0
(35)	A	none	--	0	0	0
	B	1% AMS	--	0	0	0
(39)	A	none	$3x10^5$	0	0	0
	B	none	$1.5x10^5$	3	1	0
(46)	A	none	$2x10^5$	0	0	0
	B	none	$1x10^5$	2	11	3
(50)	A		$2x10^5$	0	0	0
	B		$4x10^5$	2	6	1
SFFV						
(28)	A	none	--	0	0	0
	B	1% AMS	--	0	0	0
(35)	A	none	--	0	0	0
	B	1% AMS	--	0	0	0
(39)	A	none	$3x10^5$	2	1	0
	B	none	$3x10^5$	2	1	0
(46)	A	none	$4x10^5$	2	3	3
	B	none	$5x10^5$	6	37	20
(50)	A	none	$5x10^5$	3	5	5
	B	none	$5x10^5$	8	38	25

TABLE 8. Effect of SFFV in long term marrow cultures. Cultures from Swiss mice were established and fed on the same regimen up to day 28 as indicated in Table 7. At the time of initiation of the cultures, the indicated flasks were infected with 1.0 ml of a viral complex containing 10^6 XC PFU of Mo-MuLV and $10^{4.5}$ FFU of $SFFV_{LS}$. On day 28, the cultures were placed at 37°C and fed as indicated. The AMS was the same preparation as in Table 7. At day 28, the infected cultures were found to be producing approximately $10^{3.0}$ FFU of SFFV and $10^{6.0}$ XC PFU of Mo-MuLV. Symbols and other details of the experiment are as in Table 7. Only erythroid morphology is indicated. There were no significant differences in the granulocyte morphology in control vs SFFV infected cultures. The Lilly-Steeves polycythemic strain is the strain of SFFV used.

cells, and their continuous production over many weeks would seem to offer a useful system to study erythropoiesis in cell culture.

In addition, in cultures infected with SFFV, erythropoiesis was detected even with no AMS added to the cultures (Table 7). Benzidine positive cells and mature erythrocytes were observed. Such late erythroid cells were not seen in control cultures in this study or in many other control cultures in other experiments (17). In cultures supplemented with limiting concentrations of AMS (1%) (Table 8), a profound difference in the number of late erythroid cells (benzidine positive and mature erythrocytes) was noted in cultures infected with SFFV compared to control cultures. Over a ten-fold difference in the number of late erythroid cells was noted in the cultures treated with 1 per cent AMS and infected with SFFV, compared to control cultures fed on an identical regimen with 1 per cent AMS. The results suggest that the long-term cultures can be used as an assay for SFFV as well as an assay for F-MuLV. For SFFV, in fact, the long-term cultures seem to resemble the polycythemic state induced in mice by this strain of SFFV.

DISCUSSION

The current studies have developed approaches to two important aspects of the study of Friend virus induced diseases. First, both the helper-independent virus, F-MuLV, and the replication-defective virus, SFFV, have been molecularly cloned in an infectious DNA form. The DNA of each of these viruses has been used in gene transfer studies to begin to define rigorously the gene(s) and gene product(s) of each virus responsible for the viral-induced disease. For SFFV, Linemeyer et al. (8) have provided evidence that supports the hypothesis that gp52 plays a central role in SFFV induced disease (5,6,13). For F-MuLV, the disease-inducing property of the virus has been localized to the 3.1 kb of the 3' end of the genome. Future studies will be directed to defining whether the env gene or the C region of F-MuLV, on an unknown gene of this fragment, are required for the disease-induction. For SFFV, subgenomic pieces will be prepared and altered with site-directed mutagenesis methods to provide more rigorous proof that the gp52 is required for SFFV-induced disease.

Secondly, in long-term bone marrow cultures, effects of both F-MuLV and SFFV have been observed. Most notably, it has been possible to find culture conditions which support sustained high levels of erythropoiesis to mature erythrocytes

in the long-term cultures. Either supplementing the cultures with serum from anemic mice or infecting the cultures with SFFV can "induce" prolonged erythropoiesis. Future studies will attempt to determine in such cultures how SFFV reduces the requirements for AMS, and to see if SFFV mutants producing altered gp52 proteins will allow us to gain insights into the basic mechanisms that control normal and abnormal erythropoietic precursor cell proliferation and differentiation.

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VIRAL ENVELOPE GENES AND c REGIONS IN NON-ACUTE AVIAN LEUKOSIS VIRUS ASSOCIATED DISEASE[1]

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ABSTRACT Avian leukosis viruses (ALVs) of endogenous origin do not appear to cause disease. These viruses have similar genetic compositions to ALVs of exogenous origin which cause non-acute disease. The major genetic differences between endogenous and exogenous ALVs are found in their envelope genes and c regions. Envelope genes of endogenous origin code for subgroup E antigens ($\underline{env}^E$) whereas envelope genes of exogenous origin code for subgroups A, B, C, or D antigens ($\underline{env}^A$), etc.). Endogenous viruses have characteristic constant regions $\underline{c}^n$, that are distinct from those of exogenous viruses, $\underline{c}^x$.

We are using exogenous viruses, endogenous viruses and recombinants of exogenous and endogenous viruses to test for the role of envelope antigens and c regions in disease. Four cloned $\underline{env}^E$ $\underline{c}^x$ viruses (RAV-60s) have been compared for their oncogenic activity with a $\underline{env}^A$ $\underline{c}^x$ virus (RAV-1) and a $\underline{env}^E$ $\underline{c}^n$ virus (RAV-0).

The RAV-60s caused the same incidence of disease as RAV-1. Individual RAV-1 and RAV-60 infected chickens developed different diseases. The most frequent disease was B cell lymphomas. Anemias, a fibrosarcoma, and osteopetrosis also occurred. These results suggest that c regions, not envelope antigens, are critical for the development of disease. They also demonstrate that cloned avian leukosis viruses cause more than one disease.

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INTRODUCTION

Avian leukosis viruses are associated with the occurrence of acute (short latent period), as well as non-acute (long latent period) disease (for textbook chapter see 1). The viruses which cause acute disease appear to be recombinants of viral and cellular information, with different recombinants coding for different diseases (2,3). In contrast, viruses which cause non-acute disease appear to contain only viral genes. 5' to 3', the genomes of these viruses consist of gag genes which code for internal structural proteins, pol genes which code for RNA-directed DNA polymerase, env genes which code for envelope antigens, and c or constant regions. Not all viruses that contain gag, pol, env and c cause disease. Quite interestingly, non-acute disease is associated with avian leukosis viruses of exogenous but not endogenous origin (4,5,6,7).

If one compares the gag, pol, and env genes and c regions of exogenous and endogenous viruses, one finds the greatest differences in env and c. Env genes of exogenous origin code for subgroups A, B, C, or D antigens. Env genes of endogenous origin code for subgoup E antigens (for review see 8). Envelope antigens determine the host range of avian leukosis viruses. They also elicit the major immune response of chickens to avian leukosis virus infections.

Avian leukosis viruses of exogenous origin have c regions, c^x, (9) that are distinct from the c regions of endogenous viruses, c^n, (10,11,12). Recent work with recombinants of endogenous and exogenous viruses has demonstrated that c^x viruses grow more rapidly than c^n viruses (13). This result suggests that c regions contain sequences that control the rate of virus growth. Since in integrated proviral DNA, c region sequences are found immediately 5' of the sequences which are capped in viral mRNAs (14,15,16, for review see 18), c regions have been hypothesized to contain promotors for RNA transcription. If this is so, then the difference in growth rate of c^x and c^n viruses may reflect differences in the efficiency of promotor sequences in c^x and c^n.

In order to disassociate potential roles of envelope antigens and c regions in non-acute disease, we have compared the oncogenic potential of exogenous and endogenous viruses with that of env^E c^x recombinants of exogenous and endogenous viruses. The results of our studies suggest that c regions, not envelope antigens, are critical for the development of non-acute disease. Our results also demonstrate that cloned avian leukosis viruses cause more than one disease.

RESULTS

Viruses. The exogenous virus we used in our oncogenicity testing was RAV-1. RAV-1 is a subgroup A virus that was isolated by Dr. P.K. Vogt from a stock of the Bryan high titer strain of Rous sarcoma virus. We chose RAV-1 for our tests since RAV-1 is associated with a high incidence of non-acute disease. RAV-1 has the genetic composition gag pol env^A c^x.

For an endogenous virus, we used RAV-0. RAV-0 is the product of the endogenous viral locus ev 2 (19). We chose RAV-0 for our tests since most tests on the oncogenic activity of endogenous avian viruses have been done with RAV-0. RAV-0 has the genetic composition gag pol env^E c^n.

For env^E c^x recombinant viruses, we turned to RAV-60s. RAV-60s are subgroup E recombinants of exogenous viruses with replication defective endogenous viruses (20, for review see 8) (see Table 1). RAV-60s grow more rapidly and to higher titers ($\sim$ 4 fold) than RAV-0 (10,21). Four isolates of RAV-60s were used in our studies; NY201RAV-60, NY202RAV-60, NY203RAV-60, and NY204RAV-60. The RAV-60s were a kind gift

TABLE 1
VIRUSES

Designation	Origin	Genetic composition[a]
RAV-1	Bryan higher titer strain of Rous sarcoma virus	gag pol env^A c^x
RAV-0	Product of ev 2	gag pol env^E c^n
NY201RAV-60[b]	RAV-1 grown in gs^+chf^+ cells	gag pol env^E c^x
NY202RAV-60	RAV-2 grown in gs^+chf^+ cells	gag pol env^E c^x
NY203RAV-60	RAV-1 grown in gs^-chf^+ cells	gag pol env^E c^x
NY204RAV-60	RAV-2 grown in gs^-chf^+ cells	gag pol env^E c^x

[a] env^A codes for subgroup A envelope antigens, env^E codes for subgroup E envelope antigens. For oligonucleotide composition see Figure 1.

[b] RAV-60s are recombinants of exogenous viruses with replication defective endogenous viruses (20,8). RAV-2 is a subgroup B ALV.

of Drs. H. and T. Hanafusa, Rockefeller University, New York. Each of the isolates had been cloned by the Hanafusas by two rounds of end point purification.

The genomes of each of the viruses were mapped for T1 oligonucleotides (for methods, see 13). Each of the RAV-60s had a unique oligonucleotide composition (Figure 1). The two RAV-60s isolated from gs^+chf^+ cells had oligonucleotides in env that are characteristic of ev 3 (ev 3 is a defective endogenous virus that codes for the gs^+chf^+ phenotype (22,23, P. Tsichlis, K. Conklin, J. Coffin, and H. Robinson unpublished observations). The two RAV-60s isolated from gs^-chf^+ cells had oligonucleotides in env that appear to be characteristic of ev 9 (ev 9 is a defective virus that codes for the gs^-chf^+ phenotype; S. Astrin, H. Robinson, P. Tsichlis, K. Conklin, and J. Coffin unpublished observations). Each RAV-60 had c region oligonucleotides characteristic of c^X. Thus, these viruses had the genetic composition gag pol env^E c^X.

Chickens. Most chickens are resistant to the horizontal spread of their endogenous viruses (for review, see 8). In order to breed a chicken that is susceptible to endogenous viruses, one must breed in a dominant allele, $TV\text{-}E^S$, that is thought to code for receptors for subgroup E virus and breed

	GAG-POL	ENV	C
RAV - 1	-- 403 8 11 --- 15 29 16A 24 3 23 25 22 30 103 32	4 --- 308 -- 012 -- -- --- -- --- --- 111 402 -- -- 604 409 10	-- C - POLY(A)
NY201RAV-60	-- 403 8 11 --- 01 29 16A 24 3 23 25 22 30 103 32	4 607 308 -- --- 04 06 013 02 601 602 111 402 37 09 604 --- 10	-- C' - POLY(A)
NY202RAV-60	-- --- 8 -- 014 15 29 16A 24 3 23 25 22 30 103 32	4 607 308 -- --- 04 06 013 02 601 602 111 402 37 09 604 --- 10	-- C - POLY(A)
NY203RAV-60	-- 403 8 11 --- 15 29 16A 24 3 23 25 22 30 103 32	4 --- 308 07 012 04 06 013 02 --- --- 111 402A 37 -- 604 409 10	-- C' - POLY(A)
NY204RAV-60	-- 403 8 -- --- 15 29 16A -- 3 23 25 22 30 103 32	4 --- 308 07 012 04 06 013 02 --- --- 111 402A 37 -- 604 409 10	-- C - POLY(A)
RAV-0	13 --- 8 -- 014 01 29 16A -- 3 23 25 22 30 103 32	4 --- 308 07 012 04 06 013 02 --- --- 111 402 37 09 --- --- 10	05 08 - POLY(A)

Figure 1. T_1 oligonucleotide maps of viruses. Oligonucleotides 601, 602 and 607 are specific for information derived from gs^+chf^+ cells (see NY201 and NY202), while oligonucleotide 402A is specific for sequences derived from gs^-chf^+ cells (see NY203 and NY204). The subgroup E envelope derived from gs^-chf^+ cells is almost identical to that of RAV-0 except for the replacement of 402 by the closely related oligonucleotide 402A, the absence of 09 and the presence of 604 and 409. 604 and 409 have no role in determining subgroup as they are present in RAV-1 and the RAV-60s. Oligonucleotide C' differs from oligonucleotide C by a point mutation (C → U).

out endogenous viruses that expresses high levels of envelope antigens. Endogenous viruses that express high levels of envelope antigens appear to interfere with subgroup E virus infections (25, H. Robinson and S. Astrin unpublished observations).

We have bred a line of chickens, K28, which is susceptible to all kown avian leukosis viruses. K28 has been bred to contain TV-E^S (26). It has also been bred to contain only one endogenous avian leukosis virus ev 1 (27). Ev 1 is expressed at extremely low levels (27) and does not interfere with subgroup E virus infections.

Infections. Approximately 10^6 infectious units of virus were inoculated into the leg veins of day-old K28 chickens. Following infection, RAV-1, RAV-60, and RAV-0 infected birds and a group of uninfected control birds were maintained in separate quarantines. At 1 and 3 months of age, serum was harvested and analysed for virus (particulate RNA-directed DNA polymerase) (see Figure 2). The sera of control birds (6 out of 6 tested) did not exhibit virus. The sera of RAV-1, RAV-60, and RAV-0 infected birds exhibited similar incidences of

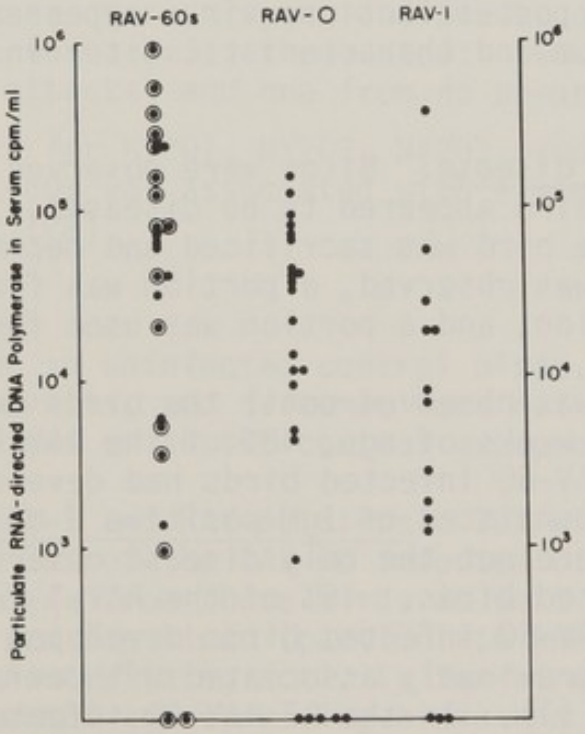

Figure 2. Virus in sera of one month old RAV-60, RAV-0, and RAV-1 infected birds. Birds were bled one month after infection and their sera analyzed for particulate RNA-directed DNA polymerase (10). ● Data from one bird. ⊙ RAV-60 birds that developed lymphomas.

particulate RNA-directed DNA polymerase. The levels of particulate RNA-directed DNA polymerase in the sera of birds within each group varied over several logs.

Our RAV-60 and RAV-0 stocks have ~50 infectious units of virus per count of particulate RNA-directed DNA polymerase. Thus, the level of infectious virus in the sera of RAV-0 and RAV-60 infected birds is approximately 50 times the level of particulate RNA-directed DNA polymerase. We do not know the ratio of infectious units to particulate RNA-directed DNA polymerase for RAV-1.

Sera of some RAV-60 infected birds (8 out of 24 tested) contained higher levels of virus than sera of RAV-0 infected birds. The median level of viremia in RAV-60 infected birds was approximately 4 times higher than the median level of viremia in RAV-0 infected birds. Despite the high levels of replication of RAV-60 in some infected birds, most RAV-60 infected birds had levels of viremia which were comparable to those observed in RAV-0 infected birds.

The levels of particulate RNA-directed DNA polymerase in the sera harvested 3 months post infection were similar to those in sera harvested one month post infection. Birds which did not mount an immune response had remarkably similar levels of virus in their sera at 1 month and 3 months post-infection. Thus, at 1 month post-infection, virus appeared to have replicated to a maximum and characteristic titer in each infected bird.

Analysis of disease: Birds were observed for disease for 44 weeks. If a bird appeared to be diseased, its serum was harvested and the bird was sacrificed and necropsied. If diseased tissue was observed, a portion was fixed for histological examination, and a portion was used for the recovery of virus.

No disease was observed until the birds were 12 weeks of age (28). By 44 weeks of age, 48% of the RAV-1 infected birds and 47% of the RAV-60 infected birds had developed lymphomas. These lymphomas consisted of IgM positive B cells (28).

Lymphomas were not the only disease observed in the RAV-1 and RAV-60 infected birds. 16% of the RAV-1 infected birds and 18% of the RAV-60 infected birds developed other diseases which have been previously associated with avian leukosis virus infections (1). In the 87 RAV-60 infected birds, these other diseases included one case of fibrosarcoma, two cases of osteopetrosis, two cases of anemia, and ten cases of wasting (28). Disease other than lymphomas developed in birds infected with each of the RAV-60s as well as RAV-1 (28).

Virus related disease occurred only in the RAV-1 and RAV-60 infected birds. No lymphomas were observed in the RAV-0

TABLE 2
INCIDENCE OF DISEASE IN RAV-1, RAV-60 AND RAV-0 INFECTED K28 CHICKENS

Virus	No. of chickens[a]	Disease, % Lymphoma	Other[b]	Miscellaneous deaths, %[c]
RAV-1	51	48	16	6
RAV-60s[d]	87	47	18	6
RAV-0	58	0	0	17
Uninfected	67	0	0	7

[a]No. of chickens in group at twelve weeks of age.

[b]Includes fibrosarcomas, osteopetrosis, anemia and wasting (see Robinson et al., 1979).

[c]Birds which were sacrificed because of trauma, birds that died of no apparent cause, missing birds. The RAV-0 group suffered a higher evidence of miscellaneous death than the other groups. 10 birds are in the RAV-0 miscellaneous group; 7 were sacrificed: 4 because of trauma, one because of limber neck, one because of an ovarian cyst, and one because we thought it was anemic; three died: two from apparent heart attacks, and one from no apparent cause.

[d]Pooled data for NY201, NY202, NY203, and NY204. Each of the RAV-60s was associated with a similar incidence of disease (28).

infected birds or in uninfected control birds. Anemias, wasting, osteopetrosis, and sarcomas were not observed in the RAV-0 infected birds or in the control group of birds.

Surviving RAV-1 and RAV-60 Birds. 30% of the RAV-1 infected birds and 29% of the RAV-60 infected birds did not develop disease. Sera from these birds as well as the diseased birds were analyzed for virus (particulate RNA-directed DNA polymerase) and neutralizing activity for virus.

Sera of diseased and surviving birds had remarkably similar incidences and levels of particulate RNA-directed DNA polymerase (28). Birds which did not express neutralizing activity had levels of viremia comparable to the levels they had expressed at one and three months of age. This result indicates that high levels of replicating virus throughout the life of a bird did not necessarily result in disease.

Sera from approximately 30% of the diseased birds and 50% of the survivors had neutralizing activity for subgroup E virus (28). This result indicates that neutralizing serum did not dramatically affect the incidence of disease.

Recovered viruses. Viruses were recovered from RAV-60 associated diseases and analyzed for oligonucleotide composition as well as the envelope mediated property of viral interference (28). Viruses recovered from 7 diseased birds had oligonucleotide fingerprints characteristic of the RAV-60 which had been used to infect the chicken. No new oligonucleotides were detected. Viruses recovered from 17 diseased tissues interfered with subgroup E but not subgroups A, B, C, D, or F avian sarcoma viruses. Thus, we were unable to recover viruses other than the infecting viruses from diseased tissues.

DISCUSSION

Our results indicate that exogenous envelope antigens are not critical for the development of non-acute disease. No difference in the incidence of disease was observed for $\underline{c}^{x}$ viruses which expressed subgroup A (exogenous) or subgroup E (endogenous) envelope antigens. No difference in oncogenicity was observed for RAV-60s which expressed slightly different subgroup E envelope antigens.

Oncogenicity tests with RAV-60 at the Regional Poultry Research Laboratory have indicated that RAV-60s were non-oncogenic (29) or had low oncogenic potentials (relative to RAV-1) (30). K28 chickens are uniformly susceptible to subgroup E viruses. The chickens which were available for testing RAV-60s at the Poultry Laboratory included 151 and 15 x 7 which have a low susceptibility to subgroup E viruses (29) or line 15_{B}, which has a high susceptibility to subgroup E viruses but a high incidence of maternal antibody to subgroup E virus (30). We consider our K28 chickens a unique asset for testing subgroup E viruses for their oncogenic potential.

The RAV-60s we used in these studies had been end point purified two times by the Hanafusas. All of the RAV-60s were associated with more than one disease. This result indicates that cloned isolates of viruses that cause non-acute disease have the potential for causing more than one disease. Evidence that cloned isolates of RAV-1 are associated with more than one disease has been recently reported by Crittenden (31).

The failure of RAV-0 to cause disease in K28 chickens is impressive. At 1 month of age, RAV-0 infected chickens had levels of virus in their sera comparable to the levels found in most RAV-60 infected birds (Figure 2). Most RAV-60 infected birds developed disease. No RAV-0 infected birds developed disease.

Examination of the oligonucleotide maps in Figure 1 reveal that all of the RAV-60s differed from RAV-0 in c region oligonucleotides. These differences in c region oligonucleotides code for a known difference in phenotype: rate and level of virus growth. Outside of the c region, the RAV-60s had only one common oligonucleotide which was not present in RAV-0. This oligonucleotide, 604, resides close to the 3' end of the env gene (as currently defined). The 3' end of env is thought to code for group specific envelope determinants (13).

We are intrigued by the possibility that c regions code for the difference in oncogenic potential of RAV-60s and RAV-0. How might c regions code for disease and code for differnt disease in different infected animals? During their life cycle, RNA tumor viruses synthesize DNA intermediates which integrate into the host genome. Integrations take place at many sites (16,17). Each integration results in a cell which is a recombinant of viral and cellular information. Viral recombinants of viral and cellular information also occur (32). We are intrigued by the possibility that non-acute disease follows the formation of recombinants in which viral promotors control the expression of cellular genes (28). The difference in oncogenic potential of $\underline{c}^x$ and $\underline{c}^n$ viruses would then reflect the difference in efficiency of promotors which reside in $\underline{c}^x$ and $\underline{c}^n$. If this is so, then $\underline{c}^x$ but not $\underline{c}^n$ would contain promotors which were sufficiently efficient to promote "oncogenic" levels of expression of a cellular gene.

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TWO REGIONS OF THE MOLONEY LEUKEMIA VIRUS GENOME ARE REQUIRED FOR EFFICIENT TRANSFORMATION BY src/sarc

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ABSTRACT

Src-containing subgenomic DNA fragments of Moloney sarcoma provirus (MSV) with high transforming activity retain two regions of the parental Moloney leukemia virus (MuLV) genome. One MuLV region is the 600 base pair terminal repeat sequence of the provirus. The presence of this sequence either 5' or 3' to an internal MSV src sequence enhances transforming activity 1000-fold. The other MuLV region represents approximately 100 nucleotides preceeding the 5' end of the src sequence. The requirement of this region for transformation is deduced by i) comparison of the endonuclease restriction maps of three MSV provirus isolates, ii) eliminating specific MuLV sequences in cloned subgenomic fragments without loss of biological activity, and iii) demonstrating that efficient transforming activity of the cell sarc sequence occurs only when MSV sequences have been substituted to replace the normal cell sequences at its 5' end.

INTRODUCTION

The Moloney sarcoma virus (MSV) produces foci of transformation in tissue culture and fibrosarcomas in animals (1,2). The genome of this virus has been shown to consist of sequences related to the parental Moloney leukemia retrovirus (MuLV) as well as sequences that are MSV specific (src) (3,4,5). Src is homologous to a single copy sequence in the genomic DNA of normal mouse cells (sarc) (6). We have cloned in phage λ, DNA fragments from transformed cells containing the integrated provirus sequences of two MSV isolates, ml and HTl (7,8). We have also cloned a normal mouse DNA fragment containing a region, sarc, homologous to the MSV src sequence (9). These DNA fragments have provided a means for determining, in a direct DNA transfection-transformation assay, what portions of the MSV genome are required for efficient transformation (10). We show that DNA fragments which transform cells efficiently contain the src or sarc

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sequence and two regions of the MSV genome presumeably acquired from the M-MuLV parent.

RESULTS AND DISCUSSION

The physical maps of the cloned Balb/c sarc containing fragment (λM sarc) and DNA fragments containing the ml (λml) and HT1 (λHT1) MSV integrated proviruses, have been described in detail (7,8,9). Maps of all three fragments are shown in Figure 1. The only endonuclease restriction sites in common between the inserted DNA fragments in λMsarc, λml and λHT1 occur in src/sarc (9). The sequences flanking the 5' and 3' ends of sarc in λMsarc are normal mouse cell sequences (9) while src, in both MSV proviruses, is immediately flanked by sequences related to MuLV (8) which in turn are flanked by normal mink cell sequences (8, McClements et al., in preparation).

We have shown that the transforming efficiency of an internal src containing fragment is enhanced approximately 1000 fold by the presence of one copy of the 600 base pair (bp) terminal repeat sequence (TRS) of the integrated provirus covalently linked to either the 5' or 3' side of the src (10). Transformation by src is also enhanced by TRS, even when they are not covalently linked, but are cotransfected on separate plasmid vectors (10). Examples are given in Table 1. The plasmid pHT10 containing the Hind III HT1 MSV src fragment tranforms with low efficiency (Table 1) (10). Addition of one TRS to either the 3' (pHT22), or 5' (pHT25) end of the MSV src sequence contained in pHT10 enhances the transforming efficiency to within 20% of the entire provirus (Table 1, 10). Alternatively co-transfection of the MSV sequences on one plasmid (pHT10) with TRS sequences on a second (pmlsp) stimulates the transforming efficiency of the src sequence 300-fold (Table 1). The elements of TRS responsible for the enhancement of src transformation are unknown, but we have speculated from nucleotide sequence data that it may provide promoter or integration functions (10, Dhar et al., in preparation).

Comparative heteroduplex analysis (11) and restriction endonuclease mapping (8) have shown differences in the conserved MuLV sequences to the 5' side of src in ml, HT1, MSV, and MSV 124. These differences suggested to us, that sequences to the 5' side of the MuLV specific Xba I site adjacent to src, are not required for transformation (8). We therefore generated from pHT10 a plasmid pHT101, which lacks the Xba I fragment (3.5-3.9 kilobase pairs (kb), HT1 map, Figure 1) preceeding the 5' end of the src sequence, and

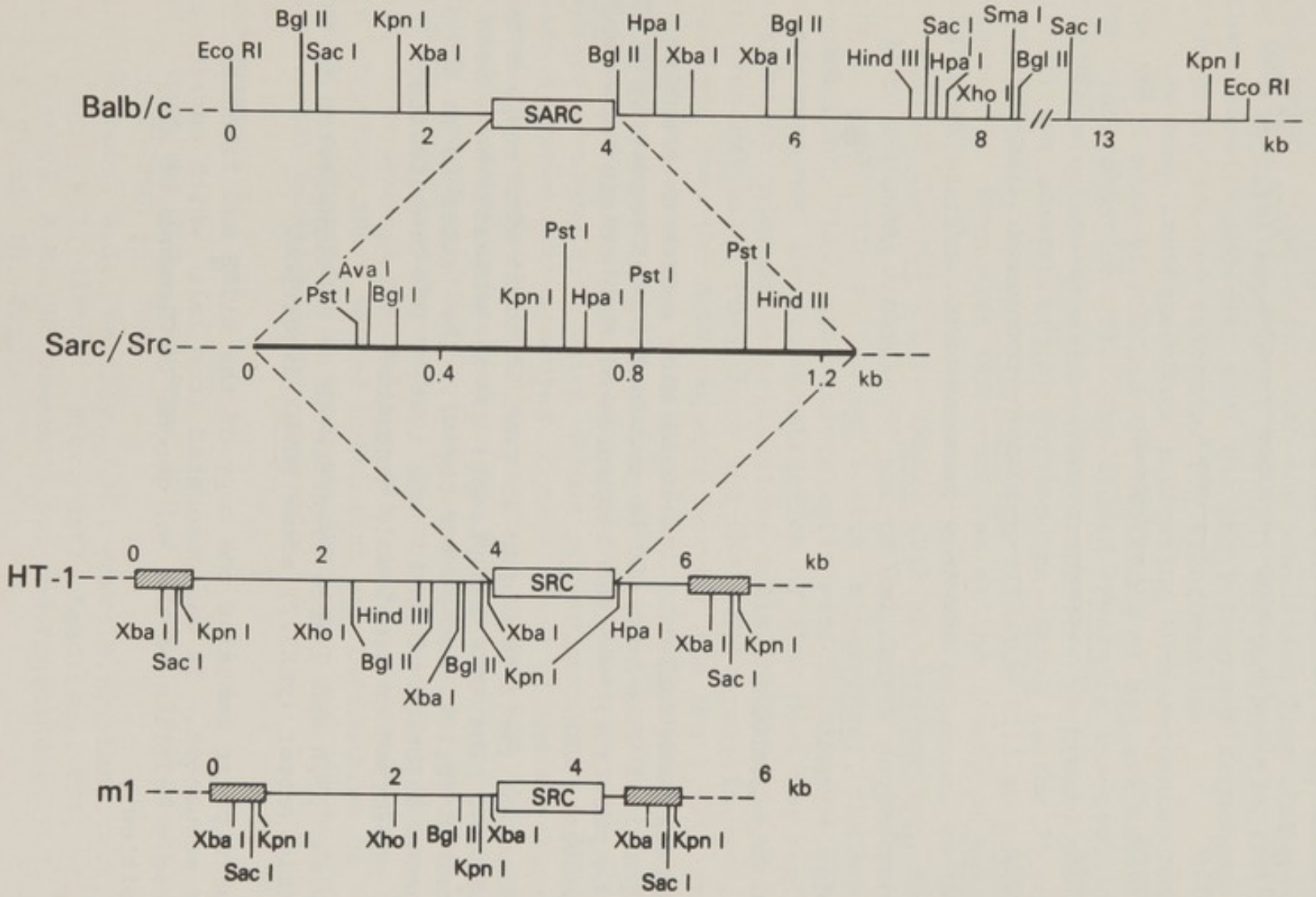

FIGURE 1. Endonuclease Restriction Maps of λMsarc, λHT1, and λm1. The orientation is from 5' to 3' (left to right) with respect to viral RNA.

TABLE 1: Transforming Fragments of MSV

Designation	Schematic of Fragment[a]	foci/pmole
1. λHT1[b]	....//...oooo——xxxx——oooo....///	39,000
2. pHT10[c]	——xxxx	7
3. pHT101	—//—xxxx	NT
4. pHT25	....//...oooo——xxxx	7,800
5. pHT22	——xxxx——oooo..../...	8,100
6. λml	...oooo——xxxx——oooo..../..	46,100
7. pmlsp[d]	...oo/ /oo..../..	< 4
8. pmlsp + pHT10		2,000
9. pmlsp + pHT101		700

[a] To approximate scale: .... normal mink sequences flanking the proviral DNA; oooo 600 bp terminal repeat sequences (TRS) of the provirus; —— MSV sequences of MuLV origin; xxxx *src* sequences (7,8).

[b] Orientation (left to right) is from 5' to 3' with respect to viral RNA. The λMSV and plasmid MSV recombinants have been described (8,10), except for pHT101. The latter is an *Xba* I fragment deletion of pHT10 (see text). DNA transfections were performed as previously described (10).

[c] Samples 2,7,8 and 9 were transfected or co-transfected in the plasmid vector (pBR322) after *Bam*Hl digestion.

[d] Plasmid pMlsp contains one copy of the mlTRS and flanking mink sequences. It was generated from λmlr^+ which carries a specific deletion of the λml genome (McClements *et al.*, submitted).

tested its efficiency in a co-transfection experiment with pmlsp (Table 1). The activity of pHT101 plus pmlsp was 100-fold higher than pHT10 alone and only 3-fold lower than pHT10 plus pmlsp. Consistent with the prediction from comparison of the physical maps of ml MSV, HT1 MSV and MSV 124 (8), these results indicate that the Xba I fragment sequences from pHT10 are not essential for tranformation.

We have presented data indicating that sequences between the Xba I site of MSV and the beginning of src are essential for transformation (9). The cloned λMsarc is inactive in the DNA transfection-transformation assay (9). A hybrid phage (λLS1) was constructed by joining the λMsarc to the 3' end of λHT1 at their common sarc/src Hind III site (1.1 kb sarc/src, Figure 1). Even with TRS sequences at the 3' end of sarc, λLS1 was inactive (9). However, when the sequences to the 5' side of the Bgl I site λMsarc (0.3 kb sarc/src map, Figure 1) were replaced with the 5' end of λml (mlMSV sequences from the 5' EcoR1 site to the Bgl I site in sarc/src), the new recombinant, λLS2, transformed cells efficiently (9). The mlMSV sequence used in construction of λLS2 does not contain sufficient information to tranform cells (McClements et al., in preparation). From these and the pHT101 results, we conclude that the MuLV sequence between the Xba I site and the beginning of src is critical for transformation by src or sarc. We can only speculate about its function. This MuLV sequence could possess a control element such as a splice acceptor site which allows src expression. A 130 base sequence corresponding to the 5' end of the envelope (env) gene region of MuLV is retained by MSV 124 (12). This sequence should correspond to the MuLV sequences preceeding the 5' end of src that we have identified to be essential for sarc/src transformation. It is possible that these sequences possess a viral env gene splice acceptor site, but in addition, there may be sufficient env information to encode a signal peptide to transport a src gene product to an unusual location within the cell.

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MOLECULAR CLONING OF MOLONEY MOUSE SARCOMA VIRUS SPECIFIC SEQUENCES FROM UNINFECTED MOUSE CELLS[1]

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ABSTRACT When uninfected mouse cell DNA is cleaved with restriction endonuclease EcoR1, a DNA fragment of the size of 14.0 kb can be identified by hybridization to cloned DNA containing sarcoma specific sequences of Moloney mouse sarcoma virus (M-MSV$_{src}$). The cellular DNA fragment contains the entire M-MSV$_{src}$ specific sequences. The 14.0 kb EcoR1 DNA fragment was cloned in bacteriophage λ. The sequence organization of a recombinant clone, λ·M$_{TX}$-1, was analyzed by restriction endonuclease mapping, S1 mapping and electronmicroscopy. The results indicate that λ·M$_{TX}$-1 contains an uninterrupted stretch of 1.0 kb similar to that found in the M-MSV genome.

INTRODUCTION

Murine sarcoma viruses can induce cellular transformation in cultured fibroblasts and fibrosarcomas in animals (1). Moloney murine sarcoma virus (M-MSV) was isolated by passage of Moloney murine leukemia virus (M-MLV) through Balb/c mice (2). The genome of a M-MSV (Clone 124) has been extensively analyzed and appears to have been derived by recombination between M-MLV and cellular genes (1,3,4). Hybridization studies have shown the presence of MSV-specific nucleotide sequences in the cellular DNA obtained from uninfected mouse cells (5). We have recently cloned unintegrated M-MSV circular DNA in bacteriophage lambda (6). A DNA fragment

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encompassing the sarcoma (src) specific region of the M-MSV genome was further subcloned in bacterial plasmid pBR322. The plasmid containing the M-MSV$_{src}$ fragment showed no hybridization to the helper M-MLV genomic RNA. When uninfected mouse cell DNA was cleaved with restriction endonuclease EcoRl and analyzed on Southern-blotting gels (7) by hybridization to cloned M-MSV$_{src}$ probe, a DNA fragment migrating with a size of 14.0 kb can be detected. The 14.0 kb band contained the entire M-MSV$_{src}$ specific sequences. We have cloned the 14.0 kb EcoRl fragment in bacteriophage lambda and characterized it by restriction endonuclease mapping, electronmicroscopy, and Sl-mapping. The results indicate that the 14.0 kb EcoRl fragment contains an uninterrupted stretch of about 1,000 nucleotides which is homologous to the src-specific region of the M-MSV genome.

RESULTS

1. Identification of M-MSV$_{src}$ specific sequences in uninfected cells. Figure 1A shows the physical map of cloned unintegrated M-MSV DNA (p·MSV-1) with Hind III cleavage site at 0 map unit. The M-MSV$_{src}$ specific fragment extending from Hind III to Xba I was subcloned in the Pst I site of plasmid pBR322 by [dC]-[dG]-tailing method (p·MSV-31) (Fig. 1B). High molecular weight DNA from uninfected NIH/3T3 cells was digested with restriction endonuclease EcoRl and electrophoresed on agarose gels. The DNA from the agarose gel was transferred onto nitrocellulose filters and hybridized to labeled p·MSV-31 DNA. A single band of a size corresponding to 14.0 kb (Fig. 1C) can be seen. In order to determine if the 14.0 kb cellular DNA fragment contains the entire M-MSV$_{src}$ region, we hybridized the EcoRl digested mouse cell DNA to restriction endonuclease Pst I cut DNA fragments obtained from p·MSV-31. Fig. 1B indicates that p·MSV-31 cleaved with Pst I generates five M-MSV$_{src}$ specific fragments. All four viral DNA fragments hybridize to the 14.0 kb DNA fragment, suggesting that the cellular DNA fragment contains a majority of the M-MSV$_{src}$ specific region.

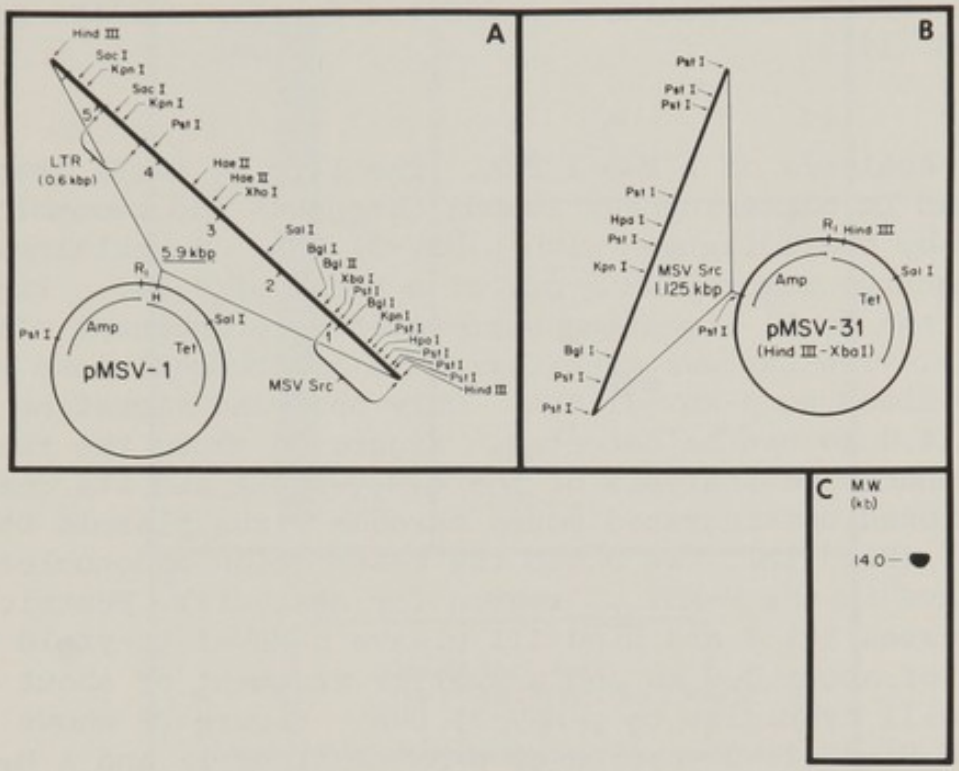

FIGURE 1. Identification of EcoR1 digested cellular DNA fragments containing M-MSV$_{src}$ specific sequences. The high molecular weight cellular DNA was prepared as described (8). In order to subclone the M-MSV$_{src}$ specific fragment, the plasmid (p·MSV-1) containing the entire M-MSV genome and two copies of long terminal repeats (LTR) was cleaved with restriction endonucleases Hind III and Xba I and fractionated on agarose gels. The Hind III-Xba I fragment was excised, DNA extracted and a stretch of 20 to 30 [dC]-residues incorporated using terminal transferase. The plasmid pBR322 was cleaved with restriction endonuclease Pst I and a stretch of 8 to 20 [dG]-residues added as described (9). The [dC]-tailed insert and [dG]-tailed plasmid were annealed and used to transform E. coli C600SF as described (9). The colonies were screened for drug resistance and Amp^{S}Tet^R colonies were further screened by hybridization to M-MSV cDNA as described (10). A recombinant plasmid (p·MSV-31) containing the Hind III to Xba I fragment in 3'→5' direction clockwise to the EcoR1 site of the plasmid was used in the studies reported here.

(A) Physical map of p·MSV-1, containing the entire M-MSV genome and long terminal repeat (LTR). (B) Physical map of p·MSV-31, containing the M-MSV$_{src}$ specific region. p·MSV-1 was cleaved with Hind III and Xba I and subcloned in the Pst I site of pBR322 by [dC]-[dG]-tailing. (C) Southern blot hybridization of EcoR1 digested uninfected mouse cell DNA with nick-translated p·MSV-31 DNA. The DNA was analyzed on 0.7% agarose gel and electrophoresis was carried out for 19

hrs at 5 volts/cm. Hybridizations were performed at 42° C for 8 hrs in 50% formamide 5 x SSC and dextran-sulfate as described (17).

2. Analysis of λ·M_{TX}-1 DNA. The 14.0 kb EcoRl fragment was cloned in bacteriophage lambda Charon 4A and recombinants screened by hybridization with p·MSV-31 DNA. We obtained three reactive recombinants out of a total of 9,000. Figure 2A shows the EcoRl digestion pattern of a recombinant clone (λ·M_{TX}-1). The DNA was transferred to a nitrocellulose paper and hybridized to p·MSV-31 DNA. Only one band migrating at a size of 14.0 kb can be detected. Figure 2B shows the restriction endonuclease analysis of the λ·M_{TX}-1 DNA and its comparison to cloned unintegrated mouse sarcoma virus plasmid DNA (p·MSV-1) (Fig. 1A). We chose the restriction endonucleases that cleave in the M-MSV_{src} region for analysis. Restriction endonucleases Bgl I and Hind III cleave p·MSV-1 to yield a fragment of about 0.9 kb and a smaller fragment of about 0.4 kb that will hybridize to p·MSV-31 DNA. Figure 2B shows that Bgl I and Hind III digestion of p·MSV-1 (lane 1) and λ·M_{TX}-1 (lane 2) show a band at about 0.85 kb in both cases. The smaller 0.4 kb fragment can be seen only in the p·MSV-1 and not in the λ·M_{TX}-1, suggesting that the sequences between p·MSV-1 and λ·M_{TX}-1 diverge between the two Bgl I sites. The larger band of about 3.0 kb in lane 2 represents the cellular DNA fragment containing a portion of M-MSV_{src} specific sequences. Digestion with Kpn I and Hind III also shows in both the p·MSV-1 (lane 3) and λ·M_{TX}-1 (lane 4) a band of 0.7 kb. The larger band in λ·M_{TX}-1 of about 1.0 kb represents a DNA fragment that contains the M-MSV_{src} specific region of λ·M_{TX}-1 and cellular sequences. The Bgl I plus Kpn I digestion also shows an identical band of an average size of 0.3 kb in both p·MSV-1 and λ·M_{TX}-1 (lanes 5 and 6 shown with an arrow). Similarly, digestion with Pst I shows the smaller fragments in both p·MSV-1 and λ·M_{TX}-1 of identical sizes (lanes 7 and 8). When p·MSV-1 and λ·M_{TX}-1 are cleaved with restriction endonucleases Hind III and Xba I, no common fragment is observed (lanes 9 and 10). Similarly, no common fragments were observed when p·MSV-1 and λ·M_{TX}-1 were cleaved with Bgl II (data not shown). Thus, it appears that the M-MSV_{src} specific sequences between p·MSV-1 and λ·M_{TX}-1 diverge between Bgl I and Xba I sites. A tentative map of the λ·M_{TX}-1 DNA is shown in Fig. 2C.

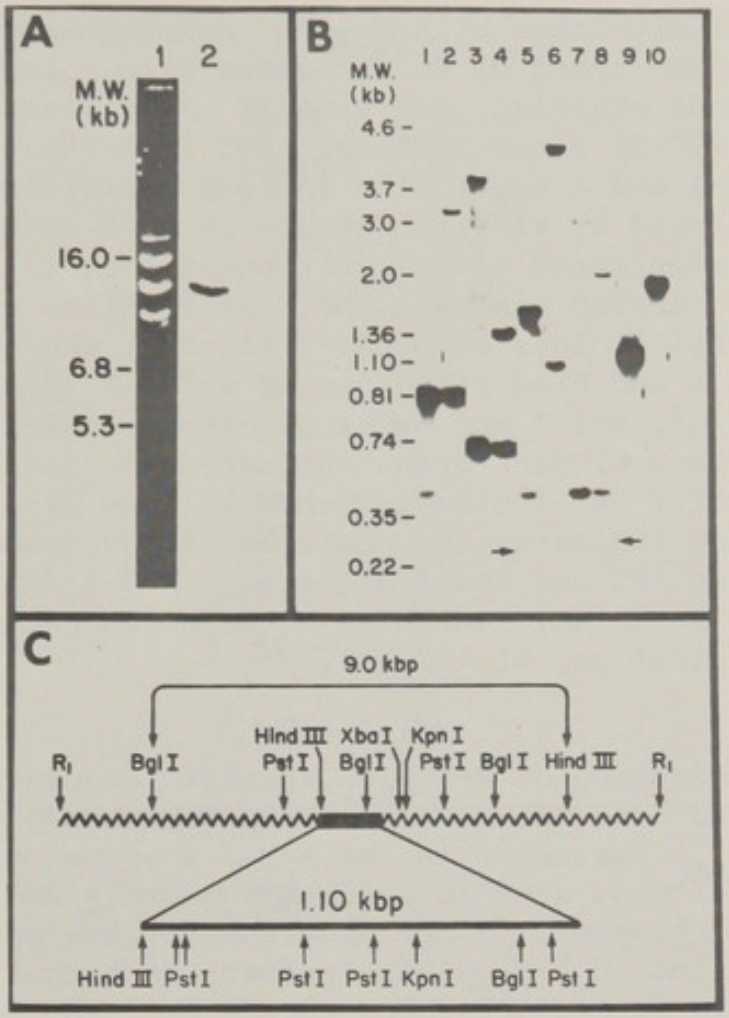

FIGURE 2. Molecular cloning and restriction endonuclease analysis of λ·M_{TX}-1. The 14.0 kb EcoR1 fragment was excised from a 0.7% agarose gel and DNA eluted by electrophoresis as described (6). About 1.0 µg of cellular DNA was joined to 3.0 µg of isolated arms of EcoR1 cleaved λ Charon 4A DNA with T4 DNA ligase (11). The ligated cellular DNA and vector DNA were then packaged in vitro into infectious lambda particles according to the protocol of Blattner and coworkers (11). About 9,000 recombinant clones were screened with labeled p·MSV-31 as the probe. Plaques that hybridized to the probe were further screened and recombinant phages of interest propagated as described (6). The nature of the cloned DNAs was examined by restriction endonucleases as described (6). Most restriction endonucleases were obtained commercially. The restriction endonuclease digested DNA was fractionated on agarose gels ranging from 0.5 to 1.2% in buffer containing 40 mM Trizma base, 1 mM EDTA and 5 mM sodium acetate, pH 7.8. The fragments were identified either by ethidium bromide staining (12) or by hybridization using the Southern blotting technique (7). The sizes of the restriction fragments were computed from the standard molecular weight marker in the same gel.

(A) EcoRl digestion of λ·M_{TX}-1. Samples were digested with EcoRl and analyzed on 0.7% agarose gels. Lane 1: Ethidium bromide staining; and lane 2: Hybridization to nick-translated p·MSV-31 after Southern blot transfer. (B) Digestion of p·MSV-1 and λ·M_{TX}-1 with various restriction endonucleases followed by electrophoresis on 1.2% agarose gel. The DNA was transferred on to a nitrocellulose filter and hybridized to labeled p·MSV-31 DNA. Restriction endonucleases Hind III and Bgl I digested p·MSV-1 (lane 1) and λ·M_{TX}-1 (lane 2); Kpn I and Hind III digested p·MSV-1 (lane 3) and λ·M_{TX}-1 (lane 4); Bgl I and Kpn I digested p·MSV-1 (lane 5) and λ·M_{TX}-1 (lane 6) (the arrow indicates the position of the common fragment); Pst I cleaved p·MSV-1 (lane 7) and λ·M_{TX}-1 (lane 8) (arrow indicates the position of the common smaII fragment); Hind III and Xba I digested p·MSV-1 (lane 9) and λ·M_{TX}-1 (lane 10). (C) Tentative restriction endonucleases generated physical map of λ·M_{TX}-1.

3. Organization of M-MSV_{src} specific sequences in λ·M_{TX}-1. Based upon the restriction endonuclease data (Fig. 2), the M-MSV_{src} sequences in the λ·M_{TX}-1 appear to have the same organization as seen in the M-MSV genomic RNA (Fig. 1A). These results, however, do not show whether the src-specific sequences present in λ·M_{TX}-1 are present as a continuous stretch or have some intervening sequences. In order to approach this question, we did the following two kinds of experiments:

a. S1-mapping. λ·M_{TX}-1 DNA was hybridized to M-MSV genomic RNA under conditions of R-loop formation. The hybrid was treated with single-strand-specific nuclease, S1, and analyzed on alkaline agarose gels. The DNA from the gel was transferred on nitrocellulose filters and hybridized to nick-translated p·MSV-1 DNA representing the entire genome. Fig. 3 (lane 1) shows that the λ·M_{TX}-1 DNA which was not annealed to M-MSV RNA shows no distinct band whereas after annealing to M-MSV genomic RNA a 1.0 kb DNA fragment can be seen (lane 2). The detection of a single DNA fragment of an average size of 1.0 kb suggests that the λ·M_{TX}-1 DNA contains the M-MSV_{src} specific sequence as a continuous stretch.

FIGURE 3. S1 mapping analysis of $\lambda \cdot M_{TX}$-1. The endonuclease S1-mapping was performed according to the procedure described by Berk and Sharp (13). The DNA and the 30S M-MSV genomic RNA were precipitated and resuspended in 10 mM EDTA. The RNA was boiled for 45 sec and kept at 4° C. The DNA preparation was boiled for 1.5 min and transferred to a 68° C waterbath, followed by addition of buffer containing 0.4 M NaCl, 0.04 M PIPES, pH 6.4, 0.001 M EDTA and formamide (final concentration, 80%). The sample was further incubated at 68° C for 15 min. The RNA was added to the DNA preparation and hybridization carried out at 56° C for 3 hr. One hundred and fifty µl of S1 buffer containing 0.25 M NaCl, 0.03 M Na acetate, 0.001 M $ZnSO_4$, 20 µg/ml denatured calf thymus DNA and 100 units of endonuclease S1 (BRL) was added to the samples. The samples were incubated at 37° C for 30 min and nucleic acids precipitated by the addition of ethanol. The precipitate was dissolved in a buffer containing 0.01 M Tris-HCl, pH 7.4, and 0.001 M EDTA, pH 7.4 and analyzed on alkaline agarose gel (14). The DNA from the gel was transferred to

nitrocellulose filters and the fragment identified by hybridization to [^{32}P]-labeled nick-translated (15) p·MSV-1 DNA followed by autoradiography.

Hybrids formed between λ·M_{TX}-1 DNA and 30S M-MSV 124 genomic RNA were digested with endonuclease S1 and analyzed on alkaline agarose gel as described in the procedures. Lane 1: λ·M_{TX}-1 DNA; and lane 2: λ·M_{TX}-1 DNA and 30S genomic M-MSV 124 RNA.

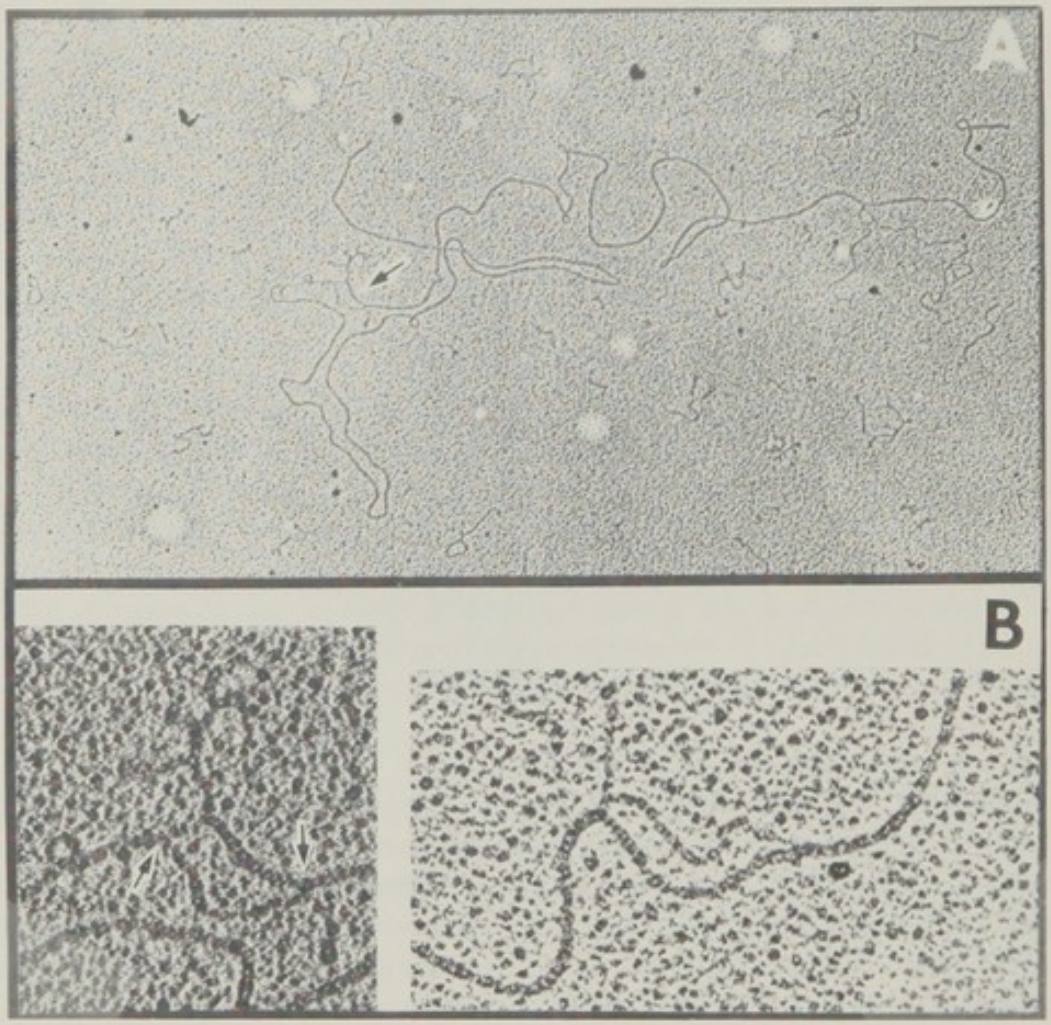

FIGURE 4. Electronmicrograph of the R-loop formed between λ·M_{TX}-1 DNA and 30S M-MSV genomic RNA. About 1 μg/ml of DNA and 3 μg/ml of RNA samples were dissolved in a solution containing 80% formamide, 0.4 M NaCl, 0.01 M PIPES, pH 6.3, and 0.001 M EDTA. The DNA was heated separately at 68° C for 5 min and then mixed with RNA, followed by incubation at temperatures which slowly decreased from 55° C to 51° C over a four-hour period. Molecules were prepared for electronmicroscopy as described (16).

(A) λ·M_{TX}-1 DNA was annealed to M-MSV genomic RNA and analyzed as described (16). The arrow indicates the R-loop formed. (B) Enlargement of the area showing the R-loop. Two different molecules are shown.

b. Characterization of $\lambda \cdot M_{TX}$-1 DNA by electron-microscopy. It is possible that there are several copies of M-MSV_{src} specific sequences in the 14.0 kb fragment. However, when $\lambda \cdot M_{TX}$-1 was heteroduplexed to MSV genomic RNA only a single R-loop structure of an average size of 1.0 kb was observed (Fig. 4A). The region showing the R-loop structure in Fig. 4A (marked with an arrow) is shown in Fig. 4B. Fig. 4B also shows the region of R-loop structure from another molecule. The unhybridized RNA appears as a condensed structure with a shorter end (presumably the 3' end) and a longer stretch (presumably the 5' end) at either end of the R-loop. Thus, it appears that $\lambda \cdot M_{TX}$-1 DNA contains only one copy of the M-MSV_{src} specific sequences.

DISCUSSION

The mouse cell DNA contains sequences homologous to the MSV_{src} specific region. Using M-MSV_{src} specific DNA as a probe, a single EcoR1 digested mouse cell DNA fragment of 14.0 kb can be identified. The 14.0 kb fragment showed no detectable hybridization to M-MLV cDNA, suggesting that it contains no viral sequences other than the M-MSV_{src} specific sequences. Occasionally, we have also observed a 5.3 kb fragment which hybridized to M-MSV_{src} PROBE. This fragment contained the entire M-MSV_{src} specific sequences. The origin of this fragment remains obscure. The 14.0 kb fragment contains a single copy of M-MSV_{src} specific sequences (Fig. 4). A 14.0 kb EcoR1 fragment in uninfected mouse cells has also been detected by Van der Woude et al. (18) and Tronick et al. (19).

The recombinant clone $\lambda \cdot M_{TX}$-1 contained a continuous stretch of 1.0 kb which was homologous to M-MSV genomic RNA. This size corresponded to the M-MSV_{src} specific sequences in the M-MSV genome. The $\lambda \cdot M_{TX}$-1 DNA was cleaved with several restriction endonucleases which also cleave the M-MSV_{src} specific region. The Bgl I to Hind III restriction fragment (Figs. 1 and 2) is present in the $\lambda \cdot M_{TX}$-1 DNA. Furthermore, analysis of $\lambda \cdot M_{TX}$-1 DNA with restriction endonucleases Kpn I, Pst I (Fig. 2) and Hpa I (data not shown) also generate the same fragments as observed for the plasmid DNA containing the entire viral genomic sequences. Restriction endonuclease Xba I cleaves p·MSV-1 DNA and is located close to the Bgl I site (Fig. 2, lanes 9 and 10). Thus it appears that endogenous transforming gene sequences in the M-MSV_{src} specific region extend between Bgl I and Hind III restriction sites and diverge between Bgl I and Xba I sites. We are now in the process of determining the nucleotide sequence of regions of

$\lambda \cdot M_{TX}$-1 DNA adjacent to Bgl I and Hind III sites to compare these with the M-MSV$_{src}$ specific sequences present in p·MSV-31.

The S1 and restriction endonuclease mapping data suggest that the cellular sequences involved in the biogenesis of Moloney mouse sarcoma virus are completely conserved in the viral genomic RNA. Earlier hybridization studies carried out by using src specific probes had suggested a large degree of homology between viral src specific and cellular DNA sequences (5). The cellular sequences containing M-MSV$_{src}$ specific sequences alone do not appear to be able to transform cells (Lai and Verma, unpublished). They apparently need to be inserted into the viral genome to become a transforming gene. The viral sequences upstream may serve as promoter for transcription of MSV$_{src}$ specific RNA. Recently, we have identified a 21S subgenomic mRNA species from M-MSV infected cells which directs the synthesis of a 37K protein in vitro (Jones, Lai, Fan and Verma, unpublished results). In vitro translation of viral genomic RNA also directs the synthesis of a 37K protein in addition to other viral proteins (Papkoff, Hunter and Beemon, personal communication). The synthesis of the 37K protein is selectively inhibited if the viral RNA is preannealed to p.MSV-31 DNA (Papkoff, Hunter and Verma, unpublished results). The 37K protein will require a coding capacity of approximately 1,100 nucleotides; the cellular DNA sequences containing M-MSV$_{src}$ sequences are about 1.0 kb.

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CHARACTERIZATION OF MOLECULARLY CLONED SPLEEN FOCUS-FORMING VIRUS DNA

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ABSTRACT Unintegrated supercoiled spleen focus-forming virus (SFFV) DNA was extracted from rat cells newly infected with a woolly monkey virus pseudotype of the Lilly-Steeves strain of SFFV. The DNA was linearized by digestion at the unique HindIII endonuclease site, inserted into the plasmid pBR322, and cloned in an approved EK2 host. Six independent clones containing SFFV DNA inserts were isolated and analyzed by restriction endonuclease digestion. One clone had an insert of 5.7 kbp and also contained the same restriction enzyme sites as the *in vivo* linear DNA except that it lacked a copy of the terminally redundant sequences. Recombinant DNA isolated from this clone was transfected into NIH 3T3 fibroblasts and rescued either by co-transfection of cloned Friend murine leukemia helper virus (F-MuLV) DNA or by superinfection with a non-erythroblastosis-inducing variant of F-MuLV. Inoculation of adult NIH Swiss mice with the virus produced from these transfections induced an erythroleukemia, characterized by splenomegaly, polycythemia and splenic foci, which was identical to that induced by the original F-MuLV pseudotype of SFFV. Also, the SFFV specific gp52 protein was synthesized in the transfected fibroblasts.

INTRODUCTION

The Friend strain of the spleen focus-forming virus (SFFV) is a replication-defective type-C RNA tumor virus which induces splenomegaly, polycythemia and splenic foci when injected as a pseudotype into susceptible mice (1,2). Originally isolated as a complex with the helper-independent Friend murine leukemia virus (F-MuLV) (2), the SFFV component has been biologically cloned free of the F-MuLV and its genome has been extensively analyzed by molecular hybridization (3,4). This analysis has demonstrated that SFFV is a recombinant between a portion of the F-MuLV genome and se-

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quences that are highly related to the envelope gene of mink cell focus-inducing (MCF) viruses. We have also examined the proteins encoded by SFFV and can detect at least two proteins, a gag gene-related 45,000 dalton protein with antigenic determinants of p15 and p12 (5), and an env gene-related 52,000 dalton glycoprotein (6). In order to determine whether either of these proteins is required for the development of the SFFV-induced disease, a genetic analysis of the SFFV genome is necessary. However, due to the replication defectiveness of the acute leukemia viruses, it has not been possible to perform genetic studies with these viruses. We now report the molecular cloning of the genome of SFFV in a biologically active form, making possible a detailed genetic analysis of this replication defective virus.

RESULTS AND DISCUSSION

Analysis of Unintegrated SFFV DNA. Since the purpose of this study was to obtain biologically active DNA homologous to SFFV which could be used as a substrate for the genetic analysis of this replication-defective virus, we set out to molecularly clone the SFFV DNA. Our approach was to isolate the closed circular form of DNA and clone the entire molecule after linearization by cleaving with an enzyme that has a single recognition site in the DNA. Unintegrated DNA was extracted by the Hirt method (7) from NRK fibroblasts infected 24 hours earlier with a woolly monkey type-C virus (WoLV) (8) pseudotype of the Lilly-Steeves strain of SFFV (9). The NRK cells were used instead of mouse cells to prevent the cloning of endogenous murine sequences with homology to SFFV. Similarly, WoLV was used as a helper virus instead of F-MuLV due to the homology between F-MuLV and SFFV. The forms of DNA present in the Hirt preparation was analysed as described in Fig. 1. Three bands of SFFV-specific DNA were detected (lane 1) after hybridization to a cDNA probe prepared to a Friend virus pseudotype of SFFV (EY10). The most prominent band, migrating at a size of 6.3 kbp, was interpreted to be the form III linear unintegrated SFFV DNA, and the lighter bands migrating with sizes of approximately 4.5 kbp and 4.1 kbp were presumed to be form I double-stranded closed circles. The autoradiograph presented in lane 2 of Fig. 1 shows the bands of DNA which hybridized to a probe prepared from WoLV, and demonstrates the lack of homology between SFFV and WoLV, allowing visualization of SFFV DNA without detection of the helper virus DNA. This figure also demonstrates the ability to separate the SFFV and WoLV DNAs by size.

A preliminary map of restriction endonuclease cleavage sites in SFFV DNA was then determined in order to identify an

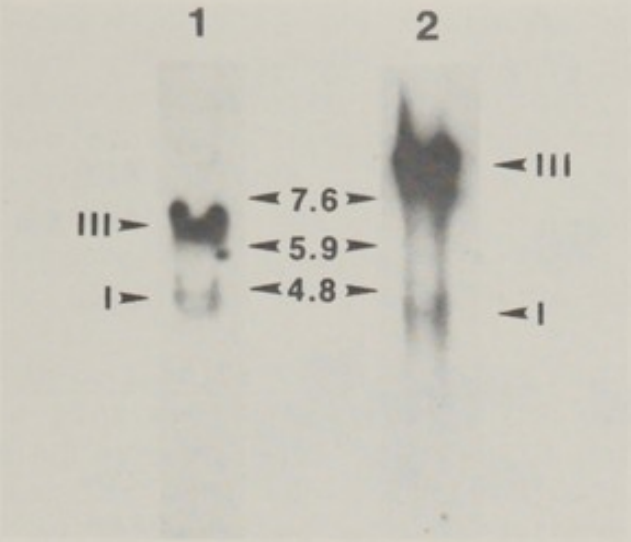

FIGURE 1. Southern blot analysis of Hirt supernatant DNA. Unfractionated Hirt supernatant DNA (30 μg) isolated from NRK cells newly infected with a WoLV pseudotype of SFFV was electrophoresed through an agarose gel, blotted by the Southern transfer technique (10) and hybridized to [^{32}P] cDNA-EY10 (lane 1) or [^{32}P] cDNA-WoLV (lane 2). The bands of hybridized DNA interpreted to be form I circular and form III linear DNA molecules are indicated at the sides of each lane. The numbers between the two lanes indicate the sizes (in kilobase pairs) of EcoRI-digested wild-type λ DNA fragments migrating on the same gel.

enzyme with only one site which could be used for cloning the form I circles, and to establish a basis for comparing known SFFV DNA with any potential cloned SFFV DNA. The linear SFFV DNA was first purified from 20 mg of Hirt supernatant DNA and analyzed as described in Fig. 2. The autoradiograph presented in Fig. 2A shows that HindIII has a single cleavage site in the linear DNA and generates two fragments which migrate at sizes of 3.7 kbp and 2.6 kbp (lane 2). Three fragments are produced after EcoRI cleavage (lane 3) indicating that this enzyme makes two cuts in the DNA. Only one band (at 5.7 kbp) was visible after KpnI digestion (lane 5); however, double digestion of the DNA with both HindIII and KpnI (lane 6) resulted in faster migration of both HindIII fragments at sizes of 3.3 kbp and 2.4 kbp. This indicates that KpnI cleaves the DNA at both ends, probably in the terminally redundant sequences as has been previously shown for other murine retroviral genomes (11,12). Other single and double digestions were performed with various enzymes to give the cleavage patterns shown in lanes 4, 7, 8, 9, and 10, and these enzyme sites were mapped on the SFFV molecule. In order to orient the map 5' and 3' according to the SFFV genomic RNA, restricted DNA was similarly analyzed after

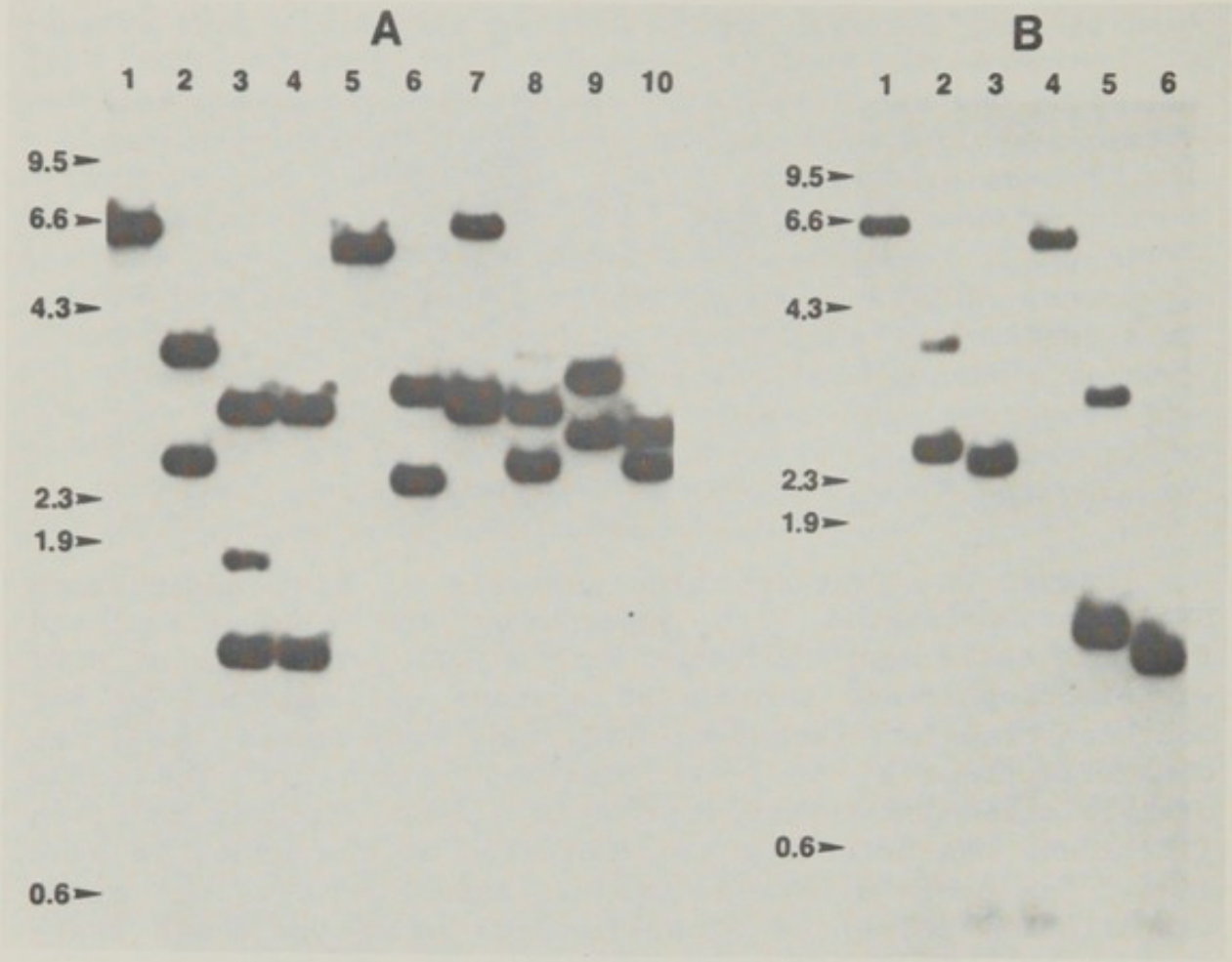

FIGURE 2. Southern blot analysis of unintegrated linear SFFV DNA after restriction enzyme digestion. The form III linear SFFV DNA was isolated from the Hirt supernatant DNA by preparative gel electrophoresis and then digested with various restriction endonucleases. The resulting DNA fragments were analyzed as in Fig. 1. (A) Autoradiograph of SFFV DNA hybridizing to cDNA-EY10. The SFFV DNA was digested with no enzyme (lane 1), HindIII (lane 2), EcoRI (lane 3), HindIII plus EcoRI (lane 4), KpnI (lane 5), HindIII plus KpnI (lane 6), XbaI (lane 7), HindIII plus XbaI (lane 8), HincII (lane 9), and HindIII plus HincII (lane 10). (B) Autoradiograph of SFFV DNA hybridizing to 3' F-MuLV cDNA. The SFFV DNA was digested with no enzyme (lane 1), HindIII (lane 2), HindIII plus KpnI (lane 3), KpnI (lane 4), EcoRI (lane 5), EcoRI plus KpnI (lane 6). The numbers at the left of each autoradiograph represent the location and size (in kilobase pairs) of HindIII-digested wild-type λ DNA fragments migrating on the same gel.

hybridization to a cDNA probe prepared from the 3' end of F-MuLV RNA, which has known homology to the 3' end of the SFFV genome (13). The autoradiograph in Fig. 2B shows that

after cleavage with Hind III, two fragments of SFFV DNA are detected with this probe. However, after the terminally redundant sequences are removed by further KpnI cleavage (lane 3), only the smaller fragment is hybridized along with a small 0.4 kbp piece of DNA. This localizes the smaller 2.6 kbp HindIII fragment to the 3' end. A similar examination of EcoRI digested DNA (lanes 5 and 6) allowed us to prepare the restriction map of the SFFV in vivo linear DNA presented in Fig. 3A.

Molecular Cloning of SFFV DNA. Since HindIII was found to have only one cleavage site in the SFFV genome, the 4 to 4.5 kbp DNA, which hybridized to cDNA-EY10 and was presumed to be form I circular DNA, was purified from 20 mg of Hirt supernatant DNA by preparative gel electrophoresis and digested with HindIII. This digested DNA was ligated to HindIII digested pBR322 plasmid DNA and cloned in E. coli strain RR1 by bacterial transformation (14). Out of 10,000 transformants screened, six were found to contain SFFV sequences. Each of the positive clones was subcloned, the plasmid DNA was amplified by culturing the bacteria in the presence of chloramphenicol, and the recombinant pBR322-SFFV was extracted and purified (15).

Analysis of Cloned DNA. The recombinant SFFV-pBR322 DNA was digested with HindIII to remove the SFFV insert and then visualized after agarose gel electrophoresis by ethidium bromide staining or by Southern transfer and hybridization to cDNA-EY10 (Fig. 4). Three types of clones were found. One clone contained a single 5.7 kbp SFFV-specific DNA insert in addition to the 4.3 kbp pBR322 DNA (lanes A1 and B1). Another clone contained a single SFFV-specific insert with a size

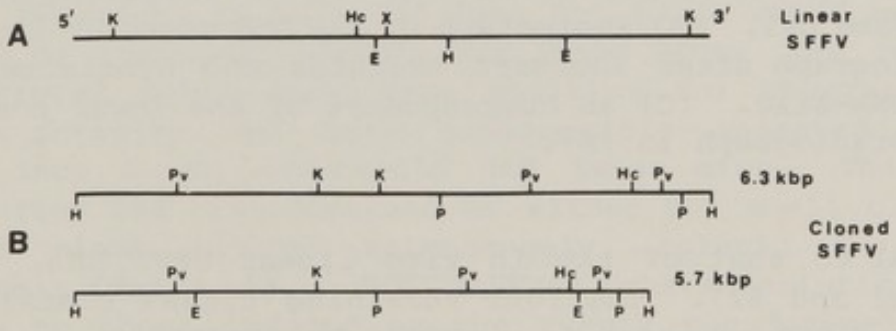

FIGURE 3. Schematic representation of restriction endonuclease cleavage sites on SFFV DNA. (A) Restriction map of unintegrated linear SFFV DNA. (B) Restriction map of the large (6.3 kbp) and small (5.7 kbp) SFFV recombinant DNA clones. The abbreviations used are as follows: H, HindIII; K, KpnI; Hc, HincII; E, EcoRI; X, XbaI; Pv, PvuII; and P, PstI.

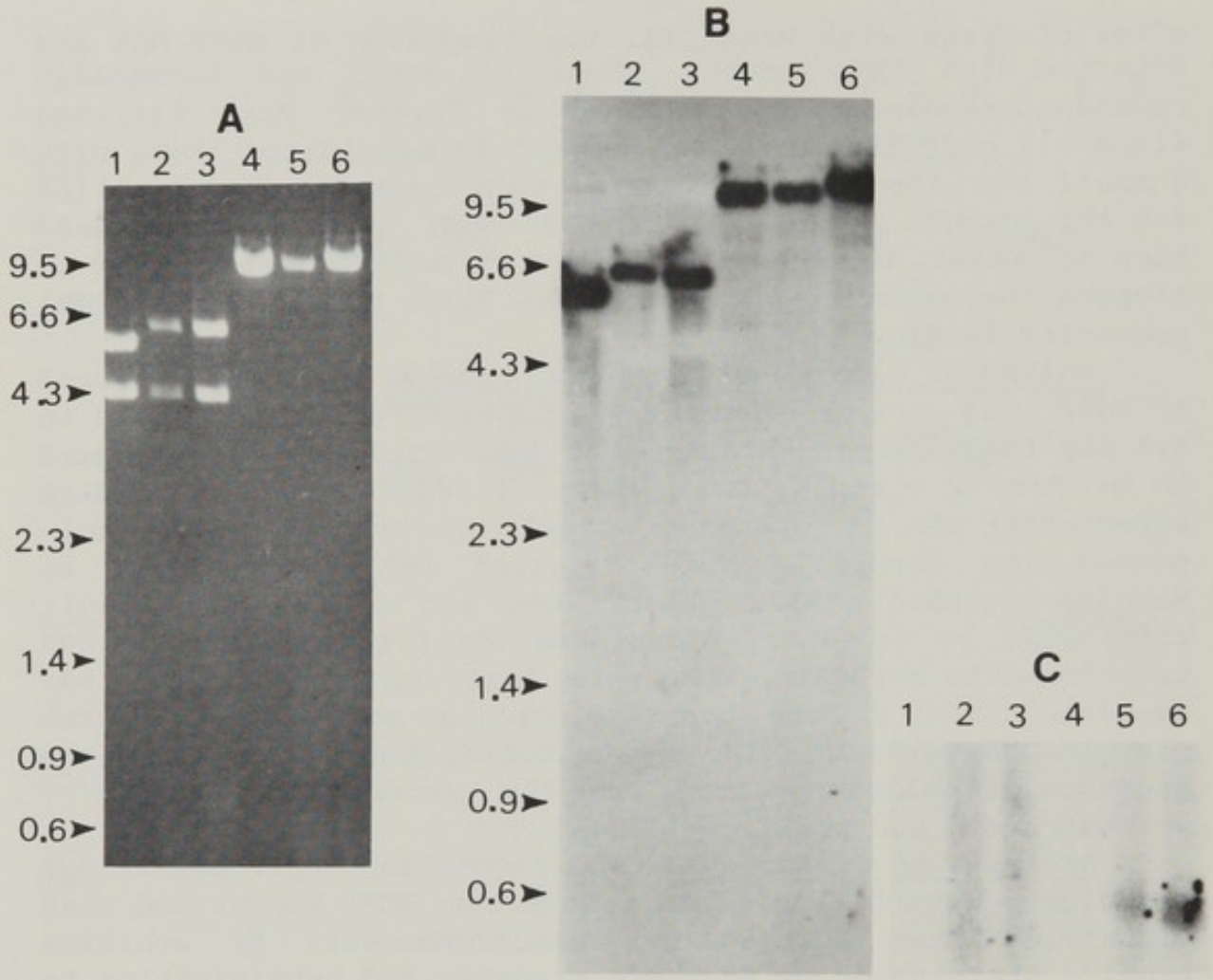

FIGURE 4. Agarose gel analysis of cloned DNA. The DNA (1 µg) isolated from the three types of recombinant SFFV-pBR322 DNAs was digested with HindIII (lanes 1, 2, and 3) or KpnI (lanes 4, 5, and 6) and electrophoresed on a 1% agarose gel containing 0.5 µg of ethidium bromide per ml. The numbers at the left represent the location and size (in kilobase pairs) of HindIII digested wild-type λ DNA fragments migrating on the same gel. (A) Photograph of the DNA under UV light. (B) Autoradiograph after Southern transfer and hybridization to [^{32}P] cDNA-EY10. (C) An overexposure of the lower portion of the autoradiograph in (B).

identical to that of the in vivo linear SFFV DNA, 6.3 kbp (lanes A2 and B2). The four remaining clones contained two inserts of DNA released from pBR322 by HindIII digestion with sizes of 6.3 kbp and 0.7 kbp (lane A3). Only the larger insert, 6.3 kbp, had SFFV-specific sequences (lane B3). The smaller 0.7 kbp insert showed no hybridization to cDNA-EY10 even in an autoradiograph exposed for a longer period of time (lane C3), and thus must represent either NRK cellular or WoLV sequences. The size of the 5.7 kbp clone was 0.6 kbp smaller than the in vivo linear DNA, a difference which

could be accounted for by the absence of one copy of the terminally redundant sequences reported for other type-C retroviruses (11,12). To examine this possibility, the clones clones were digested with KpnI which cleaves the viral DNA in the terminal redundancy but has no recognition site in pBR322 DNA. Indeed, the small clone containing the 5.7 kbp insert had only one KpnI site and when digested yielded a single band of recombinant DNA migrating at about 10 kbp (lanes 4A and 4B). KpnI digestion of the clone containing the 6.3 kbp insert generated a similar 10 kbp fragment in addition to a 0.6 kbp fragment (lanes 5A, 5B, and 5C), indicating the presence of two copies of the terminal repeat.

Further double and triple enzyme digestions were performed and the restriction maps of the two sizes of cloned SFFV DNA were deduced (Fig. 3B). Comparing these maps to the map of the in vivo linear SFFV DNA, we found the expected permuted orientation of enzyme sites for every enzyme tested except XbaI. None of the six clones could be cleaved with XbaI, which could suggest an alteration of the primary structure during the cloning process or a DNA modification by E. coli at a site overlapping the XbaI recognition site.

Transfection of Fibroblasts with Cloned DNA. To determine if the cloned SFFV DNA was biologically active, NIH 3T3 fibroblasts were transfected, by methods previously described (16), with the recombinant plasmid after HindIII digestion to remove the SFFV insert. Since SFFV does not transform fibroblasts and is replication defective, it was necessary to rescue the transfected sequences with a helper-independent virus and analyze the virus produced for oncogenicity in the mouse. To accomplish this, the fibroblasts were either co-transfected with molecularly cloned and biologically infectious F-MuLV clone 57 helper DNA (12) or superinfected after transfection with a variant of F-MuLV (F-MuLV cl B-3) which does not replicate as well and does not cause rapid erythroblastosis or anemia in newborn NIH Swiss mice. The cultures were monitored until they were positive for reverse transcriptase activity and were subsequently injected intravenously into 6- to 8-week-old NIH Swiss mice. The virus produced from the transfections of either the small clone or the large clone induced splenomegaly, splenic foci, and polycythemia (Table 1). The disease characteristics were identical to those induced by the F-MuLV pseudotype of the original SFFV (9), except that a longer latency was often seen, especially when superinfection was used to rescue the replication-defective sequences (Table 1).

Since it has previously been shown that SFFV has sequences homologous to helper F-MuLV (3), it was possible that a molecular clone of SFFV which lacked certain sequences could recombine with F-MuLV in the fibroblasts. Theoreti-

TABLE 1
BIOLOGICAL PROPERTIES OF VIRUS PRODUCED AFTER TRANSFECTION OF NIH 3T3 FIBROBLASTS WITH CLONED SFFV DNA

Cloned SFFV DNA	Rescue of SFFV activity by: Superinfection of virus	Co-transfection of DNA	Latency[a] (days)	Spleno-megaly[b]	Splenic foci	Poly-cythemia[c]
Large 6.3 kbp clone	F-MuLV cl B-3	--	33	+	+	+
	--	F-MuLV cl 57 (EcoRI cut)	15	+	+	+
Small 5.7 kbp clone	F-MuLV cl B-3	--	14-26	+	+	+
	--	F-MuLV cl 57 (EcoRI cut)	10-15	+	+	+
	Amphotropic MuLV 1504 A	--	28	+	+	+

[a]Latency is expressed as the number of days before the development of splenomegaly and splenic foci after intravenous inoculation of 6- to 8-week-old NIH Swiss mice (intraperitoneal inoculation of newborn NIH Swiss mice was used for studies involving mouse amphotropic helper virus 1504 A). Mice inoculated with helper viruses alone or with virus produced by transfection of helper virus DNA alone showed no signs of disease within 50 days.

[b] Splenomegaly is defined as spleen weights of 0.7 g or greater (normal spleen weight = 0.1 to 0.2 g).

[c]Polycythemia is defined as hematocrits of 60% or greater (normal hematocrit = 43 to 48%).

cally, such an event could allow complementation of an incomplete SFFV genome and lead to the generation of the observed disease characteristics. To insure this was not occurring, the transfection was repeated using the 5.7 kbp DNA clone and rescue by superinfection with a wild mouse amphotropic virus 1504A (17). This helper-independent virus is not highly related to SFFV and does not induce disease in newborn Swiss mice, however, it can pseudotype SFFV and allow the induction of the SFFV-specific disease when infected into newborn Swiss mice. When newborn NIH Swiss mice were injected with the virus produced after transfection of the SFFV DNA and rescue by amphotropic virus superinfection, the same disease developed (Table 1), indicating the completeness of the cloned SFFV DNA.

We were also interested in whether or not the expression of any SFFV-encoded proteins could be detected in cells transfected with cloned SFFV DNA. The fibroblasts co-transfected with the small clone of SFFV DNA and F-MuLV clone of these cells were immune precipitated and analyzed by SDS-polyacrylamide gel electrophoresis as previously described (6). The autoradiograph of such a gel is presented in Fig. 5 and shows that the SFFV-specified gp52 is expressed in the

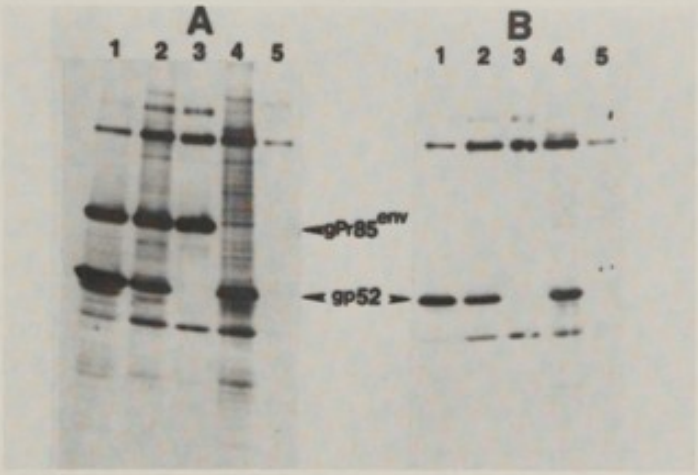

FIGURE 5. Immune precipitation of proteins from cells transfected with molecularly cloned SFFV DNA. Cells were pulse-labeled for 30 min with ^{35}S-methionine, lysed, immune precipitated and electrophoresed through 5-20% linear gradient SDS-polyacrylamide gels. (A) Goat anti-Rauscher MuLV gp70 serum or (B) goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV were used to precipitate F-MuLV/SFFV-FRE Cl 1 cells (lanes 1), NIH 3T3 cells co-transfected with molecularly cloned SFFV DNA (small clone) and F-MuLV clone 57 DNA (lanes 2), F-MuLV/NIH 3T3 cells (lanes 3), SFFV/NRK cells (lanes 4), and uninfected NIH 3T3 cells (lanes 5). The molecular weights indicated were determined from the positions of marker proteins migrating on the same gel.

transfected cells and can be precipitated by a group-specific anti-F-MuLV gp70 antiserum (lane A2) as well as by an antiserum specific for the gp70 of mink cell focus-inducing viruses (lane B2). This protein has the same molecular weight and antigenic determinants as the gp52 precipitated from EY10-infected cells (lanes A1 and B1) and SFFV/NRK nonproducer cells (lanes A4 and B4) and is not expressed in cells infected by helper F-MuLV alone (lanes A3 and B3) as previously described (6).

Genetic studies are now in progress using marker-rescue of transfected molecularly cloned fragments of the SFFV DNA as well as in vitro mutagenized DNA. These experiments should provide evidence and defined physical map locations of the SFFV sequences necessary for the induction of leukemia by this virus.

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THE NATURE AND ORIGIN OF THE TRANSFORMING GENE OF AVIAN SARCOMA VIRUSES[1]

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ABSTRACT Most of recovered avian sarcoma viruses (rASV), which were generated by recombination between transformation-defective (td) mutants of Rous sarcoma virus (RSV) and cellular sequences, were nondefective. The src sequences of these rASVs are very similar but not identical to those of standard RSV and the src gene product, pp60, is fully expressed in transformed cells. Some td mutants are not capable of generating rASV. Comparison of the RNA of various mutants suggests that the retention of the sequences at the 3' end of the src gene in the RNA of td mutants is required for the generation of rASV. One td mutant, whose deletion extends from src to the 3' end of the env region, consistently generates rASVs which have deletions in the env or in the env and pol. This implies that one of the td viral sequences required for the recombination with endogenous sarc is at the junction of the env and the src, but other similar crossover sites are located at the junctions of pol-env and gag-pol and also within the pol region.

The transforming gene of Fujinami sarcoma virus (FSV) was found to be unique and unrelated to the src gene of RSV. The genomic RNA of FSV was estimated to be 1.7×10^6 daltons or 5300 nucleotides which codes for at least one 140,000 dalton protein. The overall genetic structure of FSV was similar to those of acute leukemia viruses of avian and murine species.

INTRODUCTION

For studies on the mechanism of cell transformation, the

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findings of the nucleotide sequences related to the transforming gene of the retrovirus in DNA of uninfected host cells have profound implications. Not only could they provide possible models for the evolution of oncogenic viruses, but also they reemphasize the role of retrovirus as an agent which could drastically alter the expression of normal cellular genes. For avian systems, Stehelin et al. (1) and later Spector et al. (2) have demonstrated the presence of the sequences homologous to the src gene of avian sarcoma viruses (ASV) in DNA of various animal species of vertebrates. Similar findings have been made with the transforming genes of acute leukemia viruses (3). The cellular sequences known as endogenous sarc must be the sequences involved in the process of generation of series of ASV in the experimental system we have used.

We and others have shown that certain transformation-defective (td) mutants of Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) have incomplete deletion in the src gene (4-6). Injection of chickens or quails with such td mutants resulted in the formation of slow appearing sarcomas from which ASV was recovered (7-9). The viruses obtained were called "recovered avian sarcoma virus (rASV)", and in most cases they were nondefective in replicative functions and had sarcomagenic properties almost identical to standard laboratory strains of ASV (7). Analysis of rASV RNAs by molecular hybridization and oligonucleotide fingerprinting indicated that the genome of rASV reacquired src gene of the same size as SR-RSV. Since RNA of td mutants retain only 20 to 30% of the src sequences of SR-RSV (11), most of the src gene of rASVs must have been derived from the host cell DNA. Indeed, the src sequences of rASV are closely related, but are not identical to those of SR-RSV (8-11). The sequences outside the src region of rASV RNAs were identical to those of parental td viruses. Furthermore, the newly acquired src sequences of rASV obtained from quail tumors were distinguishable from those of rASV obtained from chicken tumors (8). These results clearly indicate that rASVs were generated by recombination between td viral genome and cellular sequences, presumably, those of the endogenous sarc.

In order to elucidate the origin of src genes present in different strains of ASV, we have compared their src sequences with those present in rASV RNAs by oligonucleotide fingerprinting (12). All ASVs and rASVs studied shared several src-specific oligonucleotides, although variations were also seen among different viruses. Interestingly, src-specific oligonucleotides present in rASVs but not in SR-RSV (thus considered to be specific to cellular sarc) were found in other strains of ASV. These results further support our interpre-

TABLE 1
PRESENCE OR ABSENCE OF src-OLIGONUCLEOTIDES
IN RNA OF VARIOUS td MUTANTS

	Oligonucleotides (src)												Formation of rASV
	---[35	13	32b	34	37	38	36	10c	8a	10a	33b	27]-	
td101	-	-	-	-	-	-	-	-	-	-	+	+	+
td103	+	+	+	-	-	-	-	-	-	-	-	-	-
td105	-	-	-	-	-	-	-	-	-	-	-	+	+
td106	-	-	-	-	-	-	-	-	-	-	-	-	-
td107A	-	-	-	-	-	-	-	-	-	-	-	-	-
td108	+	+	+	-	-	-	-	-	-	-	+	+	+
td109 (-)	-	-	-	-	-	-	-	-	-	-	+	+	+

tation for the generation of rASVs, and indicate the divergence of both viral and cellular src-related sequences. In the course of these studies, we discovered that one stock of Fujinami sarcoma virus contained a transforming gene entirely different from the src gene of RSV.

RESULTS

The src Gene of td Mutants and their Capacity to Generate rASV. As reported previously (7), td mutants of SR-RSV vary in their capacity to generate rASV. Because the sequences outside the src gene of rASV RNA are essentially the same as that of td viral RNA, a double crossover must be involved in the interaction between td virus and cellular sequences. As shown in Table 1, four out of seven td mutants thus far tested were able to form tumors from which rASVs were obtained. The oligonucleotide fingerprinting and mapping of these td viral RNAs were made by the procedures published in previous papers (8, 10), and are shown in Table 1. At least with the limited number of mutants examined, a correlation seems to exist between the presence of one oligonucleotide at the 3' end of src and the capacity to form rASV. On the other hand, the presence of oligonucleotides at the 5' end of src does not appear to be essential for the generation of rASV. In fact, one mutant, td109, was capable of producing rASV, even though the deletion of this viral RNA in the src gene is extended to the env region. In contrast, td103 is incapable of forming rASV while its RNA retains three oligonucleotides which map at the 5' terminus of the src gene.

At this stage, we are unable to determine whether the retention of oligonucleotide #27 is essential for the recombination between viral genome and cellular sequences. However, it seems reasonable that a small stretch of the sequences at the 3'-end of the src region is needed for the crossover with endogenous sarc sequences. It is interesting to note that all of rASV pp60 thus far studied have a phosphotyrosine containing peptide, near the carboxyl terminus of the molecule, which is similar to the homologous peptide of SR-RSV $pp60^{src}$ but different from that of normal cellular $pp60^{src}$ in electrophoretic mobility (13, R. Karess, unpublished). Therefore, the 3'-end sequences retained in td virus RNA appear to be responsible for coding for the portion of rASV $pp60^{src}$ containing the phospho-tyrosine residue.

Formation of rASV Defective in Replication. One mutant, td109, was unique among a series of td mutants studied. This virus replicates normally and the virus titer is the same as that of other mutants. This virus was also capable of generating rASV upon injection into chickens. However, the initial titers of about 20 isolates of rASV derived from this td mutant were always extremely low (10^2 to 10^3 FFU compared to average 10^6 FFU for rASV derived from other td mutants), and we suspected that these viruses could be defective in replication (7, 11).

In order to study the characteristics of these td109-derived rASV's, cells transformed by 12 independent isolates of these viruses were selectively cultured and fully transformed cultures were eventually obtained. The titer of virus from these cultures, was approximately 10^6 FFU/ml. When chicken cell cultures were infected with about 1000 FFU of these viruses and then plated for infectious centers on other fresh chicken cells, we found that focus formation by plated cells was virtually abolished if these infected cells were exposed to 5000 r of X-ray before plating. Since this dose of X-ray is known to block cell division but not affect virus release, the results described above strongly suggested that 12 independent isolates of td109-derived rASV thus far tested are defective in replication.

Five isolates of td109-derived rASV were chosen for further analysis. Single foci were isolated from cultures infected with each of these rASVs and virion production was examined. As shown in Table 2, none of the foci made by these five rASV, produced infectious virus. Two of them (157 and 1702) produced virions containing reverse transcriptase, and the remaining three (372, 3812, and 398) did not produce particles detectable by labeling cultures with ^{3}H-uridine. All of the foci produced 10^5 to 10^6 FFU of rASV upon super-

TABLE 2
CHARACTERISTICS OF NP CELLS TRANSFORMED BY CERTAIN ISOLATES OF rASV

Virus	Production of infectious virus	Production of physical particles	Sediment-able polyme-rase	Production of infectious ASV upon RAV-1 infection
157	-	+	+	+
1702	-	+	+	+
374	-	-	-	+
3812	-	-	-	+
398	-	-	-	+

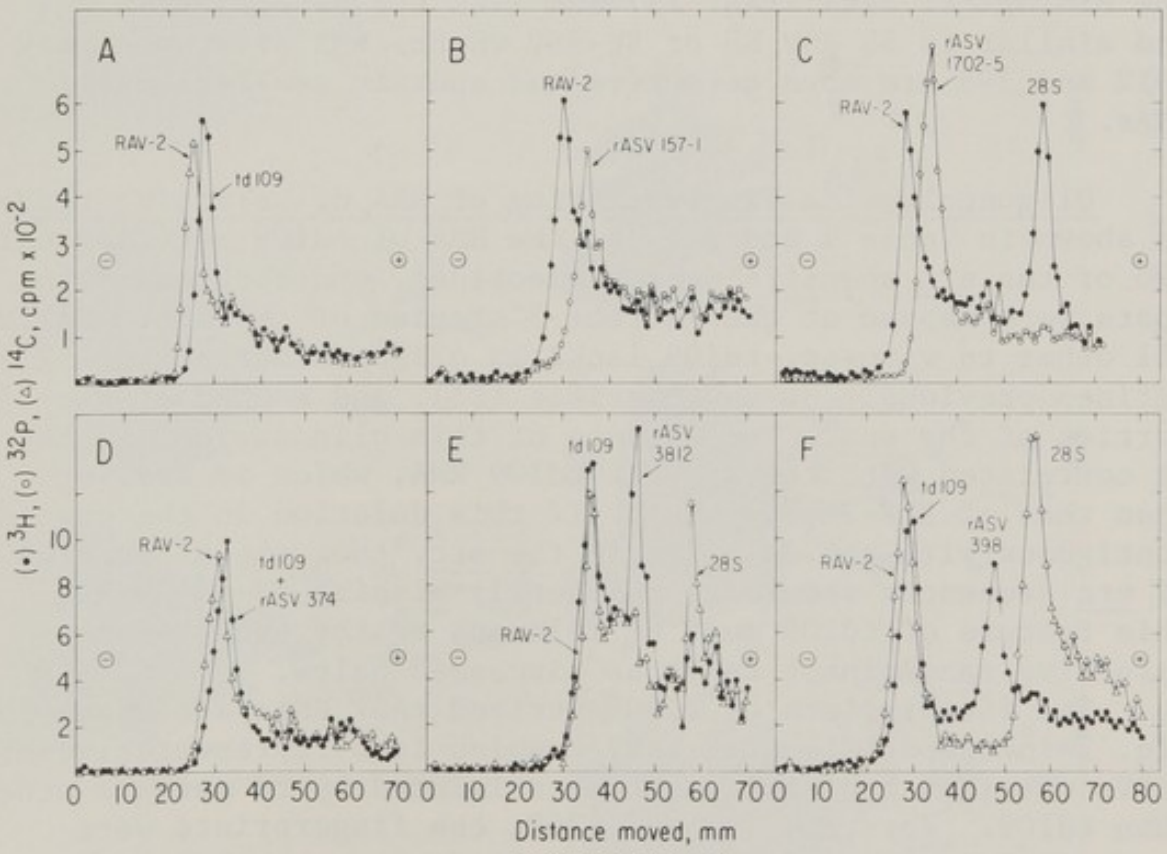

FIGURE 1. Polyacrylamide gel electrophoresis of viral RNAs. Isotope-labeled viral 60-70S RNA and marker RNAs were mixed, heat-denatured and analyzed by electrophoresis in 2.2% polyacrylamide gels.

infection with RAV-1.

The RNA of these five rASV and td 109 were sized by gel electrophoresis (Fig. 1). For rASVs 157 and 1702, clones of transformed cells were labeled with ^{32}P-orthophosphate, and RNA was extracted from ^{32}P labeled non-infectious virion. Because no physical particles were produced from foci formed by rASVs 374, 3812, and 398, cultures were superinfected with helper virus, td109, and labeled with ^{3}H, and labeled virus mixtures (rASV + td109) were analyzed for the size of RNA. As seen in Fig. 1 B and C, both rASV 157 and 1702 contain RNA smaller than RAV-2. Because these viruses contain functional src, gag and pol genes (Table 2), the results strongly suggest that the deletion occurs only in the env gene for these viruses. Fig. 1 C shows that rASV 374 probably contains small RNA similar to td109 in its size. The size of RNAs of rASV 3812 and 398 appears to be even smaller than other rASVs (Fig. 1 E and F). In sedimentation in sucrose gradients, these RNAs sedimented as 32 and 30S respectively (not shown).

The analysis of the RNA size of these rASVs thus confirms the biological data: rASV 157 and 1702 are defective in env and similar to SR RSV N8 or BH-RSV in the RNA size, and rASV 3812 and 398 are more defective and contain smaller genomic RNAs.

Oligonucleotide Fingerprinting of RNA of Defective rASVs. As shown in Table 1 and Fig. 2, the RNA of td109 retained only two of the src specific oligonucleotides, and both remaining spots were mapped at the extreme 3' region of the src. Unlike all other td viruses, td109 lacks an oligonucleotide (no. 21) defined previously as env-specific (25), and mapped at the 3' portion of the env. The absence of this oligonucleotide may be correlated with the size of td109 RNA, which is smaller than that of RAV-2 (Fig. 1A). If this deletion in the env is contiguous with the deletion in the src, then the 5' portion of src sequences should be completely missing in td109 RNA. This feature of td109 must be relevant to the generation of defective recombinant rASVs as discussed below.

The fingerprints of td109-derived rASV RNAs are shown in Fig. 2 in comparison with rASV-C which is a representative of rASVs obtained from chicken tumors induced by td mutants other than td109. For rASV 157 and 1702, the fingerprints were obtained from RNAs migrated slightly faster than RAV-2 (Fig.1). As expected for defective virus, the patterns of these two rASVs contain fewer oligonucleotides than that of rASV-C. The comparison of these patterns with the fingerprints of SR RSV, td109, and SR RSV N8 shows that 157 contains all of src-specific spots as indicated by arrows, but it lacks all of the env-specific spots. Thus, the overall pattern of 157 is very

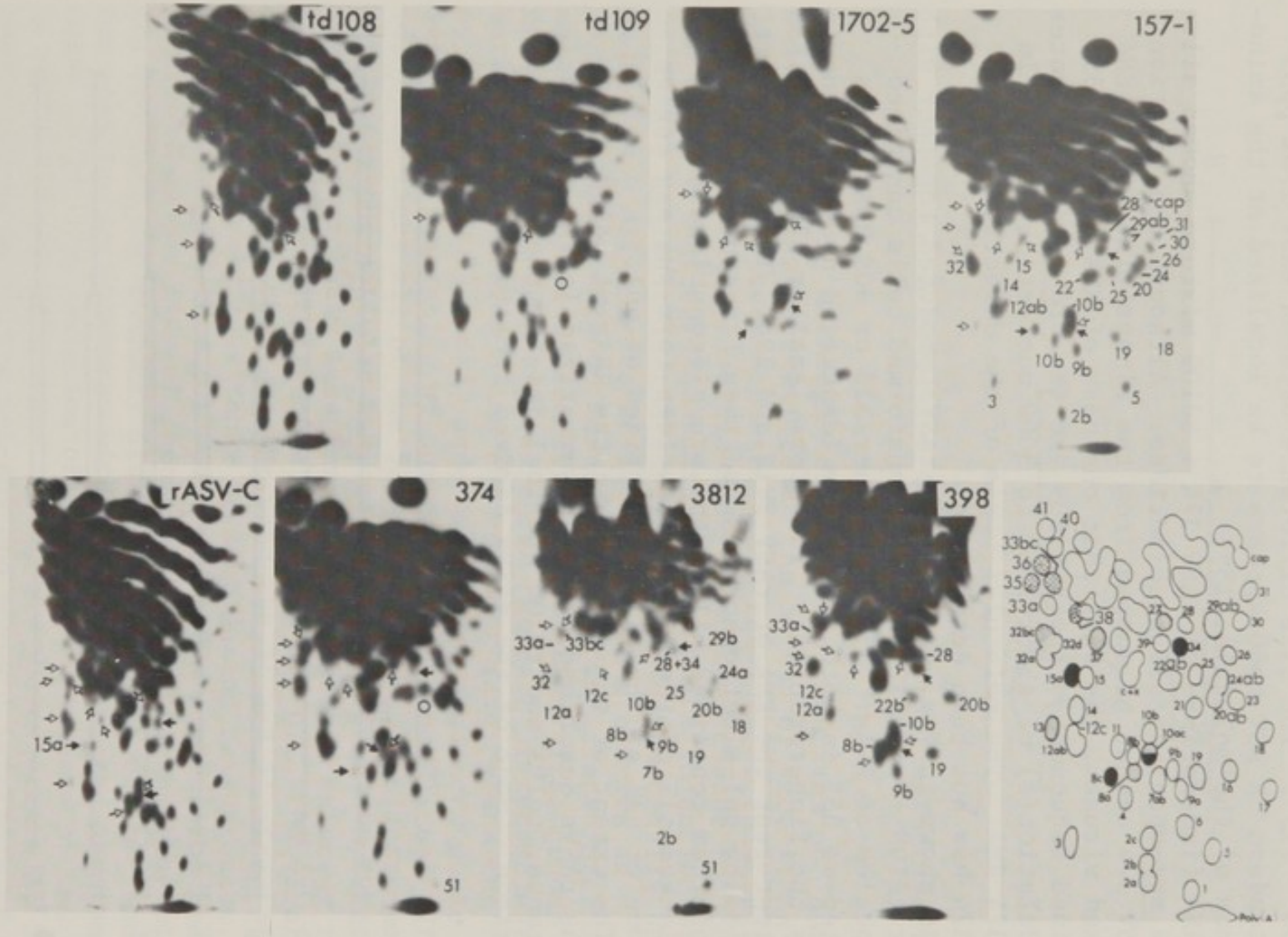

FIGURE 2. Fingerprints of td108, td109, td108-derived rASV-C and td109-derived rASVs. Open and solid arrows point to src-specific oligonucleotides.

similar to that of SR-RSV-N8 (not shown). rASV 1702 is similar to 157, but lacks 3 src-specific oligonucleotides at the 5' portion of the src. Nevertheless, rASV 1702 is fully competent in transformation and codes for a $pp55^{src}$ which is equivalent to $pp60^{src}$(13). The size difference between rASV 1702 pp55 and standard SR RSV pp60 appears to be localized at the amino-terminus of the molecule (13).

RNA fingerprints of rASV 374 + td109 mixture showed all of the src-specific oligonucleotides. Although the deletion in rASV 374 cannot be mapped from the fingerprint of the RNA of mixed viruses, the fact that one peak of RNA of about 35S contained sequences of both td109 and the src region indicates that deletion(s) of about 2500 to 3000 nucleotides exists in rASV 374 RNA. The molecular basis of the defectiveness of rASV 374 has not been determined.

As shown in Fig. 1, E and F, rASVs produced together with helper td109 from 3812 and 398 transformed cells contained two species of RNAs: 35S and either 32S or 30S RNA. RNAs migrated as 35S in both cases were identified as td109 genomic RNA by the fingerprinting. Therefore, smaller 32S and 30S RNA must represent the RNA of rASV 3812 and 398. These RNAs were purified by gel-electrophoresis and then fingerprinted (Fig.2). Both of them contained all of the src oligonucleotides but they lacked all of the oligonucleotides form the env region and some or all of the oligonucleotides of pol.

The extent of the deletion in the RNAs of td109 and various rASVs described above is summarized in Fig. 3. Two td mutants, td101 and td108 are included for the purpose of comparison. None of the rASVs derived from td101 or td108

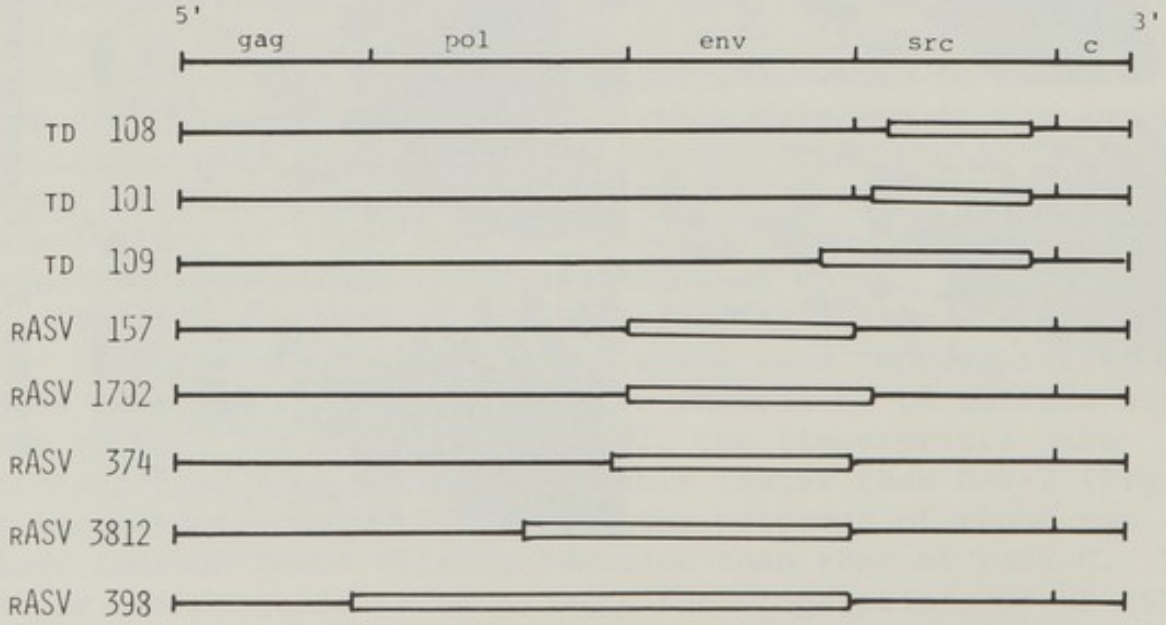

FIGURE 3. Deletion maps of td viruses and td109-derived rASVs.

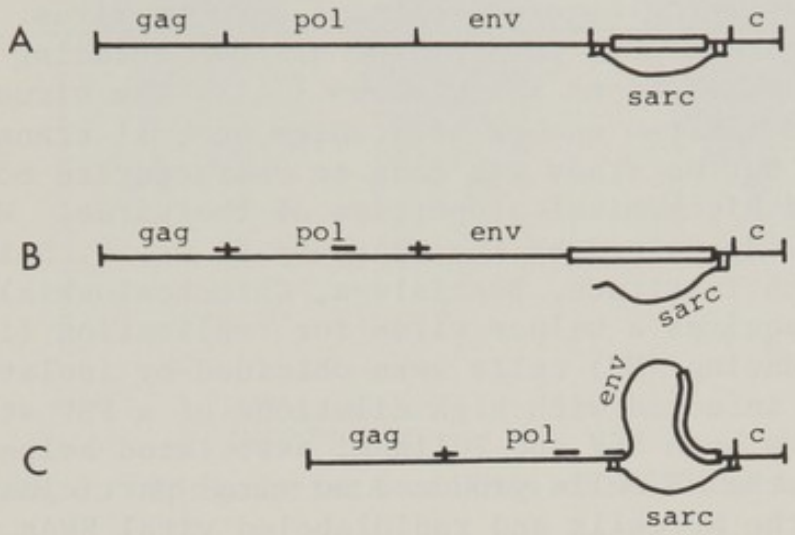

FIGURE 4. A model for the generation of defective rASVs.

were defective. Although td101 RNA also contains no detectable marker oligonucleotides at the 5' end of src, it is likely some src sequences still are retained at this region of td101 RNA. One possible model that may account for our observation that all rASV derived from td109 injection are defective at least in the env region, is schematically shown in Fig. 4. All species of nucleic acids are drawn as single-stranded without identifying the immediate intermediate molecules involved in recombination between viral genome and cellular sequences. Fig. 4A shows the case of recombination between td101 or td108 and the cellular sarc, where the viruses retain both 3' and 5' ends of the src sequences would result in the generation of nondefective sarcoma virus. If td109 RNA lacks all of the 5' src sequences, the corresponding portion of the cellular sarc would have to form a hybrid with similar but probably less homologous sequences existing further upstream in the viral genome (Fig. 4 B and C). The viral sequences, other than src, between the 3' and 5' sites of recombination would inevitably be lost during this process. Results obtained from td109-derived rASVs suggest the presence of more than one site of recombination, although these sites are probably not randomly distributed throughout the genome. For example, no crossover within the env has been observed for these rASVs. The deletion of the entire sequence of the env in rASVs 157 and 1702 seems to suggest the existence of homologous sequences at the pol-env and env-src junctions of the viral genome, which might be correlated to the sequences involved in the splicing process of the subgenomic mRNA.

The Transforming Gene of Fujinami Sarcoma Virus. Fujinami sarcoma virus (FSV) is a potent sarcoma inducing virus isolated from myxosarcoma in chickens (14). The virus has been used in a limited number of studies on cell transformation (15-18), but no study was made to characterize both biological and biochemical properties of the virus. We found that FSV (obtained by the courtesy fo Dr. V. Smidova, Cancer Research Institute, Bratislava, Chzechosloakia) is defective and requires a helper virus for replication (19). Non-virus producing (NP) cells were obtained by isolating foci from cultures infected with high dilutions of a FSV stock which contains both FSV and Fujinami associated helper virus (FAV). Since the NP cells produced no viral particles, RAV-1 was added to the NP cells and radiolabeled viral RNAs were extracted from the virus mixture thus produced. As shown in Fig. 5, the labeled RNA of this preparation was separated into two components in gel electrophroesis (Fig. 5A) and sucrose gradient sedimentation (Fig. 5C). The larger component was identified as 35S RNA of RAV-1 (see below). The smaller component cosedimented with 28S chicken rRNA, but electrophoresed slightly slower than the 28S rRNA marker. The molecular weight of 28S FSV RNA was estimated to be about 1.7×10^6 daltons or 5300 nucleotides based on its mobility in glyoxal-agarose gel electrophoresis (Fig. 5D)(19).

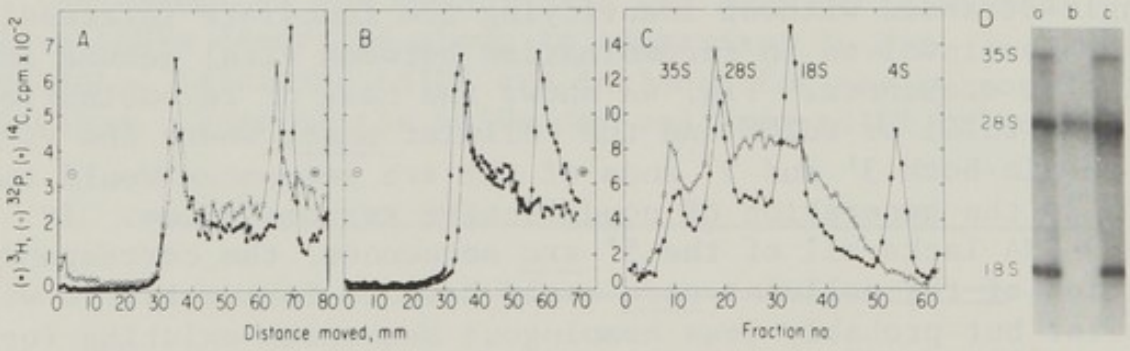

FIGURE 5. Analysis of the size of FSV and helper viral RNA. ^{32}P-labeled viral 50-70S RNA of FSV + RAV-1 mixture was mixed with ^{3}H-labeled RNA markers (35S RAV-2 RNA, 4S, 18S and 28S chicken RNAs), heat-denatured and analyzed by 2.2% polyacrylamide gel electrophoresis (A) or by sucrose gradient sedimentation (C). Panel B: ^{3}H-labeled FAV RNA was similarly analyzed in gel electrophoresis using ^{14}C-labeled RAV-2 70S RNA and 28S rRNA as markers. Panel D: ^{32}P-labeled FSV 28S RNA, purified by gel electrophoresis and sucrose gradient sedimentation, was denatured in glyoxal and analyzed in 1% agarose gel. a: RAV-1 35S RNA, 18S and 28S rRNA, b: FSV 28S RNA, c: FSV 28S RNA and the RNA markers as in a.

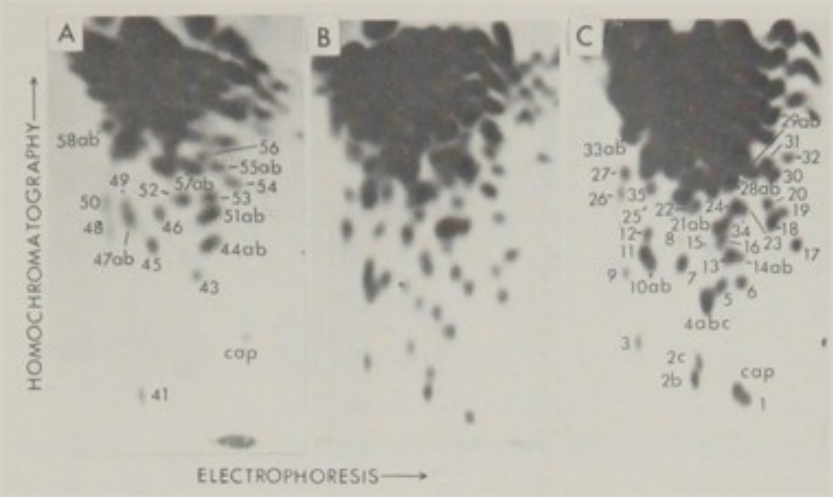

FIGURE 6. Fingerprints of RNAs of FSV and helper viruses.

^{32}P-labeled 35S and 28S components were recovered from a preparative gel and separately fingerprinted for their RNase T_1-resistant oligonucleotides. The fingerprint of 35S component was identical to that of RAV-1 RNA (Fig. 6B), whereas 28S component showed a simpler and unique pattern (Fig. 6A). Therefore the 28S component must be the FSV genome. In order to define the FSV-unique sequences, the mapping of the oligonucleotides was made with poly(A) containing RNA fragments of gel-purified FSV 28S RNA and 35S RNA of FAV. FAV RNA migrated slightly faster than RAV-2 RNA (Fig. 5B) and gave a fingerprint pattern with the complexity typical of an avian leukosis virus (Fig. 6C). The mapping studies showed that FSV and FAV RNAs shared several spots at both 3' and 5' ends, but no homology was found at the middle of the two RNAs (Fig. 7). FSV RNA contained one highly conserved gag oligonucleotide at the 5' region (spot circled in Fig. 7), but none of the highly conserved env and pol oligonucleotides of avian retroviruses. We estimated that FSV and FAV RNAs shared about 600 and 1000 nucleotides at 3' and 5' ends respectively, and FSV RNA contained, in the middle of its genome, a stretch of unique sequence of at least 3000 nucleotides (Fig. 7). The most striking feature was the total lack of the src-specific oligonucleotides in FSV 28S RNA (19).

The presence in the FSV genome of a new transforming gene unrelated to the src gene, which is present in all RSV and B77, was further substantiated by hybridization experiments. cDNA src made from SR·RSV hybridized only 3% with FSV RNA (19) and conversely cDNA specific to the unique sequences of FSV failed to hybridize with SR RSV RNA (M. Shibuya et al. unpublished). Furthermore, $pp60^{src}$ was not detectable in any of FSV transformed cells with anti pp60 sera which can recognize pp60 of various strains of RSV. Instead, a polypeptide of about 140,000 daltons (p140) was detected in FSV nonproducer and

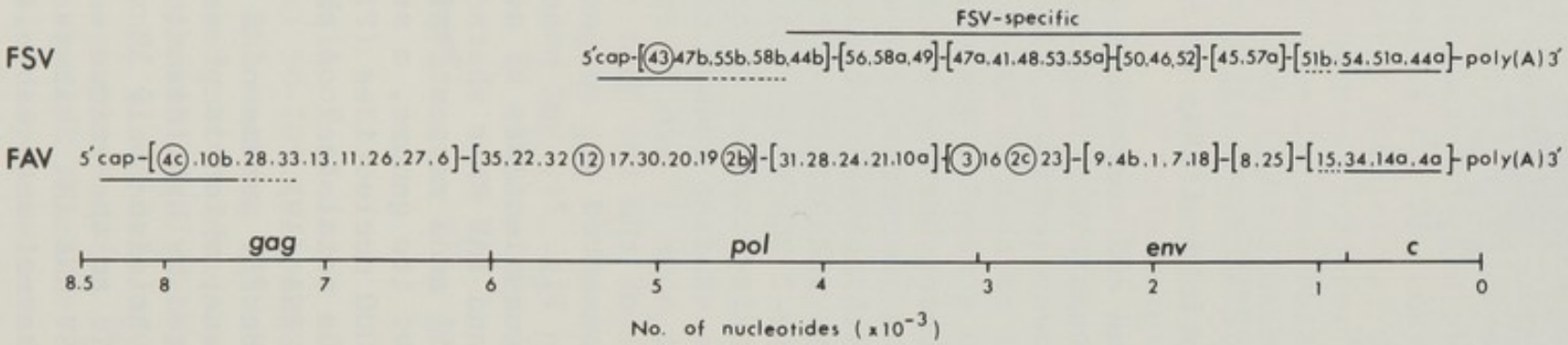

FIGURE 7. Oligonucleotide maps of FSV and FAV RNAs.

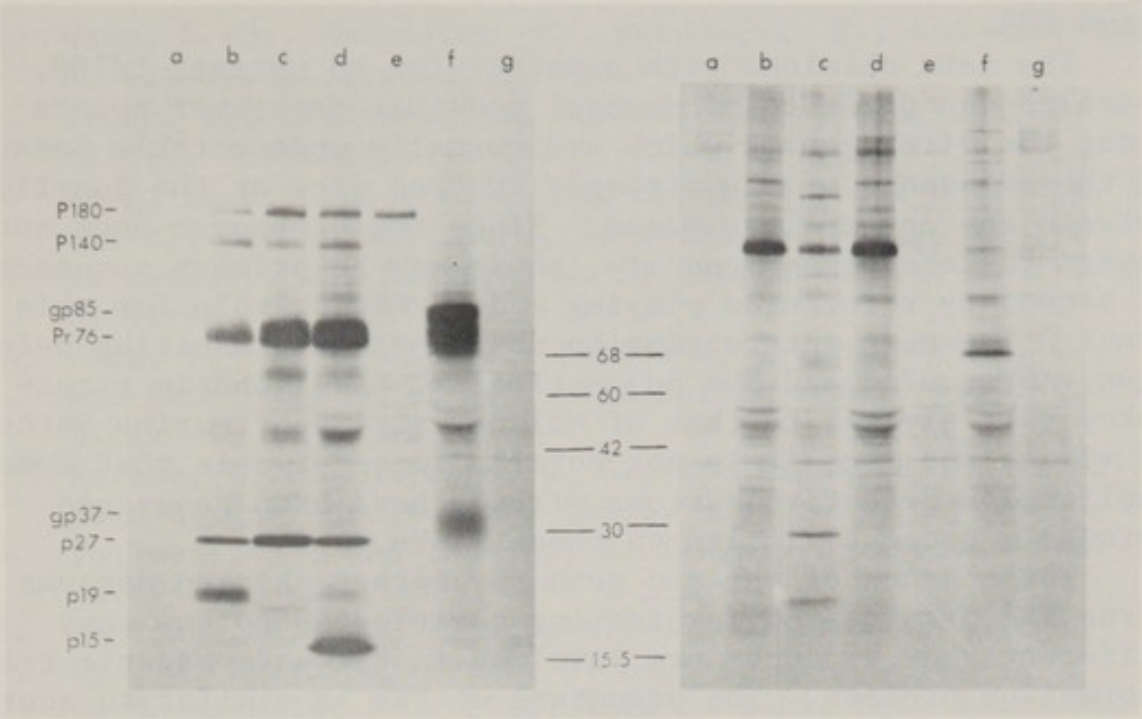

FIGURE 8. Electrophoresis of immunoprecipitates of ^{35}S-methionine-labeled viral proteins from FSV producer (left) and FSV nonproducer clones (right) in 5-15% SDS-polyacrylamide gels. Antisera used were: (a) nonimmune serum, (b) anti-p19, (c) anti-p27, (d) anti-p15, (e) anti-polymerase, (f) anti-gp85 and (g) anti-$pp60^{src}$.

producer cells by immunoprecipitation of cell extracts with anti-gag antisera (Fig.8).

The overall structure of FSV genome and its possible transforming protein seems to be very similar to those of acute leukemia viruses. However, the FSV-unique sequences as characterized by oligonucleotides are different from those of acute leukemia viruses analyzed so far (20-24). In vivo, FSV induced sarcomas at the site of injection in 5 to 10 days, and thus far there have been no signs of acute type leukemia in infected chickens.

DISCUSSION

A large amount of information has accumulated for the presence, in normal cell DNA, of the sequences homologous to the unique sequences of transforming retroviruses. The system of rASV and td mutants is thus far the only system where the genesis of transforming virus can be reproduced, albeit that the process may not be exactly the same as that involved in the genesis of transforming virus in nature because of the presence of homologous sequences in partial td

virus RNA.

The data obtained with a particular td mutant, td109, revealed the presence of several possible crossover points along the viral genome which are normally undetectable because of the existence of the strongly favored site at the junction between the env and src genes. Since the crossover does not appear to take place randomly, there may be primary sequences or secondary structures playing a determining role for this event. It seems interesting to note that the resulting defective rASVs, 3812 and 398 in particular, have genetic structures very similar to that of Moloney strain of murine sarcoma virus, and thus it is not totally inconceivable that some replication defective sarcoma viruses have been generated through a process similar to these rASVs.

The absence of the src gene in another chicken sarcoma virus RNA provides an interesting question as to how many different gene products are involved in the sarcomagenic transformation process. The structure of FSV is similar to acute leukemia viruses of murine and avian species and feline sarcoma virus, and thus the architecture of the src gene within the genomic RNA of Rous sarcoma virus appears to be more and more exceptional.

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PHOSPHORYLATION OF TYROSINE: A MECHANISM OF TRANSFORMATION SHARED BY A NUMBER OF OTHERWISE UNRELATED RNA TUMOR VIRUSES[1]

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ABSTRACT The transforming protein of Rous sarcoma virus, pp60^{src}, functions as a protein kinase which phosphorylates tyrosine when assayed in vitro. There are several observations which suggest that the modification of cellular proteins through the phosphorylation of tyrosine by pp60^{src} plays an essential role in transformation by RSV. First every type of RSV-transformed cell examined has an increased abundance of phosphotyrosine in protein. Secondly, the level of phosphotyrosine in proteins is strikingly temperature sensitive in cells infected with a mutant of RSV temperature sensitive for transformation, being high at the permissive temperature and nearly normal at the restrictive temperature. This fact coupled with the extreme rapidity with which the levels of phosphotyrosine change in mutant-infected cells upon temperature shift is consistent with the idea that phosphorylation of tyrosine is a primary event in transformation by RSV. Thirdly, two cellular proteins, which appear likely for other reasons to be substrates of pp60^{src}, both contain phosphotyrosine when isolated from RSV-transformed cells.

RSV is not unique among transforming viruses in its ability to cause elevation of phosphotyrosine in proteins. While transformation by tumor viruses such as polyoma virus, SV40, Moloney sarcoma virus and Kirsten sarcoma virus does not affect the level of phosphotyrosine, Abelson murine leukemia virus and the Snyder-Theilin strain of feline sarcoma virus do cause an increase in abundance of phosphotyrosine in proteins in transformed cells. We can conclude that augmented phosphorylation of tyrosine is not an inevitable secondary response to viral transformation. On the other hand, it is a strategy shared by at least three otherwise unrelated RNA tumor viruses.

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INTRODUCTION

Expression of the src gene of Rous sarcoma virus (RSV) is required for initiation and maintenance of the transformed state in chick fibroblasts in culture and in birds (1). The product of this gene is a 60,000 dalton phosphoprotein, pp60^{src} (2-5). Collett and Erikson, (6) showed that there was a protein kinase activity associated with pp60^{src} which was able to phosphorylate immunoglobulin heavy chains in immunoprecipitates formed between anti-tumor serum and pp60^{src}. They postulated that this protein kinase activity is an inherent property of pp60^{src} and that it plays an essential role in the malignant transformation of cells by RSV. There are now several lines of evidence that support this idea. First, pp60^{src}'s from mutants of RSV temperature sensitive for transformation possess thermolabile protein kinase activity (3,5,9). Secondly, pp60^{src} synthesized in vitro from virion RNA templates has protein kinase acivity (10,11). Finally, and most persuasively, pp60^{src} retains protein kinase activity throughout extensive purification (8). A formal proof of Collett and Erikson's hypothesis would be a demonstration of a protein(s) in RSV-transformed cells whose function is altered by new phosphorylation. This approach is complicated, however, by the fact that all normal vertebrate cells contain a gene, sarc, which has considerable homology with the viral src gene (12, 13). This gene is expressed in all cell types so far examined (14) and the product is a 60,000 dalton phosphoprotein, pp60^{sarc} (15-18). Not only does pp60^{sarc} have a structure very similar to that of viral pp60^{src}(15,16,19,20), but it too has associated protein kinase activity (16,18-20). pp60^{sarc}, however, is usually found present at only 1-5% of the level of pp60^{src} in a virally transformed cell (15,17). It is not yet known whether the two proteins are functionally equivalent or whether the viral protein has additional or different specificities. The observation of Hanafusa and colleagues (21,22) and Vogt and coworkers (23) that mutants of RSV partially deleted in the src gene can recover a fully functional src gene after passage in chickens, presumably by acquisition of cellular sarc sequences, is consistent with the notion that pp60^{src} and pp60^{sarc} are functionally equivalent. If this were the case, then transformation by RSV would be a result of overproduction of a normal cell protein kinase. Target proteins for pp60^{src} would then be characterized by a quantitative increase in phosphorylation upon transformation.

Identification of substrates of either pp60^{src} or pp60^{sarc} would be facilitated by a fuller knowledge of the enzymatic properties of the protein kinase activities of these proteins. It was reported originally that pp60^{src} phosphorylated a

threonine in the immunoglobulin heavy chain (6). During the course of identification of tyrosine as the acceptor amino acid for a phosphorylating activity present in immunoprecipitates containing polyoma virus T antigen, however, we found that phosphotyrosine was difficult to separate from phosphothreonine using the conventional technique of electrophoresis at pH 1.9 (24). Furthermore in preliminary experiments we found the chemical stability of the phosphate linkage to the immunoglobulin heavy chain to be at variance with that reported for phosphothreonine linkages. Upon re-examining the nature of the amino acid substrate for the pp60^{src} protein kinase activity, we found that tyrosine rather than threonine was the acceptor in the immunoglobulin heavy chain (25). This is an unprecedented substrate specificity for a protein kinase. Yet two observations led us to infer that this novel amino acid specificity apparent in vitro reflected that of pp60^{src} in vivo. First, pp60^{src} isolated from transformed chick cells contained a phosphotyrosine at a site thought to be subject to autophosphorylation (25). Secondly, cells transformed by RSV had 6-10 fold higher levels of phosphotyrosine in protein than uninfected cells (25).

In normal cells phosphotyrosine is an extremely rare modified amino acid, being present at only about 1/3000 the level of phosphoserine and phosphothreonine combined (25). This fact coupled with the difficulty in separating phosphothreonine and phosphotyrosine explains why phosphotyrosine was not detected previously. Although some of the elevation of phosphotyrosine in protein in RSV transformed cells is due to the pp60^{src} polypeptide itself, it seems likely that most of the increase is due to augmented phosphorylation of tyrosine in cellular proteins. For instance, the 50,000 dalton phosphoprotein which coprecipitates with pp60^{src} contains phosphotyrosine (25). In addition, removal of pp60^{src} from a transformed cell lysate caused less than a 20% decrease in the level of phosphotyrosine (26). If the increase in the abundance of phosphotyrosine were largely due to phosphorylation by pp60^{src}, the cellular content of phosphotyrosine could be used as a measurement of the activity of pp60^{src} in vivo. We have tested this hypothesis through use of mutants of RSV temperature sensitive for transformation. There is a good correlation between an elevated level of phosphotyrosine in protein, the presence of a functional pp60^{src} as measured by the immunoglobulin kinase assay and the transformed phenotype (26). Our results suggest that phosphorylation of tyrosine is necessary for transformation by RSV.

RESULTS

As an example of the specificity of the pp60^{src} associated protein kinase Figure 1 shows a two-dimensional separation of a partial acid hydrolysate from the immunoglobulin heavy chain phosphorylated by pp60^{src} upon incubation of an immunoprecipitate with γ-^{32}P-ATP. Separation in the first dimension was by electrophoresis at pH 1.9, and in the second dimension by electrophoresis at pH 3.5. As we have shown previously using other separation systems, the only detectable phosphoamino acid in the labeled heavy chain is phosphotyrosine (25).

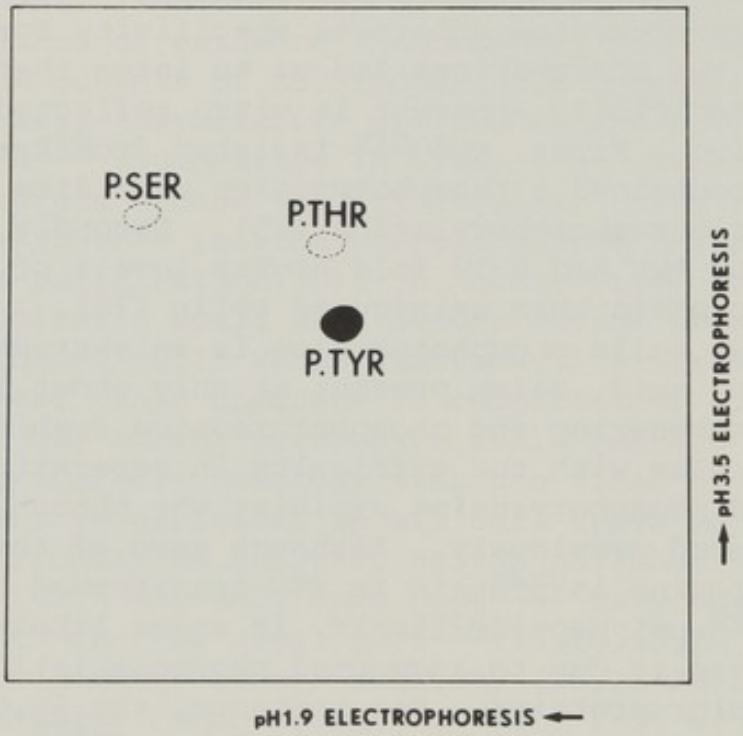

FIGURE 1. Analysis of the phosphoamino acid content of phosphorylated immunoglobulin heavy chain. The immunoglobulin heavy chain was labeled by incubation of an immunoprecipitate containing pp60^{src} with γ-^{32}P-ATP, followed by preparative gel electrophoresis. The labeled heavy chain was recovered by electroelution and TCA precipitation and hydrolysed in 6N HCl for 2 hr at 110° C. The hydrolysate was dried in vacuo and 10^4 cpm were electrophoresed on a thin layer cellulose plate first at pH 1.9 for 30 min at 3 kV and then at pH 3.5 for 30 min at 1.5 kV. Phosphoserine, phosphothreonine and phosphotyrosine were included as internal markers and detected by ninhydrin staining. The dashed circles define the positions of the marker phosphoserine and phosphothreonine. The origin in the bottom right corner is shown as a circle. The electrophoretic dimensions were as indicated. The autoradiogram was exposed for 2 hr with a fluorescent screen.

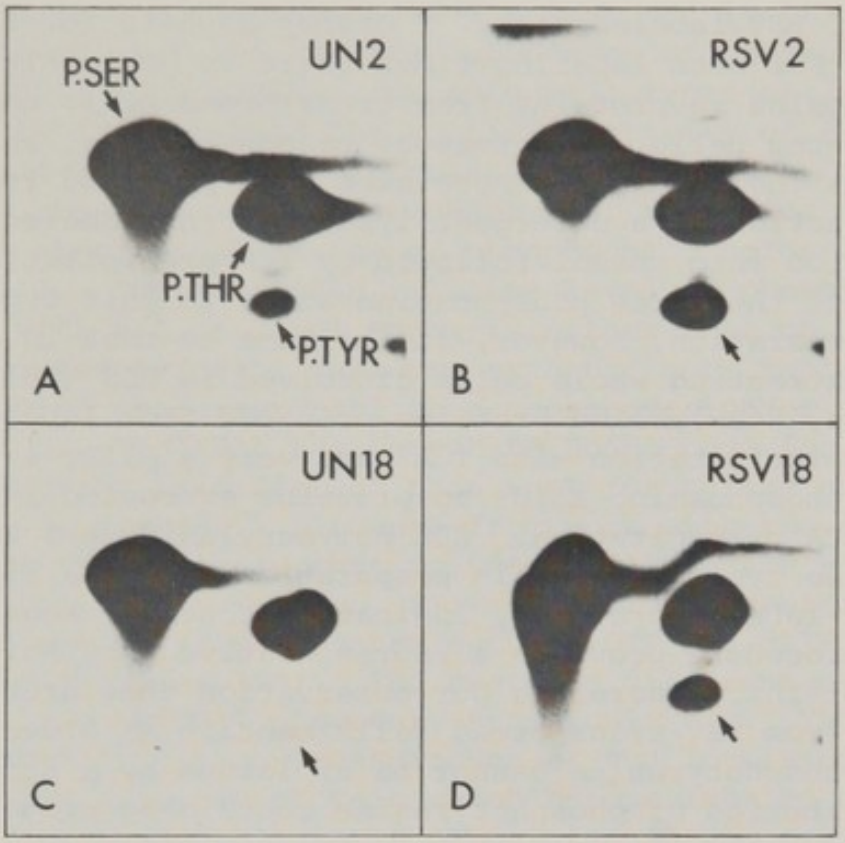

FIGURE 2. Analysis of the apparent abundance of phosphoamino acids in whole cells under different labeling conditions. 35 mm dishes of uninfected and SR-RSV-D infected chick embryo fibroblasts were labeled for either 2 hr or 18 hr with 1.2 mCi ^{32}P orthophosphate in 2 ml medium lacking phosphate. ^{32}P-labeled proteins were isolated as described (25). They were subjected to acid hydrolysis and 10^6 cpm of each sample were separated in two dimensions as described in Fig. 1. The autoradiograms were exposed for 16 hr with a fluorescent screen. Only the area containing the three phosphoamino acids is shown. Phosphotyrosine is arrowed in panels B, C and D.

A. Uninfected cells labeled for 2 hr
B. RSV-transformed cells labeled for 2 hr
C. Uninfected cells labeled for 18 hr
D. RSV-transformed cells labeled for 18 hr

Comparison of the level of phosphoamino acids in uninfected and RSV-transformed chick embryo fibroblasts demonstrates that the level of phosphotyrosine relative to phosphoserine and phosphothreonine is 5-10 fold higher in transformed cells (25). Figure 2 shows two-dimensional separations of partial acid hydrolysates of ^{32}P-labeled phosphoproteins derived from either uninfected chick cells or a parallel culture of cells transformed by the Schmidt-Ruppin

D strain of RSV labeled with ^{32}P orthophosphate for either 2 or 18 hr. For both labeling times there is obviously more phosphotyrosine in proteins from transformed cells than in the uninfected cells. For reasons of convenience, in these experiments the ^{32}P-labeled proteins were prepared from the soluble fraction of a detergent lysate of the labeled cells by extraction into phenol followed by TCA precipitation (25). The ratio of the three phosphoamino acids in this type of protein preparation, however, is the same as that in one prepared by extracting whole cells dissolved in SDS (26). Furthermore, ^{32}P-labeled proteins prepared from labeled cells by direct precipitation with TCA have very similar levels of all three phosphoamino acids to proteins extracted into phenol prior to TCA precipitation. SDS polyacrylamide gel analysis of all three types of protein preparation showed a similar profile of labeled proteins, indicating that our routine extraction procedure provides a representative population of phosphoproteins. Therefore our observation that proteins extracted from RSV-transformed cells contain an elevated level of phosphotyrosine cannot be explained by a differential distribution of phosphotyrosine containing proteins in transformed cells or a selective extraction of such proteins from transformed cells.

Table I gives the quantitation of the experiment shown in Figure 2. The data are expressed as percentages of the total radioactivity recovered after hydrolysis as phosphoamino acids. These values will differ slightly from the phosphoamino acid abundance prior to hydrolysis, because phosphotyrosine and phosphoserine are both somewhat more acid labile than phosphothreonine (27). There is no reason to believe, however, that our recoveries of phosphotyrosine are different for different types of cell.

TABLE I

APPARENT ABUNDANCE OF PHOSPHOAMINO ACIDS IN WHOLE CELLS UNDER DIFFERENT LABELING CONDITIONS

Cells	Labeling period	Phospho-serine	Phospho-threonine	Phospho-tyrosine
Uninfected	2 hr	85.95	13.94	0.102
RSV-transformed	2 hr	86.08	12.72	1.138
Uninfected	18 hr	90.25	9.69	0.057
RSV-transformed	18 hr	89.94	9.69	0.360

The amounts of radioactivity in the three phosphoamino acids for each of the samples shown in Fig. 1 were estimated by elution. The values are expressed as percentages of the total radioactivity recovered in the three phosphoamino acids in each case.

Comparison of the data for the 2 hr and 18 hr labeling periods shows that the relative abundance of phosphotyrosine is apparently much greater for both cell types at 2 hr. In an 18 hr labeling period the specific activity of ^{32}P in protein should have begun to approach a steady state, and the levels of phosphoamino acids measured at this time should reflect the absolute abundance of phosphoamino acids in protein in the cell. The increased relative level of phosphotyrosine observed with a 2 hr label suggests either that on average phosphorylated tyrosine residues exchange more rapidly with their labeled precursor pool than phosphoserine or phosphothreonine or else that the phosphate donor pool for tyrosine protein kinases is different than that for serine and threonine protein kinases. Although from work with the purified protein it seems likely that pp60^{src} utilizes ATP and/or GTP as phosphate donors in vivo (8), in an immunoprecipitate, other nucleoside triphosphates such as CTP and dATP, can serve as substrates (28). The use of a different phosphate donor pool by pp60^{src}, however, seems unlikely. This is suggested most strongly by measurement of the levels of the three phosphoamino acids in protein which become labeled during incubation of detergent lysates of uninfected and transformed cells with γ-^{32}P-ATP. In this type of experiment the added ATP provides a single phosphate donor pool, since detergent lysis abrogates any cellular compartmentalization. Although in vitro phosphorylation leads to an even higher level of phosphotyrosine than a short labeling period in vivo, the relative amount of phosphotyrosine in protein in the transformed cell lysate was still several fold higher than that in the uninfected cell lysate (Table II). We were able to show

TABLE II
ABUNDANCE OF PHOSPHOAMINO ACIDS LABELED DURING PHOSPHORYLATION IN CELL LYSATES

	Phosphoserine	Phosphothreonine	Phosphotyrosine
Uninfected	84.14	15.57	0.29
RSV-transformed	72.96	15.87	10.72

2×10^6 cells of each sort were lysed in 500 µl NP-40 buffer (7). The lysates were centrifuged at 10,000 g and the supernatants were gel-filtered over Biogel P4 in NP-40 buffer at 4° C. $MgCl_2$ was added to a final concentration of 10 mM and 75 µl of each lysate were incubated with 25 µCi of γ-^{32}P-ATP for 10 min at 30° C. The labeled proteins were precipitated with TCA at 4° C and the pellet washed twice with cold acetone. The acid hydrolysates of these preparations were analyzed as described in Figure 2 and Table I.

that this increase was due to pp60^{src} -catalyzed phosphorylation, since removal of pp60^{src} from the lysate by immunoprecipitation prior to incubation with γ-^{32}P-ATP reduced the increment of phosphotyrosine by 70%

All three phosphoamino acids resolve upon electrophoresis at pH 3.5 (25). This method of separation, however, is not satisfactory for hydrolysates of phosphoproteins labeled with ^{32}P orthophosphate in vivo. In such samples phosphotyrosine is masked by comigration with 3' UMP, which together with Cp is generated from any RNA present in the protein preparation upon hydrolysis in 6N HCl at 110° C. Because it is exceedingly difficult to isolate phosphoproteins entirely free of RNA from whole cells, it is imperative to analyze hydrolysates of ^{32}P-labeled whole cell phosphoproteins by electrophoresis in two dimensions; at pH 1.9 to resolve phosphotyrosine from Up and at pH 3.5 to separate

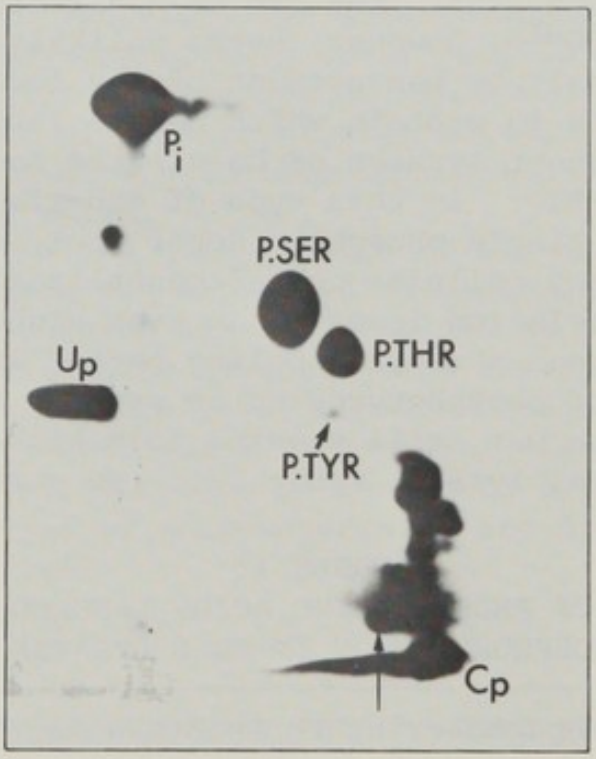

FIGURE 3. The perils of uridine. 10^6 cpm of an acid hydrolysate of ^{32}P-labeled proteins isolated from RSV-transformed cells labeled for 18 hr were separated in two dimensions by electrophoresis at pH 1.9 for 25 min at 1.5 kV and then at pH 3.5 for 25 min at 1.5 kV. Internal Up and Cp markers were detected by UV quenching. P_i designates orthophosphate. The origin is indicated by a vertical arrow. The directions of electrophoresis are the same as in Fig. 1. The autoradiogram was exposed with a fluorescent screen for 12 hr.

phosphotyrosine from phosphothreonine. To illustrate this point, a two-dimensional separation of a hydrolysate of ^{32}P-labeled proteins isolated from RSV-transformed chick cells was run under conditions which display the three phosphoamino acids, orthophosphate, Up and Cp (Figure 3). It is clear that Up and phosphotyrosine comigrate at pH 3.5. Even though the isolation of the labeled proteins used in this experiment involved steps to remove RNA, there was sufficient residual RNA to generate about 50 times as much Up as phosphotyrosine upon hydrolysis.

The finding that transformation by RSV is apparently brought about through increased levels of phosphorylation of tyrosine in protein raises the question whether this is a general mechanism of viral transformation. Several lines of evidence suggest that RSV arose through transduction of the cellular sarc gene by a non-transforming avian leukosis virus (12,21,22). This cellular genetic material has become active in transformation through introduction into the viral genome. It now seems clear that several other transforming viruses resemble RSV in the sense that they contain cellular genetic information. These include the sarcoma viruses--Kirsten sarcoma virus (29), Harvey sarcoma virus(29), Moloney sarcoma virus (30), and feline sarcoma virus (31)-- and the acute defective leukemia viruses--avian erythroblastosis virus (32), avian myelocytomatosis virus MC29 (32), avian myeloblastosis virus (32) and Abelson murine leukemia virus (33). While it has not yet been proven, expression of the transduced cellular material is probably involved in transformation by these viruses. We therefore examined whether transformation by viruses of this class other than RSV caused an elevation of the level of phosphotyrosine in protein. Typical results are shown in Table III. It can be seen that an increase in phosphotyrosine levels is not a necessary consequence of viral transformation. RSV, however, is not unique in its ability to cause an increase of abundance of phosphotyrosine in proteins. Both Abelson murine leukemia virus (A-MLV) and the Snyder-Theilin strain of feline sarcoma virus (ST-FeSV) have this effect. Interestingly, transformation by these two viruses raises the level of phosphotyrosine to essentially the same extent as transformation by RSV. In cells transformed by avian erythroblastosis virus there is a slight but reproducible increase in phosphotyrosine.

SV40 and polyoma virus differ from the viruses examined above in that they do not appear to contain recognizable cellular genetic material. Even though immunoprecipitates of the T antigens of polyoma virus have a protein kinase activity which phosphorylates tyrosine in the medium T antigen (24), several cell lines transformed by polyoma virus had normal levels of phosphotyrosine. Similar negative results were obtained with SV40 transformed cell lines.

TABLE III
ABUNDANCE OF PHOSPHOTYROSINE IN CELLS
TRANSFORMED BY DIFFERENT VIRUSES

Transforming virus	Cell type	Relative phosphotyrosine content
RSV	Chick fibroblast	6-9
RSV	Mouse 3T3 fibroblast	8
AEV	Chick fibroblast	1.5
MC29	Chick fibroblast	1
A-MLV	Mouse 3T3 fibroblast	9
A-MLV	Mouse B cell lymphoma	10*
ST-FeSV	Mink lung cell	5
Moloney sarcoma virus	Mouse TB cell	1
Kirsten sarcoma virus	Mouse 3T3 fibroblast	1
Polyoma	Hamster BHK fibroblast	1
SV40	Mouse 3T3 fibroblast	1

In each case parallel cultures of the transformed cell and the untransformed parent line were labeled for 18 hr with ^{32}P-orthophosphate. The ^{32}P-labeled proteins were isolated and analyzed as described in Figure 2 and Table I. The relative content of phosphotyrosine in the transformed cells was obtained by dividing the values for phosphotyrosine in the transformed cell by the value in the parent cell. *In the case of A-MLV B lymphoma (18-48), the value was compared to a spontaneous B lymphoma (WEHI 231).

We have also examined a number of chemically transformed cell lines and human tumor lines, but to date we have not found an instance where the abundance of phosphotyrosine was increased (26).

DISCUSSION

There are several observations which suggest that the modification of cellular proteins through phosphorylation of tyrosine by pp60^{src} plays an essential role in transformation by RSV. First, every type of RSV-transformed cell which we have examined has an increased abundance of phosphotyrosine

in protein (26). Although RSV is not unique in this respect, transformation by a number of other viruses did not cause an elevation in phosphotyrosine. Thus the notion that the increase in phosphotyrosine is an inevitable secondary response to transformation can be ruled out. Secondly, the possibility that the majority of the increased abundance of phosphotyrosine in RSV-transformed cells is due solely to the phosphotyrosine in pp60^{src} itself rather than phosphorylation of cellular proteins can be excluded, since removal of pp60^{src} by immunoprecipitation prior to determination of phosphotyrosine levels only diminishes the level of phosphotyrosine to a small extent (26). In addition two cellular proteins, which for other reasons have properties consonant with their being substrates of pp60^{src}, the 36,000 dalton phosphoprotein described by Radke and Martin (34) and the 50,000 dalton protein we have identified (25), both contain phosphotyrosine when isolated from RSV-transformed cells. Thirdly, the level of phosphotyrosine in proteins is strikingly temperature sensitive in cells infected with a mutant of RSV temperature sensitive for transformation, being high at the permissive temperature and nearly normal at the restrictive temperature (26). This fact coupled with the extreme rapidity with which the levels of phosphotyrosine change upon temperature shift in mutant-infected cells is fully consistent with the idea that the phosphorylation of tyrosine is a primary cause of transformation by RSV.

RSV is not unique among transforming viruses in its ability to cause elevation of phosphotyrosine in proteins. While transformation by tumor viruses such as polyoma virus, SV40, Moloney sarcoma virus and Kirsten sarcoma virus does not affect the level of phosphotyrosine, A-MLV and ST-FeSV do cause an increase in abundance of phosphotyrosine in transformed cells. These two viruses, like RSV, probably arose by recombination of leukemia viruses with cellular genetic information (31,33). The only recognized gene products of A-MLV and ST-FeSV are 120,000 (p120)(35,36) and 85,000 (p85)(37) daltons proteins, respectively. There is no direct proof that p120 and p85 are the viral gene products responsible for transformation. Nevertheless, because they are coded in part by the acquired cellular information in these viruses they are good candidates for the transforming function. There is an activity associated with each of the putative transforming proteins, pp60^{src}(25,26), p120 (38) and p85 (M. Barbacid, personal communication) which phosphorylates tyrosine. This fact coupled with our observation that transformation by all three viruses leads to an elevation of phosphotyrosine levels in cells suggests that pp60^{src}, p120 and p85 may all function in vivo to phosphorylate tyrosine, and that this property is

an important feature of transformation by RSV, A-MLV and ST-FeSV.

Since these three viruses seem to share a common molecular mechanism of transformation dependent upon acquisition and activation of cellular information, one might have suspected that the cellular genetic material in these three viruses would be the same. However, there are several pieces of evidence which imply that the cell sequences are different in each case. First, nucleic acid hybridization with specific probes reveals little homology between the acquired sequences (31,39). Secondly, tryptic peptide mapping of pp60^{src}, p120 and p85 does not show any relatedness (K. Beemon and T. Patschinsky, unpublished results). Thirdly, the cellular genes from which these viruses are derived appear to encode different proteins. In the case of RSV, the normal cell homologue is pp60^{sarc}, a protein found in most cell types. For A-MLV the cell homologue is NCP150, a much larger protein of 150,000 daltons whose distribution is restricted to hematopoietic cells (40). The cell homologue for ST-FeSV appears to be 92,000 daltons in size (M. Barbacid, personal communication).

Viral pp60^{src} is similar in size and apparent enzymatic activity to its cellular homologue, pp60^{sarc} (15-20, 25). It is not yet clear whether viral pp60^{src} is active in cellular transformation simply because it is expressed at much higher levels than cellular pp60^{sarc} or instead because it was altered subtly in function during acquisition by the virus. In contrast, the structure of both p120 of A-MLV and p85 of ST-FeSV is clearly different from that of the polypeptide encoded by the homologous cellular gene. Both of these viral proteins are hybrids which contain at their N-termini part of a viral precursor polypeptide and in their C-terminal halves amino acid sequences encoded by the acquired cellular sequences (35-37). Because both p120 and p85 are smaller than their cellular homologues it is probable that they contain only a portion of the normal cellular protein. It seems quite possible therefore that the precise function of these chimeric viral proteins is different from that of the cellular homologues. It would not be unreasonable, however, for the domain of the cellular polypeptide which has been acquired by the virus to retain the basic catalytic activity of the protein. We favor the hypothesis that the normal cell homologues of pp60^{src}, p120 and p85 have the same general activity in normal cells: they are protein kinases which phosphorylate tyrosine. These proteins would be members of a new class of cAMP independent protein kinase distinct from the serine and threonine protein kinases (41). This class of protein kinase would be responsible for the basal level of phosphotyrosine found in proteins in normal cells.

Clearly the most important question now is how the phosphorylation of cellular polypeptides by viral transforming proteins alters cellular metabolism and morphology. Substrates of these three virally coded protein kinases should be identifiable by virtue of their containing phosphotyrosine. Using in part this criterion two potential substrates for pp60^{src} have already been identified--the 50,000 and 36,000 dalton phosphoproteins. It will be crucial however to distinguish between those substrates whose modifications lead to cellular transformation and those which are modified incidentally.

We do not yet know whether to expect the spectrum of substrates for the viral transforming proteins to be the same as for their respective normal cell homologues or whether the viral proteins will have additional specificities. Similarly, it is not yet clear whether the substrate spectrum for any one of the transforming proteins is discrete or whether it overlaps with that of the other viral proteins. The subcellular localization of these protein kinases may to some extent dictate their substrate specificity. In this regard, it is of interest that some fraction of both pp60^{src} and p120 is associated with the plasma membrane (42,43).

Although there are several transforming viruses which cause no obvious alteration in the level of phosphotyrosine in protein upon transformation, one should not rule out the possibility that phosphorylation of tyrosine is involved in transformation by these viruses. It is conceivable that such viruses either code for tyrosine protein kinases or induce cellular tyrosine protein kinases whose effects we do not detect as gross alterations in the level of phosphotyrosine because the total number of phosphorylated protein molecules is very much less than that in a cell transformed by RSV. This situation might arise if there were a single low abundance target protein for the tyrosine protein kinase in question.

It is clear that malignant transformation can result from the alteration of cellular genetic information and does not necessarily require the introduction into the cell of new genetic information. Given that it now appears likely that the unscheduled phosphorylation of tyrosine is one mechanism of transformation, one can speculate that carcinogen-induced overproduction of a normal cellular protein kinase which phosphorylates tyrosine might lead to malignancy. This idea predicts that it may be possible to find non-virally transformed cells which contain abnormally high levels of phosphotyrosine. However, since it now appears likely that there are multiple biochemical alterations which can cause transformation, we expect that elevated phosphotyrosine will be the exception rather than the rule.

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PRCII, A NEW TYPE OF AVIAN SARCOMA VIRUS

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ABSTRACT PRCII, an avian sarcoma virus isolated from a spontaneous myxofibrosarcoma of a chicken is defective for synthesis of infectious progeny virus and codes for a transformation specific protein of 105,000 mol. wt. (p105). This p105 is a fusion protein containing gag sequences and sequences unrelated to any virion protein. The gag related domain of p105 encompasses virion proteins p19, p27 and probably p12, but not p15. The nonstructural portion of p105 is distinct from transformation specific proteins of other avian retroviruses (p110 of MC29, p100 of MH2 or p75/p40 of AEV). It appears also unrelated to pp60^{src} of Rous sarcoma virus and its relatives, and no pp60^{src} is detectable in PRCII transformed cells. It is concluded that PRCII represents a novel avian sarcoma virus with unique transformation-related genetic information.

INTRODUCTION

Avian sarcoma virus PRCII was isolated by Carr and Campbell from a spontaneous myxofibrosarcoma of a chicken (1). No detailed characterization of this virus has been published, although observations on focus formation and envelope classification may be found in the literature (2,3). In the present paper we report evidence for the defectiveness of PRCII in replication and show that PRCII codes for a transformation specific fusion protein, p105. The p105 of PRCII contains a partial gag complement plus nonstructural sequences that are distinct from transformation specific proteins of other avian retroviruses.

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RESULTS

Biological Characteristics of PRCII. Infection of chick embryo fibroblast cultures with PRCII induced the appearance of foci consisting of transformed cells (Fig. 1).

FIGURE 1. A focus of PRCII transformed chick embryo fibroblasts. Photographed at 25X magnification.

These cells were mostly fusiform in shape, grew tightly packed in several layers and predominantly in parallel orientation. In completely transformed fibroblast cultures the elongated spindle shaped cells were oriented at random. Such cultures produced large quantities of mucinous material which became visible when the growth medium was removed from the cell sheet. PRCII transformed chicken fibroblasts were also able to grow into colonies in soft agar medium. PRCII did not transform chicken yolk sac macrophages, although it replicated in these hematopoietic cells. In the animal PRCII showed a relatively low pathogenicity. Of nine 1-day old chickens injected with about 10^4 FFU, only

two came down with tumors within four weeks. Both growths were myxofibrosarcomas.

Table 1 delineates the host range of PRCII in various types of chicken cells and in Japanese quail fibroblasts.

TABLE 1
HOST RANGE OF PRCII

Cellular phenotype	EOP of infecting virus[a] PRCII	PR-A	PR-B	PR-C	SR-D
C/O	1.00	1.00	1.00	1.00	1.00
C/A	1.67	$<5.0 \times 10^{-5}$	2.64	0.25	0.82
C/B	1.52	0.72	6.7×10^{-5}	0.43	2.3×10^{-4}
Q/B	2.01	0.60	6.4×10^{-5}	4.3×10^{-5}	1.6×10^{-4}
C/AB[b]	$<8.3 \times 10^{-5}$	$<5.0 \times 10^{-5}$	7.2×10^{-5}	0.18	1.2×10^{-4}

[a] Efficiency of plating relative to C/O cells. PR-A, PR-B, PR-C = Prague strain Rous sarcoma virus of subgroups A, B and C respectively. SR-D = Schmidt-Ruppin Rous sarcoma virus of subgroup D.

[b] C/B cells preinfected with subgroup A virus RAV-3.

These data suggest the presence of subgroup A and B envelope determinants in the PRCII stocks. An associated helper virus isolated from PRCII by endpoint dilution also contains both subgroups A and B.

PRCII is defective in the production of infectious transforming progeny virus. Table 2 shows

TABLE 2
DEFECTIVENESS OF PRCII

Relative amount of virus	a FFU	b Infectious centers	Ratio $\frac{b}{a}$
1	143	156	1.09
0.5	82	91	1.11
0.25	43	37	0.86
0.125	28	16	0.57
0.063	15	4	0.27
0.031	6	0	<0.17

that while the number of particles registering as focus forming units is directly proportional to the amount of virus inoculated, infectious centers decline more steeply at high virus dilutions suggesting a requirement for double infection by transforming PRCII and a helper virus for the generation of infectious progeny. In agreement with this result of the infectious center assay some of the colonies of PRCII-transformed cells growing in nutrient agar were nonproducers. Such colonies were grown up into mass cultures and tested for the release of tritiated uridine or reverse transcriptase containing particles. No such particles were detectable in the culture fluids of the PRCII nonproducers. Superinfection of PRCII nonproducer cells with avian leukosis virus led to rescue of infectious PRCII (Table 3).

TABLE 3
RESCUE OF INFECTIOUS PRCII FROM NONPRODUCING COLONIES

PRCII nonproducer no.	Superinfection with avian leukosis virus	PRCII in culture medium (FFU/ml)
3c29	None	Ø
	RAV-7[a]	2.6×10^4
	RAV-1	2.0×10^4
	RPV	2.5×10^4

[a] RAV-7 = Rous associated virus, type 7, subgroup C.
RAV-1 = Rous associated virus, type 1, subgroup A.
RPV = Ringnecked pheasant virus, subgroup F.

Biochemical Observations on PRCII. PRCII infected cells were examined for the presence of transformation specific proteins by immunoprecipitation. Cultures were labelled with ^{35}S methionine at concentrations of 40 to 200 µC/ml for 20 min in methionine-free medium. Cell lysates were prepared and immunoprecipitates collected and resolved on polyacrylamide gels by electro-

phoresis according to published techniques (4). In chicken embryo fibroblasts transformed by PRCII, but not in cells infected with the helper virus of PRCII, a virus specific protein was detected that did not correspond to one of the known structural proteins or polyproteins of avian retroviruses. This protein had an apparent mol. wt. of 105,000 in SDS polyacrylamide gels (therefore termed p105) (Fig. 2).

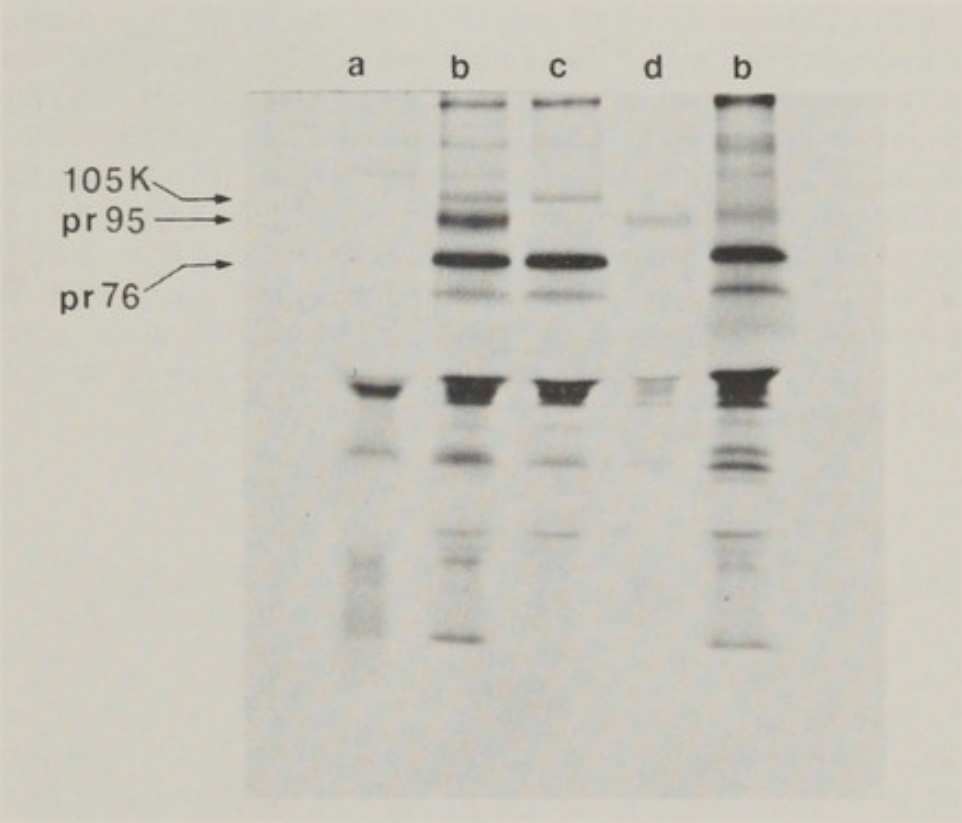

FIGURE 2. Virus-specific protein synthesized in PRCII-transformed chick embryo fibroblasts and cells infected with the helper virus of PRCII. Cells were labeled for 30 min with 100 µCi of ^{35}S methionine and lysates incubated with monospecific sera. The precipitates were resolved on a 6-18% SDS-polyacrylamide slab gel. Tracks 1-4, immunoprecipitation of PRCII-transformed cells with (a) normal rabbit serum; (b) anti-whole virus serum; (c) anti-p27 serum; (d) anti-gp serum. Track 5, immunoprecipitation of cells infected with the helper virus of PRCII using (b) anti-whole virus serum.

It could be precipitated with antiserum prepared against Prague strain Rous sarcoma virus (RSV) and with a serum specific for the gag protein p27, but it did not react with antiserum against the envelope glycoprotein, or with serum against the transforming protein pp60^{src} of RSV. The p105 of PRCII was demonstrable in producing and non-producing transformed cells, in the latter it was the only detectable viral gene product. No evidence was found for the presence of pp60^{src} in PRCII transformed cells as tested by immuno-precipitation of ^{35}S labeled cell lysates followed by electrophoresis and autoradiography (5,6). The p105 of PRCII was also produced in infected macrophages, although these cells were not overtly transformed. In order to characterize the genetic origin of p105, two dimensional tryptic peptide maps of the ^{35}S methionine labeled protein were prepared and compared to the maps of the structural viral proteins. Figures 3 and 4 show the results for p105 and the gag precursor pr76.

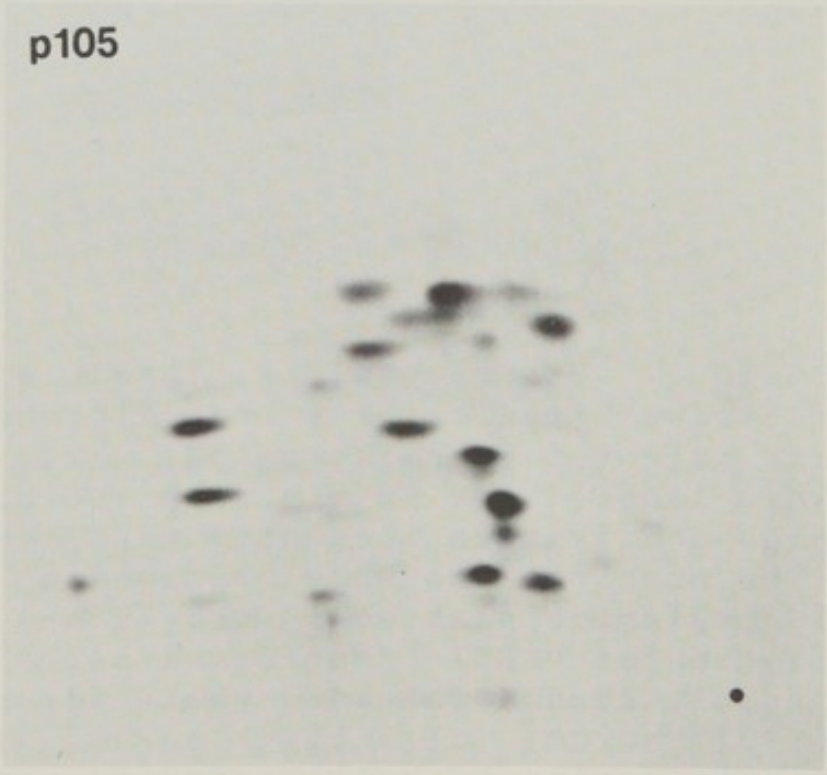

FIGURE 3. Tryptic peptide fingerprint of the p105 polyprotein of PRCII. The ^{35}S methionine labeled protein was separated by SDS-polyacrylamide gel electrophoresis, eluted,

formic acid and digested with TPCK-trypsin. The resultant peptides were separated by TLC-electrophoresis (pH 4.5) and chromatography in n-butanol: water:pyridine:acetic acid (75:60:60:15) and detected by autoradiography on Kodak NS film.

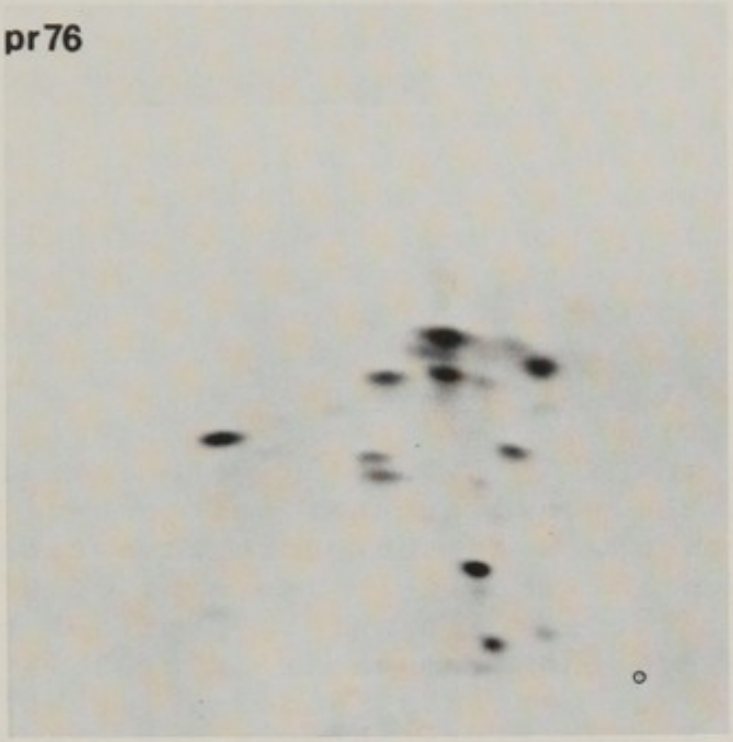

FIGURE 4. Tryptic peptide map of the ^{35}S methionine labeled pr76^{gag} polyprotein of PRCII helper virus. This map was prepared according to the procedure outlined in Figure 3.

Some of the peptides of pr76 and p105 overlap as would be expected from the immunological cross-reactivity. These overlaps have been confirmed by preparing a map of a mixture of pr76 and p105 peptides. Tryptic peptide maps of the individual gag proteins p27, p19 and p15 have also been obtained, and by comparing these maps with those of p105, it was determined that p105 contained all the peptides of p19 and p27 but none of p15. P12, the fourth virion protein derived from pr76, was not analyzed, because it lacked clear methionine containing peptides. In addition to the peptides of p19 and p27, p105 also contained peptides that were absent from pr76. The possibility existed that these represented sequences of the pol or env

genes. Tryptic maps were therefore prepared of the intracellular env precursor pr95 and of the gag-pol precursor pr180, precipitated with the respective specific antobodies from cells infected with PRCII helper virus. No overlap was found between the peptides of p105 and those of pr95. However, one of the pol-specific peptides of pr180 appeared to comigrate with a p105 peptide. Since the pr180 map is very complex, the apparent overlap with p105 could be fortuitous. This question requires further investigation. However, even if one p105 peptide was pol-derived, enough p105-specific peptides remain to support the conclusion that a considerable portion of the p105 protein is unrelated to any helper viral gene product.

PRCII thus codes for a fusion protein that combines partial gag with nonstructural sequences, similar to the transformation specific fusion proteins of avian acute defective leukemia viruses MC29, MH2 and AEV (7,8,9,10). Potential homologies in the nonstructural portions of these fusion proteins were therefore investigated by tryptic peptide mapping. The maps of MC29 p110 and MH2 p100 contained mainly peptides that were clearly different from those of p105, a possible overlap in two peptides is being investigated further. A comparison between the p75 and p40 AEV specific proteins and p105 of PRCII also failed to reveal relatedness except in the gag portions (data not shown, see ref. 4). Since PRCII is a sarcoma virus, it was also conceivable that the p105 fusion protein contained all or part of the $pp60^{src}$ protein common to all avian sarcoma viruses tested so far (11). Two dimensional tryptic peptide maps of various src proteins have been prepared in this laboratory (12) and are also available in the published literature (11). The peptides common to these $pp60^{src}$ proteins show no overlaps with peptides of p105 (Fig. 5). These data lead to the conclusion that the transformation-specific protein p105 of PRCII contains nonstructural, possibly oncogenic sequences which are different from those of known avian sarcoma and acute leukemia viruses and may represent a new class of sarcoma inducing genes.

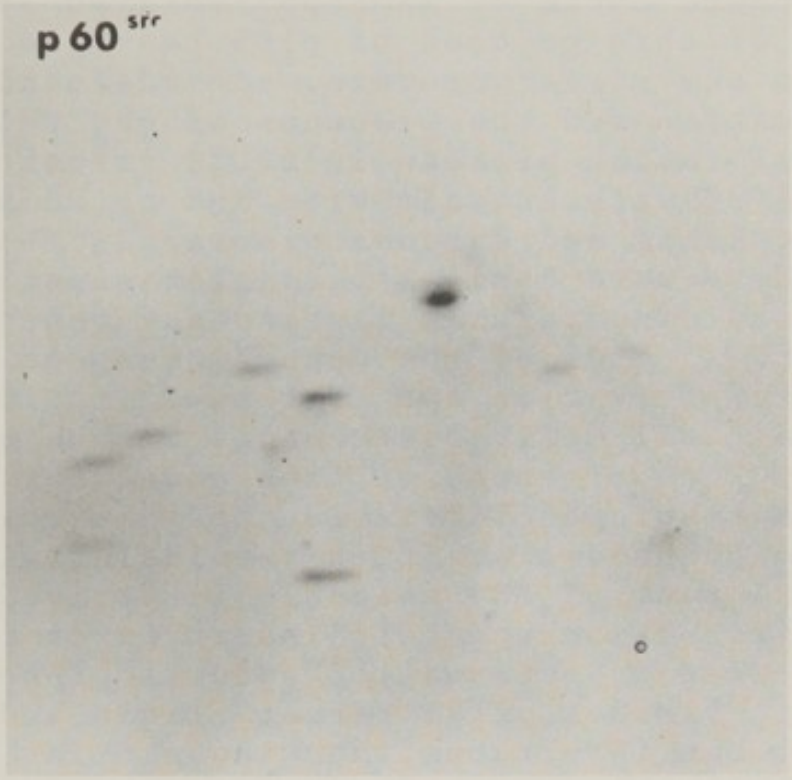

FIGURE 5. Tryptic peptide fingerprint of ^{35}S methionine labeled $p60^{src}$. The $p60^{src}$ protein was generated by *in vitro* translation of virion RNA from recovered avian sarcoma virus 14-6 (12) and processed by the procedure outlined in Figure 3.

DISCUSSION

PRCII induces exclusively sarcomas in the chicken, and compared to RSV and its relatives it appears to be a less efficient oncogenic agent. Stocks of PRCII contain a transforming virus which is defective in the synthesis of infections progeny. This transforming PRCII codes for a fusion protein of 105,000 mol. wt. referred to as p105. P105 contains the *gag* sequences representing the structural avian retrovirus proteins p19 and p27. The sequences of p12 are located between p19 and p27 in functional *gag* (13) and, by analogy, are probably also present in p105. However proof for this assumption could not be obtained, since p12 lacks methionine containing peptides. Assuming that the *gag* sequences in p105 are not reiterated, they would account for (19+12+27):105 = 55% of the apparent molecular weight of p105. In the remain-

ing 45% there could be some pol-derived sequences, but most of this portion of p105 bears no resemblance to any virion protein. The defectiveness in replication and the presence of gag related nonstructural fusion protein in PRCII transformed cells are reminiscent of avian and of some mammalian leukemia and sarcoma viruses (4,7-10,14-16). All of these have basically similar genetic structures, retaining partial gag and env genes at the 5' and 3' ends of the genomes respectively, with a transformation specific substitution of nonstructural cell-derived sequences in the center (17-23). Each of these viruses codes for a transformation specific fusion protein which contains gag sequences at its N-terminal region, the C terminal rest of the molecule is derived from the transformation specific insertion of the genome. That transformation specific insertion differs in viruses of different origin and pathogenicity, but in viruses inducing similar spectra of tumors in the same host species the transformation specific genome insertions are genetically related (24). These observations suggest a role for the fusion proteins in oncogenesis and in the tissue tropism of tumor induction. The p105 of PRCII is distinct from the fusion proteins of other rapidly transforming, defective avian retroviruses. It is also unrelated to the pp60^{src} of RSV, and pp60^{src} cannot be detected in PRCII transformed cells. Thus p105 appears to be the product of a new transformation specific gene in avian viral sarcoma, and PRCII an avian sarcoma virus with a genetic structure that is unlike that of RSV and is similar to the genomes of avian acute leukemia viruses.

Recently a new avian sarcoma virus obtained from a spontaneous chicken tumor has been described (25). This new isolate, Y73, is also different from RSV in its genetic structure and more like avian acute leukemia. A further unusual avian sarcoma virus is that of Fujinami (26) which, like PRCII, is defective for replication and codes for a transformation specific fusion protein (Hanafusa 1980, personal communication, Duesberg 1980, personal communication). A comparison of PRCII with Y73 and Fujinami sarcoma virus will be instructive.

ABBREVIATIONS

PRC	Poultry Research Centre
RSV	Rous sarcoma virus
MC29	Avian myelocytomatosis virus MC29
CMII	Avian myelocytomatosis virus CMII
MH2	Avian carcinoma virus Mill Hill 2
AEV	Avian erythroblastosis virus
RAV	Rous associated virus

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FUJINAMI SARCOMA VIRUS AND SARCOMAGENIC, AVIAN ACUTE LEUKEMIA VIRUSES HAVE SIMILAR GENETIC STRUCTURES

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ABSTRACT The structure of the genomic RNAs and the gene products of avian Fujinami sarcoma virus (FSV) and of avian acute leukemia viruses with sarcomagenic potential, i.e., viruses of the myelocytomatosis (MC29)- and erythroblastosis (AEV)-subgroups, are compared. The genome of replication-defective FSV is a 4.5 kilobase (kb) RNA and, like the RNAs of defective acute leukemia viruses, consists of three distinct sections: an internal FSV-specific section flanked by 5' and 3' helper-virus-related sequences. Their related terminal sequences identify each of these viruses as belonging to the avian tumor virus group. The internal specific sequences are the structural basis for the distinction of the different subgroups of avian oncogenic viruses, i.e., the MC29-, AEV-, and FSV-subgroups. These sequences are not shared between members of different subgroups, and are not related to the *src* gene of the Rous subgroup of sarcoma viruses. In each of these viruses, the 5'-*gag* gene-related and internal specific sequences together form a genetic unit that codes for a nonstructural phosphoprotein which is probably involved in transformation. These genetic units represent less than the total coding capacity of the acute leukemia viral RNAs. However, in the case of FSV, the genetic complexities of the 4.5-kb RNA and of the 140,000-dalton, FSV-specific protein are nearly identical, a fact which excludes the synthesis of other major proteins, in the same reading frame, and suggests that this protein would be sufficient for transforming function. The 140,000-dalton FSV protein is phosphorylated in an *in vitro* kinase reaction by itself or an associated phosphokinase.

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INTRODUCTION

Replication-defective avian acute leukemia viruses have a common characteristic genetic structure: the genomic RNA measures between 5.5 and 6 kilobases (kb), and contains an internal specific section of 2 to 3 kb, which is flanked by 5' and 3' helper virus-related sequences (1-6). The specific sequences are thought to be essential elements of the transforming *onc* genes of these viruses and are used as a basis for subclassification of avian oncogenic viruses (1, 4, 7). Acute leukemia viruses with closely-related oncogenic spectra (8-10), like the myelocytomatosis viruses MC29 and CMII, and Mill Hill virus (MH2), were found to have related internal, specific RNA sequences (4, 11-13), and, consequently, were grouped together in the MC29-subgroup of avian oncogenic viruses (1, 4). Avian erythroblastosis virus (AEV) causes predominantly erythroblastosis and sarcomas in the chicken. Its RNA contains a specific sequence unrelated to that of the MC29-subgroup, and therefore defines a new subgroup of oncogenic viruses, the AEV-subgroup (1, 5). The defective *gag* genes and specific sequences of these viruses together code for nonstructural, *gag* gene-related proteins, which are thought to be the transforming proteins (1, 3, 4, 6, 14-16).

In contrast to the acute leukemia viruses described above, the well-characterized prototype of avian sarcoma viruses, Rous sarcoma virus (RSV), is nondefective and contains a specific gene responsible for sarcomagenicity (*src*), near the 3' end of viral RNA, which is unrelated to replicative genes or to the specific sequences of the MC29- or AEV-subgroups. (For review, see refs. 7, 9, 17.)

In this report, we extend our recent investigation (18) of Fujinami sarcoma virus (FSV). The sarcoma, from which FSV was subsequently isolated, was discovered in Japan at approximately the same time in 1909 as RSV was isolated in the United States. However, the viral nature of the Fujinami agent was only clearly recognized later (19, 20). Although the oncogenic spectra of FSV and of RSV are very similar (19-21), we have found that RSV and FSV have completely different genetic structures (18). Here, we compare the genetic structure and gene product of FSV with those of RSV and of avian acute leukemia viruses with sarcomagenic potential.

RESULTS

Oncogenic Potential of FSV. A stock of FSV, obtained from H. M. Temin, was shown to induce foci of transformed cells in chicken embryo fibroblast (CEF) cultures. The titer reached 10^5-10^6 focus-forming units (FFU)/ml after repeated

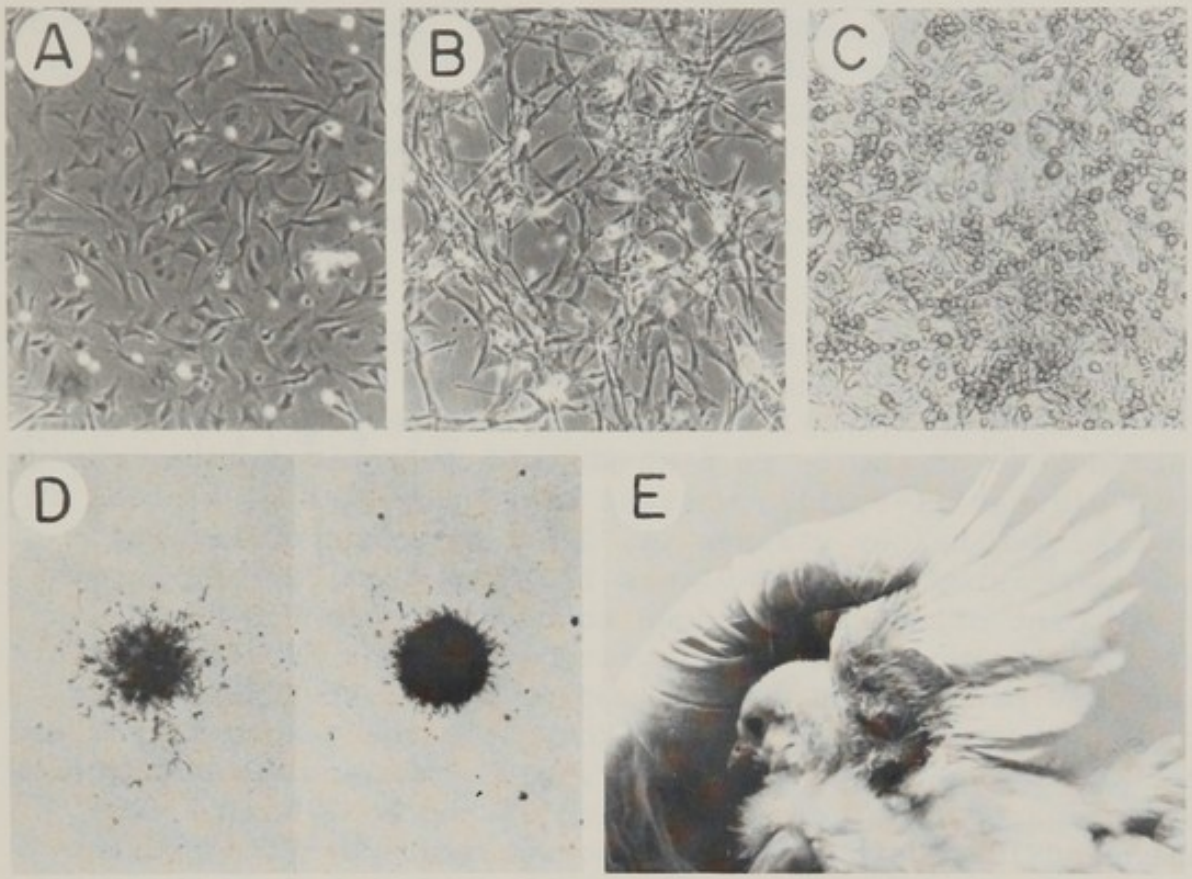

FIGURE 1. Comparison of (A) normal chicken embryo fibroblasts (CEF), (B) CEF transformed by FSV, and (C) CEF transformed by RSV (Schmidt-Ruppin strain of envelope subgroup D). (D) Colonies of FSV-transformed CEF in soft agar. (E) Fibromyxosarcoma in the wing of a four-week-old chicken 26 days after inoculation of 0.1 ml FSV stock ($\sim 10^4$ FFU) into the wing web.

cloning through selection for virus-producing foci. Cells in these foci and CEF cultures transformed by FSV in mass infection had a characteristic fusiform morphology (Fig. 1B), which could easily be distinguished from the morphology of normal or RSV-transformed CEF (Figs. 1A and 1C). FSV-infected CEF also grew into colonies in semi-solid agar (Fig. 1D) under assay conditions described before (14, 22). The majority of cultures derived from these single-cell colonies were nonproducers, i.e., did not release infectious transforming virus into the culture medium. This and the biochemical analysis of FSV RNA and protein (see below) indicated that FSV is replication-defective. The oncogenic properties *in vivo* were assayed by inoculating 0.1-0.3 ml of filtered (0.45 μm) culture medium from FSV-producing CEF into the wing web of 1- to 5-day-old

C/E chickens. Tumors at the site of inoculation occurred in all infected chickens within 10-15 days (Fig. 1E), and proved to be fibromyxosarcomas upon histological examination (18). Examination of the blood failed to show any evidence of leukemia in surviving chickens up to 6 weeks after infection. Thus, oncogenic properties of the FSV stock used here appear to be very similar to those described for the original isolate (19-21).

The RNA of FSV. RNA preparations of FSV contained a 4.5-kb and a 8.5-kb species as demonstrated by polyacrylamide gel electrophoresis (Fig. 2A). The size of the 4.5-kb RNA is similar to that of the 5- to 6-kb RNAs of defective acute leukemia viruses (1-5, 11), whereas the 8.5-kb RNA is typical of nondefective helper viruses (2, 22). Nontransforming Fujinami-associated virus (FAV) was isolated from FSV(FAV) stocks by endpoint dilution and shown to contain only 8.5-kb RNA. This and the positive correlation between the FFU-titer of a given FSV preparation and the amount of 4.5-kb RNA prepared from it indicated that the 4.5-kb RNA is the genome of FSV and the 8.5-kb RNA the genome of FAV. Both ^{32}P-labeled RNA species were prepared from gels as in Fig. 2A and analyzed by two-dimensional electrophoresis-homochromatography (fingerprinting) of RNase T_1-resistant oligonucleotides as described (2-5, 17, 23) (Figs. 2B and 2C). 15 FSV-oligonucleotides and 24 FAV-oligonucleotides were identified, analyzed by RNase A digestion (Table 1), and ordered on a linear map according to their distance from the 3' poly(A)-coordinate, as described (17, 23). This analysis showed that FSV-RNA (Fig. 2D) consists of three sections: (i) a 5' section of 1 kb, which is identically shared with the 5' region of the FAV *gag* gene (cf., ref. 18 for a map of FAV); (ii) an internal section of 3 kb, which is unrelated to any other viral RNA tested, including RSV and MC29- and AEV-subgroup viruses as well as their helper viruses (Table 1); and (iii) a 3' section of about 0.5 kb, containing two oligonucleotides, i.e., 3 and 14, which were subject to competition by unlabeled FAV RNA during hybridization of FSV(FAV) [^{32}P] RNA with FSV(FAV) cDNAs (18). No. 14 is a closely-related homolog of the C-oligonucleotide common to exogenous avian tumor viruses (17, 23). Highly-conserved oligonucleotides of the *gag*, *pol*, and *env* genes of nondefective viruses were lacking in FSV RNA (Table 1; Fig. 2D), but present in FAV RNA (Table 1), which is a direct explanation for the replication-defectiveness of FSV.

FSV Codes for a 140,000-dalton, *gag* Gene-related, Nonstructural Protein. Immunoprecipitation with antiserum directed against disrupted RSV (Prague strain) and subsequent

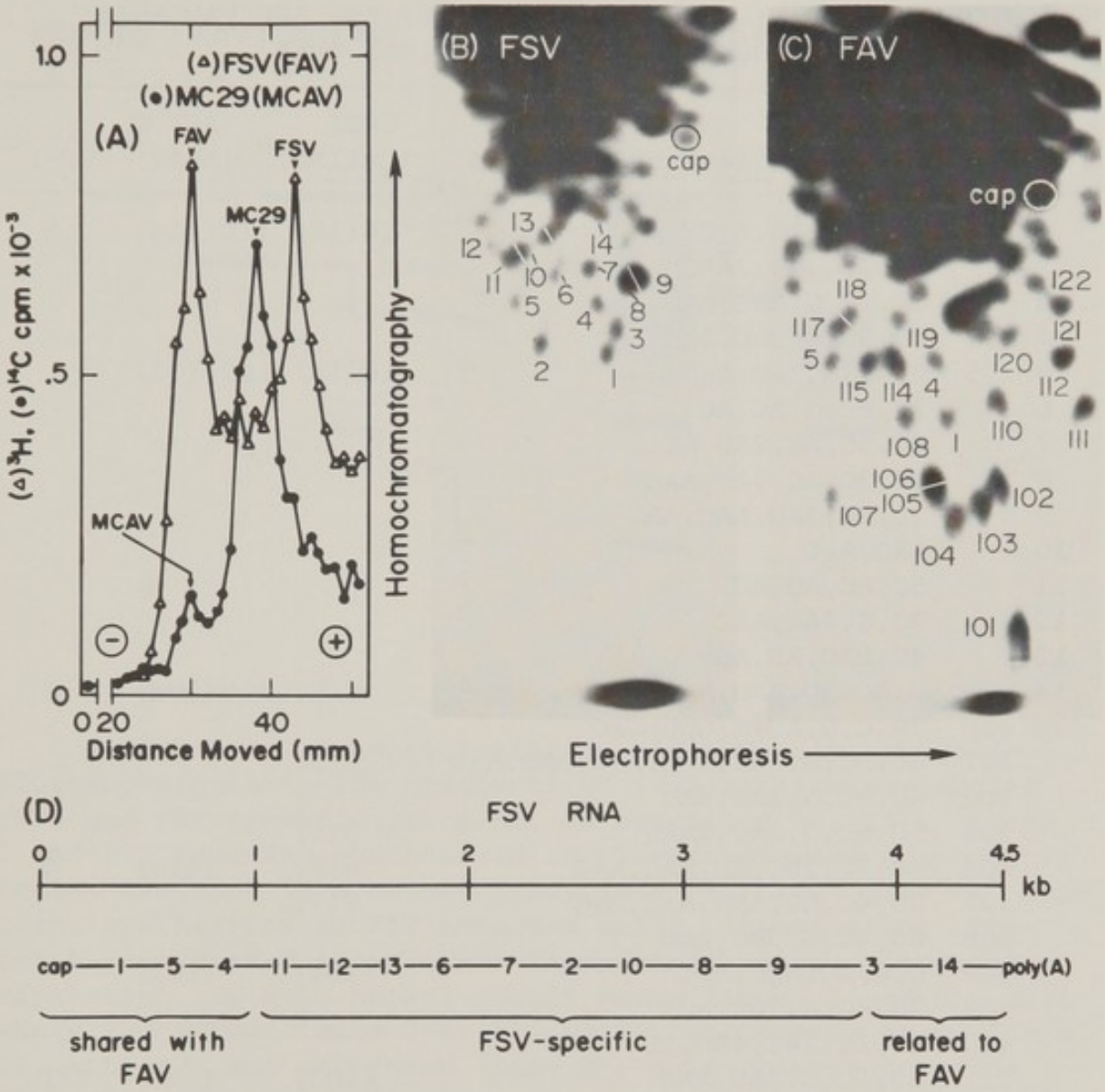

FIGURE 2. The RNA of FSV. (A) Coelectrophoresis of [^{3}H] RNA monomers of FSV(FAV) with [^{14}C] RNA monomers of MC29(MCAV) in 2.1% polyacrylamide (2, 22). (B) and (C) T_1-oligonucleotide-fingerprinting of ^{32}P-labeled RNAs of FSV and FAV prepared from a gel, as in (A), as described (2-5). (D) Oligonucleotides of 4.5-kb FSV RNA were ordered on a map of 4.5 kb according to the size of the smallest poly(A)-tagged RNA fragment from which they could be isolated (1, 17).

SDS-polyacrylamide gel electrophoresis (4, 14) demonstrated the presence of a 140,000-dalton protein in lysates of [^{35}S] methionine-labeled FSV-transformed nonproducer CEF cultures (Fig. 3). The typical precursor proteins of nondefective viruses, Pr76 *gag*, Pr180 *gag-pol*, and Pr95 *env*, were not

TABLE I
COMPOSITION OF T_1-OLIGONUCLEOTIDES OF FSV AND FAV RNAs AND HOMOLOGS IN OTHER VIRAL RNAs

FSV	FAV	RNase A Digestion Products	CMII (CMIIAV)[a]	AEV (AEAV)[b]	PR-B[c]
1	1	4U,5C,AAG,A_4C	16(16)	114(114)	8
2		U,6C,G,AU,2AAC			
3		3U,2C,G,AAC,AAU,A_3C			
4	4	2U,3C,G,3AC,2AU			
5	5	U,4C,G,2AC,A_4C	14(14)	102(102)	
6		2U,8C,G,AC,AU			
7		3U,5C,AC,2AU,AG			
8		2U,3C,AC,2AU,AAG			
9		2U,2C,3AU,AAU,AAG			
10		AAG,A_5C			
11		5C,AC,AU,AG			
12		3C,G,2AC,AAC			
13		4C,2AC,AU,AG			
14		G,AC,AU,AAU,A_3U	C(C)	108(108)	C
cap	cap	2U,C,G,AU,m^7GpppGmC			
	101	8U,5C,G,2AC,2AU,AAC,AAU,A_3U	3(3)		
	102	4U,5C,G,2AC,4AU			
	103	5U,6C,G,2AC,2AAU	5(5)		
	104	6U,7C,2AC,AU,AAC,AAG	6(6)	(110)	4
	105	2U,4C,AC,AAU,A_3C,A_4G	7(7)		
	106	6U,4C,G,3AC,2AU			
	107	C,G,AC,A_4C,A_5C	(101)	(101)	
	108	2U,4C,G,2AC,AU			
	110	4U,2C,2AC,4AU,AG			
	111	4U,2C,G,3AU,AAU	(103)	(119)	21
	112	7U,5C,G,AU	(104)	(120)	11
	114	2U,4C,G,2AC,AU,AAC		(105)	14
	115	U,4C,2AC,AU,AG,AAC	12(102a)	(107)	
	117	2U,5C,3AC,AAG	13(13)	(109)	20a
	118	2C,AU,AG,A_4U/C	(107)	(108b)	20b
	119	2U,G,2AC,A_4U/C			
	120	3U,C,AC,AU,A_4G			
	121	5U,2C,G,AU,AAU	24(24)	(122)	18
	122	3U,2C,G,2AU,AAU	(108)	(127)	
	C	G,AC,AU,AAU,A_3C	C(C)	108(108)	C

[a]Oligonucleotides of CMII and CMIIAV (in parentheses) are numbered as in ref. 4.
[b]AEV and AEAV oligonucleotides are numbered as in ref. 5.
[c]Oligonucleotides of Prague RSV of envelope subgroup B (PR-B) are numbered as in ref. 17, 23.

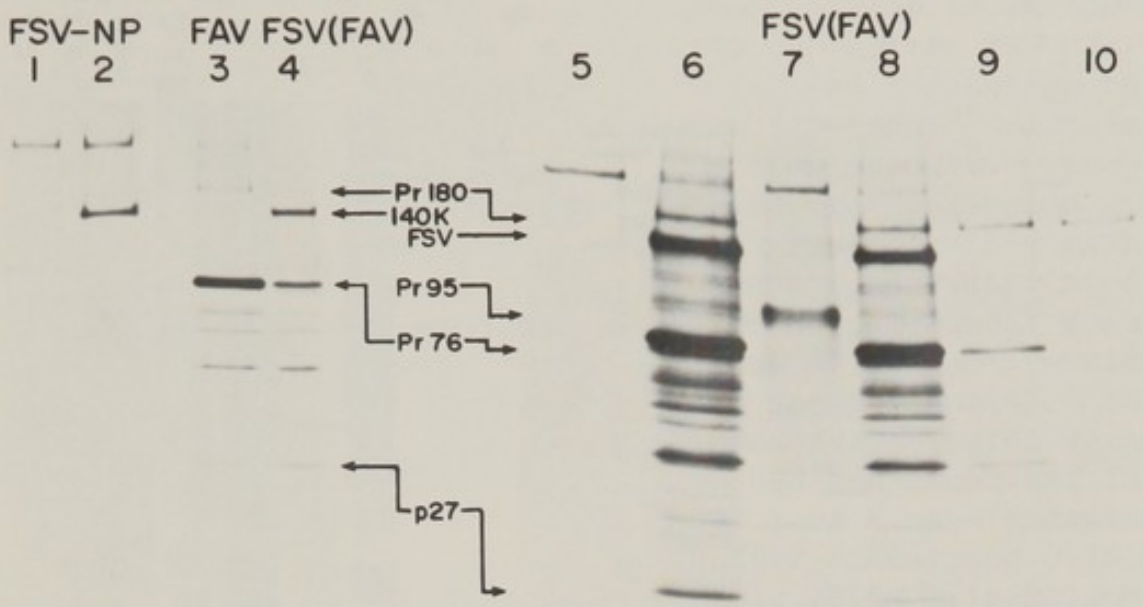

FIGURE 3. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis (6-18% gradient) of [^{35}S]methionine-labeled FSV- and FAV-specific proteins, as described (4, 14). Lanes 1 and 2: Proteins synthesized in FSV-transformed nonproducer cells, isolated in a colony assay (see Fig. 1). Lane 3: Proteins synthesized in FAV-infected cells. Lanes 4-10: Proteins synthesized in FSV(FAV)-transformed cells. Immunoprecipitation was with normal rabbit serum (lanes 1 and 5), anti-whole RSV serum (lanes 2-4, 6), anti-glycoprotein serum (lane 7), anti-p27 and p19 serum (lane 8), anti-polymerase serum (lane 9), and anti-polymerase serum preabsorbed with 1 µg of NP-40-disrupted RSV per 1 µl serum (lane 10).

observed in these cells, but were present in FAV-infected CEF. FSV(FAV)-infected cells contained the 140,000-dalton protein in addition to the FAV proteins (Fig. 3). Therefore, it is concluded that the 140,000-dalton protein is coded by FSV and represents the only gene product detectable by antibodies against viral structural proteins. Analysis with specific antisera demonstrated that the 140,000-dalton FSV protein is related to the *gag* gene, but unrelated to the *env* and *pol* genes of avian RNA tumor viruses (Fig. 3). It is not found as a major component in FSV(FAV) pseudotype virions (not shown).

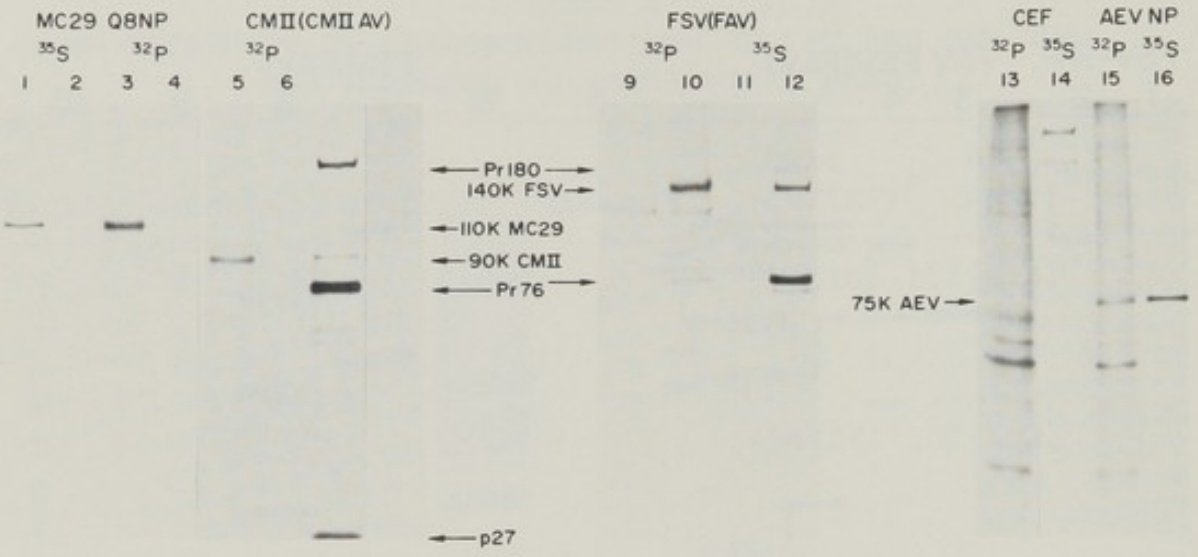

FIGURE 4. ^{32}P- or ^{35}S-labeled proteins immunoprecipitated from extracts of cells transformed by acute leukemia viruses MC29, CMII, AEV, or by FSV. Control: uninfected CEF. Q8NP is a nonproducer line of MC29-transformed quail cells (14), and AEV NP is a nonproducer line of AEV-transformed chicken cells, clone C23 (kind gift of G. S. Martin, Berkeley). Labeling was for 2 hr with $H_3{}^{32}PO_4$ (500 μCi/ml) or [^{35}S]methionine (100 μCi/ml). Cell extracts were prepared in lysis buffer containing EDTA or unlabeled ATP to prevent phosphorylation of proteins with labeled ATP during preparation. Immunoprecipitation was carried out with normal rabbit serum (lanes 2, 4, 6, 8, 9, 11), anti-whole RSV serum (lanes 1, 3, 5, 7, 10, 12), or anti-p27/p19 serum (lanes 13-16). Gel electrophoresis was in 7.5% SDS-polyacrylamide gels and otherwise, as described (4, 14).

The Nonstructural Proteins of FSV and of the Acute Leukemia Viruses are Phosphorylated. Quail cells or CEF transformed by MC29, CMII, AEV, and FSV were labeled with [^{32}P]orthophosphate or [^{35}S]methionine, and cell lysates were subjected to immunoprecipitation with either normal rabbit serum, rabbit antiserum against disrupted RSV, or rabbit antiserum against *gag* proteins p27 and p19. The immunoprecipitates were analyzed by gel electrophoresis and fluorography, as described (4, 14). Fig. 4 shows that the *gag* gene-related proteins coded by MC29, CMII, AEV, and FSV can be labeled by [^{32}P]orthophosphate, and are electrophoretically and immunologically indistinguishable from the [^{35}S]methionine-labeled proteins in cells from parallel culture dishes. Some ^{32}P-labeled cellular proteins are seen in immunoprecipitates from uninfected CEF upon prolonged autoradiographic exposure of the gel (Fig. 4,

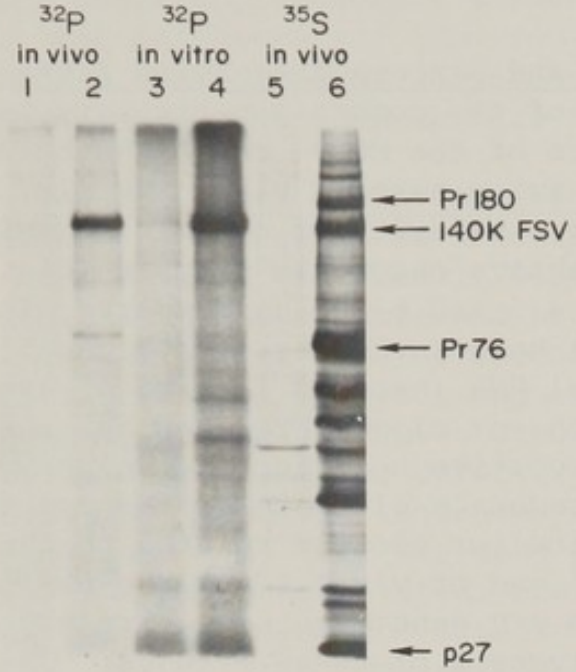

FIGURE 5. *In vitro* phosphorylation of the 140,000-dalton FSV protein, and comparison to the ^{32}P- and ^{35}S-labeled *in vivo* product. Proteins from unlabeled CEF transformed by FSV(FAV) were immunoprecipitated with normal rabbit serum (lane 3) and anti-whole RSV serum (lane 4). The immunoprecipitates were washed and incubated in 50 µl of kinase reaction buffer containing 0.15 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.1% NP-40 and 10 µCi γ-[^{32}P]ATP (∿4000 Ci/mmole). In parallel, [^{32}P] and [^{35}S] proteins labeled *in vivo*, as described in Fig. 4, were precipitated with normal rabbit serum (lanes 1 and 5) or anti-p27 serum (lanes 2 and 6). The reaction products were electrophoresed together on a 7.5% SDS-polyacrylamide gel.

lane 13). The Pr76 *gag* precursor protein of CMIIAV and FAV in CMII(CMIIAV)- and FSV(FAV)-infected cells, respectively, is also phosphorylated (Fig. 4) like the *gag* gene product of other nondefective viruses (24). However, the specific [^{32}P] radioactivity of the 90,000-dalton CMII- and 140,000-dalton-FSV proteins is significantly higher than that of the helper virus Pr76 proteins based on the respective $^{32}P/^{35}S$ ratios (Fig. 4). This suggests the presence of specific, non-*gag*-related phosphorylation sites in the CMII- and FSV-proteins.

In vitro Phosphorylation of the 140,000-dalton FSV Protein. Immunoprecipitates of proteins from unlabeled cells infected by MC29, CMII, AEV, and FSV were incubated in a phosphokinase reaction mixture containing γ-[^{32}P]ATP under conditions described for the *in vitro* kinase assay of the *src* gene product (25-27). Analysis of the reaction products by SDS polyacrylamide gel electrophoresis showed that the 140,000-dalton FSV protein was phosphorylated (Fig. 5), whereas the immunoprecipitates of proteins from cells infected with the acute leukemia viruses did not exhibit phosphokinase activity under our assay conditions (not shown).

DISCUSSION

Genetic Structure of FSV and Sarcomagenic Avian Acute Leukemia Viruses. Comparison of the common genetic structure of avian acute leukemia viruses of the MC29- and AEV-subgroups (1) with the RNA structure of avian sarcoma virus FSV (18; this paper) reveals a striking similarity of genomic organization in these replication-defective oncogenic viruses (Fig. 6). The RNAs measure between 4.5 and 6 kb, as compared with the 8.5 kb-RNA of nondefective helper viruses. Both the 5' and the 3' regions of the viral RNA (hatched in Fig. 6) are either isogenically shared with, or closely related to, analogous RNA sequences of the respective nondefective helper virus. They represent partial sequence elements of the *gag* and *env* genes and sequences identical or closely related to the common c-region (17) at the 3' end of viral RNA. The partial or total lack of *gag*, *pol*, and *env* gene sequences directly explains the replication-defectiveness of these viruses. The internal specific RNA sequences of the MC29- and AEV-subgroup viruses, and of FSV, are unrelated to each other, to the RNAs

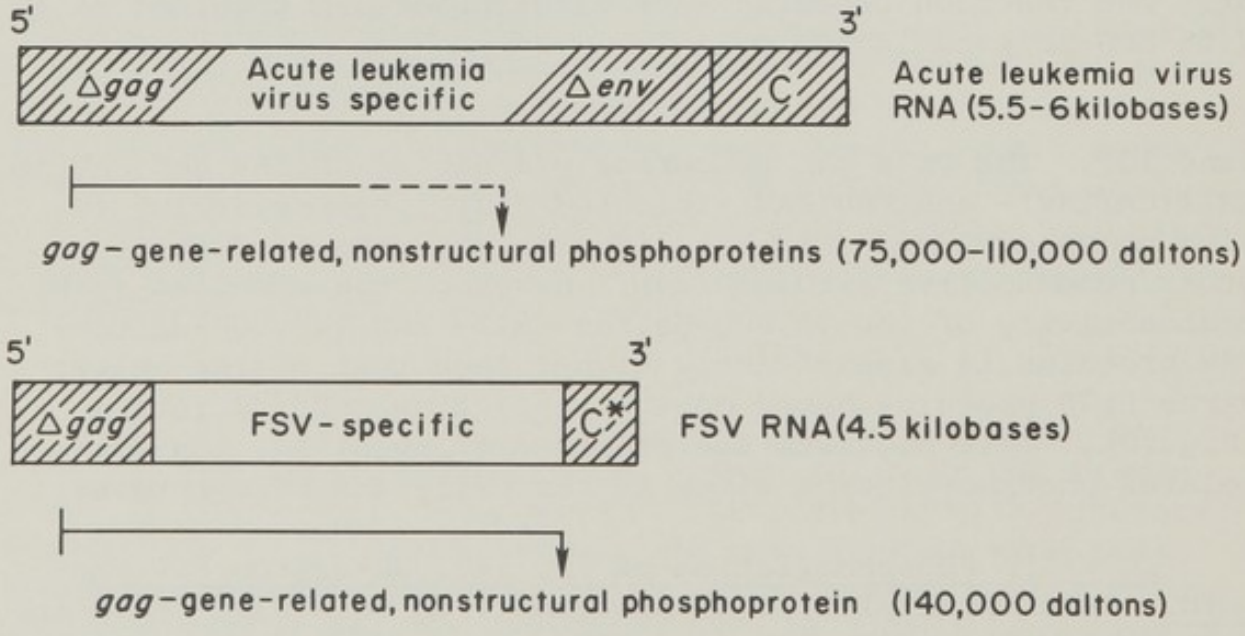

FIGURE 6. Genetic structure and gene products of acute leukemia virus and FSV RNAs. Hatched areas indicate common RNA sequences shared with or closely related to helper virus RNA. The upper and lower limits of these common sequences for the different members of the MC29- and AEV-subgroups of acute leukemia viruses are indicated by diagonal borders. Δ represents partial sequence elements of the gene indicated.

of the nondefective helper viruses, and to the *src* gene of RSV. The defective *gag* gene and all or part of these specific sequences together code for nonstructural *gag* gene-related phosphoproteins ranging in size from 75,000-140,000 daltons.

The *onc* Genes of FSV and of Acute Leukemia Viruses. The transforming *onc* genes of defective transforming viruses have not been defined genetically because *onc* deletion mutants are biologically undetectable and because recombinants are difficult to define for lack of secondary markers (1, 6, 7). However, biochemical analyses and comparison of the RNAs of several acute leukemia viruses strongly indicate that the genetic units consisting of the defective *gag* gene and part or all of the internal specific sequences represent the *onc* gene of these viruses (1, 7). The gene products of these genetic units, the *gag* gene-related, nonstructural phosphoproteins, would then represent the transforming proteins. In the case of FSV, the genetic complexities of the 4.5-kb RNA and the 140,000-dalton phosphoprotein approximately coincide. This strongly suggests that the gene coding for the 140,000-dalton protein is nearly identical with FSV RNA. Therefore, barring translation in different reading frames, the 140,000-dalton FSV protein appears to be a transforming protein, and is probably sufficient for transformation. The *gag* gene-related, nonstructural proteins of each of the defective transforming viruses analyzed here were shown to be phosphoproteins *in vivo*. Moreover, the 140,000-dalton FSV protein was phosphorylated *in vitro* by an associated kinase activity. Further analysis is necessary to decide whether this is due to an intrinsic enzymatic activity of the FSV protein itself or due to a cellular phosphokinase present in the immunoprecipitate.

RNA Sequences as a Basis for Avian Tumor Virus Classification. On the basis of specific RNA sequences, four classes of transforming genes can be distinguished in the avian tumor virus group which define distinct subgroups of transforming viruses: the *src* gene of the RSV subgroup, and the *onc* genes of the MC29-, AEV-, and FSV-subgroups of avian tumor viruses (1, 7, 18). In the case of avian myeloblastosis virus (AMV), which differs from the viruses of the four defined subgroups by its inability to transform fibroblasts in culture and to induce sarcomas in the animal, another class of transforming gene might be expected. It is remarkable that the classification of avian oncogenic viruses according to their biochemically-defined transforming genes is in close agreement with the prototype classes of avian tumor viruses (sarcoma, myeloblastosis, myelocytomatosis, and erythroblastosis) defined according to their predominant oncogenic properties by Beard

and his colleagues (8) and extended more recently by others (10). However, the cases of FSV and RSV present a clear example in which a very similar oncogenic phenotype is produced by two structurally-unrelated RNA sequences. Based on their different *onc* genes, RSV and FSV now define two distinct subgroups of sarcoma viruses. Overlaps among the oncogenic spectra of the four different subgroups of avian transforming viruses suggest that multiple mechanisms for neoplastic transformation, like sarcoma formation, exist, unless the products of each of these different *onc* genes are functionally similar.

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A MODEL FOR FOCMA EXPRESSION IN CELLS TRANSFORMED BY FELINE LEUKEMIA AND SARCOMA VIRUSES

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ABSTRACT A model is presented to interpret why lymphoid tumor cells induced by FeLV and fibroblasts transformed by different FeSV strains express the tumor-specific antigen, FOCMA. A central prediction is that a genetically variant (recombinant) FeLV is generated *in vivo* and contains sequences not found in standard replication-competent FeLV strains. Variant FeLV sequences encoding FOCMA are proposed to contribute to the formation of sarcoma viruses and, although not localized within *src*, are postulated to appear within the "sarcoma viral-specific regions" of FeSV genomes. Thus, certain sequences involved in tumorigenesis may be common to both FeLV and FeSV.

INTRODUCTION

Cats exposed to feline leukemia virus (FeLV) or to viruses of the feline sarcoma-leukemia virus complex (FeSV [FeLV]) synthesize antibodies directed not only to viral components but also to virus-induced tumor cells. Certain such sera react with a tumor-specific antigen (the feline oncornavirus-associated cell membrane antigen or "FOCMA" [1]) which is not detected on the surfaces of untransformed, FeLV-infected fibroblasts or on normal cat lymphoid cells (2,3). By contrast, lymphoid tumor cells induced by FeLV or cat fibroblasts transformed by FeSV (FeLV) are FOCMA-positive whether they produce FeLV or not (1,2,4,5). Using an immunofluorescence test performed with FeLV-producing lymphoblastoid target cells established from cats with virus-induced lymphoma, it has been shown that absorbtion of cat antisera with either FeLV (6) or purified FeLV structural proteins (7) does not diminish reactivity to FOCMA. In an extensive survey of cat sera from animals exposed experimentally or naturally to FeLV, no concordance has been seen between titers of antibodies to FOCMA and those to the major FeLV

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structural proteins, p30 and gp70 (6,7). Antibodies to FOCMA, apparently directed to antigens not encoded by replication-competent FeLV, are believed to be important in determining whether cats can resist tumors caused by oncogenic feline leukemia and sarcoma viruses (1,4).

Cells of heterologous species, productively or nonproductively transformed by the Gardner-Arnstein strain of FeSV (FeLV), are FOCMA-positive, suggesting that the antigen is encoded by sequences present in FeSV (3). Three independent isolates of FeSV [the Snyder-Theilen (ST), Gardner-Arnstein (GA), and McDonough-Sarma (MS) strains (8-10)] are genetic recombinants between portions of FeLV and other sarcoma-viral specific sequences (designated src) (11,12). The src sequences are transduced from normal cat cells and presumably confer the properties of morphological transformation. As yet, however, no direct genetic evidence implicates these sequences in the transformation process. Two different sets of src sequences are represented in the three prototype FeSV strains: a closely related set shared by ST- and GA-FeSV and an apparently non-homologous set found in MS-FeSV (11). Each FeSV strain encodes a high molecular weight polyprotein containing antigens related to a portion of the FeLV gag gene product ($Pr60^{gag}$) as well as unique antigenic determinants (designated "x") unrelated to those of FeLV (13-15). Several laboratories have shown that at least certain FeLV-absorbed cat antisera with anti-FOCMA reactivity can precipitate the gag-x polyprotein encoded by GA-FeSV (5,13), suggesting that FOCMA may be specified by FeSV src sequences. If this were the case, FeLV-induced transformation of lymphoid target cells might involve activation of endogenous cat cellular sarc sequences homologous to the src element of ST- and GA-FeSV (Model #1).

DNA transcripts representing sequences specific to FeSV genomes have been prepared by hybridizing FeSV (FeLV) cDNA with RNA obtained from standard stocks of FeLV, recovering the single-stranded cDNA, and subsequently selecting only those transcripts which anneal to FeSV RNA (11,12). Using these probes (operationally defined as $cDNA_{src}$), src sequences were shown to be contiguous with gag sequences in the ST-FeSV genome showing that, indeed, the x-portion of this polyprotein is encoded by FeSV-specific sequences (12). Recent heteroduplexing experiments performed with cloned ST-FeSV and ST-FeLV DNA confirmed both the complexity and topology of these sequences and showed that the order of genes in ST-FeSV is 5'-gag-src-env-c-region-3', where only 1000 bases of gag and ~600 bases of env are represented (16). However, it is still not formally established that all sequences hybridizing to $cDNA_{src}$ (1300-1500 bases) derive from a single, continuous gene sequence in normal cat cellular DNA. Thus, the "FeSV-specific" sequences currently defined as src could in fact

represent two distinct genetic regions, one derived from cellular sarc genes and the other derived from novel FeLV sequences not found in prototype FeLV strains. If the latter sequences were to encode FOCMA, FeLV-induced disease need not involve activation of cellular sarc elements (Model #2). Preliminary efforts to distinguish between these models are discussed below.

RESULTS AND DISCUSSION

Recent Studies with FeSV Polyproteins. Figure 1 (panels B,C,D) shows that each strain of FeSV codes for a different gag-x polyprotein. The products encoded by ST-, GA-, and MS-FeSV have apparent molecular weights of 78,000, 92,000, and 130,000 daltons, respectively. The values are approximate since both the ST- and GA-polyproteins are phosphorylated and all three appear to be glycosylated (Ruscetti and Sherr, unpublished data). The proteins can each be precipitated with antisera to FeLV gag gene antigens (p15, p12 and some p30 antisera) (15; Ruscetti, Turek, and Sherr, submitted) and also by rat antisera prepared against FeSV-transformed rat nonproducer cells (panels B-D, lanes 1,3,5). Although rat antisera directed to any one polyprotein can precipitate the others, absorbtion with disrupted FeLV (subgroups A, B, and C) removes antibodies to FeLV gag products and renders each serum specific for the x-portion of the molecule. Using FeLV-absorbed sera (panels A-E, lanes 2,4,6), the 78K ST-FeSV and 130K MS-FeSV polyproteins were precipitated only by antisera raised to cells transformed by the identical FeSV strain. By contrast, antisera to the x-portion of GA-FeSV reacted both with the GA- and ST-FeSV products. The type-specific reactivities of these sera show that the x-portions of each of these molecules cannot be identical.

A second product (~110K) was identified in either mink or rat cells transformed by MS-FeSV (see panel D). This protein was precipitated only by rat antiserum to the MS-FeSV polyprotein either before (lane 3) or after (lane 4) absorbtion with FeLV. Similar results were obtained by Barbacid et al. (15) who reported higher molecular weights for the two MS-FeSV coded products and showed that tryptic peptides in the lower molecular weight species were represented in the larger gag-x molecule. Pulse-chase analyses show no definitive precursor-product relationship between the two MS-FeSV encoded proteins (15; Ruscetti, Turek, and Sherr, submitted) suggesting that the smaller, non-gag-containing species might be synthesized from a different mRNA molecule.

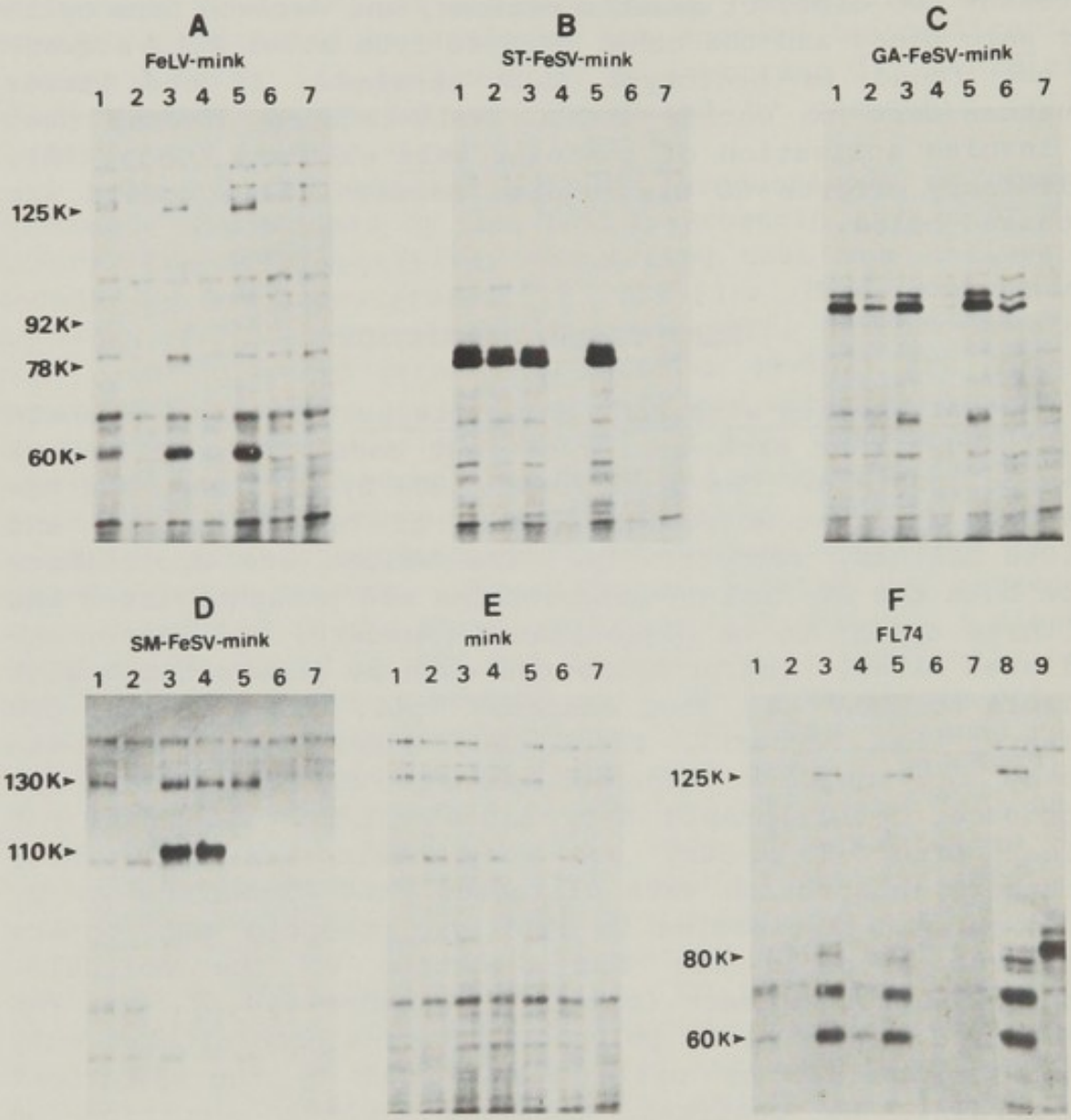

FIGURE 1. Immune precipitation of FeLV-infected or FeSV nonproducer cells with rat anti-gag-x sera. Lysates from the indicated cells were obtained after a 30-min pulse with ^{35}S-methionine and were reacted with the following sera: rat anti-ST-FeSV (lanes 1); rat anti-ST-FeSV absorbed with FeLV (lanes 2); rat anti-MS-FeSV (lanes 3); rat anti-MS-FeSV absorbed with FeLV (lanes 4); rat anti-GA-FeSV (lanes 5); rat anti-GA-FeSV absorbed with FeLV (lanes 6); normal rat serum (lanes 7); goat anti-FeLV p12 (lane 8); and goat anti-FeLV gp70 (lane 9). Molecular weights are in thousands (K) of daltons.

FeLV-induced Tumor Cells Appear Not to Express sarc Products. When rat antibodies to gag-x polyproteins were used to precipitate radiolabeled lysates from FOCMA-positive, FeLV-infected FL74 cells (panel F), two major gag-containing polypeptides were detected (lanes 1,3,5). Each of these products was precipitated with antibodies to FeLV p12 (lane 8) but not with a reference antiserum prepared to the envelope glycoprotein (gp70) of FeLV subgroup A (lane 9). Absorbtion of rat anti-ST- and GA-gag-x sera with FeLV (subgroup A) eliminated all reactivity to FL74 products (lanes 2 and 6). While rat anti-MS-gag-x sera absorbed with FeLV (subgroup A) still precipitated some $Pr60^{gag}$ (lane 4), absorbtion with FeLV (subgroup C) eliminated the residual reactivity. Unlike certain cat antisera to FOCMA, FeLV-absorbed rat anti-gag-x sera failed to precipitate any proteins from the plasma membranes of lactoperoxidase-iodinated FL74 cells and did not react by immunofluorescence with FeSV-transformed mink cells. We conclude, then, that rat antibodies to antigens within the x-portions of the different FeSV polyproteins lack reactivity to FOCMA determinants. Assuming that at least some antibodies in these sera are directed to polypeptide domains encoded by sequences homologous to *sarc*, the results fail to support the hypothesis that *src*-related proteins are expressed in FOCMA-positive tumor cells. Consistent data were obtained by Frankel *et al.* (11) who failed to detect augmented levels of either $src^{ST/GA}$ or src^{MS} RNA in such cells using liquid hybridization analyses.

Cells Transformed by ST-, GA-, and MS-FeSV Express FOCMA Because rat anti-gag-x sera lacked reactivity to FOCMA, we obtained several coded cat antisera from Dr. M. Essex (Harvard School of Public Health) and screened them "blind" for immunofluorescence reactivity against cat FL74 cells and mink cells transformed by the different FeSV strains. Sera (0.2 ml aliquots) were each absorbed with 8 mg of viral protein from isopycnically banded FeLV virions (subgroups A, B and C) produced by FL74 cells. This procedure removed all detectable antibodies to FeLV components as determined by immunoprecipitation of radiolabeled lysates of FeLV-infected mink cells. As expected, neither serum showed indirect immunofluorescence staining with normal mink cells or FeLV-infected mink cells. By contrast, two FOCMA-positive cat sera exhibited strong punctate fluorescence with mink cells transformed by either ST-, GA-, or MS-FeSV. The anti-FOCMA sera reacted with more than 80% of cells transformed by ST- and GA-FeSV but to only 30-50% of cells transformed by the MS-strain. The two anti-FOCMA sera also showed strong fluorescence reactivity with FL74 target cells. The data are consistent with a more extensive survey showing that most, if not all, cat sera with anti-FOCMA reactivity to FL74 cells stain GA-FeSV mink

cell transformants (17). The fact that MS-FeSV transformed cells react with anti-FOCMA sera suggest, however, that FOCMA is specified by sequences shared in common between FeSV strains containing distinctly different src elements.

A Model for FOCMA Expression. Although different FeSV strains appear to encode FOCMA, FeLV-producing but FeSV-negative cat lymphoid tumor cells express this antigen as well. The anti-FOCMA reactivity to FeLV-induced tumor cells is not diminished by absorbtion of cat sera with viruses released from the same cells, suggesting that FOCMA could be encoded by cellular sarc sequences. Yet, we have been unable to demonstrate induction of sarc gene RNA or proteins in such cells. A possible explanation could be that FOCMA determinants are encoded by certain sequences in genetically variant FeLV genomes which are also represented in the different FeSV strains (see Model, Figure 2). Since these sequences would not be present in prototype replication-competent FeLV, they would appear to map only within regions operationally defined as "FeSV-specific." If the postulated, genetically variant FeLVs represented a minority population in standard FeLV stocks, this could explain why FeLV-infected fibroblasts score as FOCMA-negative. Alternatively, the FeLV variants encoding FOCMA could be replication-defective, requiring replication-competent FeLV genomes for efficient propagation in vitro; the failure to detect the products of these viruses in infected fibroblasts might then involve standard interference phenomena. A necessary correlate of this model is that although FeLV variants might be transmissible, either as rare replication-competent genomes or as defective pseudotypes formed with competent helpers, the FOCMA antigen encoded by them cannot be efficiently packaged into extracellular FeLV particles. Thus, absorbtion of anti-FOCMA sera with FeLV virions would fail to diminish anti-FOCMA reactivity. By contrast, FOCMA does appear in certain FeSV pseudotypes as a component of packaged gag-x products (14).

A precedent for such an interpretation derives from the Friend murine leukemia virus (F-MuLV) system. The replication-defective Friend spleen focus-forming virus (SFFV) encodes a glycoprotein (gp52) which is not packaged in virions but is expressed on the surfaces of SFFV-induced erythroleukemia cells (18). The glycoprotein is specified by recombined env sequences acquired by passage of F-MuLV in mice (19) and shares crossreactive antigenic determinants only with gp70 products encoded by helper-independent Friend mink cell focus-inducing (F-MCF) recombinants (18,20). By contrast, ecotropic F-MuLV gp70 fails to cross-react with SFFV gp52 (18,20).

Genetic mapping experiments performed with ST-FeSV show that FOCMA cannot be encoded by the portions of gag and pol

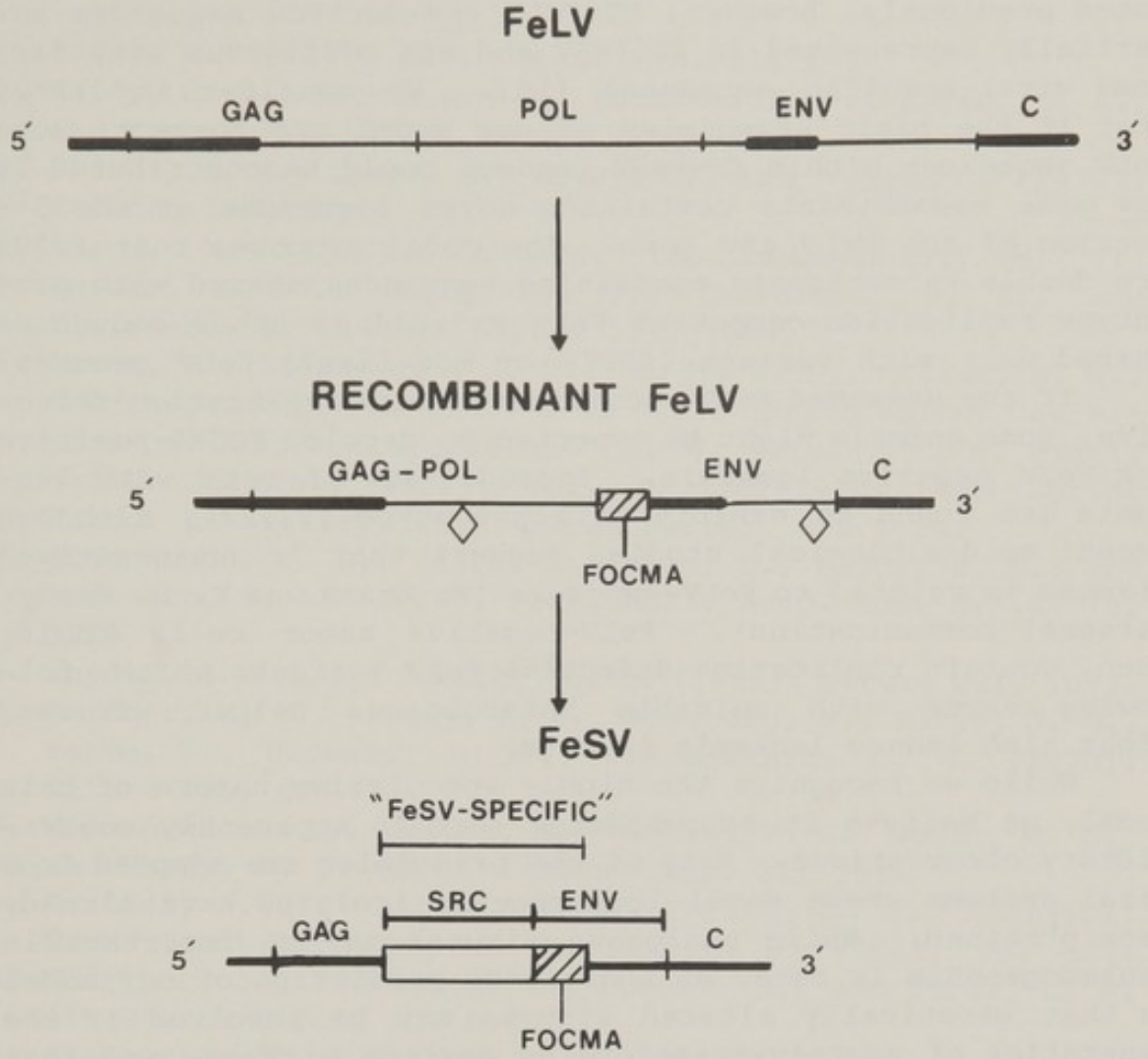

FIGURE 2. A model postulating that genetically variant FeLVs contribute to the formation of FeSVs. A critical aspect of the model is that sarcoma viral-specific sequences currently defined as src contain two distinct subsets of sequences, only one of which derives from cat cellular sarc genes. Sequences encoding FOCMA are presumed to be novel env sequences (cross-hatched rectangle) contained in variant FeLV strains and also mapping within the sarcoma-specific sequences of FeSV. The solid lines represent sequences shared in common between a prototype FeLV (subgroup B) and ST-FeSV (16). Diamonds indicate arbitrary sites of deletion which would occur if the putative intermediate were replication-defective.

sequences deleted in the formation of this virus (12,16). As noted previously, however, ST-FeLV env-derived sequences are partially represented in ST-FeSV and are contiguous with sarcoma viral-specific sequences (16). We consider it likely that if the basic principles of our model are correct, some FeLV sequences within ST-FeSV genomes could be contributed by env gene recombinants containing novel sequences at the 5'-portion of the FeLV env gene. The model proposes that FeSVs are double recombinants containing sequences shared with prototype replication-competent FeLV as well as other sequences shared only with variant (SFFV- or MCF-like?) FeLV genomes.

If the presumed FeLV recombinants were replication-defective, some animals might be expected to develop FOCMA-positive but FeLV negative leukemia. Indeed, ~30% of cats with leukemia are found to exhibit this phenotype (21,22), although recent epidemiological studies suggest that "virus-negative" disease is related to FeLV-exposure (M. Essex and W. D. Hardy; personal communication). FeLV-negative tumor cells could, then, contain replication-defective FeLV variants which, following rescue with suitable heterologous helper viruses, might also induce leukemia in cats.

While we recognize the highly speculative nature of this model, we believe it accommodates certain apparently contradictory observations. Many of the principles are adapted from viral systems where novel leukemogenic isolates have already been obtained. While analogous viruses may be important in leukemogenesis in cats, an additional prediction of our model is that genetically altered viruses may be involved in the generation of acutely-transforming sarcoma viruses, and that some sequences necessary for tumorigenesis may be common to both FeLV and FeSV. Many aspects of the model are directly testable, and further experiments should resolve relatively quickly whether the predictions are valid.

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TRANSFORMATION DEFECTIVE MUTANTS OF AEV AND MC29 AVIAN LEUKEMIA VIRUSES SYNTHESIZE SMALLER GAG-RELATED PROTEINS

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ABSTRACT Avian erythroblastosis virus (AEV) and myelocytomatosis virus 29 (MC29) are replication defective oncoviruses capable of causing acute leukemias as well as other neoplasms in the chicken. Hematopoietic cells transformed by AEV have properties of erythroblasts, those transformed by MC29 resemble macrophages. The uninfected target cells of these viruses are similar to the corresponding transformed cells in their phenotype of differentiation. AEV and MC29 viruses are also capable of transforming fibroblasts in vitro. Clones of fibroblasts transformed by AEV and MC29 can be distinguished by the pattern of transformation parameters they express. The different biological properties of the two virus strains have been attributed to the specific action of two new oncogenes designated as erb for AEV and mac for MC29.

Candidate transforming proteins of these viruses are p75 AEV and p110 MC29, consisting of a portion related to the gag-gene product and a non-gag related portion (different for each virus). A mutant of AEV was isolated which had lost the ability to transform erythroblasts but which retained the ability to transform fibroblasts. This mutant, td 359 AEV, was found to have a small deletion in the non-gag related portion of p75 AEV, suggesting that this molecule is responsible for erythroblast transformation. The p40 AEV protein synthesized in an in vitro translation system using RNA from td 359 AEV was unaltered in size.

Three mutants of MC29 were found which synthesize altered gag-related proteins of approximately 100k, 95k and 90k daltons, respectively. Preliminary experiments indicate that these mutants have a reduced potential to transform macrophages while having retained their ability to transform fibroblasts.

INTRODUCTION

Replication defective avian leukemia viruses (DLV's) induce a variety of different types of acute leukemias within a short period

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of latency. They are also capable of causing nonhematopoietic neoplasms such as sarcomas (for review see ref.1). The two virus strains which have been characterized most extensively, AEV and MC29, retain their multiple oncogenic potential even after clone purification (2,3,4). Both strains are capable of transforming hematopoietic cells as well as fibroblasts in vitro (for review see 1). This enabled us to study a series of questions which are the subject of the present communication.

In the first part we will review some evidence demonstrating that AEV and MC29 viruses specifically interact with different hematopoietic target cells and that fibroblasts transformed by these viruses can be distinguished with respect to the pattern of transformation parameters they express. In the second part, mutants of AEV and MC29 will be described which have lost their ability to transform hematopoietic cells but are still capable of transforming fibroblasts. The gag-gene related proteins synthesized by these mutants were preliminarily characterized with the aim to determine whether or not they play a role in cell transformation.

RESULTS

AEV and MC29 specifically interact with erythroid and myeloid hematopoietic cells. Infection of bone marrow cells with AEV or MC29 leads to the formation of rapidly proliferating colonies of transformed cells (for review see 1). These colonies can be isolated and cells grown into homogeneous mass cultures (Fig.1) which are amenable to characterization.

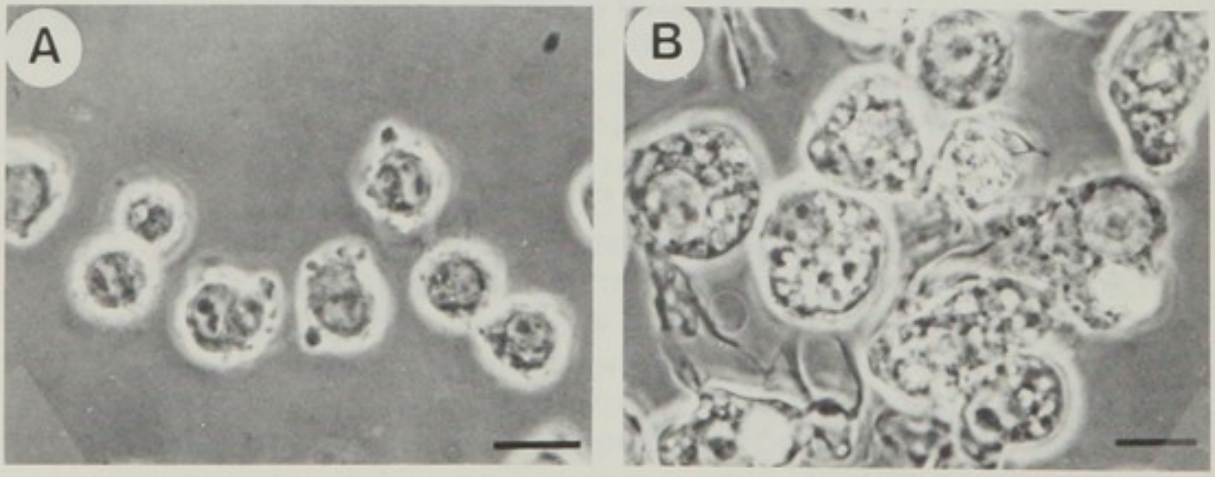

FIGURE 1. Clones of bone marrow cells transformed in vitro by AEV (A) and MC29 (B). Bar: 10 um.

As reviewed in Table 1 the phenotype of differentiation of cells transformed by AEV corresponds to that of erythroblasts, whereas cells transformed by MC29 resemble macrophages.

TABLE 1
DIFFERENTIATION PARAMETERS IN DLV-TRANSFORMED AND DLV-TARGET HEMATOPOIETIC CELLS[a]

Differentiation parameter	Cell	Infecting virus: AEV	Infecting virus: MC29
Substrate adherence	Tra	-	+, -
	Tar	-	+, -
Phagocytic capacity	Tra	-	+
	Tar	-	+, -
Cell surface antigens	Tra	Erb,(Ery)	Mac,(Myb)
	Tar	Erb,(Ery)	Myb,(Mac)

[a]Summary of data described in ref. 5,6,7 and Graf et al., in preparation. Tra=transformed cells; Tar=target cells. Erb=erythroblast-, Ery=erythrocyte-, Myb=myeloblast- and Mac=macrophage antigen. +=positive;-=negative; +,-=some positive, some negative. In parenthesis: weak expression.

This observation raised the question, whether or not DLV's transform cells already committed to a particular lineage of hematopoietic differentiaton. To answer this, normal chick bone marrow cells were subjected to various treatments in an attempt to separate and characterize the target cells before infection. These treatments included: separation of adherent from nonadherent cells; incubation with iron particles and removal of phagocytic cells with a magnet; and incubation with lineage specific cell surface directed antisera plus complement. A compilation of the results obtained, in which properties of the target cells are compared with those of the transformed cells, is shown in Table 1. As can be seen, target cells for AEV have properties of immature erythroid cells whereas target cells for MC29 have properties of immature macrophages. Interestingly, target cells for MC29 seem to be somewhat more immature than the corresponding transformed cells as judged by their expression of myeloblast-specific antigen and relatively low phagocytic capacity.

Fibroblasts transformed by AEV and MC29 exhibit distinguishable phenotypes of transformation. Knowing that AEV and MC29 specifically interact with different hematopoietic target cells it was surprising to find that both were also capable of transforming fibroblasts. In the course of these studies we found that MC29 transforms two types of cells present in chicken embryo "fibroblast" cultures that is, contaminating macrophages as well as fibroblastic cells (4). It was therefore important to study fibroblast transfor-

mation by these viruses using cloned cell strains (8). Clones of chicken fibroblasts transformed by AEV and by MC29 were not only distinguishable in their morphology (Fig.2) but also displayed a widely different pattern in their expression of seven other transformation parameters.

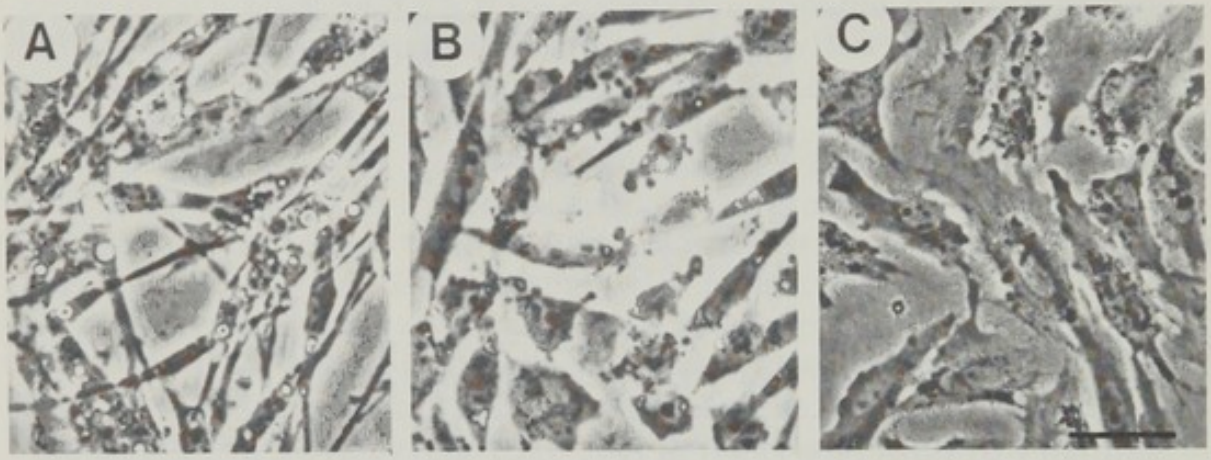

FIGURE 2. Chicken fibroblast clone (1/77) transformed with AEV (A) and MC29 (B). C, uninfected control. Bar: 50 µm.

These data, summarized in Table 2, suggest that AEV and MC29 employ different pathways for cell transformation.

TABLE 2
TRANSFORMATION PARAMETERS OF CHICKEN FIBROBLASTS TRANSFORMED BY AEV AND MC29[a]

Transformation parameter	Fibroblasts transformed by AEV	MC29
Disarrangement of actin cables	+	+
Altered fibronectin network	+	(+)
Increased hexose uptake rate	+	-
Increased plasminogen activator	+	-
Increased growth rate	-	+
Anchorage independence	+	+
Sarcomagenicity	+	-

[a]Summary of data described elsewhere (8).

To study the important question whether or not AEV (and MC29) transform hematopoietic and nonhematopoietic target cells using different gene products, efforts were made to isolate mutants capable of transforming one type of target cells but unable to transform those of the other type. As will be described in the following, we have been able to isolate a set of mutants of both AEV and MC29 defective for hematopoietic cell transformation.

Mutant of AEV defective for erythroblast transformation which is still capable of fibroblast transformation. Fibroblast cultures were infected with 5-azacytidine-treated AEV under the conditions of the colony assay (2), and individual transformed clones tested for the type of virus produced after superinfection with helper virus. From about 500 clones screened one could be isolated which produced a virus (td359 AEV) with restricted target cell specificity (9). Biological properties of this mutant are listed in Table 3.

TABLE 3
TRANSFORMING SPECIFICITY OF td 359 AEV

Virus	Transformation in vitro[a]		Transformation in vivo[b]	
	Bone marrow	Fibroblasts	Erythro-blastosis	Sarcomas
wt AEV	7.5×10^2	2.5×10^4	7/7	7/7
td 359 AEV	0	5.8×10^4	0/6	6/6

[a] CFU/ml; [b] In these experiments, fibroblasts ($1\text{-}10 \times 10^6$) transformed in vitro with wt AEV (RAV-1) and td 359 AEV (RAV-1) were injected i.m. into 5 to 7 day-old chicks. Values shown: incidence of positive over total number of animals.

As can be seen, td359 AEV, although unable to induce an erythroblastosis, it is still capable of inducing sarcomas (see Fig.3). These properties remained unchanged even after clone-purification of the mutant by isolating transformed nonproducer cells and rescue of infectious virus with the use of various standard helper viruses (9).

The gag-related protein of td359 AEV carries a small deletion. An analysis of AEV-transformed cells infected in the absence of helper viruses using antisera against virus structural antigens revealed the presence of a 75k protein (10,11,12,14,15). This protein consists of part of the gag-gene product pr76 and a unique portion (11,14). That p75 is coded for by the virus has been shown by in vitro translation experiments where besides p75 also a 40k protein is synthesized (13,15,16).

Several lines of indirect evidence have implicated p75 AEV in the process of cell transformation. Studies with a temperature-sensitive mutant for transformation indicate that a viral gene product must be continuously synthesized to maintain the transformed phenotype of the cell (18). In addition, heteroduplex mapping and oligonucleotide fingerprinting analyses indicate that p75 AEV is derived in its majority from that portion of the genome in which the non gag-related, presumably transformation specific sequences are located (19,20).

FIGURE 3. Sarcoma induced by td 359 AEV in the left wing of a chick 70 days after infection.

It was therefore interesting to determine whether or not p75 AEV or p40 AEV of td359 AEV were altered. For this purpose, several clones of fibroblasts independently transformed by the mutant were labeled with ^{35}S-methionine, immunoprecipitated with a chicken anti-p75 serum as well as a rabbit anti-virus serum and subjected to gelelectrophoresis and autoradiography. The results in Fig.4 show that td359 AEV synthesizes in all clones a protein which migrates slightly faster than p75 of wild type AEV. That the change seen was due to a deletion is indicated by results obtained after tryptic peptide analysis of this protein, designated Δ p75. The results in Fig.5 show that out of 53 lysine-arginine labeled tryptic peptides which were resolved in p75, 3 of the peptides labeled less intensively disappeared in Δ p75 whereas one additional peptide appeared. This result was reproducible in three independent experiments, two of which were performed with a different td 359 AEV-transformed fibroblast clone. Since the gag-related tryptic peptides of p75 AEV labeled with lysine-arginine have not yet unambiguosly identified, the above results did not allow us to locate the deletion in the gag-or in the non-gag portion of p75 AEV. We have therefore recurred to an alternative approach to map the deletion. This approach was based on the observation of Vogt et al. (12) that p75 AEV can be cleaved with the p15 protease of avian retroviruses into two discrete fragments of approximately 45k and 30k molecular weight, designated in the following as F45 and F30.

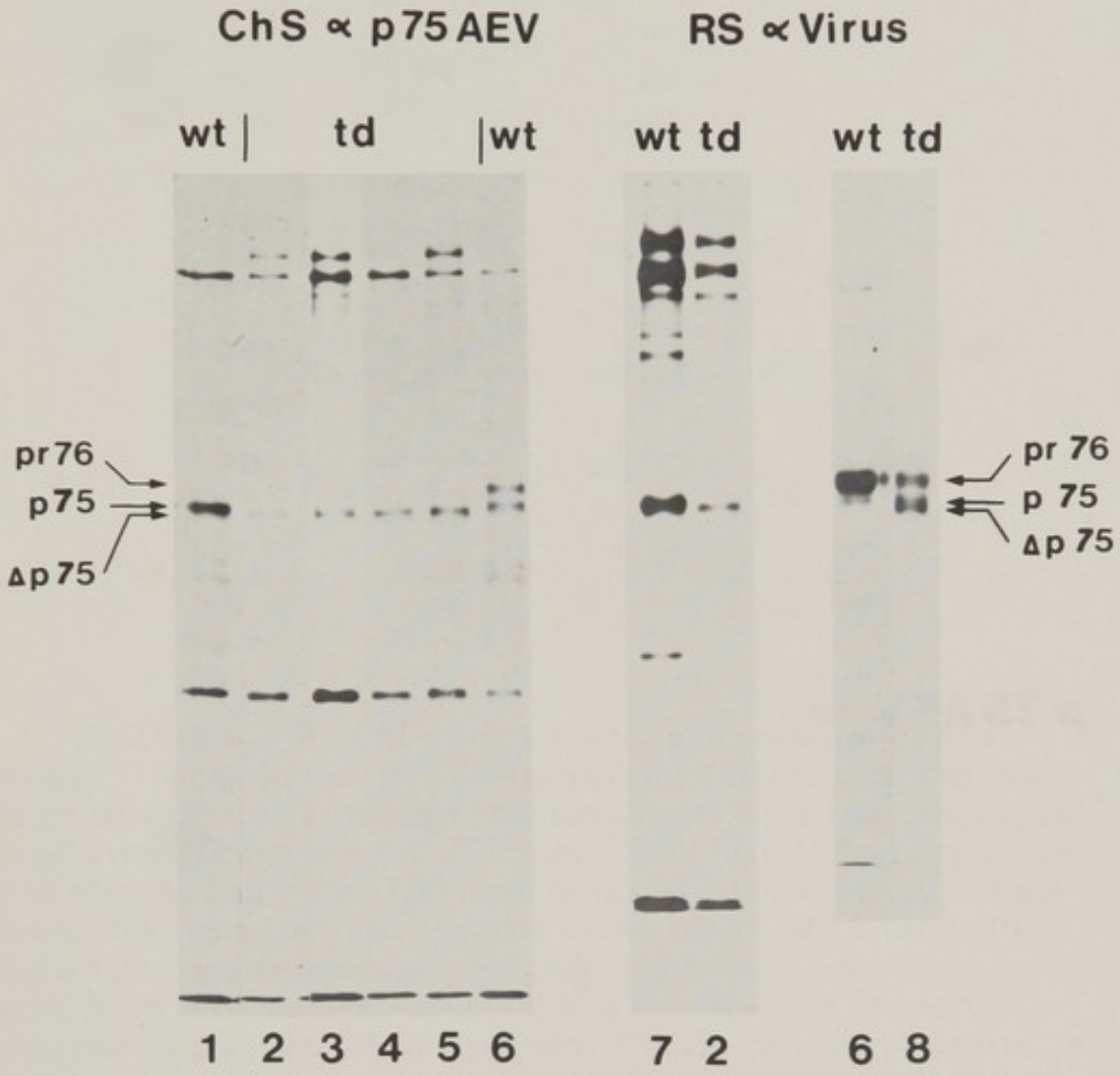

FIGURE 4. Expression of p75 and Δ p75 in chicken cells transformed with wtAEV and td359 AEV. Cells (4 clones of td359 AEV transformed fibroblasts, numbers 2,3,5,8; fibroblasts from a td359 AEV-induced sarcoma, 4; wtAEV-erythroblasts, 1,6; and wtAEV-fibroblasts, 7) were labeled with methionine and processed for immunoprecipitation analysis as described earlier (11). Samples were run on a 6-15% PAGE gradient. The sera used were a rabbit serum against the structural proteins of AMV and B77 viruses (RS∝Virus) and a chicken serum obtained from animals surviving a ts34 AEV-induced erythroleukemia (18) (ChS∝ p75 AEV), which reacted mainly with non-gag determinants of p75 AEV but also with the gag-gene product pr76. The rabbit serum was immunoprecipitated with protein A containing Staphylococcus aureus; the chicken serum with affinity-purified goat anti chicken IgG-antibody (Beug et al., in preparation).

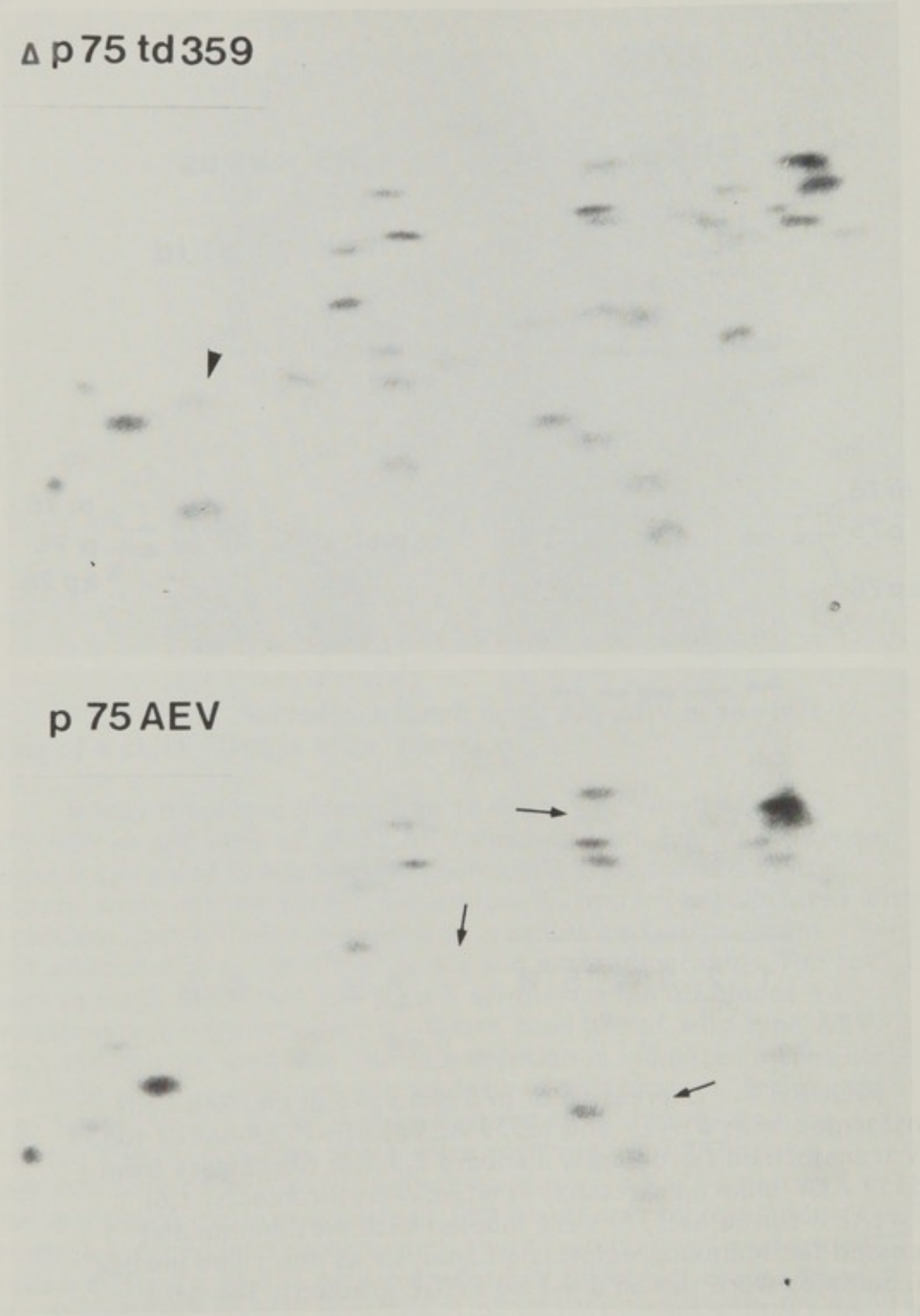

FIGURE 5. Comparison by tryptic peptide mapping of the gag-related proteins induced by td359 AEV and wtAEV. Cells were labeled with lysine and arginine and immunoprecipitated and processed according to procedures described earlier (17). Black arrows: Peptides missing in Δ p75; arrowhead: new peptide in Δ p75.

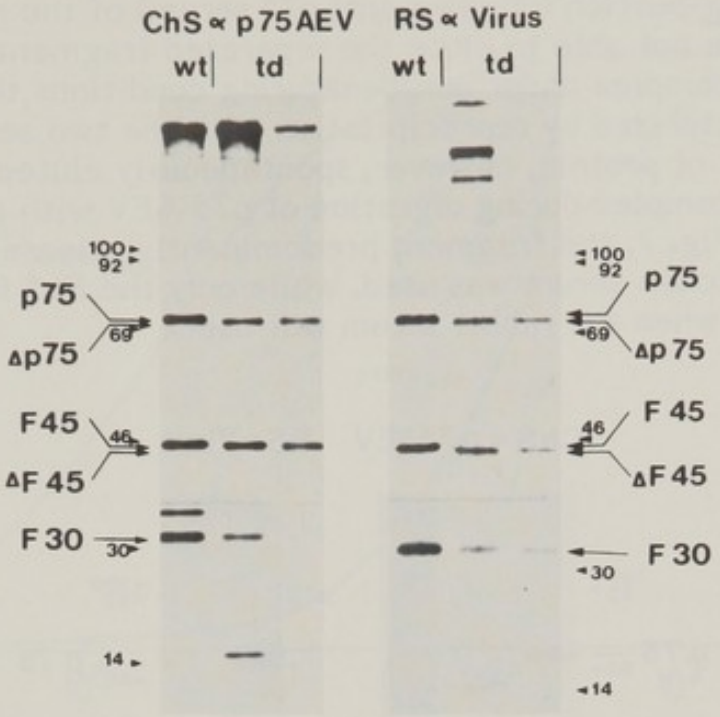

FIGURE 6. Partial digestion with p15 protease of p75 and Δ p75. Nonproducer wtAEV-transformed fibroblasts and two clones of td359 AEV transformed fibroblasts were immunoprecipitated with chicken and rabbit antisera (see Fig.4). Anti gag-activity of the chicken serum had been removed completely by absorption with virus structural proteins in the serum batch used. The washed immune complexes were digested with p15 protease prepared from AMV as described (12) and the cleavage products analyzed on a 6-15% PAGE gradient. The figure is a composite of the same gel exposed for 2 days (p75 and F45) and 6 days (F30) to compensate for the large differences in radioactivity of the F45 and F30 bands. Positions of molecular weight standards are indicated in small numerals.

The results obtained after partial digestion with p15 of the gag-related proteins of two td359 AEV-transformed fibroblast clones and a wtAEV-transformed fibroblast clone are shown in Fig. 6. As can be seen, the mobility of ΔF45 from td359 AEV was slightly higher in both clones than that of F45 from wtAEV, whereas the mobility of F30 was unchanged in both mutant clones. Similar results were obtained regardless of whether the chicken or the rabbit serum was used. This shows that the deletion in Δ p75 is located in the region of the 45k fragment.

The F45 fragment of p75 corresponds to the non-gag portion of the molecule. To map the two p15 cleavage fragments of p75 AEV, we took advantage of our two types of sera recognizing

determinants of the gag-portion (rabbit anti virus serum) and of the non gag-portion (chicken anti p75 serum) of the molecule. Since we were not able to elute the separated fragments from the immune complex under non-denaturing conditions, they could not be characterized by reprecipitation with the two sera. A small amount of protein, however, spontaneously eluted from the immune complex during digestion of p75 AEV with p15 protease. As shown in Fig. 7, the fragment predominantly released was F30 if the chicken serum was used, while only the F45 fragment was released when the rabbit serum was used.

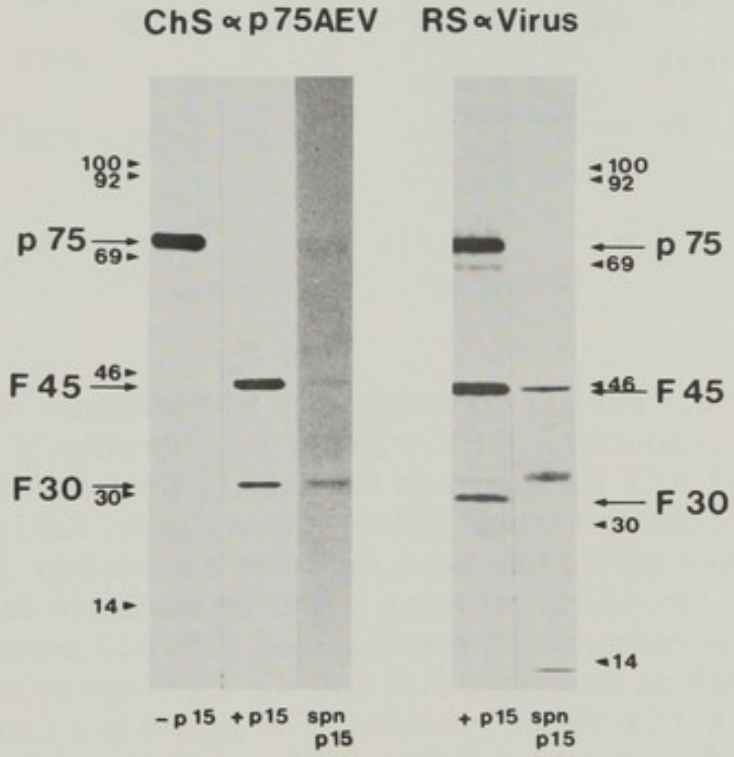

FIGURE 7. Characterization of p15-generated fragments released from the immune complex. wtAEV-transformed nonproducer-erythroblasts were used as a source of p75. After incubation of the immune complex with p15, the reaction mixture was spun for 10 min at 10,000 xg and the proteins released into the supernatant analyzed as described in the legend of Fig. 6. Photographs represent a composite of 2 day (p15 digestion mixtures) and 7 day (p15 supernatants) exposures of the same gel.

These results are compatible with the interpretation that F45 corresponds to the non-gag and F30 to the gag-portion of p75 AEV, and that the deletion in Δp75 of td359 AEV is located in the non-gag portion. This conclusion was confirmed by tryptic peptide mapping experiments in that F30 contains predominantly gag specific peptides whereas F45 contains only non-gag peptides (data not shown).

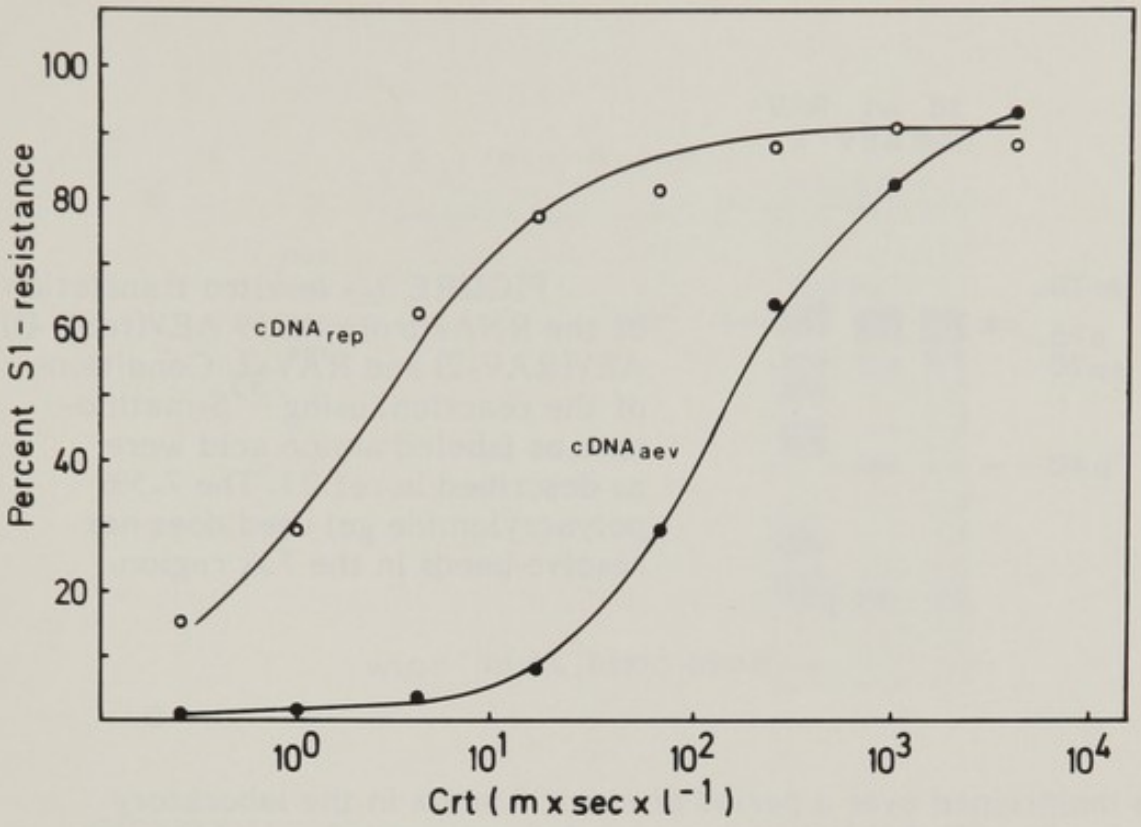

FIGURE 8. Hybridization of ^{3}H cDNA aev (complementary to AEV specific sequences) and ^{32}p cDNA rep (complementary to virus structural gene sequences) (21,22) with the RNA of td359 AEV. The reaction with cDNA rep is due to helper virus present in excess in the stock of td359 AEV used.

The deletion in the genome of td359 does not extend into the region coding for p40. Evidence available so far suggests that p40 AEV is coded for by the 5' half of the AEV-specific sequences of the virus (13,14,19). It was therefore interesting to determine whether or not the deletion in td359 AEV would reach from the p75 region of the genome into the region coding for p40. Two lines of evidence indicate that this is not the case. First, almost 100% of cDNA aev (21) reassociates with the RNA of td359 AEV (Fig.8). Similarly, as shown by Northern blotting, the size of the mutant RNA (about 30S) was indistinguishable from that of wtAEV (data not shown). Second, in vitro translation experiments using td359 AEV RNA showed that the mutant directs the synthesis of a p40 protein indistinguishable in electrophoretic mobility from that of parental wtAEV (Fig.9).

Mutants of MC29 altered in the gag-related p110 protein. MC29 synthesizes a 110k protein which, like p75 AEV consists of a gag-related portion and a non-gag portion (12,14,24). This protein was first detected in quail fibroblast clones transformed by MC29 (24). A line derived from one of these clones, MC29-Q10

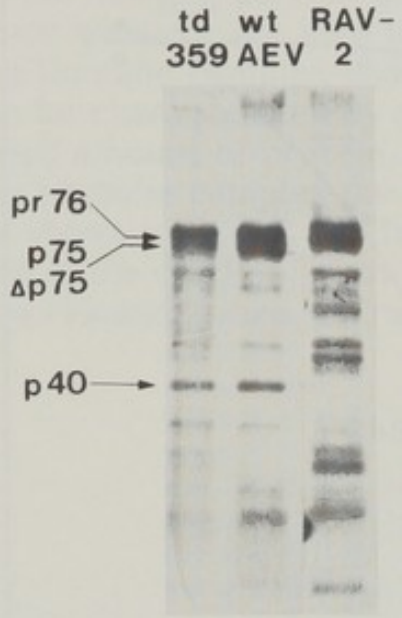

FIGURE 9. In vitro translation of the RNAs from td359 AEV(tdSR-D), AEV(RAV-2) and RAV-2. Conditions of the reaction, using ^{35}S-methionine as labeled amino acid were as described in ref.23. The 7.5% polyacrylamide gel used does not resolve bands in the 75k region.

was maintained over a period of about 3 years in the laboratory of one of us (M.J.H.). With increasing passage number, additional proteins with molecular weights smaller than 110k appeared in cells of this line. Attempts were therefore made to obtain variants which would stably induce altered gag-related proteins. This was done by screening with a rabbit anti virus serum a series of transformed quail fibroblast clones derived after infection at low m.o.i. with MC29 virus from the Q10 line. We were indeed able to isolate three such mutants which synthesize gag-related proteins of approximately 100k, 95k and 90k, respectively (Fig.10). That these proteins are homologous to p110 MC29 was demonstrated by tryptic peptide analyses (data not shown). Virus rescued from nonproducer fibroblasts transformed by these mutants still induced the corresponding altered gag-related protein upon reinfection of fresh quail cells. Preliminary experiments done to characterize the biological properties of the MC29 mutants indicate that in all cases they have a reduced ability to transform hematopoietic cells (macrophages) (Table 4).

DISCUSSION

The data summarized in the first part of this paper show that AEV and MC29 exhibit a remarkable specificity of transformation in both hematopoietic and fibroblastic cells. AEV recognizes an erythroid bone marrow target cell with the outcome of transformed cells resembling erythroblasts. Likewise, MC29 and other members of the MC29 group (1) hit immature myeloid target cells giving rise to macrophage-like transformed cells. In fibroblasts, AEV and MC29 induce different, specific patterns of transformation parameters,

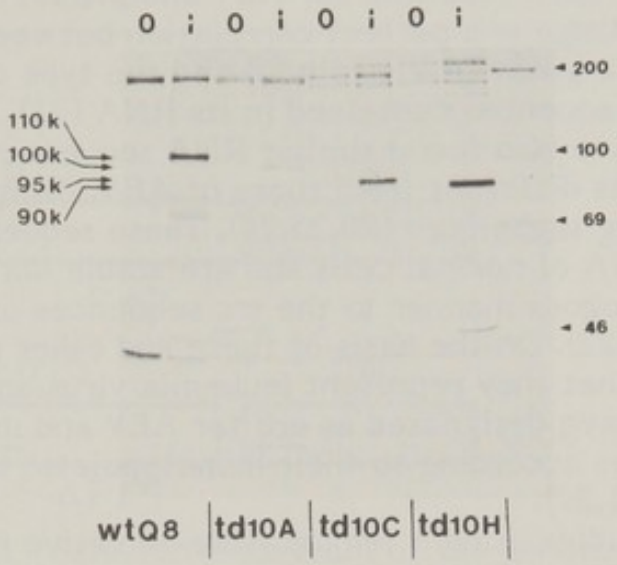

FIGURE 10. Gag-related proteins synthesized in Q8 nonproducer cells transformed by wild type MC29 (24), and three mutants of MC29 derived from the MC29 transformed Q10 line. Cells were labeled as described (11) and immunoprecipitated with rabbit anti virus serum (i) or with non-immune serum (o). Standard proteins were run as molecular weight markers in the right-hand lane.

TABLE 4
TRANSFORMING SPECIFICTY OF MC29 MUTANTS WITH ALTERED GAG-RELATED PROTEINS

Virus[a]	Transforming activity (CFU/ml) in Bone marrow	Fibroblasts
wt MC29	5×10^3	2×10^4
td 10A MC29	0	3.4×10^4
td 10C MC29	12	6×10^3
td 10H MC29	8	2.5×10^4

[a] td B77 was used as helper virus for the mutants. wt MC29 contained RAV-2 as helper virus and was derived from a transformed macrophage cell line (5).

even if cloned fibroblast strains are used (8).

Results obtained by screening seven independently isolated DLV-strains with cDNA specific for AEV and MC29, respectively, have shown that there is a perfect correlation between the biological properties of a given DLV strain (5) and the type of transformation-specific sequences contained in its RNA (21). Duesberg and colleagues have also found similar RNA sequences of the MC29-type viruses different from those of AEV, using oligonucleotide fingerprinting techniques (20,25,26). These sequences are present in the DNA of normal cells and are stable during evolution (21,29) in an analogous manner to the src sequences of avian sarcoma viruses (22). On the basis of these and other results, we have postulated that they represent leukemia virus-specific oncogenes which we have designated as erb for AEV and mac for MC29-type viruses according to their hematopoietic target cell specificity (5,7,21,27).

Previous studies using a temperature-sensitive mutant of AEV indicate that a virus gene product has to be continuously synthesized for the maintenance of erythroblast and fibroblast transformation (18,28). Several lines of indirect evidence have suggested a function of the gag-related proteins of DLV's in cell transformation (for review, see 7). The perhaps strongest argument comes from the finding that the non gag-portions of these proteins are similar in DLV-strains with the same biological specificity and different in strains with different specificity (17). The results obtained with td359 AEV are the first direct evidence in favor of this notion. The diagram in Fig.11 summarizes our findings. We conclude that the non gag-portion of p75 AEV is essential for erythroblast transformation and that part or all of it is not required for fibroblast transformation. As indicated in the diagram, only about 2,000 of the 3,500 AEV specific nucleotides are accounted for by the "erb" portion of p75AEV. Whether or not the remaining 1,500 nucleotides, presumably coding for p40 AEV, are also required for erythroblast transformation, for fibroblast transformation or for both is still unclear. At present, p40 AEV has only been detected by in vitro translation experiments and there is no evidence to suggest a functional role of this protein whatsoever. Chickens which survive a ts34 AEV-induced erythroleukemia develop antibodies against the erb portion of p75 AEV but not against p40 AEV (Beug et al., in preparation).

The size of the deletion in td359 AEV has not yet been accurately determined. As judged from the difference in mobility on SDS-gels between pr76, p75 and Δp75 and from the loss of about three out of 53 lysine-arginine labeled tryptic peptides it is probably only a hundred nucleotides or so long. This might also explain why we have not been able to detect a difference between the complexities of the AEV specific RNA sequences of wtAEV and td359 AEV.

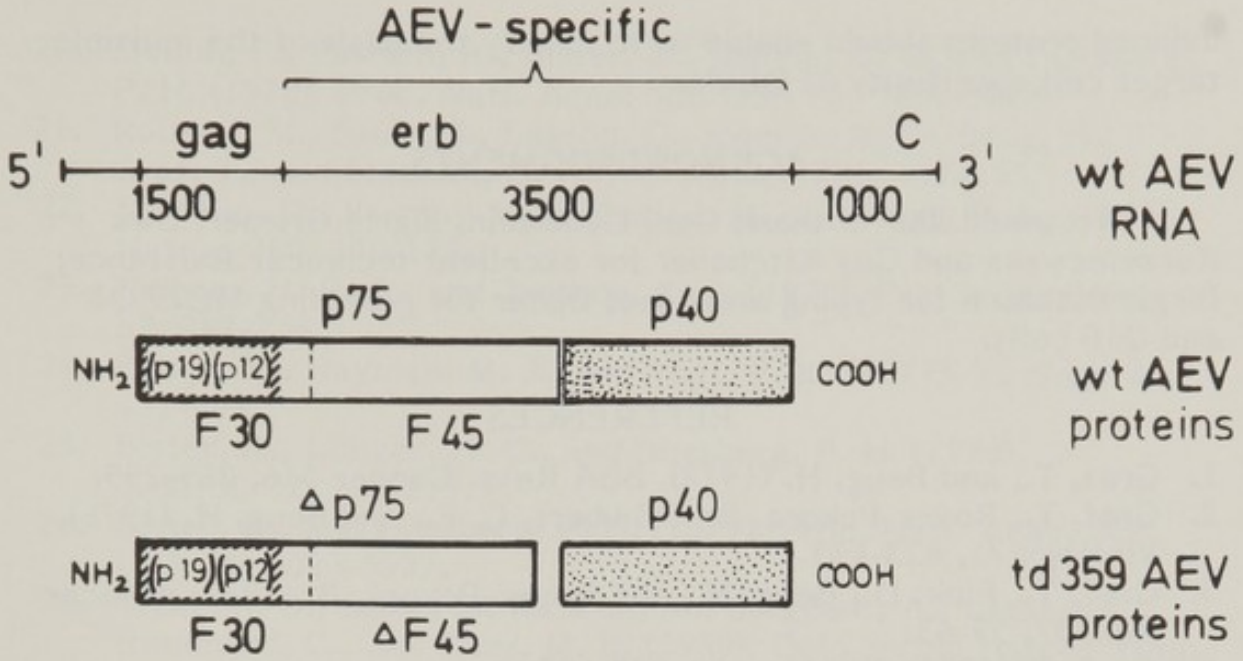

FIGURE 11. Schematic diagram of the genome structure and coding capacity of wtAEV and td359 AEV. The approximate length of the different regions on the genome (given as number of nucleotides) was compiled from the literature (14,19,21).

Our results with td359 AEV, although suggesting that separate genes exist for erythroblast and fibroblast transformation, do not rule out the possibility that p75 AEV consists of a domain necessary for erythroblast transformation and a subdomain sufficient for fibroblast transformation. The observation that ts34 AEV is not only temperature-sensitive for erythroblast, but also for fibroblast transformation (18,28) supports this idea.

It is interesting that td359 AEV has biological properties essentially indistinguishable from avian sarcoma viruses in that it is capable of inducing sarcomas but no leukemias. This shows that different viral oncogenes can induce very similar types of neoplasms.

The observed correlation between the impaired macrophage transforming ability of the td mutants of MC29 and their synthesis of gag-related proteins smaller than p110 MC29, suggest that in analogy to the findings with p75 AEV this protein is responsible for hematopoietic cell transformation by MC29. However, biological characterization of these mutants is still incomplete. Thus, it remains to be shown that MC29 isolates with an unaltered p110 protein derived from the original Q10 line are capable of inducing a transformation of macrophages in addition to fibroblasts. Preliminary characterization of the mutant 100k, 95k and 90k proteins indicate that they have lost some of the non gag-peptides, attributed to the mac sequences of MC29 (17) while they have gained others (unpublished data).

The kind of approach described in this communication, that is, correlating altered biological functions with changes in virus-

induced proteins should enable us to clarify the basis of the multiple target cell specificity of DLV's.

ACKNOWLEDGMENTS

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ISOLATION AND CHARACTERIZATION OF PHENOTYPIC REVERTANTS FROM MOLONEY MURINE SARCOMA VIRUS-TRANSFORMED CELLS [1]

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ABSTRACT Several characteristics of NRK cells transformed with Moloney murine sarcoma virus have been studied and compared to those of independent clonal isolates which have reverted to a normal phenotype.

Molecular level analysis of cell DNA by means of endonuclease restriction, electrophoresis, blotting, and hybridization to radioactive DNA containing sequences specific for the transforming gene of MSV, revealed that revertants maintain a complete copy of the viral genome. Similar studies of isolated cytoplasmic poly(A)-containing RNA indicate that revertants do not contain detectable quantities of mRNA that can hybridize with the *src*-specific DNA. Revertants, when stained with an antiserum cross-reacting with MSV-specific proteins, stain as does the non-transformed control. These studies support the conclusion that reversion is related to a block in viral gene transcription. The relevance of this defect along with others for non-transformed revertants is discussed in terms of cellular and viral factors contributing to the neoplastic state.

INTRODUCTION

RNA tumor viruses have been used extensively in an effort to decipher those factors which result in the establishment and maintenance of a transformed cellular phenotype. During the course of virus infection of animal cells the viral RNA replicates into a double-stranded DNA molecule by the action of a virally encoded RNA-dependent DNA polymerase known as reverse transcriptase (1). This DNA molecule is stably integrated into the host cell DNA, is replicated and transcribed

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into virus-specific genomic messenger RNA by cellular enzymes. Expression of the viral mRNAs is required for the induction of the transformed phenotype (2).

To understand the role of viral gene expression in malignant transformation we have generated variants from virally-transformed cells that have reverted to a non-transformed phenotype. The analysis of these revertants and their transformed precursors will determine which cellular and/or viral parameters are responsible for the change in cell morphology and growth pattern. Such a system can provide the means of dissecting individual cellular and viral factors involved in cellular transformation and the interaction between the two.

The present study describes the isolation and initial characterization of phenotypic revertants from Moloney murine sarcoma virus (Mo-MSV)-transformed cells.

METHODS

All cells were maintained in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells grown in spinner culture were maintained in DME buffered with HEPES. All cells were incubated at 37°C, 5% CO_2 unless stated otherwise.

Clone 2TS. Clone 2 was obtained by infecting normal rat kidney fibroblasts (NRK) with Mo-MSV clone 124 (3). Clone 2 cells, at a dosage of 10^6 cells, were injected subcutaneously into both sides of nude mice. Resulting tumors were explanted by grinding through mesh and plated into 100 mm tissue culture dishes (Falcon Co.). One resulting clone, 2TS, was found to grow well in spinner culture.

Selection of 60^+H^-. 2TS cells were grown as a spinner culture to a final density of 10^5 cells/ml in a volume of 1.5 liters. Cells were pelleted and resuspended as a spinner culture supplemented with 1 mM oubain (Sigma Co.). The concentration of oubain was raised to 2 mM after one week. After a total of four weeks of selection in oubain survivors were plated into T25 flasks (Falcon Co.) with media containing 2 mM oubain. After cells reached confluence they were fed medium containing thioguanine (30 µg/ml) for three weeks. Surviving clones were picked, one of which, 60^+H^-, grew as a spinner culture. Cells were confirmed to be HPRT deficient by their nonviability in medium containing 15 µg/ml hypoxanthine, 0.2 µg/ml aminopterin and 5 µg/ml thymidine (HAT medium) (Sigma Co.).

Selection of Non-transformed Revertants. The phenotypic

revertants that were isolated were done so with the initial intent of obtaining cells temperature-sensitive for transformation (4). $60^{+}H^{-}$ cells were propagated as a spinner culture to a final density of 10^5 cells/ml in a volume of 1.7 liters. Cell medium was supplemented with ethyl methane sulfonate (1 mg/ml, Sigma Co.) for 2 hours. Cells were pelleted and washed in Hank's Balanced Salt Soltuion (GIBCO) and returned to spinner culture and maintained at a density of 1.2×10^5 cells/ml for 2 days at 34°C. After shifting cells to 40°C for 4 hours, 5 mCi of ^{3}H-thymidine (54 Ci/mmol, New England Nuclear) were added to the medium. Cells were pelleted and washed three times after 50% of the cells were killed (60 hours). They were then plated into 100 mm tissue culture dishes and incubated for 12 hours after which they were placed at 34°C. Surviving cells were grown to confluence and then shifted to 40°C and subjected to 25 µg/ml FUdR plus 125 µg/ml uridine (Sigma Co.) for 48 hours. Plates were then washed extensively with fresh medium and placed at 34°C. Colonies appeared within three weeks and were isolated with cloning rings, grown up, and examined for morphology and growth in methyl cellulose.

Cellular DNA and RNA. Cellular DNA was obtained by pronase digestion of a lysate of isolated cell nuclei. Cells were lysed with a buffer containing 0.25 M sucrose, 10 mM magnesium acetate, 250 mM KCl, 10 mM Tris HCl, pH 7.4, 100 µg/ml heparin, 100 µg/ml cyclohexamide, and 0.5% Triton X-100. The nuclei were pelleted at 10,000 rpm and the supernatant saved to obtain cytoplasmic RNA (see below). Nuclei were resuspended in 25 ml of a solution which contained 0.1 M NaCl, 10 mM Tris HCl, pH 7.4, 50 mM EDTA, 1% sodium dodecyl sulfate (SDS) and 1.5 mg/ml pronase (Calbiochem), and incubated at 37°C for 4 hours. The DNA was extracted with chloroform and phenol saturated with 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris HCl, pH 7.4, followed by extensive dialysis against 10 mM Tris HCl, pH 7.4. Cytoplasmic RNA in the supernatant from the lysed cell centrifugation was extracted with chloroform and saturated phenol and then precipitated in ethanol. Poly(A)-containing RNA was selected by oligo (dT) column chromatography followed by ethanol precipitation in the presence of carrier yeast tRNA (10 µg/ml).

Analysis of cellular DNA involved enzyme restriction, gel electrophoresis, blotting and hybridization, the procedures for which have been described (5). The restriction endonuclease *Sac* I was chosen since it cleaves at both ends of an integrated MSV genome to yield a nearly full size viral genome (6). Hybridization was achieved using a P^{32}-labelled DNA probe which represents a cloned DNA segment of the *src*-specific region of the MSV genome (7; Dina, manuscript in preparation).

RNA samples were similarly analyzed by gel electrophor-

esis, blotting and hybridization to the same *src*-specific DNA probe. After electrophoresis RNA gels were washed in 50 mM NaOH for 45 minutes and neutralized in 1 M sodium acetate, pH 4.5. The RNA was then transferred to activated diazobenzyloxymethyl paper (Schleicher and Schuell) as described by Alwine et al. (8). For each DNA-DNA and DNA-RNA hybridization experiment, 2.5 x 10^6 cpm of P^{32}-labelled DNA probe (10^8 cpm/µg) was used.

MSV Antibody Stain. Cells to be tested were grown on glass cover slips to semi-confluence in tissue culture dishes. Cells were fixed for 10 minutes with a solution of one part methanol and two parts acetone and then air dried. Fixed cells were stained for ½ hour at 37°C, washed and the cover slips blotted dry. Staining was achieved with a fluorescein isothiocyanate-conjugated goat anti-MuLV antiserum cross reactive to MSV-specific proteins. Cover slips were then mounted onto microscope slides and examined under ultraviolet light.

RESULTS

Examination of Transformed Phenotype. Clone 2TS, an NRK-derived Mo-MSV-transformed cell line, was used for a series of selections in oubain and HAT medium. One surviving clone, termed 60^+H^- (O^+ for oubain resistance, H^- for loss of HPRT enzyme activity), retained the ability to be propagated in spinner culture. These cells display morphological characteristics typical of Mo-MSV transformed cells (Fig. 1a), are spherical in shape and light refractile. In contrast, the NRK progenitor cells which have never been exposed to virus are flat, non-refractile and contact inhibited (Fig. 1b). Both 2TS and 60^+H^- cells form colonies in methyl cellulose. These cell lines have been shown to be transformed non-producers.

Suicide selection in spinner cultures with ^3H-thymidine followed by similar selection in tissue culture dishes with FUdR resulted in the selection of survivor cells, several of which reveal characteristics of the NRK parental cells. Cloned survivor cells grow as flat, non-refractile cells which are subject to contact inhibition (Fig. 1c). Although not all clones have been tested, those that have, namely C3, C7, C9 and C10, do not grow in methyl cellulose at either 34°C or 40°C. Some of the revertant clones display a leakiness of the non-transformed phenotype. Cells with a transformed morphology often appear and may overgrow the cells of a nontransformed phenotype. For these cells, occasional recloning is required to maintain relatively homogenous cell populations.

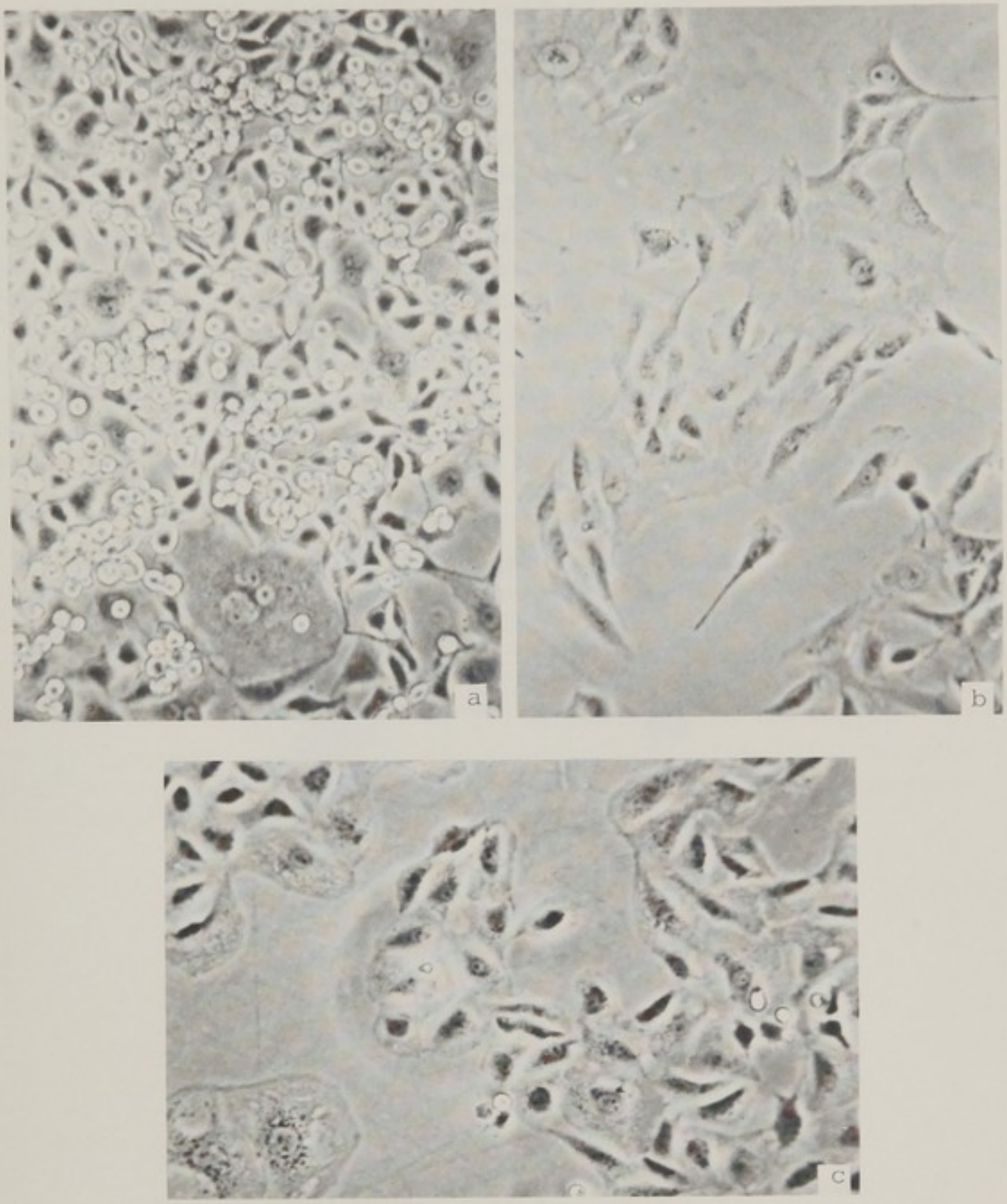

FIGURE 1. Phase contrast micrographs of transformed and reverted NRK cells grown on tissue culture dishes. a, $60^{+}H^{-}$; b, NRK (normal); c, C10 (revertant).

Characterization of cellular DNA. To compare the genomes of non-transformed revertant cells to those of the transformed $60^{+}H^{-}$ cells and the uninfected NRK cells, DNA from each cell type was isolated and electrophoresed followed by blotting and

hybridization to sarcoma-specific DNA. The resulting X-ray autoradiograph is shown in Fig. 2. It can be seen that the revertant cells (lanes c-e) contain two very closely juxtaposed *Sac* I bands that also appear in the $60^{+}H^{-}$ cells, whereas only one such band appears in NRK DNA (lanes b,f). All revertant, $60^{+}H^{-}$ cells and NRK cells have in common a single band corresponding to endogenous MSV-like sequences (9,10) whereas the second band migrating slightly slower in the $60^{+}H^{-}$ and revertant lanes corresponds to integrated viral sequences. Additional controls are provided with 3T3 DNA (lane a) and DNA of MSV (lane g), a mouse MSV-infected fibroblast cell line derived from thymus-bone mouse cells chronically infected with Mo-MSV clone 124 (Ball et al., 1973). Although lane g is overexposed it is possible to detect an additional band pres-

FIGURE 2. Hybridization of *Sac* I digested DNA blotted to nitrocellulose paper following gel electrophoresis in 0.7% agarose for 24 hr at 60V. DNA was blotted, baked, and then hybridized to 2.5×10^{6} cpm P^{32}-labelled *src*-specific DNA for 48 hr. Lane a, 3T3 (NIH); lane b, NRK; lane c, C10; lane d, C9; lane e, C8; lane f, $60^{+}H^{-}$; lane g, MSV. Restriction fragments are visualized by X-ray autoradiography.

ent in MSV-infected mouse cells which is not present in uninfected cells. This band migrates slightly faster than the one common to both cell types and represents an integrated MSV viral genome which resulted from infection of the mouse cells.

The uninfected NRK DNA lane (lane b) contains a fast migrating band also present in both uninfected and infected mouse DNA (lanes a and g), but absent in the $60^{+}H^{-}$ and revertant cell DNA. Studies are presently under way to determine if this band, perhaps representing an additional endogenous MSV-like sequence, is present in other NRK derived transformed cells.

The organization of exogenous viral sequences for the phenotypic revertants and parental $60^{+}H^{-}$ cells is not identical. The presence of anomalously migrating bands in C10 and C8 DNA (lanes c and e) which do not appear in the $60^{+}H^{-}$ or NRK DNA suggests that rearrangements of these sequences have occurred.

Cytoplasmic mRNA Hybridization. To determine if the reversion phenomenon is causally related to a block in virus gene expression, poly(A)-containing cytoplasmic RNA was isolated from revertant cells by oligo (dT) chromatography. The RNA was then electrophoresed, blotted and hybridized to *src*-specific DNA. In this manner it is possible to determine whether or not mRNA coding for transforming protein(s) is produced in revertant cells. Isolated viral MSV RNA was co-electrophoresed as a positive control.

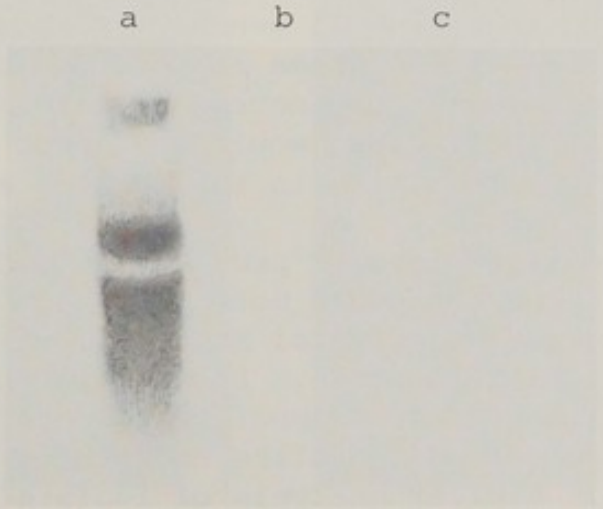

FIGURE 3. Hybridization of cellular poly(A)-containing RNA blotted to activated diazobenzyloxymethyl paper following gel electrophoresis in 1.5% denaturing agarose (10 mM methyl mercury) for 5 hr at 40 V. Blotted RNA was hybridized to 2.5 x 10^{6} cpm P^{32}-labelled *src*-specific DNA for 48 hr and visualized by X-ray autoradiography. Lane a, control MSV RNA; lane b, C8 mRNA; lane c, C10 mRNA.

The X-ray autoradiograph of the RNA blot hybridized to the *src*-specific DNA probe is shown in Fig. 3. The only RNA bands visualized were those in the MSV RNA control lane (lane a). The absence of mRNA bands from the phenotypic revertants (lanes b and c) suggests that in such cells no MSV-specific mRNA is produced.

Expression of MSV-specific proteins. To test for the expression of MSV-specific proteins, revertant cells along with control cells were stained with an antiserum containing antibodies cross-reactive to MSV-specific proteins. $60^{+}H^{-}$ cells serving as a positive control fluoresced evenly and intensely when examined under U.V. light (Fig. 4a). Of all the revertant cells tested most (C10, C9, C8, C3) fluoresced to a degree similar in intensity to that of the NRK cells serving as a negative control (Fig. 4b,c). In the case of two revertant clones, a1 and a2, a heterogeneous staining pattern was observed with some cells fluorescing as the $60^{+}H^{-}$ positive control and some as the NRK negative control with both types on the same cover slip (Fig. 4d). Such negative staining results are not surprising given the inability to detect *src*-hybridizing mRNA from the revertants.

DISCUSSION

Cells derived from transformed cells and exhibiting a non-transformed phenotype may fall into a variety of mutant classes. The first is one in which the integrated viral genome is lost from the host cell genome. A second class would be one in which the viral genome is retained but is not properly transcribed to yield the necessary transformation-specific protein(s). A third class would represent proper viral gene expression but resulting in the lack of a functional transforming factor.

The first mutant class of proviral sequence loss is the simplest to envisage and would not yield much information concerning the interaction of viral and cellular factors responsible for the neoplastic state. Restriction of cell DNA and hybridization with sequences specific for the virus genome was carried out to detect and localize endogenous and exogenous viral sequences. Similar analysis of isolated cell mRNA was performed to investigate viral genome transcription. Antibody staining was used to visualize and localize virus-specific polypeptides.

The results of DNA cleavage, electrophoresis, blotting and hybridization indicate that of the three non-transformed phenotypic revertants, C8, C9 and C10, all seem to maintain both exogenous and endogenous integrated viral sequences as does the parental $60^{+}H^{-}$ cell line. This result is consistent

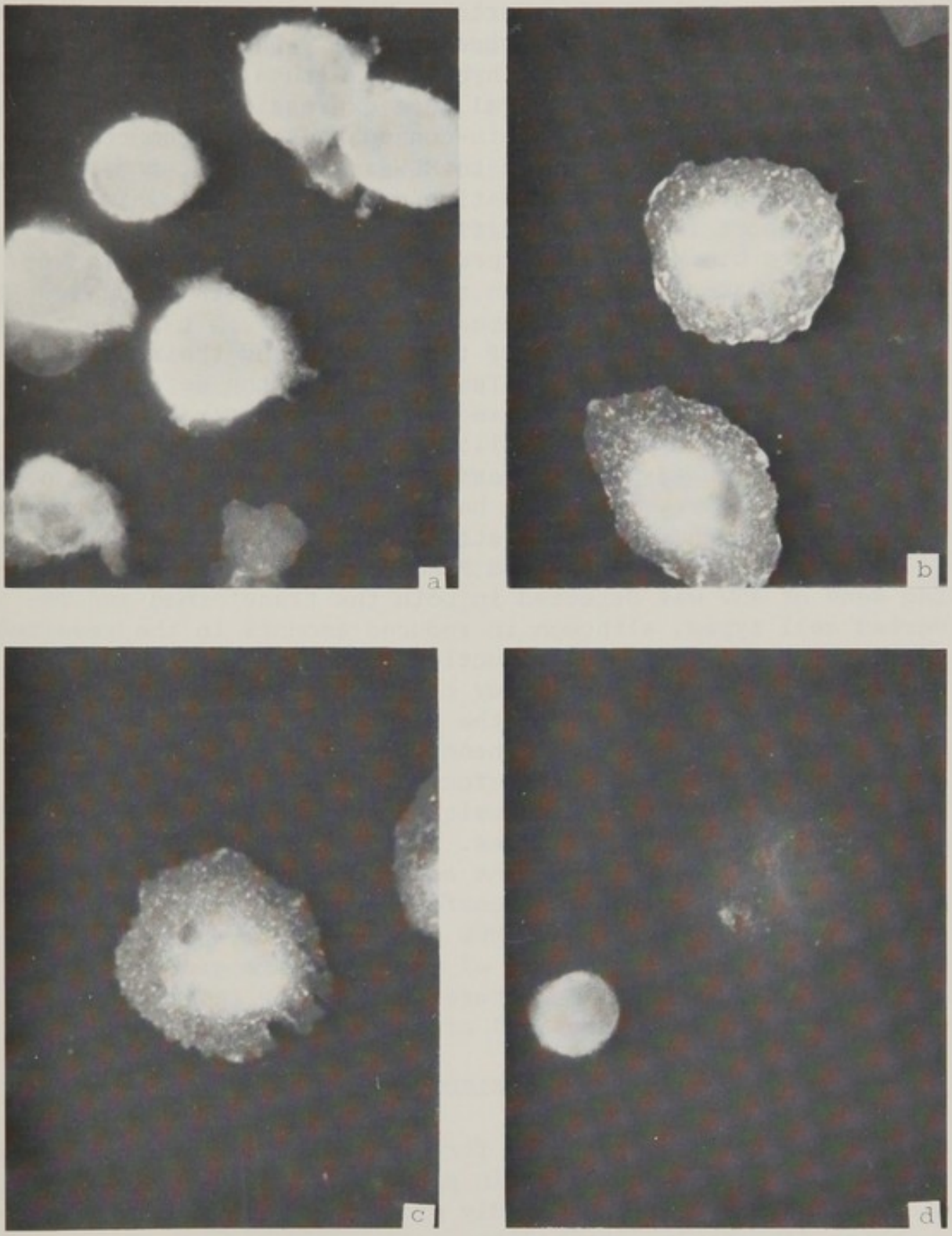

FIGURE 4. U.V. light micrographs of transformed and reverted NRK cells grown on glass cover slips. Cells were stained with a fluorescein-conjugated goat anti-MuLV antiserum containing cross-reacting anti-MSV antibodies. a, $60^{+}H^{-}$ cells; b, NRK; c, C9 (revertant); d, a2 (revertant). Note heterogeneous staining pattern in Fig. 4d (see DISCUSSION).

with the mutant class of revertants characterized by retention of the integrated exogenous viral genome. The finding that revertant cell mRNA does not hybridize with a *src*-specific DNA probe suggests a block in viral gene expression. These cells, when stained with a fluorescein-conjugated antiserum containing antibodies cross-reactive to MSV-specific proteins, fluoresce similarly to the NRK negative control cells. This is further support for the classification of the revertants as containing a block in gene expression. This block would result in the lack of production of the transforming gene product(s).

A detailed examination of these cells and their integrated viral sequences could further define the defect(s) in question. These include altered viral promoter regions and alterations in the cell's ability to process nuclear RNA.

A similar analysis was performed by Deng et al. (11) using transformed and reverted baby hamster kidney (BHK) cells transformed by the Schmidt-Ruppin strain of Avian Sarcoma virus (12). Polyribosome-associated RNA specific for the transforming gene of ASV was detected in both the transformed and reverted cell types, although in reduced amounts in the reverted cells. For this system a reduction in the concentration of viral RNA in reverted cells may cause the reversion phenomenon.

As stated above some of the revertant clones show a leakiness of the non-transformed phenotype. These cells show colonies of morphologically transformed cells which appear at random. These colonies are positive when stained with virus-specific fluorescent antibodies. This indicates that within cloned populations of revertant cells occasional cells exist which can be recognized as transformed by the criteria of morphology and expression of MSV proteins. It would be of interest to develop populations of transformed segregants from a mixed population and to compare them to the revertant parental cells by the biochemical criteria discussed above.

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GENETICS OF ACYCLOGUANOSINE RESISTANCE AND THE THYMIDINE KINASE GENE IN HSV-1[1]

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ABSTRACT We have identified a locus of HSV-1 which, when mutated, confers resistance to acycloguanosine (ACG); this locus corresponds to the HSV thymidine kinase (TK) gene. ACG-resistant mutants exhibit reduced levels of HSV-specific TK activity, and the lower the TK activity, the greater the degree of ACG-resistance. Moreover, in a conditionally resistant mutant, KG-111, resistance to ACG at 39° and sensitivity at 34° is associated with thermolabile TK activity. Eleven TK^-, ACG-resistant mutants behaved in genetic crosses as alleles of a single locus mapping between the A and G loci on the HSV-1 strain KOS genetic map. Analysis of the fine structure of this locus in now possible with the aid of a recombinant plasmid bearing the KOS TK gene.

INTRODUCTION

The herpes simplex virus (HSV) thymidine kinase (TK) gene encodes a defined polypeptide[1,2] and has been mapped by biochemical transformation experiments to a sequence of two

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kilobase pairs (kbp)[3]. Of twenty-nine complementation groups of temperature-sensitive (ts) mutants of HSV-1[4,5], mutants in thirteen groups of strain KOS have been arranged in a linkage map by means of two-factor crosses and nine of the thirteen have been mapped physically by marker rescue and the analysis of HSV-1 x HSV-2 recombinants[6]. We wished to map the TK gene genetically and physically relative to other genes in preparation for studies of its expression and regulation.

A substantial amount of data has accumulated indicating that HSV TK is essential for the antiviral effect of the antiherpetic drug acycloguanosine [9(2-hydroxyethoxymethylguanine); generic name, acyclovir; ACG][7-9]. ACG is relatively non-toxic to primate cells in culture, especially Vero cells[10]. We chose to isolate mutants resistant to ACG on Vero cell monolayers as a way of isolating TK^- mutants, and to use the drug-resistance phenotype in genetic analyses of the TK gene.

We report here our findings identifying and mapping the TK gene as a locus which, when mutated, confers resistance to ACG in HSV-1. The construction of a recombinant plasmid which contains the KOS TK gene is also described. Elsewhere, we have presented data demonstrating the existence of a second locus which, when mutated, confers resistance to ACG; this second locus appears to correspond to the HSV DNA polymerase gene[11].

MATERIALS AND METHODS

Procedures used for the propagation of cells and viruses, the use of drugs, virus assays, recombination analysis, and TK assays are described in Coen and Schaffer (1980)[11]. Methods used to prepare extracts from cells infected with mutant KG-111 for TK assays are described in the Legend to Figure 2.

Bam HI fragments of KOS DNA were incorporated into bacterial plasmid pBR322[12] using a "shotgun" procedure. KOS DNA (5 µg) and pBR322 DNA (0.5 µg) were cleaved with restriction enzyme Bam HI (New England Biolabs). The mixture was then rendered 6.6 mM $MgCl_2$, 66 mM Tris-HCl, pH 7.6 2 mM dithiothreitol, 1 mM ATP (ligation buffer). The total DNA concentration was adjusted to 50 µg/ml. DNA ligase (Miles) was added and the mixture was incubated at 15° for 8 hrs. The DNA concentration was then adjusted to 2 µg/ml by addition of ligation buffer, additional ligase was added, and ligation was permitted to proceed for an additional 18 hrs at 15°.

E. coli K-12 strain HB-101 was transformed using 0.1 ml of the DNA mixture by the procedure of Cohen *et al.*[13] under P2, EK-1 (CV) containment. Transformed bacteria were plated

on nutrient agar plates supplemented with 100 μg/ml ampicillin. Colonies which arose were screened for ampicillin resistance (amp^r) and tetracycline sensitivity (tet^s). $Amp^r tet^s$ colonies were further screened by a rapid isolation method for plasmid DNA (G.M. Rubin, personal communication). DNA from recombinant clone pKOS 17 was purified by CsCl-Ethidium bromide centrifugation; restriction fragments were purified as described[5].

Biochemical transformation of TK-deficient cells to the TK^+ phenotype was performed as described by Wigler *et al.*[14] using Ltk^-, $aprt^-$ cells (the generous gift of S. Silverstein) as recipients and salmon sperm DNA as carrier. TK^+ colonies appearing after three weeks of HAT-selection were counted after staining with crystal violet.

RESULTS

Table 1 summarizes the relative resistances to 100 μM ACG of the wild-type virus, KOS, and four mutants derived from KOS. These data are taken from an experiment reported elsewhere[11] similar to that depicted in Figure 3 in which the effect of varying concentrations of ACG on the plating efficiencies of the five viruses was measured. The wild-type virus, KOS, was highly sensitive to ACG--the plating efficency being 0.1% at 100 μM ACG. $ACG^r 8$ was resistant with a plating efficiency of 45% in 100 μM ACG. Mutants $ACG^r 2$, 3, 5, 9, 19, 20, 31 and 34 behaved like $ACG^r 8$ in these kinds of experiments (not shown). Mutants $ACG^r 18$, 33 and 35 were partially resistant, exhibiting plating efficiencies similar to that of $ACG^r 8$ at 20 μM[11] but much lower plating efficiencies at 100 μM.

TABLE 1
DRUG RESISTANCE PHENOTYPES AND TK ACTIVITIES

Virus	Resistance to ACG[a]	% TK Activity[b]
KOS	0.1 (ACG^s)	100
$ACG^r 8$	45 (ACG^r)	0.5
$ACG^r 18$	4 (partially ACG^r)	5
$ACG^r 35$	2 (partially ACG^r)	14
$ACG^r 33$	1 (partially ACG^r)	16

[a] $EOP = \left(\frac{\text{PFU/ml in 100 μM ACG}}{\text{PFU/ml without ACG}}\right) \times 100.$

[b] TK assays were performed as described[11]. The activity induced by each virus was normalized to that induced by KOS. Mock-infected activity was 0.7% of KOS-induced activity.

TABLE 2
RECOMBINATION BETWEEN ACG^r MUTANTS AND PAA^{r}5[a]

Cross: PAA^{r}5 x	Recombination Frequency %
ACG^{r}2	4.7
ACG^{r}3	4.8
ACG^{r}8	4.2
ACG^{r}9	3.6
ACG^{r}19	4.0
ACG^{r}20	4.4
ACG^{r}31	4.4
ACG^{r}35	4.6

[a]Recombination was performed and recombination frequencies were calculated as described[11].

TK Activities The TK activities induced by the ACG-resistant mutants were substantially reduced compared to that of the wild-type virus (Table 1). For these five viruses the lower the TK activity, the higher the degree of ACG-resistance. This relationship also held true for the other eight mutants described above. Thus, ACG-resistance is strongly associated with reduction in TK activity.

Recombination Between ACG-Resistant Mutants and a PAA-Resistant Mutant Mutants ACG^{r}2, 3, 8, 9, 20, 31 and 35 were crossed with the phosphonoacetic acid (PAA)-resistant mutant, PAA^{r}5[15], and progeny virus was assayed for resistance to both ACG and PAA. All seven of the ACG-resistant mutants tested recombined with nearly equal frequencies with the PAA^r mutant (Table 2).

Recombination Among ts Mutations and ACG-Resistance Mutations: Map Position of the acg^r-tk Locus Aron *et al.*[16] showed that ts mutants A1, E6 and G3, derived from strain KOS, were highly deficient in the induction of TK activity. This led us to attempt to map the ACG-resistance locus associated with the deficiencies in TK of these mutants relative to their ts mutations, by performing back-crosses between these mutants and KOS at 34°. Progeny virus was selected for growth at 39° and for ACG-resistance (20 μM ACG) (see bold-line, Figure 1, top). Simultaneously, the three ts mutants were crossed with each other (ts$^+$ progeny were scored) and tsA1, tsE6, and ACG^{r}20 were crossed with PAA^{r}5 (progeny were scored for resistance to both PAA and ACG at 34°). The recombination

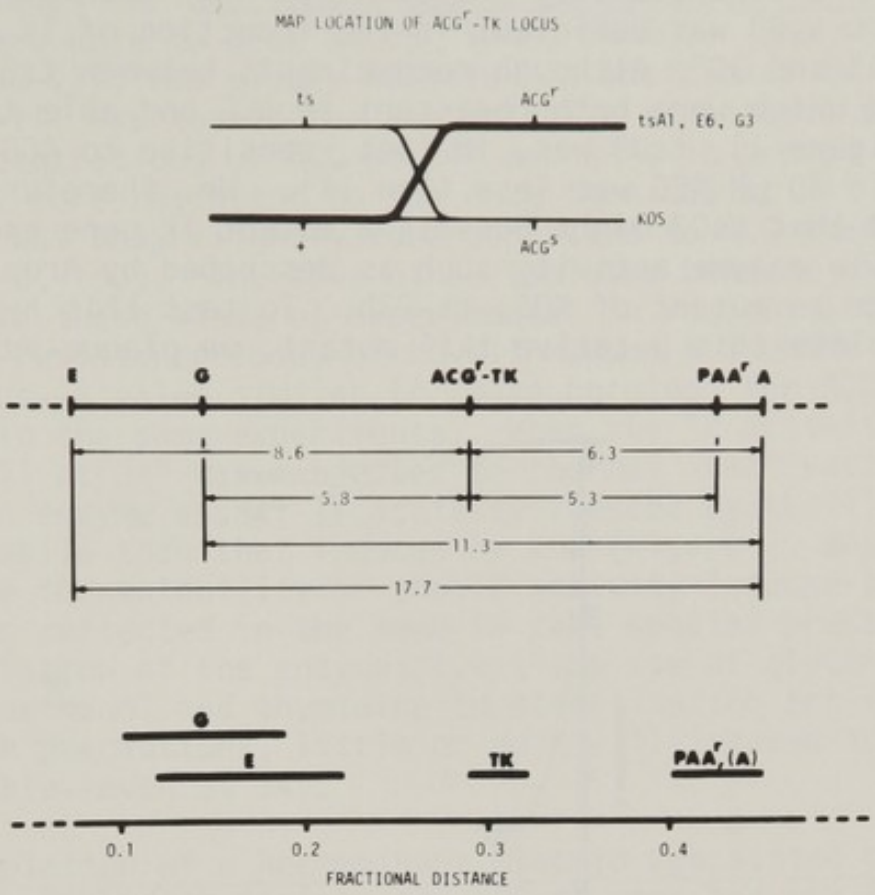

Figure 1. (Top) Temperature-sensitive mutants tsA1, tsE6 and tsG3 which are also TK$^-$ were crossed with wild-type virus, KOS, and ts$^+$, ACG^r progeny were scored. (Middle) Linkage map derived from cross depicted at top and others described in text. Recombination was performed and scored as described in the text. Recombination frequencies were calculated by multiplying the percent recombinant progeny scored by two. (Bottom) Physical map position of tsG, tsE, tsA, PAA^r and the TK gene on the HSV-1 DNA molecule[6] (and S. P. Little, personal communication).

frequencies obtained in these crosses are shown in the middle portion of Figure 1. As can be seen, a linkage map was derived which was linear, additive and consistent with previous genetic mapping experiments[6,15].

The physical map position of the three ts mutants, the PAA^r locus and the TK gene as determined by marker rescue, analysis of HSV-1 x HSV-2 recombinants, and biochemical transformation experiments[6] (and S. P. Little, personal communication) are shown in Figure 1 (bottom). The TK gene lies between the E and G loci on the left, and the PAA^r and A loci on the right consistent with its location on the genetic map.

A Mutant Conditionally Resistant to ACG Aron *et al.*[16] showed that tsG3 was deficient in the induction of TK activity at both 34° and 39°. Although recombinants between tsG3 and KOS were found which were both resistant to ACG and able to grow at 39° (Figure 1), tsG3 was, in fact, sensitive to ACG at 34° (its EOP in 20 μM ACG was less than 1%). We, therefore, hypothesized that tsG3 might possess a mutant TK gene encoding thermolabile enzyme activity such as described by Aron *et al.*[16] for another ts mutant of KOS, ts-23b. To test this hypothesis and to isolate this putative tk^{ts} mutant, we plaque-purified

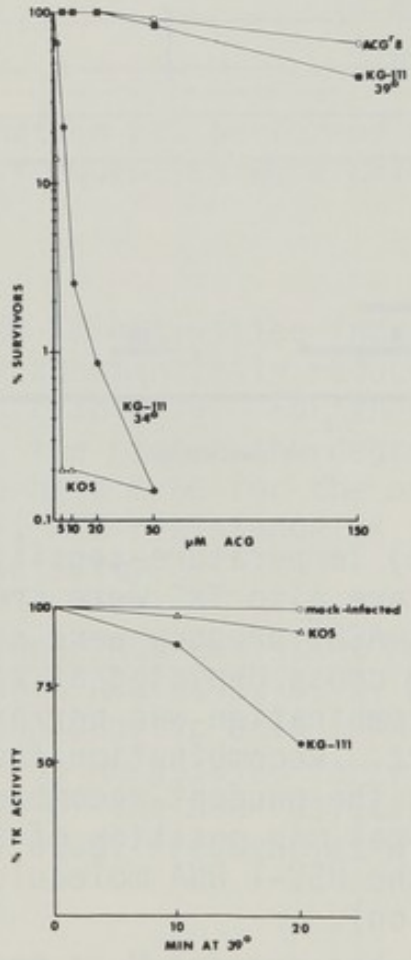

Figure 2. (Top) Effect of varying concentrations of ACG on the plating efficiency of KOS, $ACG^r 8$ and KG-111 at 34° and 39°. Viruses were assayed as described[11]. (Bottom) Frozen mock-infected or infected Vero cell pellets were prepared as described[11]. Pellets were thawed and resuspended in 0.25 ml of 10 mM sodium phosphate buffer, pH 6.0 containing 5 mM 2-mercaptoethanol, 50 μM thymidine and 10% glycerol. Cell extracts were prepared from these suspensions as described[11]. Aliquots of the cell extracts were held either on ice or at 39° for the times indicated. TK assays were performed as described[11] except that mock-infected activity was assayed in the absence of TTP.

six isolates derived from a back-cross between KOS and tsG3 which were able to grow at 39° in 20 μM ACG. One of these mutants, KG-111, was plaque purified an additional two times. When KG-111 was tested for its relative resistance to different concentrations of ACG at 34° and 39°, it proved to be slightly more resistant to the drug than KOS at 34° whereas at 39°, it was only slightly more sensitive to ACG than ACG^r8 (Figure 2, top). The five other isolates behaved similarly to KG-111 in these kinds of experiments. In recombination tests, the ACG-resistance locus of KG-111 mapped 5.5 units from the PAA^rlocus--a value similar to those obtained for ACG^r8 and ACG^r35 in the same experiments. When the TK activity induced by KG-111 at 34° was subjected to thermal inactivation at 39° prior to enzyme assay, TK activity induced by KG-111 was more thermolabile than that induced by KOS (Figure 2, bottom).

The thermolability of the TK activity induced by KG-111 was also reflected in the need to take special precautions in the isolation of the enzyme; i.e., the use of glycerol, 2-mercaptoethanol and thymidine to stabilize the activity. Without such precautions, little or no KG-111-induced activity was detectable--even at 34°.

Isolation of a Recombinant Plasmid Containing the KOS TK Gene We wished to isolate the KOS TK gene and its flanking sequences by molecular cloning to enable us to map TK^- mutants more precisely and to aid in our studies of TK gene expression. Figure 3 shows a restriction enzyme map of pKOS 17, a recombinant plasmid isolated by ligation of a total Bam HI digest of HSV-1 strain KOS DNA and pBR322 DNA. This plasmid contains two Bam HI fragments inserted into pBR322 DNA. One insert measures

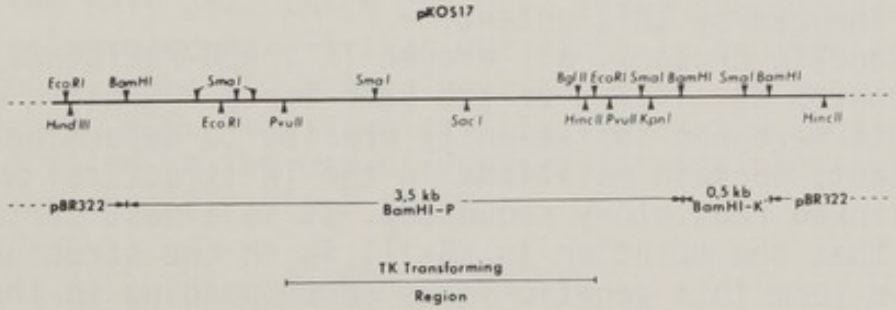

Figure 3. (Top) Restriction enzyme cleavage map of pKOS 17 DNA. (Middle) Regions of pKOS 17 corresponding to pBR322, the 3.5 kbp insert which co-migrates with KOS Bam HI fragment P, and the 0.5 kbp insert which co-migrates with KOS Bam HI fragment K'. (Bottom) The region of pKOS 17 which transforms TK cells to the TK^+ phenotype based on the experiments described in the text and elsewhere[3].

about 0.5 kbp and co-migrates with KOS Bam HI fragment K' upon agarose gel electrophoresis. The other measures 3.5 kbp, contains HSV DNA sequences detectable by Southern blotting hybridization experiments[17] using KOS DNA labelled *in vivo* with ^{32}P as probe, and co-migrates with KOS Bam HI fragment P, which maps at about 0.30 on the KOS physical map (data not shown). The restriction sites shown in Figure 3 for the 3.5 kbp insert are similar, if not identical, to those described for the TK gene-containing Bam HI fragment by other groups[3,18-20]. The 3.5 kbp insert lacks restriction sites for Hpa I, Xba I, Xho I and Hind III, also in agreement with others' results.

To show that the 3.5 kbp insert encodes an active TK gene, we performed biochemical transformation experiments using a) pKOS 17 DNA cleaved with Bam HI and b) the purified 2.4 kbp Eco RI fragment derived from pKOS 17. Both DNA samples transformed Ltk$^-$ cells to the TK$^+$ (HAT^r) phenotype. The Eco RI fragment transformed at a frequency of 8 colonies per microgram, while pBR322 DNA did not transform detectably.

Preliminary marker transfer experiments have shown that the 2.4 kbp Eco RI fragment derived from pKOS 17, when mutagenized *in vitro* by the procedure of Sandri and Levine (personal communication), will transfer the acg^r phenotype to intact KOS DNA at frequencies significantly above background.

DISCUSSION

The data presented herein identify the HSV TK gene as a locus which, when mutated, can confer resistance to ACG. These data include the correlation of ACG-resistance with reduced TK activity--particularly in a conditionally resistant mutant, KG-111--where resistance to ACG at high temperature and sensitivity at low temperature were consistent with thermolabile TK activity induced by this mutant.

In genetic crosses, all eleven TK$^-$, ACG-resistant mutants tested behaved as alleles of the same locus. Genetic mapping experiments were not sufficiently precise to determine whether these mutants contain mutations in the TK structural gene or in closely-linked regulatory sequences. It is almost certain, however, that the mutation in KG-111 is in the structural gene for TK. We term this genetic locus corresponding to the TK gene, acg^r-tk (small letters are used because mutants in this locus are recessive to the wild-type phenotype, ACG^{s11}).

The genetic map position of the acg^r-tk locus derived from two-factor crosses corresponds roughly to the physical map location of the TK gene relative to the other genetic markers used (Figure 1). This finding is in sharp contrast to that of Brown and Jamieson[2] who placed resistance to bromodeoxycytidine (their marker for TK) at one end of the genetic map and

the PAA^r locus at the other. All ten mutants tested, including tsD which has been shown to map physically in the short arm of the HSV DNA molecule[6] mapped genetically between the TK and PAA^r loci. The differences between our results and those of Brown and Jamieson may be due to their reliance on *syn* markers in their three-factor crosses. There is now considerable evidence indicating that there are at least four different *syn* loci[6].

Our studies of the recombinant plasmid pKOS 17 indicate that this plasmid contains sequences encoding the HSV TK gene as well as flanking sequences. pKOS 17 contains a Bam HI fragment of HSV DNA of the same size and with many of the same restriction enzyme cleavage sites as the Bam HI fragments of other HSV-1 strains shown by others to contain the TK gene. pKOS 17 DNA was shown to contain sequences able to transform Ltk$^-$ cells to the TK$^+$ phenotype. The 2.4 kbp Eco RI fragment derived from this plasmid also transformed cells to the TK$^+$ phenotype. This result contrasts with the failure of Wigler *et al.*[22] and Wilkie *et al.*[20] to transform cells to the TK$^+$ phenotype using Eco RI cleaved HSV DNA or plasmid DNA, but agrees with the findings of Colbere-Garapin *et al.*[3] that a plasmid bearing a similar Eco RI fragment was able to transfer the TK$^+$ phenotype, albeit at low frequency. The discrepancies in these results are probably due to the low frequency of transformation by this fragment such that it was not detected by Wigler *et al.* and Wilkie *et al.*

We hope to use pKOS 17 to map more finely the acg^r-tk locus. Our preliminary findings that the 2.4 kbp Eco RI fragment derived from pKOS 17 when mutagenized *in vitro* will transfer the acg^r phenotype, strengthen the link between the genetic and physical data defining the acg^r-tk locus.

It should be emphasized that mutations in the HSV TK gene are not the only ones which can give rise to resistance to ACG. We have shown elsewhere[11] that mutations in another locus (most probably the HSV DNA polymerase gene) can also confer resistance to ACG. This finding illustrates the caution which must be applied in the use of drug-resistance phenotypes as markers for specific viral genes.

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STUDY OF GENETIC VARIABILITY OF VIRUSES THROUGH THE USE OF MONOCLONAL ANTIBODIES[1]

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ABSTRACT Monoclonal antibodies secreted by hybridomas and directed either against the hemagglutinin (HA) molecule of influenza virus or against the nucleocapsid or glycoprotein of rabies virus were used to detect antigenic changes characterizing viral mutants that arose in nature or were produced experimentally. In the case of the HA molecule, it may also be possible to analyze those mutants through changes in the polypeptide map and in amino acid composition. Antigenic variations among rabies viruses may account for failures in cross-protection between experimental variants and parental viruses on the one hand, and between the vaccine strain and street virus variants on the other.

INTRODUCTION

Since the first hybridoma that secreted antiviral antibodies was produced (1), monoclonal antibodies have been used extensively for the analysis of antigenic variations among many species of viruses. In this report we will discuss results of studies of antigenic variations of influenza and rabies viruses that were recognized by monoclonal antibodies. Since most of the data dealing with influenza virus are already available in the literature, only a summary of the results of investigations of this virus will be presented. Conversely, results of the study of variants of rabies virus will be presented in greater detail since most of the information is only now being published.

Antigenic variants of influenza virus PR8 could readily be selected by a single passage of the parental virus in the presence of monoclonal anti-hemagglutinin (HA) hybridoma antibody. Using five different monoclonal antibodies, the average frequency of variants in several cloned PR8-virus seeds was found to be $10^{-5.5}$ per infectious unit of parental

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virus (2,3). The general characteristics of influenza variants selected with monoclonal anti-HA antibodies are exemplified in Table 1 with one parental-variant virus pair. Thus, the variants exhibit an antigenic change that abolishes or strongly reduces the capacity of the selecting monoclonal antibody to react with the variant. However, the vast majority (65-90%) of presumably randomly selected monoclonal hybridoma antibodies and consequently also heterogeneous antisera react, in general, equally well with parental and variant virus. Biochemical comparison of parental and variant HA-molecules has made possible to pinpoint, in the case of many variants, single amino acid substitutions which presumably are responsible for the antigenic change exhibited by the variants (4,5).

TABLE 1

CHARACTERISTICS OF ANTIGENIC VARIANTS OF INFLUENZA SELECTED BY MEANS OF MONOCLONAL ANTI-HA ANTIBODIES[a]

	Parental Virus (PR8)	Variant Virus (PV2)
Hybridoma Antibody used for selection[b]:		
HI-titer (log 10)	4.34	< 1.80
Neutralization titer (log 10)	2.39	< 1.0
Rabbit antiserum against HA molecule of parental virus, HI-titer (log 10)	4.52	4.73
Rabbit antiserum against variant PV2, HI-titer (log 10)	4.10	4.43
Peptide map of HA1 polypeptides[c]	single peptide difference	
Amino acid composition[c]	identical except for single amino acid difference in changed peptide. Ser → Leu	

[a]See refs. 5 and 6.

[b]Hybridoma PEG-1 ascitic fluid.

[c]Peptide mapping and amino acid composition analysis was performed on variant PV12 which seemed to be antigenically identical to PV2. Approximately 1/3 of the HA1 polypeptide was analyzed.

Eleven different monoclonal anti-HA antibodies were used to select variants from cloned PR8 virus seed. Five to 12 variants were selected with each antibody. As shown in Table 2, antigenic analysis of 71 PR8 variants by means of 60 anti-HA hybridoma antibodies showed that at least 32 of these variants were antigenically unique. This demonstrates that the HA molecule of influenza has an extremely large freedom for antigenic variation particularly if one considers that the 32 distinct variants may represent only a small fraction of the total repertoire of single point mutants of PR8.

TABLE 2

REPERTOIRE OF ANTIGENIC HA-MUTANTS DERIVED FROM CLONED HA/PR/8/34

Number of antigenic variants analyzed:	71
Minimum number of antigenically unique variants:	32

Comparison of the HA-polypeptides of influenza field strains of the Hong Kong subtypes (H3 N2), isolated from humans between 1968 and 1977, by peptide-mapping and analysis of amino acid composition has clearly established the fact that interpandemic field strains derive from each other in a sequential manner by accumulation of point mutations (7).

Which or how many of these point mutations are actually required for producing an epidemiologically relevant antigenic change in the HA molecule has remained essentially unknown, but determination of the position of the amino acid substitutions of in vitro selected single point antigenic mutants has made possible to pinpoint some positions of the HA polypeptide which must be involved in the formation of the antigenic structure of the HA molecule (4). Comparison of field strains and in vitro selected variants indicates that they exhibit changes in the same regions of the HA polypeptide, though, in general, not at the same positions (4) of the HA polypeptide (see Table 3). For instance, analysis of more than 10 H3 variants selected in vitro by means of monoclonal anti-HA antibodies showed that the proline (pos. 143) was replaced by either serine, leucine, threonine or histidine (8). None of the 8 field strains (1968 to 1977) analyzed showed a change of the proline (pos. 143). Instead, changes occurred in the neighboring amino acids (gly-ser-gly; pos. 144 to 146). In one variant, however, glycine (pos. 144) changed to aspartic acid, a change which also occurred in the course of antigenic drift in nature. Interestingly, the substitution of the glycine at pos. 144 in-

TABLE 3

COMPARISON BETWEEN INFLUENZA FIELD STRAINS AND VARIANTS SELECTED IN VITRO WITH MONOCLONAL ANTIBODIES

	In vitro selected variants	Field strains
Antigenic difference to parental[a] virus:	detected, in general, with: monoclonal antibodies only	monoclonal antibodies and antisera
Difference in HA protein sequence compared to parental virus[b]:	single amino acid substitution	several
Location of amino acid substitutions[c]	occur in similar regions of HA1 polypeptide but often in different positions	

[a] In the case of field strains, the first virus strain that introduced the new hemagglutinin subtype is regarded as the parental virus strain.

[b] See refs. 4 and 7.

[c] See ref. 4 and 8.

TABLE 4
PRODUCTION OF HYBRIDOMAS SECRETING ANTIBODIES AGAINST RABIES VIRUS COMPONENTS

Virus used for immunization of mice	Ratio of hybrid cultures[b] secreting antiviral antibodies	Ratio of hybridomas[c] recognizing virus components: G	N	NS or M
ERA	56/95	8/16	6/16	2/16
CVS	26/52	4/26	7/26	15/26
Kelev	45/74	8/29	7/29	14/29
Street (AF)	5/18	1/3	0/3	2/3
Lagos bat[a]	3/18	3/3	0/3	0/3
Mokola[a]	9/24	0/9	2/9	7/9

[a]Rabies-associated viruses; all others are rabies viruses.

[b]Initial, uncloned hybrid cultures.

[c]Cloned hybridomas.

Antibodies produced by hybridomas were first screened for binding activity in RIA to rabies-infected cells, followed by indirect immunofluorescence tests on live and acetone-fixed virus-infected cells. Antibodies staining fixed cells were further checked for binding in RIA to purified nucleocapsid (N). Antibodies staining live cells are checked for binding in RIA to purified glycoprotein (G) and in neutralization tests. Antibodies which bound to rabies-infected cells in RIA but did not react with either G or N components of rabies virus were tentatively classified as directed against either NS or M proteins.

duced an antigenic modification that resulted in a considerable inhibition of the HI activity by antiparental antiserum. This finding suggests that single point mutations at certain positions may be sufficient to produce an epidemiologically relevant antigenic drift (8). The qualitative difference between experimental variants and field strains results probably from different selection requirements, monoclonal antibodies of murine origin and antibody populations (probably of restricted heterogeneity) of human origin, respectively (see Table 3).

In contrast to influenza virus, strains of rabies virus isolated during the past 100 years from different animal species in various parts of the world were considered to be closely related on the basis of their reactivity with animal sera. Production of monoclonal antibodies directed against various components of rabies virus (9) has made it possible to re-examine antigenic relationship(s) between different field strains of rabies and to delineate the relationship of field strains or of laboratory-developed variants to strains used for vaccination purposes and to parental strains from which mutants were selected in vitro, respectively. Since protection in a lethal infection such as rabies is based on the immunization of an exposed host with an efficient vaccine, a study of the ability of vaccine strains to protect against challenge with mutant strains became quite important to undertake.

As shown in Table 4, no difficulties were encountered in producing hybridomas secreting antibodies (HAb) reacting with various components of rabies virus. It is interesting to note that the number of hybridomas produced after immunization of mice with street virus was rather low and that only one such hybridoma secreted antibody reacting with the glycoprotein of rabies. Immunization of mice with rabies-associated viruses, Lagos bat or Mokola, resulted in the production of hybridomas which secreted antibodies reacting with only the rabies-associated viruses but not with rabies viruses. Contrariwise, immunization with rabies virus resulted in the production of HAb, some of which showed cross-reactivity with rabies-associated viruses.

ANTINUCLEOCAPSID HYBRIDOMAS

The specificity of hybridomas producing antibodies directed against the nucleocapsid of rabies virus (10) was determined in binding assays using purified nucleocapsids isolated from various laboratory (fixed) strains of rabies.

TABLE 5
REPERTOIRE OF ANTINUCLEOCAPSID HYBRIDOMAS

HAbs		Binding in RIA[a] of HAbs to nucleocapsids of:					
		Rabies viruses			Rabies-associated viruses		
Group	No. in group	ERA, LEP Kelev	CVS	HEP	Mokola	Lagos bat	Duvenhage
A	1	+	+	+	+	+	+
B	11	+	+	+	-	-	-
B-1	1	+	+	+	-	-	+
B-2	1	+	-	+	-	-	-
B-3	1	+	+	-	+	-	-
B-4	2	+	+	-	+	+	-
B-5	1	+	+	-	-	+	+
B-6	3	+	+	-	+	+	+
C	1	-	-	-	+	+	+

HAb = hybridoma antibody.
[a]Based on cpm of ^{125}I anti-mouse $F(ab')_2$ added to a nucleocapsid mixed with hybridoma antibody; a background of 100 cpm was subtracted from each value.
+cpm > 200
-cpm < 200

The results (shown in Table 5) indicate that half of the hybridomas (B) delineated antigenic determinants expressed by the five rabies viruses but not by any one of the three rabies-associated viruses. Next in frequency were seven Habs (B-3 - B-6) which reacted with all rabies virus nucleocapsids but not with those of the Flury-HEP virus in spite of the fact that Flury-HEP is a direct derivative of the LEP strain. The same group (B-3 - B-6) cross-reacted with nucleocapsids of either the two or three rabies-associated viruses. Only one HAb (B-2) distinguished the nucleocapsids of CVS from those of the other four rabies viruses. CVS is a Pasteur-derived strain most frequently used in laboratory assays. Only one HAb (group A) bound specifically to nucleocapsids of both rabies and rabies-associated viruses. One HAb (group C) obtained by fusion of splenocytes of a mouse immunized with the rabies-associated virus Mokola differentiated nucleocapsids of rabies-associated from nucleocapsids of rabies viruses.

After establishing that antinucleocapsid hybridomas were able to delineate antigenic differences between fixed viruses, we further investigated field strains of rabies.

ANALYSIS OF FIELD STRAINS OF RABIES BY MEANS OF ANTINUCLEOCAPSID HYBRIDOMAS

In this study, a panel of 21 antinucleo apsid HAbs was tested for FA staining of nucleocapsid present in the cytoplasm of brain cells of mice infected with field strains of rabies obtained from different geographical areas (11) as shown in Table 6. The results indicate that the vast majority (81%) of HAbs recognize specificities expressed by nucleocapsids of all viruses listed in the Table. Only four out of the 21 hybridomas revealed antigenic differences among the various field isolates. Given the small number of hybridomas able to differentiate field strains on the basis of their nucleocapsid, it seems premature to draw any general conclusion. It is interesting, however, that nucleocapsids of viruses isolated from the same geographical region, such as those of the two isolates from the USSR, one from France, one from FGR, and three from Chile (dogs) and Brazil (man, bat), tend to show similar reactivity with the four differentiating antinucleocapsid HAb's.

On the other hand, limitations on such deductions are clearly illustrated by the fact that the two isolates from

TABLE 6
VARIANTS OF FIELD STRAINS OF RABIES RECOGNIZED BY ANTINUCLEOCAPSID HYBRIDOMAS

Virus isolates			FA of virus-infected cells exposed to hybridoma antibodies			
Continent	Country	Species	364-11	377-7	102-27	193
Europe	France, FGR	Fox (2)	-	+	+	+
	Czechoslovakia	Mouse (1)	+	-	+	+
	USSR	Man (2)	+	+	+	+
Africa	Burundi	Dog, goat (2)	+	+	+	+
	Nigeria	Horse (1)	+	-	+	+
South America	Chile	Dog (1)	+	-	+	+
		Man (1)	+	-	+	-
	Brazil	Man, bat (2)	+	-	+	+
North America	USA	Dog (1)	+	+	+	+
		Dog, fox (2)	+	+	+	-
		Bat (1)	+	+	-	-
Control	Fixed virus	CVS	+	-	+	+

Mice were injected intracerebrally with the respective virus preparations and sacrificed when moribund; their brain smears were stained with a fluorescein conjugated anti-mouse-immunoglobulin reagent after exposure to hybridoma antibody.
( ) = Number of isolates.
Note: Antibodies secreted by seventeen hybridomas reacted with nucleocapsids of all viruses listed in this Table.

TABLE 7
REPERTOIRE OF ANTIGLYCOPROTEIN HYBRIDOMA ANTIBODIES

Hybridoma		Reactivity of antibodies with fixed strains of rabies:				
Group	No. per group	CVS	PM	ERA	HEP	Kelev
I	12	+	+	+	+	+
II	1	+	+	+	+	-
III	2	+	+	+	-	-
IV	2	+	-	+	+	-
V	2	-	-	-	+	+
VI	1	-	-	+	-	+
VII	2	+	-	-	-	-
VIII	2	-	-	-	-	+
IX	1[a]	-	-	-	-	-

[a]Reacts with the three rabies-associated viruses.

Reaction of antibodies secreted by hybridomas with rabies viruses was studied in two ways: 1) binding to virus-infected cells in RIA or 2) in a neutralization test in which serial dilutions of virus were mixed with hybridoma medium and the mixture added to cultures of BHK-21 cells (the results were expressed in terms of neutralization index by dividing number of PFU/ml of virus mixed with normal medium over number of PFU/ml of virus mixed with antibody).

Positive (+)

Negative (-)

man in the USSR, the one isolate from a dog in the USA and the two isolates from a dog and a goat in Burundi showed identical patterns of cross-reactivity of their nucleocapsids with the 21 HAbs.

ANTIGLYCOPROTEIN HYBRIDOMAS

Turning from antinucleocapsid hybridomas to the study of antiglycoprotein hybridomas (12), the five fixed virus strains listed in Table 7 were tested in RIA and in neutralization tests for binding of HAb. The results show that approximately half of the HAbs (13) reacted with all viruses. HAbs of group II reacted with all viruses except Kelev strain, whereas HAbs of group III reacted with CVS, PM and ERA, and those of group IV reacted with CVS, ERA and HEP. HAbs of the remaining groups reacted with either one or two rabies strains only. The HAb of group IX was produced after immunization of mice with rabies-associated (Mokola) virus and did not bind to rabies viruses but to rabies-associated viruses. Glycoprotein of each of the five rabies viruses tested showed its own distinct pattern of binding of HAbs.

VARIANTS OF FIELD STRAINS OF RABIES VIRUS RECOGNIZED BY ANTIGLYCOPROTEIN HYBRIDOMAS

Seven HAbs were tested for their ability to neutralize 16 rabies isolates obtained from different species in countries representing four continents: North and South America, Africa and Europe (11) (see Table 8). Included in this study were seven street virus strains isolated from brain tissue of seven fatal cases of human rabies in the USA (13) that occurred during the past six years. Eleven variant groups could be differentiated. For instance, the isolates from foxes in France and FGR and the isolates from the USSR formed two groups, each consisting of two cross-reacting variants. The remaining five groups from different geographical regions were represented by one variant expressing individual specificity. Lastly, the viruses isolated from the seven fatal human cases in the USA formed four groups, comprising 3, 2, 2, and one isolate, respectively. Since a significant number of variants was found among field strains of rabies including street viruses isolated from fatal human cases it seems pertinent to perform cross-protection experiments in which animals were immunized by viruses representing one group of variants and were challenged by variants representing another group.

TABLE 9

NEUTRALIZATION OF PARENTAL CVS AND CVS-DERIVED VARIANTS BY DIFFERENT HYBRIDOMAS

Hybridoma clone	Neutralization index of hybridoma antibodies reacting with parental virus and virus variants					
	Parent CVS	RV 101-1	RV 220-8	RV 231-22	RV 240-3	RV 226-11
101-1	4.2	0	4.0	4.0	> 4.0	> 4.0
220-8	4.3	0	0	0	> 4.0	> 4.0
162-5	4.3	0	0	3.3	> 4.0	> 4.0
231-22	4.3	4.5	0	0	> 4.0	> 4.0
240-3	4.0	4.5	4.0	4.0	0	0
226-11	4.3	> 4.0	> 4.0	> 4.0	0	0
194-2	4.2	4.5	> 4.0	4.0	> 4.0	3.0

Dilutions of CVS and of variant strains were mixed with a 1:5 dilutions of medium from the respective hybridomas and the mixtures used to infect BHK-21 cells. Neutralization index was determined by dividing the titer of virus mixed with normal medium by the titer of virus mixed with antibody containing medium.

The Neutralization index is expressed in log 10.

TABLE 8
VARIANTS OF FIELD STRAINS OF RABIES VIRUS RECOGNIZED BY ANTIGLYCOPROTEIN HYBRIDOMAS

Hybridoma antibody	Ratio of virus strains neutralized by hybridoma antibody isolated in various geographical regions from man and animals								
	USA		South America		South Africa		France	FGR	USSR
	Man	Bat	Man	Bat	Man	Dog	Fox	Fox	Man
231-22	3/7[b]	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/2
248-2	7/7	0/1	1/1	1/1	0/1	1/1	1/1	1/1	2/2
101-1	7/7	1/1	1/1	0/1	0/1	1/1	1/1	1/1	2/2
110-3	5/7	0/1	1/1	1/1	0/1	1/1	1/1	1/1	0/2
120-6	3/7	0/1	1/1	0/1	0/1	0/1	1/1	1/1	0/2
194-2	7/7	1/1	0/1	1/1	0/1	0/1	1/1	1/1	2/2
613-2[a]	7/7	0/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2

[a]Hybridoma produced from mouse immunized with street virus.

[b]Denominator = number of virus strains tested.
Numerator = number of virus strains neutralized by the respective hybridoma.

One hundred infectious doses of rabies virus strains isolated in various geographical regions were mixed with a 1:5 dilution of the respective hybridoma antibody, and the mixture was used either for infection of tissue cultures (only in case of human isolates in USA) or intracerebral inoculation of mice: survival of all mice or absence of t.c. foci indicated positive neutralization. Death of all mice or presence of infectious foci in t.c. indicated no neutralization.

SELECTION OF VARIANTS

Rabies variants were selected experimentally in a manner similar to that employed in the case of the PR 8 strain of influenza virus (6,11,13). Dilutions of CVS-11 strain were mixed with a 1:5 dilution of individual hybridoma antibodies and were added to BHK-21 monolayers. Virus plaques that developed in the presence of antibody were picked and the plaque progeny virus was analyzed for its antigenic specificities with the panel of hybridoma antibodies. The variant frequency, calculated as the ratio of the CVS titer in the absence of antibodies to the CVS titer in the presence of HAb (the latter including only plaques giving rise to variants) ranged between $10^{-4.0}$ to $10^{-4.5}$ per infectious unit of CVS. This variant frequency was somewhat higher than that obtained with influenza virus.

The analysis of variants (13) is shown in Table 9. All variants are characterized by the fact that they cannot be neutralized by antibodies used for their selection (neutralization index = 0). The variants can be differentiated from each other by means of hybridoma antibodies. For instance, 101-1 is a unique variant since HAb 101-1 neutralizes all variants except for RV-101-1. RV-220-8 and RV-231-22 can be differentiated from each other and from other variants by their reactivity with HAbs 231-22 and 162-5. As judged from the more than 10-fold difference in the neutralization index of HAb 194-2, it seems likely also that variants RV-240-3 and RV-226-11 represent distinct variants. Furthermore, the variants can be arranged on the basis of this analysis into two groups: the variants of the first group (RV 101-1, RV 220-8 and RV 231-22) exhibit antigenic changes that prevent, for instance, HAb 220-8 from neutralizing these variants but do not affect the neutralizing potency of HAbs 240-3 and 226-11. Contrariwise, the antigenic changes of the variants of the second group (RV 240-3, RV 226-11) abolish the neutralizing potency of the latter HAb's but not of HAb 220-8, 101-1, 162-5 or 231-22. Based on these findings it seems that the changes in the two groups of variants occurred probably in distinct antigenic sites of the viral glycoprotein which seem to vary independently from each other. Production of a larger number of hybridomas and selection of a larger number of variants as in the case of influenza virus, may provide further insight into antigenicity and mutability of the rabies virus glycoprotein.

Successful selection of variants of rabies virus in the laboratory made it possible to check the efficacy of the variant viruses to immunize mice against challenge with either the parent virus or variants that differ antigenically from the "vaccine" variant.

THE RANGE OF CROSS-PROTECTION AMONG CVS-VARIANTS SELECTED IN VITRO

As shown in Table 10, mice immunized with parental CVS-11 vaccine were protected against challenge either with homologous virus or with CVS-derived variant viruses RV-231-22 or RV-240 (13). In contrast, mice vaccinated with variant viruses showed good protection only against challenge with homologous virus but not against challenge with parental virus or another variant. The only exception was observed in the case of mice vaccinated with variant RV 101-1 which showed resistance to challenge with variant RV-231-22. These mice remained, however, quite susceptible to challenge with the parental virus. (The reason why the reverse situation, i.e., vaccination with RV 231-22 did not protect against challenge with RV 101-1, is not understood.) At the time of challenge, vaccinated mice were bled and the sera were then tested for the presence of neutralizing antibodies against CVS-11 and variant viruses. The results, shown in Table 11, juxtapose the mortality ratio vis-a-vis titers of neutralizing antibody. Surprisingly, mice showed as good an antibody response to the challenge virus to which they had become resistant as to the one which caused their death (13).

THE ABILITY OF A STANDARD VACCINE STRAIN TO PROTECT MICE AGAINST CHALLENGE WITH STREET VARIANTS

Since the experimental variants of CVS were unable to protect mice against challenge with either parental virus or other variants, and since street viruses isolated throughout the world have been found in this study to show the presence of at least 11 variant groups, it became necessary to investigate whether standard rabies vaccines (which are produced throughout the world from two or three strains of fixed viruses) will protect against challenge with street virus variants. We chose as challenge inocula for this purpose eight coded samples of street viruses isolated from fatal human cases in the USA since it was possible to obtain these viruses as original human brain isolates and to use the isolates within one or two passages in the laboratory. These eight samples were then tested in a neutralization test with a panel of fourteen antiglycoprotein HAbs (13). This analysis permitted us to group the virus isolates into four groups (see Table 12): Virus isolates 4, 5, 7 comprised group A; 1 and 8 formed group B; 2 and 6 comprised group C; and No. 3 formed group D. Each of the street virus groups differed from the fixed virus used for production of vaccine.

Identification of the origin of the street viruses (Table 13)

TABLE 10
CROSS-PROTECTION OF MICE IMMUNIZED WITH CVS-11 STRAIN OF RABIES OR WITH VARIANT STRAINS DERIVED FROM CVS-11

Vaccine	Challenge virus	Ratio of mice dying after inoculation with challenge virus dilution						Cumulative mortality %
		Virus dilutions (log 10)						
		0	-1	-2	-3	-4	-5	
None	CVS-11	5/5	5/5	5/5	5/5	5/5	0/5	100
	RV 231-22	5/5	5/5	5/5	5/5	5/5	1/5	100
	RV 101-1	5/5	5/5	5/5	5/5	5/5	1/5	100
	RV 240	5/5	5/5	5/5	5/5	5/5	2/5	100
CVS-11	CVS-11	1/6	1/6	2/6	0/6	0/6	0/5	13.3
	RV 231-22	1/6	2/6	0/6	0/6	0/6	0/6	10.0
	RV 240	1/6	1/6	2/6	0/6	0/6	0/6	13.3
RV 231-22	RV 231-22	0/5	0/5	1/5	0/5	0/5	0/5	4.0
	RV 101-1	4/5	2/5	5/5	4/5	1/5	0/5	64.0
	RV 240	3/6	3/6	5/6	5/6	2/6	0/5	72.0
	CVS-11	4/5	5/5	3/5	2/5			70.0
RV 240	RV-240	0/6	0/6	2/6	2/6	0/6	0/6	13.3
	RV-231-22	3/5	2/5	4/6	4/6	0/6	0/6	50.0
	CVS-11	5/6	4/6	5/6	4/6	1/6	0/6	63.3
RV 101-1	RV 101-1	1/5	1/5	2/5	1/5	0/5	0/5	20.0
	RV 231-22	0/5	0/5	2/5	1/5	0/5	0/5	12.0
	CVS-11	2/5	4/5	5/5	2/5	0/5	0/5	52.0

ICR stock mice were vaccinated twice at one-week intervals with 0.5 ml of a 1:50 dilution of a vaccine containing β-propiolactone inactivated CVS-11 or variant virus. Seven days later the animals were bled and challenged i.c. with dilutions of virus.

TABLE 11
PRESENCE OF CROSS-REACTING ANTIBODIES IN SERA OF VACCINATED MICE AT THE TIME OF CHALLENGE

Mice Vaccinated with	Antibody titer of time of challenge and cumulative mortality after challenge with:							
	CVS-11		RV V 231-22		RV 101-1		RV 240-3	
	CM	AT	CM	AT	CM	AT	CM	AT
CVS-11	13	2400	10	800	NT	800	13	800
231-22	70	800	4	800	64	800	72	800
240-3	63	2400	50	2400	NT	2400	13	2400
101-1	52	2400	12	2400	20	6000	NT	2400

CM = Percent cumulative mortality.

AT = Final dilution of serum antibody reducing the number of fluorescent foci of the virus on BHK-21 monolayers by 50%.

TABLE 12
ANTIGENIC DIFFERENCES AMONG STREET VIRUSES ISOLATED FROM FATAL HUMAN CASES

	HAbs	Neutralization of human rabies isolates by hybridoma antibodies				
Group	No. per group	4,5,7 (A)	1,8 (B)	2,6 (C)	3 (D)	Vaccine Virus
A	4	0	+	+	+	+
B	2	0	0	+	+	+
C	2	0	0	+	+	0
D	1	+	0	0	+	0

+ = more than 50% of cells showing fluorescence.

- = no fluorescing cells.

One hundred tissue culture infective doses of virus were mixed with a 1:5 dilution of the respective hybridoma added to BHK-21 monolayers and, after incubation for 3 days at 37°C, the number of foci of virus-infected cells was determined by FA staining.

TABLE 13

ORIGIN OF STREET VIRUSES ISOLATED FROM HUMAN BRAINS[a]

Street No.		Group
4	Oklahoma, 1979, exposure unknown	
5	Maryland, 1976, infection by bat bite[b]	A
7	The same as no. 5	
1	Origin, 1978, exposure unknown; donor of corneal transplant for case no. 8	
8	Idaho, 1978, recipient of corneal transplant from case no. 1	B
2	Texas, 1976, infection by dog bite in Mexico	
6	Texas, 1976, infection by dog bite in Mexico[c]	C
3	Minnesota, 1974, infection by cat bite[d]	D

All strains of virus received through the kindness of Dr. George Baer, Center for Disease Control, Atlanta, Georgia.

[a]From Wiktor, T. J. and Koprowski, H. (1980). J. Exp. Med. in press.

[b]Patient received human rabies immune globulin (HRIG) and 21 doses of vaccine starting from day 2 after exposure.

[c]Patient received HRIG and vaccination after development of symptoms.

[d]Patient received 14 doses of vaccine starting three weeks after exposure.

Note: All other cases were not treated.

was in excellent agreement with the grouping based on the antigenicity of the viral glycoproteins. Thus, viruses 5 and 7 were isolated from the same fatal case in Maryland which developed after a bat bite. The patient had received the full course of treatment with anti-rabies serum and vaccine. Strain No. 8 is one human passage removed from strain No. 1, representing a recipient of corneal transplant from Case No. 1. Our results show complete antigenic relatedness between the two strains. It is worth noting also that antinucleocapsid HAb grouped strains 1, 4, 5, 7 and 8 together, whereas antiglycoprotein HAbs separated these viruses into two groups.

In order to find out whether mice vaccinated with standard vaccine would be protected against challenge with street virus variants and whether cross-protection would reflect antigenic relatedness between vaccine and challenge virus, vaccinated mice were challenged with strain 6 representing group C (closely related) and strain 7 representing group A (more distantly related). The results shown in Fig. 1 indicate that vaccine protected mice against challenge with fixed virus used for the production of vaccine and against street virus of group C, but it protected only partially against challenge with virus of group A since approximately 40% of mice died after challenge.

In summary, the results presented here show that monoclonal antibodies are powerful tools in the recognition of minor antigenic dissimilarities in viral proteins. This, in turn, permits a thorough investigation of viral mutants either occurring in nature or produced experimentally. Biochemical analysis of these mutants may make possible to establish the correlation between primary protein structure and antigenicity of viral proteins. Finally, mutations in the viral genome(s) may be related to antigenic changes relevant to the epidemiology and epizoology of a given virus infection.

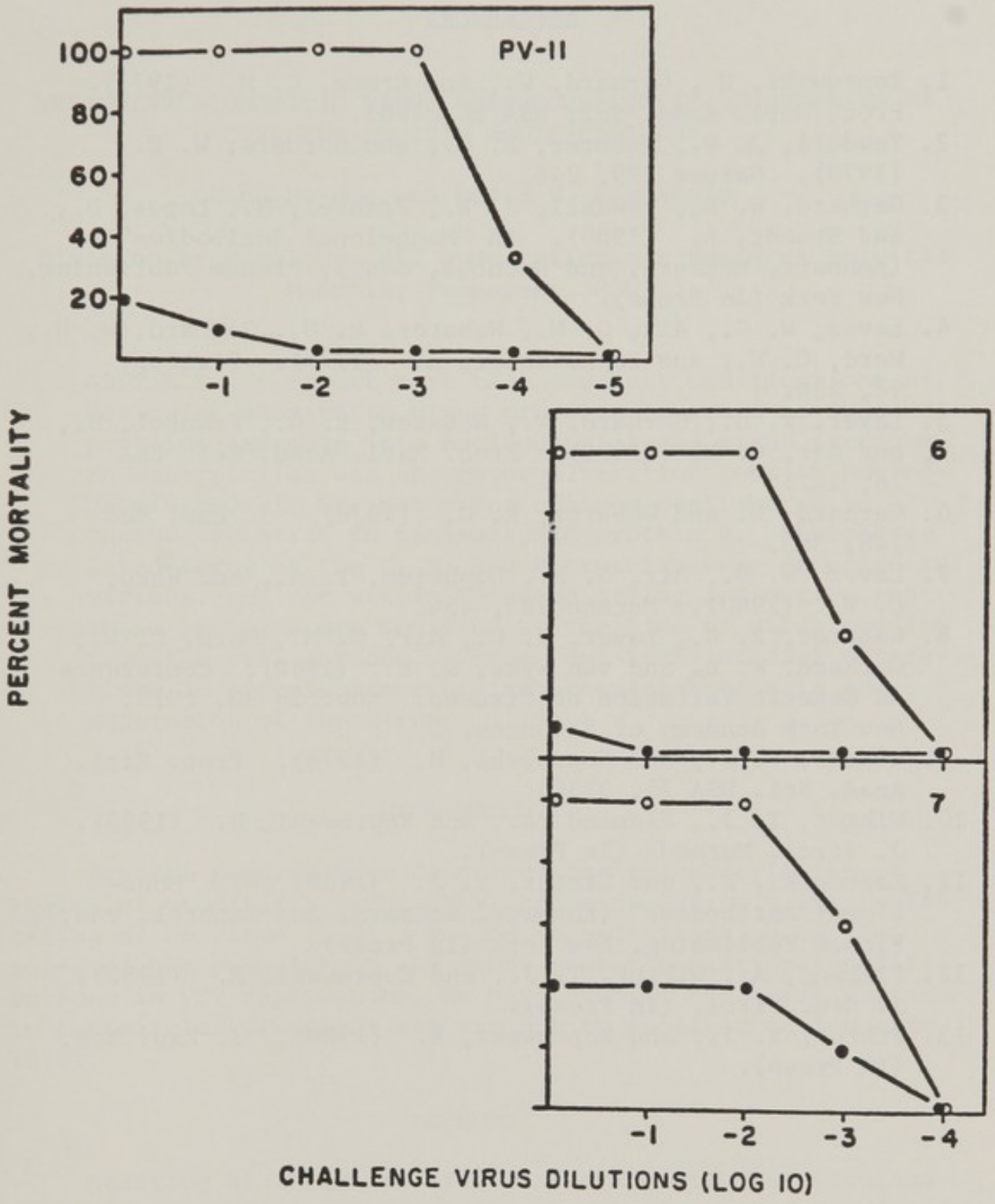

Fig. 1. Protection of mice by vaccine prepared from PV-11 virus against challenge with homologous of virus or with street strains (6,7) of rabies virus. Percent mortality after challenge with indicated virus dilutions. (●), vaccinated mice; (o),unvaccinated controls.

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VESICULAR STOMATITIS VIRUS MORPHOGENESIS IS ACCOMPANIED BY COVALENT PROTEIN MODIFICATIONS[1]

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ABSTRACT We report here that covalent modifications of vesicular stomatitis virus proteins increase as these proteins assemble into nucleocapsids and virus particles. Phosphorylation was the major alteration seen in nucleocapsid protein NS, but other changes contributed to charge variation in nucleocapsid protein N. Phosphorylated forms of the G, N, and M proteins were detected in virions, but not within infected cells, indicating that these proteins are modified at the time of virus budding from the cell surface. These post-translational alterations in protein structure may regulate steps in the maturation of the virus.

INTRODUCTION

Several reports have described the phosphorylation of vesicular stomatitis virus (VSV) proteins during virus replication or *in vitro* (1-4). To add to understanding of the roles of phosphorylation and other post-translational modifications in VSV replication, we have studied the viral proteins in subcellular fractions and virions by isoelectric focusing (5,6).

METHODS

Starting at three hrs after infection with VSV (Indiana strain), BHK cell monolayer cultures were incubated for 4 hrs with [^{35}S]methionine or with [^{32}P]phosphate.

Our standard protocol (7) was used for isolating radiolabeled virus particles and for separating the cells into 3 fractions: a "soluble" fraction (containing Triton X-100-

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solubilized membrane proteins and any other proteins not attached to virus nucleocapsids or to cellular organelles), and two populations of nucleocapsids, a "free" fraction, and a "bound" fraction attached to rapidly-sedimenting cellular structures.

Two-dimensional isoelectric focusing and SDS gel electrophoresis were performed essentially as described before (5,6), except the second-dimension gel was made with 9% acrylamide and 0.45% N,N'-methylene bis-acrylamide. Sample preparation for isoelectric focusing involved preliminary dissolution in sodium dodecyl sulfate (8).

RESULTS

Modified Viral Proteins in Infected Cells and Virions. We have now resolved VSV gene products G, N, NS, and M in two-dimensional separations (Fig. 1), whereas we had only seen species N and G in a previous study (6). Besides two subspecies of N seen before, designated by numerals 1 and 2 in Fig. 1, we have now identified a third electronegative N subspecies designated by numeral 3. There may be an additional electropositive subspecies to the left of N_1, but we have not provided a numerical designation for this less discrete component. The other nucleocapsid protein resolved in these gels is NS, which comprises two subspecies that we designate NS_a and NS_b, the latter being the more electronegative. We use alphabetical subscripts for NS subspecies to avoid confusion with the subspecies that have been separated by other means (4,9,10), since homologies cannot be assumed without direct comparisons. As we described before, glycoprotein G was resolved into a large number of variants by isoelectric focusing; we had shown that this heterogeneity was not caused by differences in sialic acid content, but its actual basis is still unknown (6). Nucleocapsid protein L was not seen, possibly because it has an isoelectric point beyond the range of pH 4-8 employed in our first dimension. The nonglycosylated envelope protein M was not resolved into discrete components, but was spread over a range of alkaline pH values (Fig. 1E). M is thus a rather basic protein, a behavior exhibited also by the analogous "M" proteins of Sendai virus and influenza virus (6,11).

Figure 1 also shows that the degree of modification of each viral protein depends on its location within a cellular compartment or within virions. Envelope proteins M and G were recovered only in the "soluble" fraction, because cell membranes were dissolved by the Triton X-100 used for cell disruption (Fig. 1B). Comparison with panel A, which shows the soluble fraction from uninfected cells, assists recognition of these virus-specific proteins. The nucleocapsid

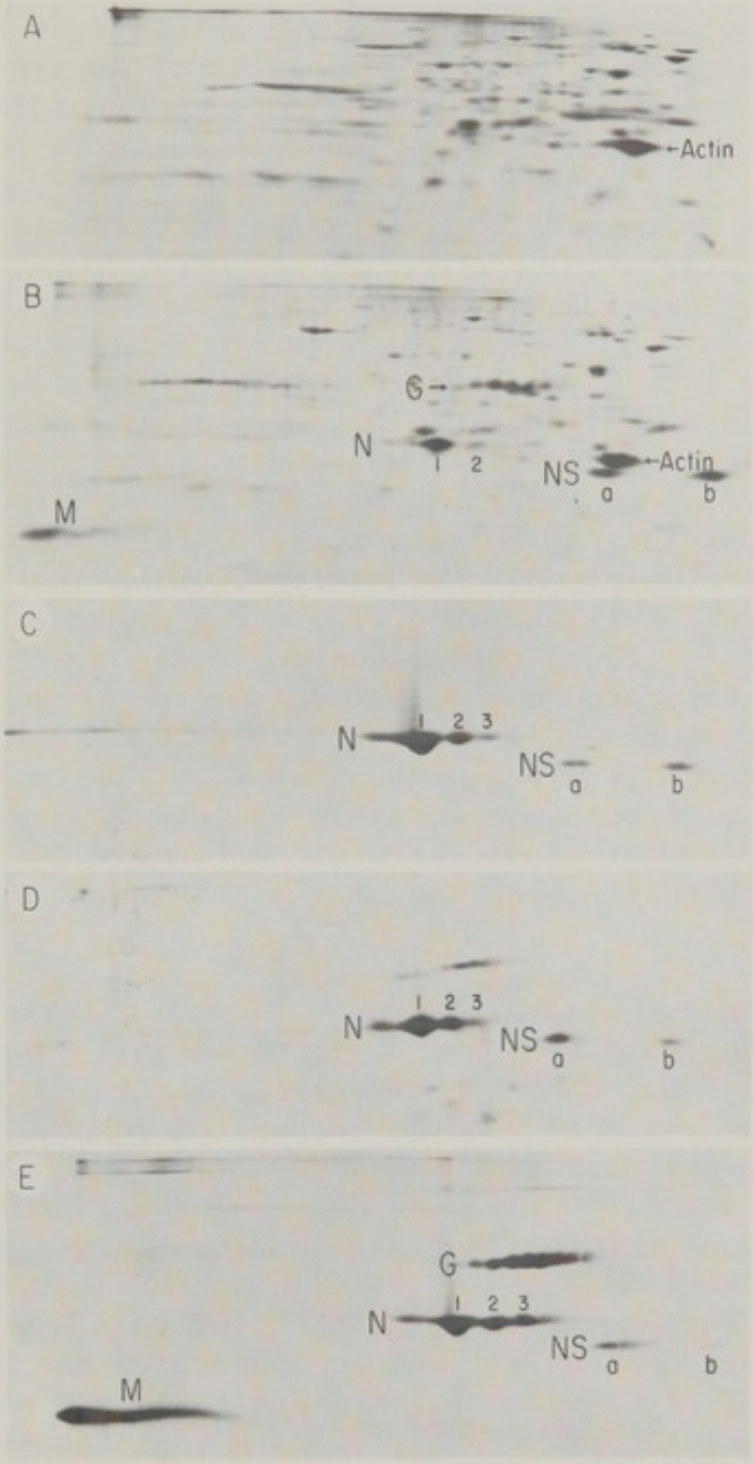

FIGURE 1. Two-dimensional separation of [^{35}S]methionine-labeled VSV proteins synthesized by BHK cells. In all figures, the isoelectric focusing dimension is horizontal, from pH 8 on the left to pH 4 on the right. Autoradiograms of dried polyacrylamide gels are shown. (A) Soluble proteins from uninfected BHK cells; (B) soluble proteins from infected cells; (C) free nucleocapsids from cells; (D) bound nucleocapsids released from cellular organelles by salt extraction (7); (E) virions.

proteins N and NS can also be identified in the soluble fraction: N displays less isoelectric complexity here than in virions, whereas the a and b components of NS are represented in both compartments. The two NS components migrated just ahead of the prominent cellular protein actin in the second dimension (7). The abundance ratio of NS_a to NS_b in the soluble fraction was unity, contrasting with the predominance of NS_a in virions (Fig. 1B,E).

In nucleocapsids from both "free" and "bound" cellular fractions, the major structural protein N was more heterogeneous than it was in the soluble compartment, but N was most heterogeneous in virions (Fig. 1C-E). In the freely sedimenting nucleocapsids (Fig. 1C), NS_a and NS_b were present in equal amounts, similar to their proportions in the soluble compartment. In contrast, NS molecules in the bound nucleocapsids (Fig. 1D) were mainly of the NS_a type, which was also the predominant type in virions (Fig. 1E). Few cellular proteins contaminated the bound nucleocapsids, because we used salt extraction to separate nucleocapsids from the large amounts of cellular structures co-sedimenting in this fraction (7).

In summary, Figure 1 demonstrates considerable charge heterogeneity of both nucleocapsid and envelope proteins of VSV, and the number of variants of proteins G, N, and M increased as these proteins associated with nucleocapsids and virions.

Patterns of Phosphorylation. Autoradiograms of [^{32}P]-phosphate-labeled proteins from infected cells were markedly simplified, compared to the ^{35}S-labeled proteins (Fig. 2). NS was the only virus protein that was phosphorylated in any of the three cellular fractions. Relative amounts of radioactive phosphate in subspecies NS_a and NS_b resembled the relative amounts of protein in these two forms revealed by [^{35}S] methionine labeling. In the soluble compartment, components NS_a and NS_b were equally represented, whereas NS_a became increasingly predominant in free nucleocapsids and bound nucleocapsids (Fig. 2A-C). In virions (Fig. 2D), NS_b was not seen at all. Thus, the ^{32}P results confirm that the NS_a/NS_b composition of bound nucleocapsids is like the NS_a/NS_b composition of virions, whereas the proportions of NS subspecies in free nucleocapsids resemble the proportions in the soluble pool more closely.

After long autoradiographic exposures, a phosphorylated form of the structural nucleocapsid protein N was detected in virions. This species corresponded in isoelectric position to component N_3 seen among the ^{35}S-labeled proteins (Figs. 1E, 2D). Evidently, a post-translational modification other than phosphorylation generates the other electronegative subspecies

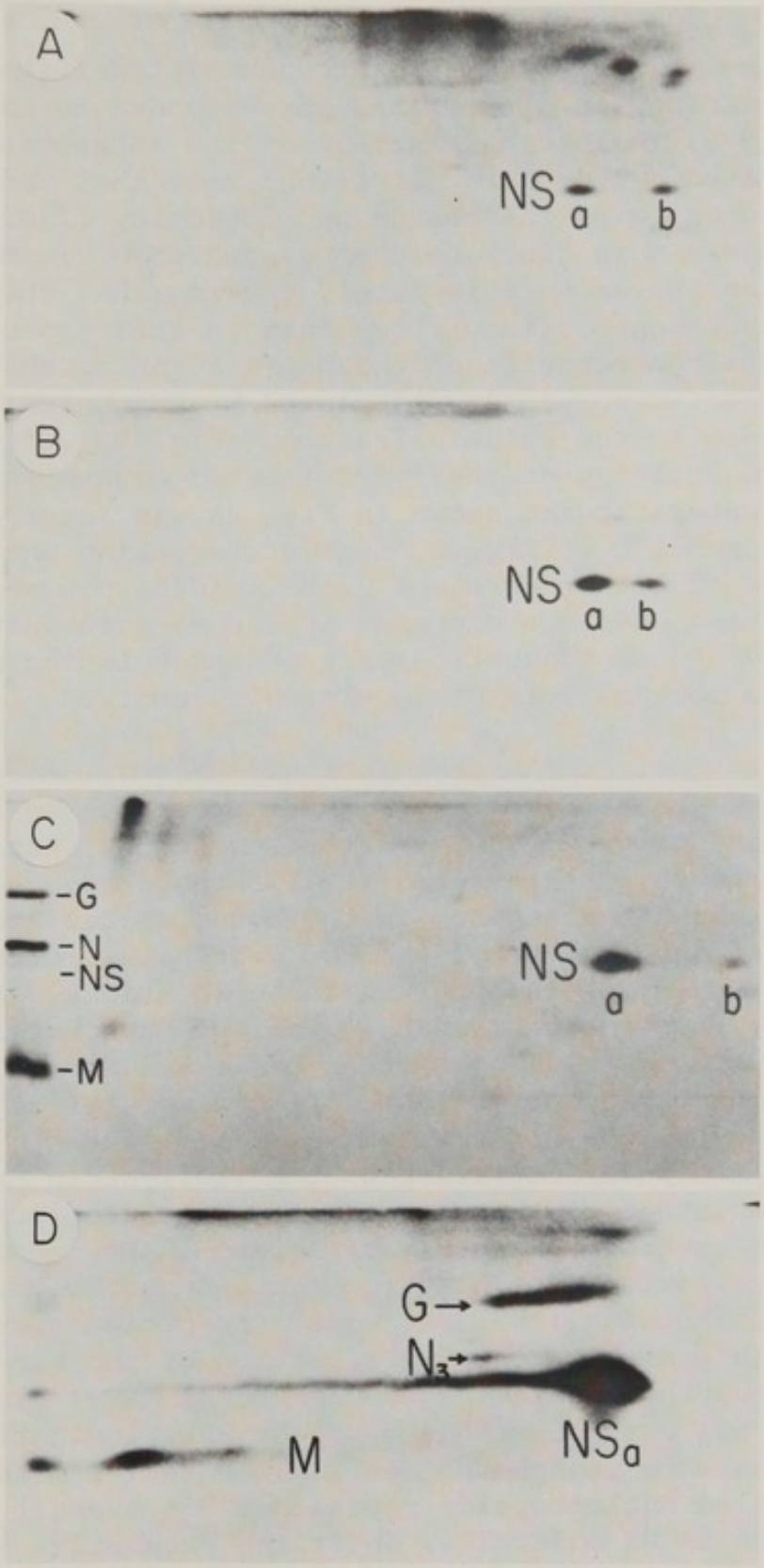

FIGURE 2. Phosphorylated VSV proteins made by BHK cells. Shown are autoradiograms of two-dimensional gels of [^{32}]phosphate-labeled samples, all from infected cells. (A) Soluble proteins; (B) free nucleocapsids; (C) bound nucleocapsids; (D) virions.

of N, designated N_2 (Fig. 1E).

Also seen in virions were phosphorylated forms of M and G. The distribution of ^{32}P in G corresponded to the distribution of ^{35}S, indicating that all of the subspecies of G are phosphorylated. Therefore, it is unlikely that variable phosphorylation explains the charge heterogeneity of G. Phosphate in M was more discretely separated into two major components than the amino acid label, showing that this protein is more heterogeneous in virions than in infected cells and suggesting a contribution of phosphorylation to that heterogeneity. Furthermore, phosphorylated M and phosphorylated G were not seen in the soluble fraction (Fig. 2A), despite clear representation of their ^{35}S-labeled counterparts (Fig. 1B). The autoradiogram shown in Fig. 2A was later exposed as heavily as panel D of Fig. 2, without revealing phosphorylated forms of either envelope protein (data not shown). Phosphorylated envelope proteins appear to accumulate exclusively in virions, suggesting that phosphorylation of these proteins is somehow related to virion assembly.

The State of NS Phosphorylation. The evidence presented above demonstrates progressive increases in isoelectric heterogeneity of G, M, and N, depending on the state of association of these proteins with viral structures. In contrast, NS became less heterogeneous in the bound nucleocapsid and virion fractions, as one of the two subspecies, electronegative NS_b, tended to disappear in those locations. Taken at face value, this suggests that an initial phosphorylation of NS molecules, occurring soon after their synthesis, is reversed during virion morphogenesis. However, as we now show, the opposite is true.

To relate phosphorylation to the isoelectric properties of NS components a and b, we treated ^{35}S-labeled nucleocapsids with bacterial alkaline phosphatase (BAP), an enzyme capable of cleaving phosphoserine and phosphothreonine ester linkages in proteins (12). We added the protease inhibitor Aprotinin (Sigma) to prevent destruction of the viral proteins by a protease that contaminates commercial BAP. Figure 3 shows that NS_a was converted to NS_b by BAP, indicating that NS_a contains more phosphate than NS_b. Parallel measurements of ^{32}P-labeled nucleocapsids revealed a 75% overall decrease of phosphate in NS molecules, under the exhaustive digestion conditions used in Fig. 3C. The specific activity ($^{32}P/^{35}S$) of NS_a remained constant, indicating that a fraction (25%) of NS_a molecules is completely resistant to BAP. At the same time, the specific activity of NS_b decreased four-fold, confirming that NS_b is less phosphorylated than NS_a. It follows

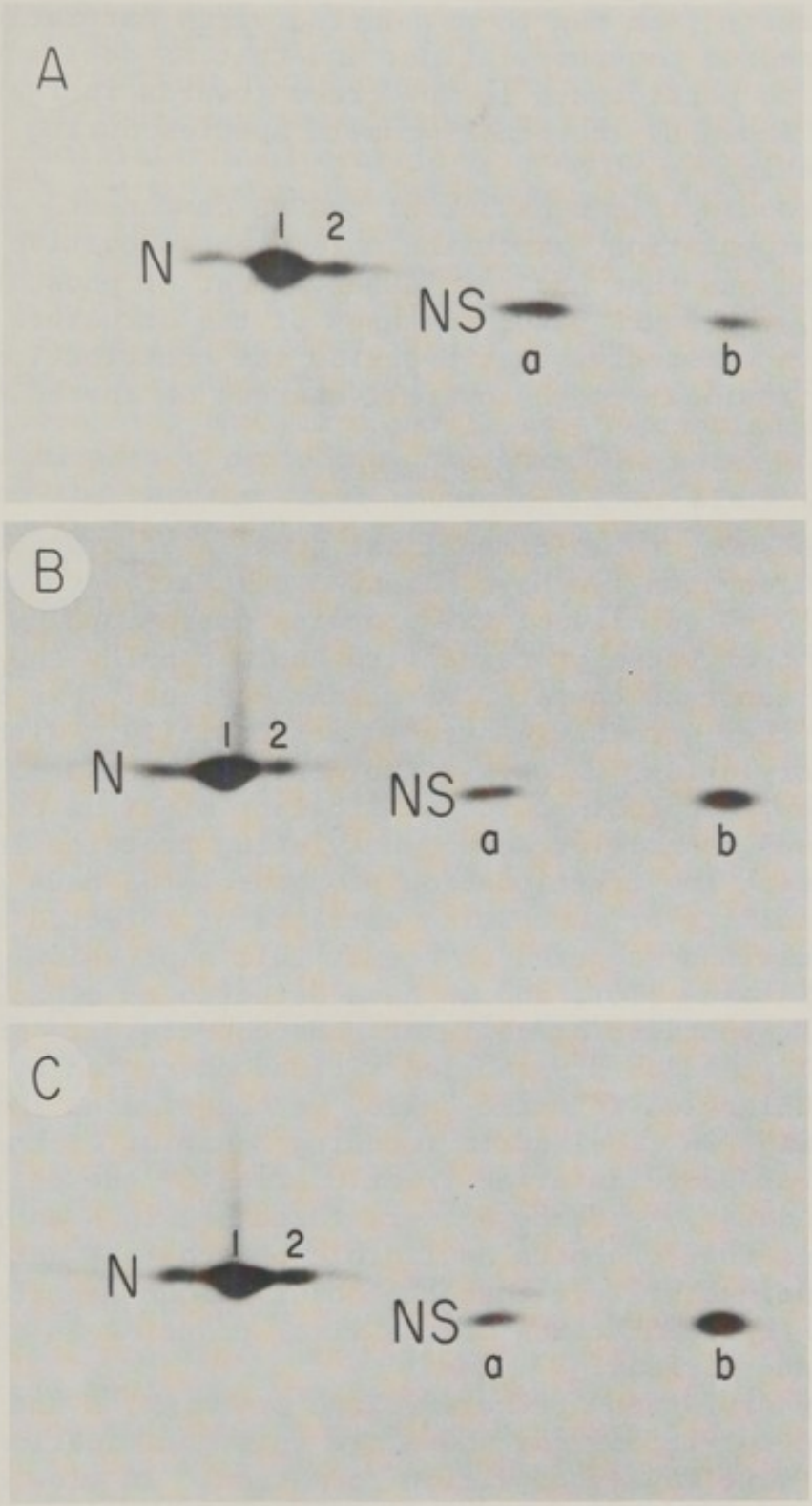

FIGURE 3. Conversion of NS_a to NS_b by dephosphorylation. [^{35}S]methionine-labeled free nucleocapsids from BHK cells were incubated for 1 hr at 37°C with 2 units of the protease inhibitor Aprotinin (Sigma) per ml and with *E. coli* alkaline phosphatase (Worthington) before separation in two-dimensional gels. (A) Untreated control; (B) 25 µg of alkaline phosphatase per ml; (C) 100 µg of alkaline phosphatase per ml.

that the shift from NS_b to NS_a during virus maturation represents *increased* phosphorylation, and that NS does not fail after all to participate in the trend towards increasing modification shown by the other protein species during virion morphogenesis.

The isoelectric behavior of the NS components was contrary to expectation, since the more electronegative species NS_b was the one that had the lesser amount of phosphate. Evidently, there are other features of the structure of these two protein subspecies that override the contributions of phosphate residues to the overall charges on the molecules.

DISCUSSION

By the use of two-dimensional protein separation and cell fractionation, we have observed cumulative covalent modifications of 4 species of VSV proteins corresponding to the association of these proteins with nucleocapsids and virions. Before we can take these observations seriously, we must be sure that they were not generated artificially. With respect to phosphorylation, which was the most widespread modification that we saw, a potent source of artifact might be cellular phosphatases capable of dephosphorylating proteins in cell extracts during the fractionation procedure. We have evaluated this possibility by incubating extracts of infected BHK cells under conditions reported to permit full expression of cellular phosphatases (10), and we have detected no dephosphorylation of VSV-specific or cellular phosphoproteins. In addition, when we added ^{32}P-labeled virions disrupted by Triton X-100 to unlabeled infected cells, we observed no loss of label or change in isoelectric focusing behavior of the radioactive viral proteins after fractionation of the cells (unpublished data). Perhaps we were fortunate in a choice of cell system that exhibits negligible phosphatase activity. In any case, we believe that the observations reported here are valid representations of the viral proteins as they exist in cells and virions.

Phosphorylation of the envelope proteins, M and G, was restricted to virions and therefore this modification may be linked in some manner to virion formation. In view of the existence of a protein kinase in VSV particles (2,3), it might be questioned whether phosphorylation of G and M actually occurs before virus release; instead, the phosphorylation might result from the action of the kinase within the completed virions. We have found no increase in the specific ^{32}P-activity of intact pulse-labeled virions after extensive incubation at 37°C (unpublished data), which seems to rule out this explanation. We are left with the interesting possibility that there is a cause-effect relationship between

phosphorylation of one or more of these proteins species and budding of virus from the cell membrane.

Modifications of nucleocapsid proteins N and NS were not limited to virions, but were also seen in the soluble pool and in intracellular nucleocapsids. However, nucleocapsid-associated N and NS molecules exhibited more extensive modifications than their free counterparts, and incorporation of nucleocapsids into virions was accompanied by still more alterations. At each of these steps in virion morphogenesis, regulatory roles for covalent modifications are conceivable. The hyperphosphorylated form NS_a was predominant in both bound nucleocapsids and virus particles, suggesting that this form is required for productive interactions between nucleocapsids and the nascent viral envelope. Ever since we observed that nucleocapsids of Sendai virus and VSV separated into free and bound fractions, we have been seeking such evidence of a structural or functional difference between these fractions (7,13). Aside from morphogenesis, NS is known to play one or more roles in viral RNA synthesis, and the phosphorylation state of the protein might well have regulatory consequences for that kind of activity (4,14).

Clinton *et al.* (4,10) have resolved two phosphorylated NS subspecies, designated NS_1 and NS_2, by electrophoresis. These authors observed both species in the soluble protein fraction from infected cells and in a nucleocapsid-free fraction from virions, but only NS_1 was seen in nucleocapsids derived from either source (4). This resembles our results, which revealed a preponderance of one component (NS_a) in virions. However, these authors concluded that NS_1 was *less* phosphorylated than NS_2, which did not associate with nucleocapsids. In contrast, our data indicate that the major form of NS in virions, NS_a, is the more highly phosphorylated form. These differences may depend on the procedures used to separate the variant protein molecules. In this regard, it is important to bear in mind that any single method of protein separation has limitations, and there may be more complexity in these viral proteins than has been revealed so far. In addition, the anomalous isoelectric behavior of our two NS subspecies underscores the need for better understanding of the structural basis of that separation. It seems that a variety of analytical approaches will be required to define the structural bases and functional significance of these post-translational modifications in viral proteins.

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UNCOUPLING OF THE HEMAGGLUTINATING AND NEURAMINIDASE ACTIVITIES OF NEWCASTLE DISEASE VIRUS (NDV)[1]

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ABSTRACT We have isolated and characterized revertants of an RNA$^+$ temperature-sensitive (ts) mutant of the virulent strain Australia Victoria (AV) of NDV. Among the stable isolates was a revertant with wild-type levels of hemagglutinating activity (HA) but with only 3% of the neuraminidase activity (NA) of strain AV. The uncoupling of HA and NA in this revertant raises the possibility that the activities occupy different sites on the HN glycoprotein. The deficiency in the NA of this revertant did not impair its reproduction in cultured chicken embryo (CE) cells not its virulence *in ovo*.

INTRODUCTION

The envelope of the avian paramyxovirus NDV is derived from the cellular plasma membrane during assembly. The envelope contains at least two antigenically (1) and physically (2) distinct populations of external projections. One population is composed of hemagglutinin-neuraminidase (HN) glycopolypeptides that are involved in agglutination of erythrocytes, attachment to cellular receptors, and elution of virions from mucins and cellular receptors accompanied by the release of neuraminic acid. The other population contains the disulfide-linked fusion glycopolypeptides F_1 and F_2 (3). These $F_{1,2}$ projections are required for hemolysis, penetration of host cells, and the cellular fusion activity associated with virions (fusion from without) (4). Cellular surfaces that have accumulated HN and $F_{1,2}$ proteins during infection also acquire hemadsorbing, neuraminic acid-cleaving, and cell-fusing activities (fusion from within, FFWI) (5).

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The fine structure of the external projections of virions is unknown. It is not known whether the HN glycopolypeptides are arranged in two physically similar types of spikes, each having only one activity, or whether HN assembles into bifunctional spikes with either overlapping active sites or separate ones. The tendency of the glycoproteins to aggregate following the dissolution of the envelope has added to the difficulties in analyzing the structure of the projections. It is known that the HA and NA of NDV have similar rate constants for thermal inactivation (6), an observation which lends support to the existence of a single population of bifunctional spikes. And some evidence of two active sites comes from immunological studies in which HA and NA appear to occupy antigenically distinct sites (7, 8).

We have obtained data which bear on the arrangement of active sites for HA and NA and on the role of the latter activity in viral infections through the isolation and characterization of revertants of an RNA^+ ts mutant originally isolated by J.E. Tsipis (9). In our hands the Cl mutant, a member of complementation group C, formed plaques and infectious virions at 37.5°C (permissive temperature) in cultured chicken embryo (CE) cells but not at 42.5°C (nonpermissive temperature). Both Cl virions and the plasma membranes of infected cells had reduced levels of HA and NA compared to the wild-type strain AV at 37.5°C and 42.5°C. In addition cultures infected by Cl did not undergo measurable levels of FFWI at either temperature.

Starting with Cl we isolated a series of revertants which regained in discrete steps plaque-forming ability and the capacity to produce infectious virus at 42.5°C. Analyses of the revertants included measurement of the biological activities of purified virions and of host cell surfaces, comparisons of viral proteins on polyacrylamide gels, cytopathic effects in culture, mean embryo death times, growth yields, and plaque formation.

RESULTS

Isolation, Growth Characteristics, Virulence, and Cytopathology of Revertants. The Cl ts mutant appeared to carry multiple lesions since it had deficiencies in functions of both the F and HN genes. Our strategy for identifying the individual lesions was to isolate a series of spontaneous revertants from populations of Cl. The phenotype originally used to isolate Cl was the capacity to form plaques at 37.5°C but not at 42.5°C. To isolate revertants

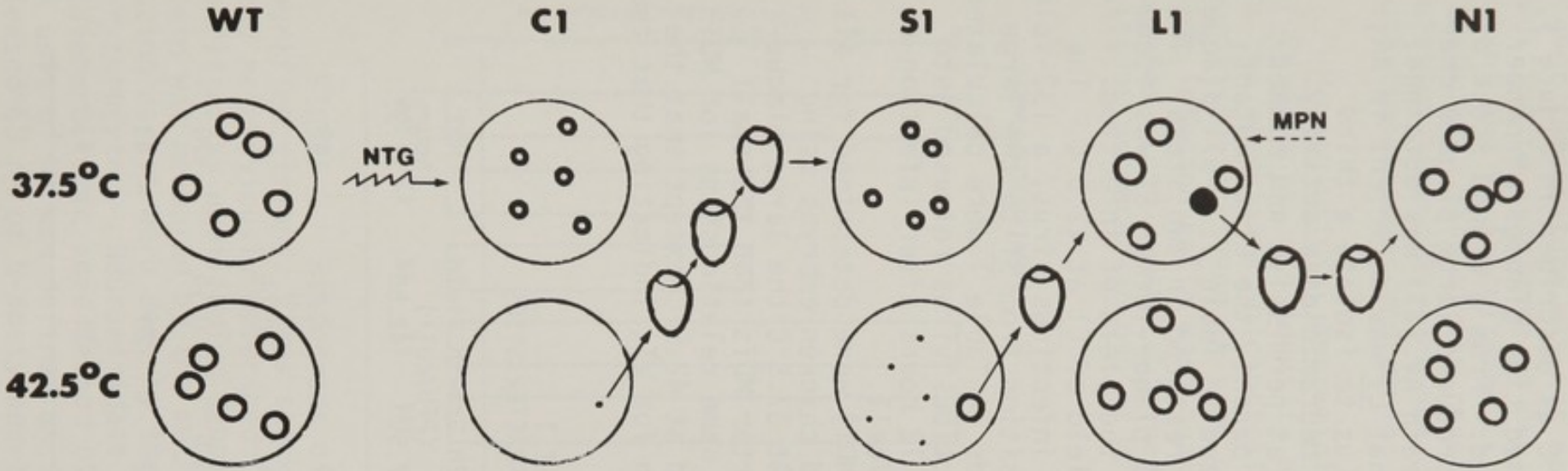

FIGURE 1. Diagram of procedure for isolation of revertants of ts C1. Relative sizes of plaques formed by each virus on CE cells incubated at permissive (upper row) and nonpermissive (lower row) temperatures are shown. Confluent secondary cultures of CE cells prepared according to published methods (10) and cultivated in NCI medium (Gibco) supplemented with 5% calf serum were used. For plaque assays infected cultures were incubated for 3 days at 37.5°C or 42.5°C in NCI medium supplemented with 5% calf serum and 0.8% agarose. The C1 mutant was originally derived from a cloned stock of strain AV which had been mutagenized with nitrosoguanidine (NTG) (9). The agarose plug above a spontaneously-occurring small plaque was picked from a C1 plaque assay at 42.5°C. The virus was eluted from the plug and injected into an embryonated chicken egg (Θ) for amplification. The virus (S1) was plaque-purified between egg passages. A second-step revertant (L1) was derived from S1 using a similar procedure. Revertants (N1) which had regained NA were obtained from L1 using a chromogenic neuraminidase substrate 2-(3' methoxyphenyl)-N-acetyl-α-neuraminic acid, MPN, which was added to the agar overlay (11). Dark red plaques (●) indicative of neuraminidase activity were picked from a plaque assay of L1 and cloned.

we screened the C1 stock for viruses which had regained plaque-forming ability at 42.5°C as described in Figure 1. A stable revertant S1 that formed tiny plaques at nonpermissive temperature was isolated. From a cloned stock of S1 a second revertant L1 that produced large plaques characteristic of the wild-type strain AV (WT) was obtained. Subsequent analyses revealed that L1 was deficient in NA, a characteristic which enabled us to isolate a third revertant N1 that had regained neuraminidase activity.

The relative plaque sizes at permissive and nonpermissive temperatures of the WT parent, the C1 ts mutant, and the revertants are summarized in Table 1. Full plaque-forming capacity at 42.5°C was restored in two steps. The transition from C1 to the small plaque-former S1 was accompanied by a 3-fold increase in the yield of infectious virus in CE cell cultures over the yield of C1 at 42.5°C. The main recovery in production of infectious virus, a 133-fold increase, occurred in the transition from S1 to the large plaque-former L1. Growth yields in Table 1 were calculated from plaque assays of the infectious virus released into the medium of CE cell cultures 12 hours after infection at an input multiplicity of 3 PFU/cell.

Mean embryo death times (MDT) were determined for all of the revertants in 10-day old chicken embryos using established procedures (12). At 37.5°C the large plaque-formers WT, L1, and N1 had shorter MDTs than the small plaque-formers C1 and S1. The same relationships for MDT among the viruses were obtained at 42.5°C except that the death times were prolonged even for WT, indicating that the

TABLE 1
PHENOTYPIC CHARACTERISTICS

Virus	Relative Plaque size		12h Growth Yield (PFU/cell)		MDT (hours)
	37.5C	42.5C	37.5C	42.5C	37.5C
WT	○	○	11	2.6	43
C1	o		4.2	0.05	75
S1	o	∘	4.6	0.15	70
L1	○	○	12	20	43
N1	○	○	12	11	43

higher temperature was not optimal for the infection. In addition to virulence *in ovo*, the cytopathic effects of each mutant and WT in cultured CE cells were examined by light microscopy. All of the revertants, WT, and Cl caused vacuolation, rounding, and detachment of cells from plates at both temperatures and during similar time intervals.

Hemagglutinin, Neuraminidase, and Hemolytic Activity of Revertants. To identify additional phenotypic changes in the revertants, biological activities associated with purified stocks and in some cases activities associated with infected cells were quantified. The data shown in Figures 2 and 3 were obtained from stocks of purified virions produced *in ovo* at 37.5°C. The assays of virion-

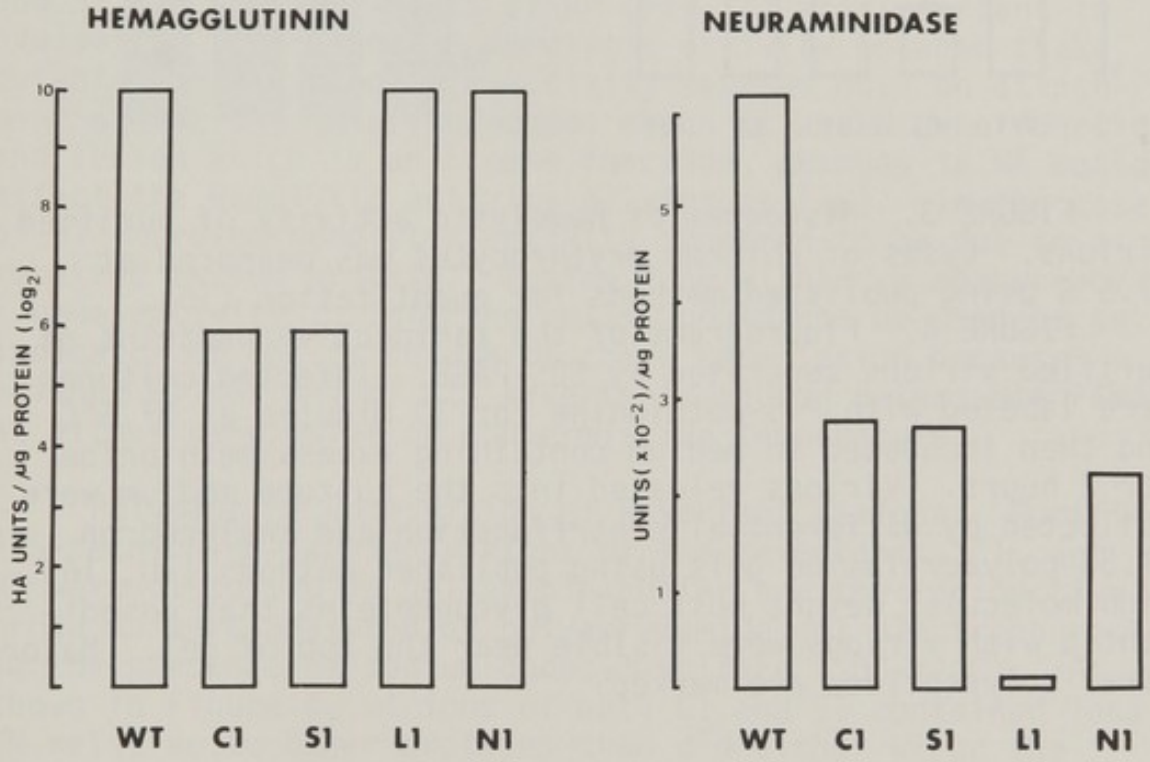

FIGURE 2. Histograms of HA and NA associated with purified virions. Virus stocks were prepared and concentrated as previously described (10). All stocks were further purified by equilibrium density ultracentrifugation. Protein was measured by the method of Lowry (13). Hemagglutination assays were done by the pattern method using chicken erythrocytes. Neuraminidase assays were done by the thiobarbituric acid method using fetuin as a substrate (14). A unit of NA was defined as the amount of enzyme that released 60 nM of N-acetylneuraminic acid per hour at 37.5°C.

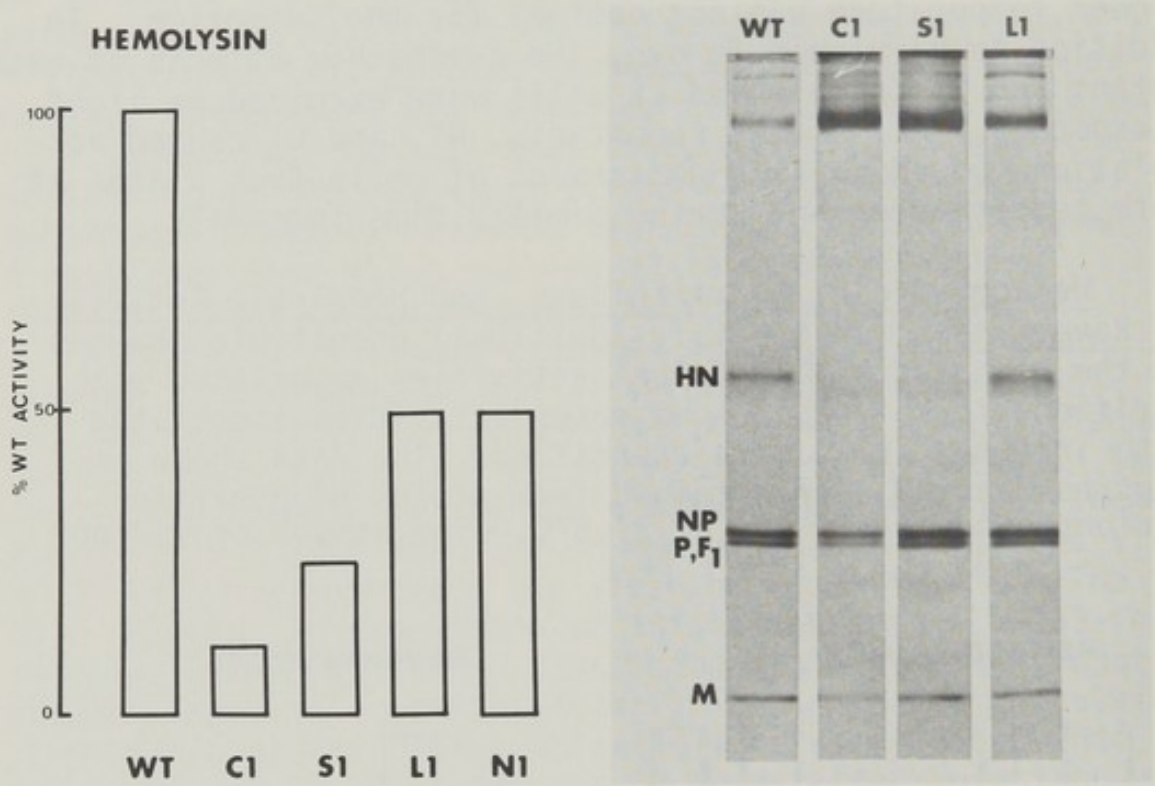

FIGURE 3. Histogram of hemolytic activity of purified virions. Lysis of chicken erythrocytes was measured at 37.5°C using published methods for quantitation (15).

FIGURE 4. Fluorograms of the radioactive proteins of purified virions separated by SDS-PAGE. Infected cultures were labeled with ^{35}S-methionine for 30 minutes at 37.5°C and then incubated in medium containing excess methionine for 2 hours. Virions released into the culture medium were collected by differential centrifugation and analyzed on 11.5% polyacrylamide gels using published methods (10, 16). High molecular weight host cell glycoproteins that cosedimented with virions were visible near the top of gel. Major viral polypeptides are marked.

associated activities were more sensitive and reproducible indicators of changes in phenotypes than assays of infected cultures; however, the latter system allowed evaluation of the temperature-sensitive character of the phenotypes.

The levels of HA and NA in stocks of purified virus were measured at 37.5°C (Figure 2). C1 and S1 were deficient in both activities relative to WT even at the permissive temperature. In contrast L1 regained wild-type levels of HA but lost additional NA. The N1 revertant that was selected on the basis of neuraminidase function showed a 10-fold increase in NA over that of L1, which was a 35% recovery of wild-type activity. To evaluate the ts nature

of these phenotypes, the surfaces of CE cells that had been infected for 10 hours at 37.5°C and 42.5°C were assayed. Instead of HA the hemadsorbing capacity of infected cells, which is another measure of HN gene activity, was assayed by published methods (17). Regardless of temperature, results similar to those shown in Figure 2 for virions were obtained. Cells infected by Cl and Sl had about 30% of the levels of hemadsorption and NA measured in WT-infected cultures. Little NA could be detected on Ll-infected cells at either temperature, and Nl showed about a 30% recovery of NA relative to WT.

As a measure of F gene activity, the hemolytic activity of the revertants was compared to WT (Fig. 3). The hemolytic activity associated with Cl virions was 12% of WT levels at permissive temperature. The hemolytic activity of the Sl revertant was twice that of Cl. The fact that both Cl and Sl had similar levels of HA (Fig. 2) was important in evaluating the change in hemolytic activity between these mutants. Since hemolytic activity depends both on attachment of virions to erythrocytes which is an HN gene function and fusion which is an F gene function, changes in HA could affect the hemolytic activity of virions (18). In the case of Sl improved hemolytic activity appeared to be due to a repaired fusion protein. Fusion from within, a measure of F gene activity in infected cells, could not be used as an indicator of the ts nature of the F gene lesion because neither Cl nor any of its revertants caused detectable FFWI at either permissive or nonpermissive temperature.

Polypeptide Composition of Revertant Virions. Purified, ^{35}S-methionine-labeled virions of the revertants, Cl, and WT were dissolved in gel sample buffer and the radioactive proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). As shown in Figure 4, virions of both Cl and Sl contained less HN relative to other proteins than did either WT or the large-plaque revertant Ll. The amount of radioactive HN in Cl and Sl virions was about 20-35% of the level in WT or Ll, based on quantification of the radioactivity in the gel by liquid scintillation counting. This reduction in the number of HN molecules in virions correlated with the 60-70% reduction in NA of Cl and Sl (Fig. 2). Therefore the deficiency in NA of these mutants was due to reduced accumulation of HN glycopolypeptides in virions and on the cell surface and not to major changes in the specific activity of the enzyme.

DISCUSSION

Our analyses of the revertants indicate that the original ts C1 mutant probably carried lesions in both the F and HN genes. A 2-fold increase in hemolytic activity of the revertant S1 signaled the repair of the $F_{1,2}$ glycoprotein. The reverting mutation in S1 did not affect activities directed by the HN gene; like C1, the S1 revertant had lower NA and HA than wild-type. The extent of recovery of $F_{1,2}$ activity in S1 is not known since our hemolysis assay required both an attachment function which is impaired in S1, and a fusion activity. Sufficient $F_{1,2}$ activity was regained to confer wild-type reproductive properties on revertants L1 and N1, both of which had regained HA completely and had recovered 3% and 35% of wild-type levels of NA respectively. Recovery of $F_{1,2}$ activity was also accompanied by partial recovery of plaque-forming ability and a 3-fold increase in virus yield at 42.5°C. An active fusion protein is required for both plaque formation and infectivity (19).

Analyses of the polypeptide composition of C1 virions coupled with assays of HA and NA showed that the mutant was deficient in these activities because it contained approximately one-third of the HN glycopolypeptides found in WT virions. The synthesis of HN glycopolypeptides in C1-infected cells was similar to WT (unpublished observations). Therefore the cause of the dearth of HN in C1 virions could have been metabolic instability of the glycopolypeptides, impaired maturation, or an assembly problem. The defect in the accumulation of HN was repaired in the second-step revertant L1. This revertant completely regained HA, and WT levels of HN glycoproteins accumulated in virions. However, the conformational change that must have occurred in the HN glycopolypeptide to stabilize it or to relieve the block in assembly destroyed most of the NA of L1.

Despite the fact that L1 had only 3% of WT levels of neuraminidase activity, it made large plaques and produced 8-fold higher yields of infectious virus in CE cells at 42.5°C than did WT. Perhaps most surprising, L1 was as virulent in chicken embryos as WT and had similar cytopathic effects in CE cells. Recovery of WT-levels of HA appeared to be the crucial factor in increasing virulence *in ovo*. For NDV strains, good but not absolute correlations exist among plaque size in CE cells, virulence in embryos, and virulence in adult birds (20, 21). Our revertants showed the expected increases in virulence as plaque sizes increased. Previous attempts at similar correlations between NA and virulence yielded contradictory results (22,

23). In the present study we found no correspondence between NA and either virulence *in ovo* or cytopathogenicity. At least 97% of virion-associated NA was dispensable in *L1*, and modulation of NA over a broad range among *L1*, *N1*, and *WT* had no effect on MDT for embryos.

Studies of ts mutants of influenza virus (24) and Sendai virus (25) showed that NA is not required for assembly of virions. However influenza mutants deficient in NA aggregated and had low HA and low infectivity which was not true of the *L1* revertant of NDV. The Sendai mutant ts 271 was deficient in HN glycoproteins and mutant-infected cells experienced much less cytopathology than wild-type infections. By analogy with *L1* which had normal HA, very little NA, but still was cytopathic, we suggest that loss of NA may not have been the significant factor in decreasing the cytopathology of the Sendai mutant.

An essential role for neuraminidase in natural infections remains to be defined. In birds NDV may require NA to avoid respiratory mucins and unfavorable reproductive environments such as erythrocytes, factors which are of no consequence in cell culture and perhaps not very important *in ovo*.

The fact that both NA and the accumulation of HN in virions are affected in the *L1* revertant argues against a conservative change in the enzymatic site. It is likely that the reverting lesion caused a significant conformational change in the HN glycopolypeptide resulting in the loss of NA and the restoration of normal amounts of HN in *L1* virions. The uncoupling of HA and NA in this revertant is consistent with the existence of different sites for these activities on the HN glycoprotein. In addition the isolation of the neuraminidase revertant *N1*, which should carry at least three lesions in its HN gene barring same-site and pseudo-reversions, provides evidence that HA and NA can be independently modulated.

To assess the integrity of the attachment sites on the HN glycoproteins of *L1* we are currently determining the first order rate constants for the attachment of *L1* and *WT* virions to cellular receptors and the kinetics of neutralization of their respective infectivities by anti-*WT* sera. These experiments will provide more rigorous tests than HA for the possibility that NA and attachment functions occupy different sites on the HN glycoprotein.

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SUPPRESSION OF TEMPERATURE-SENSITIVE PHENOTYPE IN REOVIRUS: AN ALTERNATE PATHWAY FROM ts TO ts^+ PHENOTYPE

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ABSTRACT Backcross analysis of a revertant of a ts mutant of reovirus has demonstrated that the revertant contains an extragenic suppressor mutation and is therefore a suppressed pseudorevertant. Studies are summarized which show that (a) extragenic suppression is the most common pathway from ts to ts^+ phenotype in reovirus. (b) Nonparental ts mutations can be isolated from reovirus pseudorevertants and some of these mutants represent new recombination groups. (c) Suppressed ts lesions can complicate the mapping of ts lesions, but the ability to identify suppressed recombinant mapping clones makes unambiguous mapping possible. (d) The new ts mutant groups tsH, tsI and tsJ, isolated from suppressed pseudorevertants, map to genome segments M1, L3 and S1, respectively. (e) Suppressed pseudorevertants can be rapidly and reliably identified. (f) Extragenically suppressed virus can be shed from cells persistently infected with a ts mutant of reovirus. (g) Extragenic suppressor mutations in pseudorevertants of reovirus ts mutants can be mapped. In addition, models for the mechanism of suppression in reovirus are presented and discussed. These results indicate that it is critical to distinguish between ts^+ phenotype and ts^+ genotype when revertants of animal virus mutants are used in biochemical or genetic studies.

INTRODUCTION

Conditional-lethal mutations are useful tools for the study of virus replication and genetics. The growth of conditional-lethal mutants under nonpermissive conditions provides an absolute selection for reverse mutation or reversion. Revertants may not be true revertants but may represent the acquisition of second, suppressor mutations that bypass the original defect (1-3). In many systems, these suppressor mutations are often in genes other than the one with the original mutation and the revertants are more properly termed extragenically suppressed pseudorevertants. The process of reversion has not been studied in detail in animal virus systems. Here, studies of reversion in reovirus temperature-sensitive (ts) mutants are briefly

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reviewed. These studies reveal that: (a) ts mutations can be suppressed by extragenic suppressor mutations. (b) Suppression by extragenic suppressor mutations is the most common pathway from ts to wild type (ts^+) phenotype in revertants of reovirus ts mutants. (c) Nonparental ts lesions are rescued from suppressed pseudorevertants at high frequency following backcross to wild type. (d) Suppression can complicate the interpretation of mapping data if some of the recombinant progeny contain suppressed ts lesions. (e) Suppression of ts phenotype can occur in cells persistently infected with ts mutants. In addition, a method for mapping suppressor mutations and possible mechanisms of suppression in reovirus pseudorevertants will be discussed.

METHODS

The methods used in these studies are outlined in detail in the papers cited or will be discussed below. Permissive temperature is 31° and restrictive temperature is 39° for all experiments.

RESULTS

Reovirus ts Mutations Can Be Suppressed by Extragenic Suppressor Mutations. The group A mutant tsA(201) of reovirus type 3 was isolated following mutagenesis with proflavin (4). It contains μ1 and μ1C polypeptides with altered electrophoretic mobility (5). Reversion of tsA(201) to ts^+ phenotype is generally accompanied by a change in the mobility of the μ1 and μ1C polypeptides (6). In one revertant of tsA(201), clone 101, reversion was not accompanied by a change in the mobility of μ1 and μ1C. The possibility that this revertant was not a true revertant but contained the tsA(201) lesion in a suppressed form was examined (7). To show that the revertant clone 101 contained a suppressed ts lesion, it was necessary to show (a) that the reversion event occurred outside the gene with the ts lesion and (b) that the clone still contained the original tsA(201) lesion.

Since the genome of reovirus is segmented (8) and recombination occurs by reassortment of genome segments (4,9), we reasoned that the suppressed ts lesion could be separated from its suppressor by reassortment, if the ts lesion and the suppressor lay on different genome segments. Once separated from the suppressor mutation, the ts phenotype of the ts lesion would be expressed. However, if the suppressor mutation and the ts mutation resided in the same gene (genome segment), they could not be separated by reassortment and such intragenic suppressors could not be distinguished from true original site reversion events. Therefore, all suppressor mutations detected in the reovirus system are of the extragenic type, and the two very different intragenic events are indistinguishable and are referred to as intragenic reversion events.

To determine if the revertant clone 101 contained a suppressed ts lesion, the clone was backcrossed to wild type and the temperature phenotype of progeny clones was examined (7). As controls, the two parental viruses were self-crossed and the temperature phenotype of progeny was examined. All progeny derived from wild type and clone 101 had a ts^+ temperature phenotype. The progeny from the backcross had a bimodal distribution of temperature phenotypes. One group of progeny clones had an efficiency of plating (EOP) similar to that seen for the parents and was definitely ts^+. The other group had an EOP characteristic of ts virus and similar to the tsA(201) parent. This result provided unequivocal evidence that clone 101, although phenotypically ts^+, contained a suppressed ts lesion.

All of the ts progeny clones rescued from the backcross to wild type were characterized by recombination tests. All of the ts mutations rescued were in the recombination group A. Thus, clone 101 contained an extragenically suppressed ts lesion, and this lesion was in group A as expected. The failure to rescue any ts lesions except group A lesions in the progeny of the backcross indicated that the suppressor lesion of clone 101 did not have an intrinsic temperature phenotype.

Extragenic Suppression Is The Most Common Pathway From ts to ts^+ Phenotype in Reovirus. To determine if extragenic suppression was common among revertants of reovirus ts mutants, three independent, spontaneous revertants were selected from each of the 7 reovirus mutant groups and tested for extragenic suppression by backcross analysis (10). ts lesions could be rescued, following backcross, in 25 of 28 revertants examined in this study. This result indicated that approximately 90% of the spontaneous revertants examined regained ts^+ phenotype through the generation of extragenic suppressor mutations. In all but one of the cases the parental ts lesion was rescued from the backcross progeny as expected. In about half of the pseudorevertants examined, only the parental ts lesion was rescued, indicating that the suppressor mutations present in those clones did not have an intrinsic ts phenotype. However, in the remaining pseudorevertant clones both the parental and nonparental ts lesions could be isolated following backcross to wild type. The nonparental ts lesions rescued from these pseudorevertants are candidates for suppressor mutations with ts phenotype. This possibility has not yet been examined.

Isolation of New Mutants From Suppressed Pseudorevertants. The nonparental ts mutations isolated from the backcrosses described above were placed into mutant groups by recombination tests with the 7 existing reovirus mutant groups. This analysis showed that about half of the rescued nonparental ts mutants were in previously identified recombination groups. However, 10 of the nonparental ts lesions recombined with the prototype mutants of all the known mutant groups. This indicated that these mutants represented recombination groups not previously identified in reovirus. The new mutants were

crossed in all pairwise combinations to determine the number of new groups that they defined. Five of the new mutants fell into a group, designated tsH, within which recombination did not occur. Four mutants fell into a second group, designated tsI, and one mutant defined a third group, designated tsJ (10, 12; unpublished data). This result indicated that the selective forces involved in mutation from ts to ts^+ phenotype were different from those active in mutation from ts^+ to ts phenotype. Since only 7 mutant groups had been defined by mutants isolated following mutagenesis of wild type virus, the rescue of mutations from suppressed pseudorevertants may represent a very useful tool for the selection of new mutants.

Mapping New ts Mutants of Reovirus. The three new mutant groups isolated from suppressed pseudorevertant clones were mapped by standard mapping techniques used for viruses with segmented genomes (16,17). The analysis of genome segment segregation in the recombinant mapping clones was complicated by the occurrence of suppression of the ts phenotype of a ts lesion in some of the ts^+ recombinant progeny (Ramig, Ahmed and Fields, unpublished).

Each of the new mutants (type 3) was crossed with a ts mutant of type 1 or type 2. ts^+ recombinants were picked from the progeny of the crosses and the parent of origin for each of the genome segments was determined by polyacrylamide gel electrophoresis. In the clones analyzed to map the tsH(26/8) mutation, segment M1 was derived from the type 1 or type 2 parent in all ts^+ progeny and from the type 3 parent in all ts progeny. This indicated that the ts lesion of the type 3 tsH(26/8) parent resides on segment M1.

When ts^+ recombinants from crosses to map tsI(138) and tsJ(128) were analyzed, no single type 1 or type 2 segment segregated with ts^+ phenotype in all of the clones examined. Each of the ts^+ recombinant clones was tested for suppression to see if suppressed parental ts lesions could account for the failure of segregation patterns to reveal a clear site for the tsI(138) and tsJ(128) lesions. Backcross analysis revealed that some of the ts^+ recombinant clones did indeed contain suppressed ts lesions. In the case of the clones to map tsI(138), all of the suppressed recombinant clones were shown to contain tsI as the suppressed ts lesion. The nature of the ts lesion rescued from the suppressed recombinant mapping clones with tsJ(128) has not yet been determined, but we predict that the ts lesion contained in these clones will be tsJ. When the true ts^+ recombinant clones were separated from the suppressed ts^+ recombinant clones, the segregation patterns of the genome segments revealed very clearly that the site of tsI(138) was genome segment L3 and that of tsJ(128) was S1.

Thus, suppression of ts phenotype can complicate the analysis of genome segment segregation in mapping crosses. The ability to test for, and recognize, suppressed clones makes unambiguous assignment of mutations to genome segments possible when some of the progeny contain suppressed ts lesions.

Rapid Detection of Suppressed Pseudorevertants. Detection of suppressed pseudorevertant clones is a tedious process if each suspected pseudorevertant must be backcrossed, progeny picked and passaged and the temperature phenotype of each progeny clone determined. A rapid method for detection of pseudorevertants of reovirus ts mutants, that has high reliability, has been described (11). This method relies on the fact that many of the reovirus ts mutants are "leaky", that is, the ts lesion is not completely lethal at the nonpermissive temperature (4). As a result of this incomplete lethality, two populations of plaques are observed when a "leaky" ts mutant is plated at nonpermissive temperature. The revertant plaques are large, clear, lytic plaques whereas the majority of the plaques are small and faint with diffuse edges. These plaques often have a clump of viable cells in their centers and represent limited growth by the ts mutant at the restrictive temperature. The "leaky" character of ts mutants can be used to advantage for the detection of suppressed pseudorevertants. The revertant to be tested is backcrossed to wild type and the progeny of the cross are plated at nonpermissive temperature. Any ts lesions that are rescued in the backcross will make plaques having "leak" morphology at the nonpermissive temperature, whereas the parental virus will make lytic plaques. The difference in plaque morphology is great enough that a few "leak" plaques can be detected on a plate with many lytic plaques. The rescue of progeny having leak morphology indicates the presence of ts mutations among the progeny, the classic demonstration of suppression by backcross.

Suppression of ts Phenotype Can Occur in Cells Persistently Infected with ts Mutants. Suppression of ts phenotype has been shown to be one of the genetic changes that occur during the establishment of persistent infection with reovirus (12). When persistently infected L cells were established following infection with a highly defective population of tsC(447), the virus released from the cells gradually changed from ts to ts^+ phenotype (13). Backcross analysis of the ts^+ virus released from the tsC(447)-persistently-infected cells showed that 2 of the 7 clones examined contained suppressed ts lesions, while the remaining 5 clones had gained ts^+ phenotype through intragenic events. Recombination analysis showed that the parental tsC(447) lesion was not rescued from either of the suppressed clones, and that most of the nonparental ts lesions fell into previously defined recombination groups. However, one pseudorevertant yielded ts mutations in the new recombination group designated tsH (see above).

These results indicated that, although extragenic suppression of ts phenotype can occur, suppression alone does not account for the shift from ts to ts^+ phenotype observed in the virus shed from the cells persistently infected with a ts mutant.

Mapping Suppressor Mutations. A technique to map the location of suppressor mutations has recently been formulated. A suppressed pseudorevertant (type 3) is crossed with wild type of either type 1 or

type 2. The ts recombinant progeny are selected from the progeny of the cross and recombination tested to confirm that they contain the parental ts lesion. The parent of origin is determined for each genome segment by polyacrylamide gel electrophoresis. The analysis of only those ts recombinant progeny that contain the parental ts lesion places two constraints on the segregation pattern of the genome segments. (a) All ts recombinants must contain the segment bearing the ts lesion from type 3, since only the type 3 parent contained a ts lesion. (b) Expression of the ts phenotype of the ts lesion requires that the ts recombinants not contain the type 3 segment bearing the suppressor mutation. Thus, the type 3 segment bearing the suppressor lesion must always be replaced by the corresponding segment from type 1 or type 2. If a sufficiently large number of ts recombinants are analyzed, one segment (that bearing the ts lesion) will be derived from the type 3 parent in every case and one segment (that bearing the suppressor lesion) will be derived from type 1 or type 2 in all recombinants. This method for mapping the sites of suppressor mutations has the advantage that the segment bearing the suppressor mutation is selected only on the basis of suppressor activity, allowing the suppressor mutations having no intrinsic temperature phenotype to be mapped.

This mapping method has been applied to one pseudorevertant at this time. The suppressor mutation of the tsA(201) pseudorevertant clone 101 has been mapped by this technique. In all ts recombinants analyzed, segment M2 was derived from the type 3 parent, confirming this segment as the location of the tsA(201) lesion. Segment L3 was derived from type 1 or type 2 in all of the recombinants analyzed, indicating that this segment was the site of the suppressor lesion in clone 101 (Ramig, unpublished). Studies are currently underway to map the sites of the suppressor lesions in a number of other pseudorevertant clones isolated from mutants representing all 10 of the reovirus recombination groups.

Mechanism of Suppression of ts Phenotype. The mechanism by which the suppressor mutations overcome the defect of the mutations they mask is unknown. The hypotheses of others are attractive. The suppressor mutations may restore stoichiometric relationships altered by the original mutation (14), or the suppressor mutation may produce compensating alterations in a second (suppressor) protein that is in physical contact with the parental (ts) protein (15). In the absence of any evidence for stoichiometric alterations of protein synthesis in any of the reovirus ts mutants (5), the second of these hypotheses must be favored. The possibility of translational suppression has been excluded by several lines of evidence discussed in detail in (10).

Two models are currently considered to be reasonable and testable hypotheses for the mechanism of suppression. (a) The compensating protein interactions model. The ts lesion causes an alteration in the structure of a protein such that the protein interacts with a second protein in a manner that is thermolabile. In the suppressed pseudorevertant, the second protein is also altered so that its interaction

with the original protein is thermostable. This compensating interaction yields a complex in which both proteins are mutant but which has wild type thermostability. This model predicts that a ts lesion could be suppressed by suppressor mutations in any protein with which the product of the ts gene interacts. In reovirus, where many of the gene products are structural components of the virion, suppressor mutations would be expected in any protein with which the ts protein interacts either in the virion or during morphogenesis. In the case of the tsA(201) pseudorevertant clone 101, this model predicts that the interactions between protein μ1C (product of segment M2, the ts protein) and protein λ1 (product of segment L3, the suppressor protein) are altered in tsA(201) relative to their interactions in either wild type or the suppressed pseudorevertant clone 101. This notion is currently being tested. (b) The mutator transcriptase model. In this model the ts lesion makes a protein thermolabile in its interactions with another protein(s). The defect in the interactions between proteins is corrected by a compensating alteration in a second protein as described above. However, in this case the second, suppressor mutation is the direct result of a virion transcriptase that reads with lower fidelity than the wild type enzyme. Since the transcription products of reovirus function as both messenger RNA and template for the synthesis of progeny genomes, any mutations introduced in the transcriptase product could be "locked in" to the genome of progeny virions. This model would account for both the high frequency of suppressed pseudorevertants among revertants of reovirus ts mutants and the high frequency of nonparental ts lesions that are isolated from suppressed pseudorevertants.

DISCUSSION

The experiments described here demonstrate that ts mutations of reovirus can be suppressed by extragenic suppressors. Indeed, the generation of extragenic suppressors represents the major pathway from ts to ts^+ phenotype in reovirus. These results suggest a general mechanism by which RNA viruses can bypass the effects of deleterious mutations in the absence of intramolecular recombination.

No temperature-sensitive phenotype has been associated with many of the suppressor mutations identified. In those pseudorevertants from which nonparental ts lesions could be rescued, the nonparental ts lesions have not been linked to suppressor activity. It is of interest to note that about half of the nonparental ts lesions rescued from pseudorevertants were found to represent the three previously undefined reovirus mutant groups. The mapping of these new mutant groups was complicated by the presence of suppressed ts lesions in the progeny of the mapping crosses. However, the ability to detect and differentiate pseudorevertant recombinants from true ts^+ recombinants made an unambiguous assignment of the new mutant groups to genome segments possible. The three new mutant groups tsH, tsI and

tsJ map to genome segments M1, L3 and S1, respectively. The high frequency of new mutants rescued from suppressed pseudorevertants indicates that the selective pressures active in mutation from ts to ts^+ pseudorevertant phenotype are very different from those active in mutation from ts^+ to ts phenotype during standard mutant searches. This suggests that rescue of ts lesions from suppressed pseudorevertants may provide a useful tool for the selection of new mutants in a system where many mutants exist but the map is not saturated.

The new mutants isolated in the studies described here have allowed a ts mutant group to be assigned to each of the 10 reovirus genome segments. The "genetic map" of reovirus is summarized in Table I. The tsF mutant has not been unambiguously mapped to segment M3, but this location is consistent with the limited mapping data available (Ramig, Ahmed, Mustoe and Fields, unpublished). Data from other sources on the polypeptide species encoded in each genome segment and the location of each polypeptide species within the reovirion has been included in Table I to give a more complete overview of what is known about the function of each genome segment.

TABLE I

Genome segment	ts mutant group[a]	Polypeptide encoded[b]	Polypeptide location (function)[c]
L1	tsD	λ3	core
L2	tsB	λ2	core
L3	tsI	λ1	core
M1	tsH	μ2	core
M2	tsA	μ1 → μ1C	outer capsid
M3	(tsF)	μNS	nonstructural
S1	tsJ	σ1	outer capsid (HA)
S2	tsC	σ2	core
S3	tsE	σNS	nonstructural
S4	tsG	σ3	outer capsid

[a]References: (16, 17, this work).

[b]References: (18, 19).

[c]References (20, 21, 22, 23).

Suppressed pseudorevertants are found among the ts^+ virus shed from cells persistently infected with a ts mutant of reovirus. The role, if any, of pseudoreversion in the establishment and maintenance of the persistent infection remains to be determined.

A method for mapping the suppressor mutations of the various pseudorevertants has been presented. This method has been used to unambiguously map the suppressor mutation of one tsA(201) pseudorevertant to genome segment L3.

Two models for the mechanism of suppression of ts phenotype in reovirus have been presented. The mapping of suppressor mutations and biophysical studies currently underway should help to distinguish the correct model.

Finally, these experiments suggest that reversion in animal viruses can occur by mechanisms similar to those elucidated in other biological systems. Thus, it is critical to distinguish between ts^+ phenotype and ts^+ genotype when virus revertants are used in biochemical or genetic studies.

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TRANSLATION PRODUCTS OF THE 124 STRAIN OF MOLONEY MURINE SARCOMA VIRUS (Mo-MuSV): CHARACTERIZATION OF A 23,000 DALTON CANDIDATE 'src' GENE PRODUCT

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ABSTRACT Analysis of translation products of Mo-MuSV RNA indicated that at the 5' half of the viral RNA codes for a 63,000 dalton polypeptide ($P63^{gag}$) derived from the 'gag' gene; the 3' half of the RNA codes a P38 and a P23 polypeptide. Immunoprecipitation and peptide mapping experiments showed that P23 is not a product of the 'gag', 'pol' or 'env' genes, suggesting it to be a product of the 'src' gene. $P63^{gag}$ was readily detectable in Mo-MuSV-124 infected cells. No fused 'gag-src' gene products were detected in these cells. A mutant sarcoma virus derived from a subclone of Mo-MuSV-124 containing a temperature sensitive lesion in the transforming function contains a candidate 'gag-src' protein (P85). P85 is detectable at 33°, the transformed temperature but not at 40°, the normal phenotype. Part of the sequences in P85 are derived from p15, pp12 and p30 whereas the origin of the remaining ~25,000 to 40,000 daltons is as yet unknown. Initial peptide mapping studies suggest that part of the unidentified sequences in P85 may be present in P23.

INTRODUCTION

Murine sarcoma viruses are a class of retroviruses that are able to induce transformation of fibroblasts *in vitro* and fibrosarcomas *in vivo*. However, these viruses are defective in replication since they require a helper virus in order to form an infectious transforming virus (1,2). Nucleic acid hybridization studies indicate that murine sarcoma viruses probably originated by a process of genetic recombination between non-defective murine leukemia virus (MuLV) sequences and specific cellular sequences present in uninfected mouse cells (3-5).

The Moloney murine sarcoma virus (Mo-MuSV) produced by

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the G-124 cell clone overproduces the sarcoma virus relative to the helper virus in a ratio of more than 20 to 1. This Mo-MuSV-124 virus has been compared with Mo-MuLV by RNase T1 fingerprint analysis (7), hybridization analyses (8), and by heteroduplex analysis (5). Seventy percent of the Mo-MuSV-124 genome is shared with Mo-MuLV (clone 1) and the remaining is sarcoma virus specific. The arrangement of these sequences within the Mo-MuSV-124 genome is believed to be as follows: a 3'-terminal region about 1,000 nucleotides long containing sequences shared with the Mo-MuLV genome; a sarcoma-specific region ('src') of about 1,500 nucleotides in length which is located between 1,000 and 2,500 bases from the 3' end of the viral genome; and a 5' region of about 3,500 bases in length that contains sequences shared with Mo-MuLV.

A specific gene product has not as yet been implicated in MuSV-induced transformation. Thus, we examined the viral gene products present in G8-124 MuSV infected cells with the aim of identifying the MuSV specified proteins and possibly the transforming protein. We had originally proposed that unique viral polyproteins might be found in MuSV transformed cells that would contain both viral structural proteins and the sarcoma virus specific protein (9). This appears to be the case with several transforming viruses such as feline sarcoma virus (FeSV) (10,11), Abelson leukemia (AbLV) (12), and mink cell focus forming (MCF) viruses (13). Our results indicate that Mo-MuSV-124 transformed mouse TB cells do not contain a fused protein such as a 'gag-src' gene product. However, upon mutagenesis and infection of NRK cells a candidate 'gag-src' gene product has been detected.

METHODS

The methods used to generate the results presented here have been presented (14,15).

RESULTS

Characterization of Intracellular Proteins Present in Mouse TB Cells Transformed and Producing Mo-MuSV-124. The murine retrovirus-related proteins were detected following 20 min incubations of cells with ^{3}H-leucine by radioimmunoprecipitation experiments combined with electrophoresis of the immunoprecipitates in SDS-polyacrylamide gels (Fig. 1). Gene products derived from the 'gag' gene included at least three polypeptides identified as $P63^{gag}$, $Pr67^{gag}$ and $gPr85^{gag}$. $P63^{gag}$ lacked detectable p10 antigenic determi-

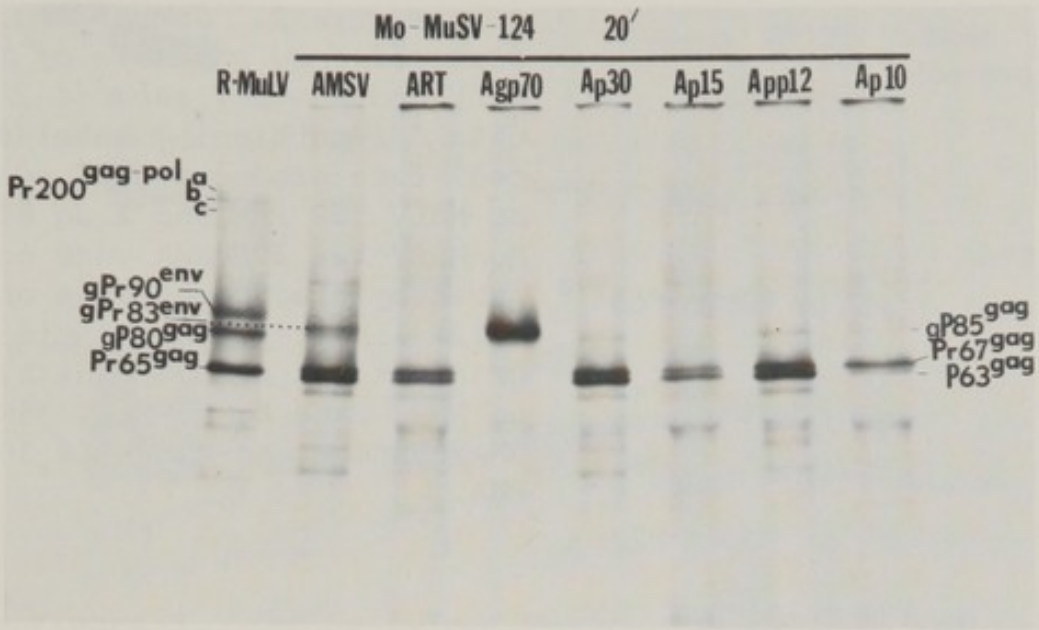

Figure 1. *Resolution by SDS-PAGE of polyproteins present in Mo-MuSV-l24 transformed cells isolated by immunoprecipitation with antisera prepared against purified viral proteins.* The first lane on the left is a 15 min pulse-labeling (^{3}H-leucine) of R-MuLV infected JLS-V16 cells immunoprecipitated with anti-R-MuLV. The other seven lanes are 20 min pulse-labelings of Mo-MuSV-l24 cells with ^{3}H-leucine followed by immunoprecipitations with the following antisera as marked: anti-MuSV-l24, anti-gp70, anti-p30, anti-pl5, anti-ppl2 and anti-pl0.

nants. The normal size 'gag-pol' and 'env' precursors were also detected. In an effort to detect fused proteins such as 'gag-src' which might be rapidly cleaved during pulse-labeling experiments, cells were incubated in 4 mM canavanine, an arginine analog, to inhibit processing (16,17). The results failed to show any unusual 'gag' gene precursor (18).

Translation Products of 30S Mo-MuSV-l24 RNA and 35S Mo-MuLV RNA. Mo-MuSV 50S RNA and Mo-MuLV 70S RNA were heat-denatured (85°C-2 min) and the intact subunit RNAs were translated *in vitro*. Fig. 2, Lane C, shows that the cell-free product of 30S Mo-MuSV RNA contained major polypeptides with mol. wts. of 63,000 ($P63^{gag}$), 42,000 (P42), 40,000 (P40), 38,000 (P38) and 23,000 (P23). In some experiments a 68,000 to 70,000 M_r ($P70^{gag}$) polypeptide was synthesized in small amounts. The number and mobility of the bands around 40,000 M_r were variable. Usually, at least two bands were resolved. The pattern of polypeptides synthesized from 35S

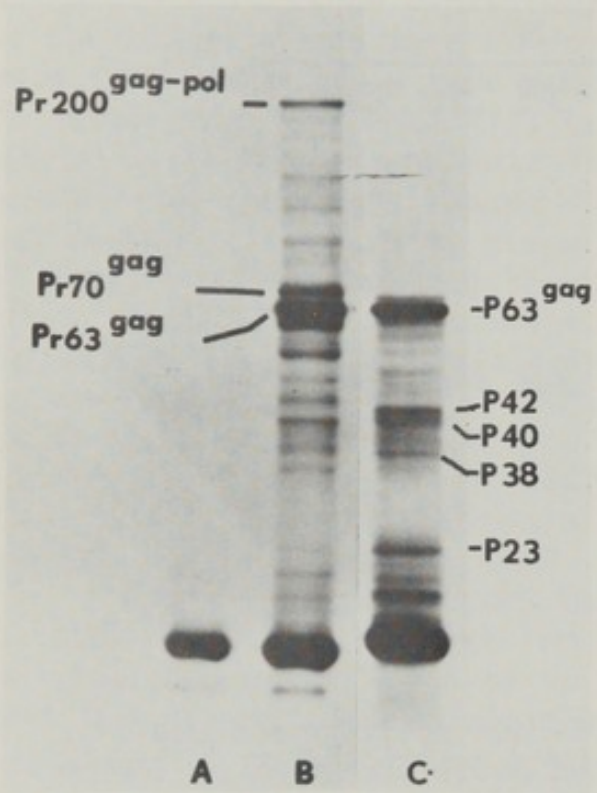

Figure 2. *Comparison of the cell-free products of Mo-MuLV and Mo-MuSV subunit RNAs.* The ^{35}S-methionine labeled cell-free products of 1 µg of Mo-MuLV 35S RNA and 1 µg of Mo-MuSV-124 30S RNA were compared by electrophoresis on a 6-12% linear polyacrylamide gradient gel slab. Lane A, no RNA; Lane B, Mo-MuLV 35S RNA; Lane C, Mo-MuSV-124 30S RNA.

Mo-MuLV RNA was quite different (Fig. 2, Lane B). Polypeptides of 200,000 M_r ($Pr200^{gag-pol}$), 70,000 M_r ($Pr70^{gag}$) and 63,000 M_r ($Pr63^{gag}$) were synthesized. These polypeptides have been characterized and found to be analogous to those polypeptides synthesized from 35S R-MuLV RNA (19). The remaining minor bands appear to be premature termination products, all containing 5' 'gag' gene coded products (e.g. p15). We note that a faint band in the MuLV product similar in size to P23 synthesized from MuSV RNA has been analyzed by O'Farrell 2-dimensional gels and found to be different in charge.

Translation of Poly(A) Selected MuSV RNAs. Heat-denatured Mo-MuSV 50S RNA was fractionated on oligo(dT) cellulose. The poly(A)-containing and poly(A)-minus RNA fractions were fractionated by velocity sedimentation on 5-25% sucrose gradients. Various size classes of RNA in each of the gradients were translated and the product examined by gel electrophoresis. The results show that $P63^{gag}$ was synthesized mainly from full-sized (30S) poly(A)-containing RNA (Fig. 3, Lane 6), while both P23 and P38 were quite abundant in the cell-free product of 18-25S poly(A)-containing RNA (Fig. 3, Lanes 4 and 5). The cell-free product of the poly(A)-minus RNA contained primarily $P63^{gag}$, very little P42, P40, and P38, and virtually no P23 (20).

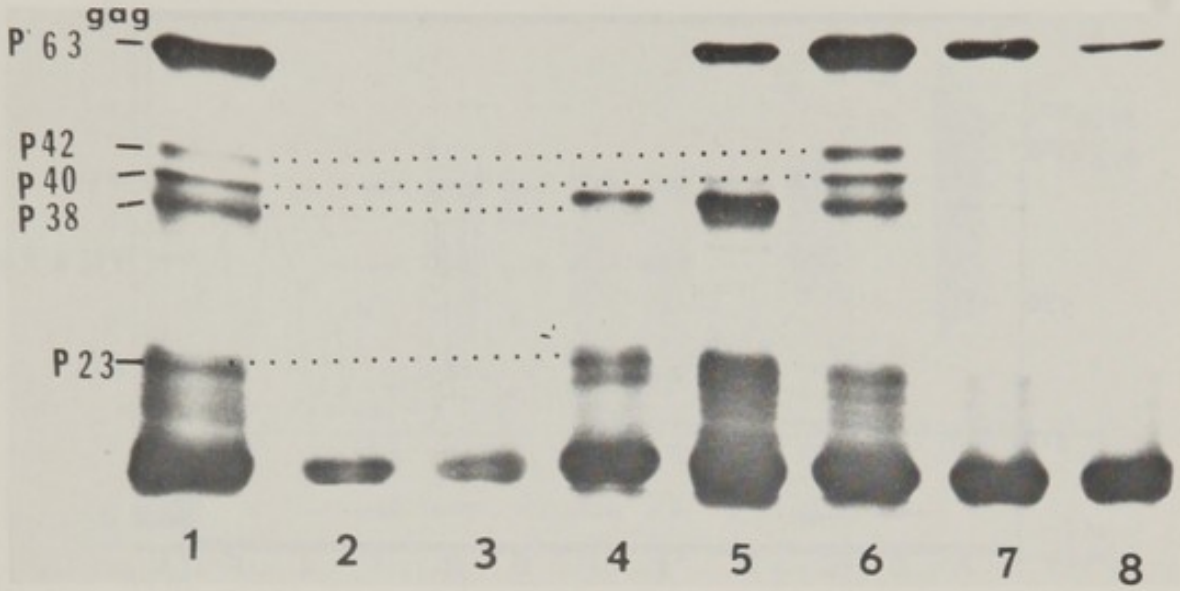

Figure 3. *The Cell-Free Product of MuSV-124 Poly(A)-containing RNA.* MuSV-124 50S RNA was denatured, poly(A)-selected and fractionated on a sucrose gradient as described under Methods. The cell-free product of 1 µg of various size classes of the poly(A)+ RNA was subjected to electrophoresis in a 6-12% polyacrylamide gel slab. (Panel A) MuSV-124 Poly(A)+ RNA: (Lane 1) Total poly(A)+ 50S RNA; (Lane 2) 4S RNA; (Lane 3) 10-15S RNA; (Lane 4) 18-20S RNA; (Lane 5) 21-25S RNA; (Lane 6) 28-30S RNA; (Lane 7) >30S RNA; (Lane 8) pelleted RNA.

Immunological Characterization of the Cell-Free Translation Products of Mo-MuSV-124 RNA. To examine the virus-specificity of the polypeptides translated from Mo-MuSV RNA, the cell-free products were tested for immunoprecipitability with antisera to MuLV core, envelope, and reverse transcriptase proteins (20). The product was analyzed by gel electrophoresis (Fig. 4, Lane L) and found to contain $P63^{gag}$, P42-P38, P23, and trace amounts of a 68,000 to 70,000 M_r polypeptide ($P70^{gag}$). In this experiment, the polypeptides near 40,000 M_r were not as well resolved as in other experiments. Immunoprecipitation with normal goat serum precipitated only small amounts of the cell-free product (Fig. 4, Lane B). MuLV anti-30 precipitated $P63^{gag}$ and the P42-P38 fraction (as well as $P70^{gag}$), but not P23 (Fig. 4, Lane C) to any greater extent than did normal goat serum. Competition with 50 µg of excess disrupted Mo-MuSV reduced the precipitation of $P63^{gag}$ and the P42-P38 polypeptides by anti-p30 (Fig. 4, Lane D) to the level precipitated by normal serum (Fig. 4, Lane B), and demonstrated that the products identified were in fact viral specific products. The low level of P23 precipitated by anti-p30 was not significantly

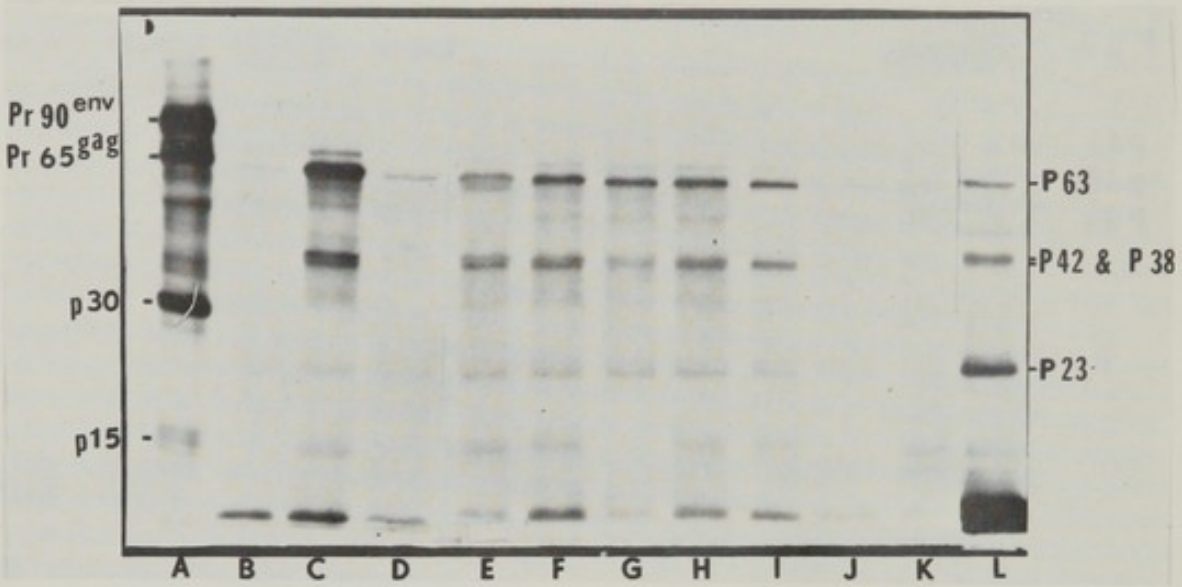

Figure 4. *Immunoprecipitation of Mo-MuSV-l24 translation products.* The products of the reaction mixture were first 'cleared' by precipitating with normal goat serum to reduce non-specific precipitation. (L) Polypeptides generated by Mo-MuSV-124 RNA; (B) immunoprecipitation with normal goat serum; (C) with anti-p30 serum; (D) with anti-p30 serum in the presence of an excess of disrupted Mo-MuSV; (E) with anti-R-MuLV; (F) with anti-p15; (G) with anti-p12; (H) with anti-p30; (I) with anti-p10; (J) with anti-gp69/71 absorbed with disrupted Mo-MuSV to reduce contaminating antibodies to core proteins; (K) with anti-RT absorbed with disrupted Mo-MuSV. (A) A standard of R-MuLV precursor proteins was prepared as described previously (15).

reduced by competition with disrupted virions. To test further the antigenicity of the products of MuSV RNA, antisera to MuLV, p15, p12, p30, p10, gp69/71 and reverse transcriptase were used to immunoprecipitate other aliquots of the Mo-MuSV cell-free product reactions. The results (Fig. 4, Lanes E-K) showed that $P63^{gag}$ and the P42-P38 polypeptides (as well as P70) shared antigenic determinants with all the MuLV core proteins (Fig. 5, Lanes E-I), but not with MuLV gp69/71 (Fig. 4, Lane J) or MuLV reverse transcriptase (Fig. 4, Lane K). The results show that P23 is not immunlogically related to any of the MuLV gene products, but $P63^{gag}$ and components of the P42-P38 class contain core protein determinants.

Peptide Mapping Studies of the Mo-MuSV Specified Polypeptide. We have previously shown that the tyrosine-containing tryptic peptides of R-MuLV p30, p15, pp12 and p10 can be resolved by cation exchange chromatography, and tryptic

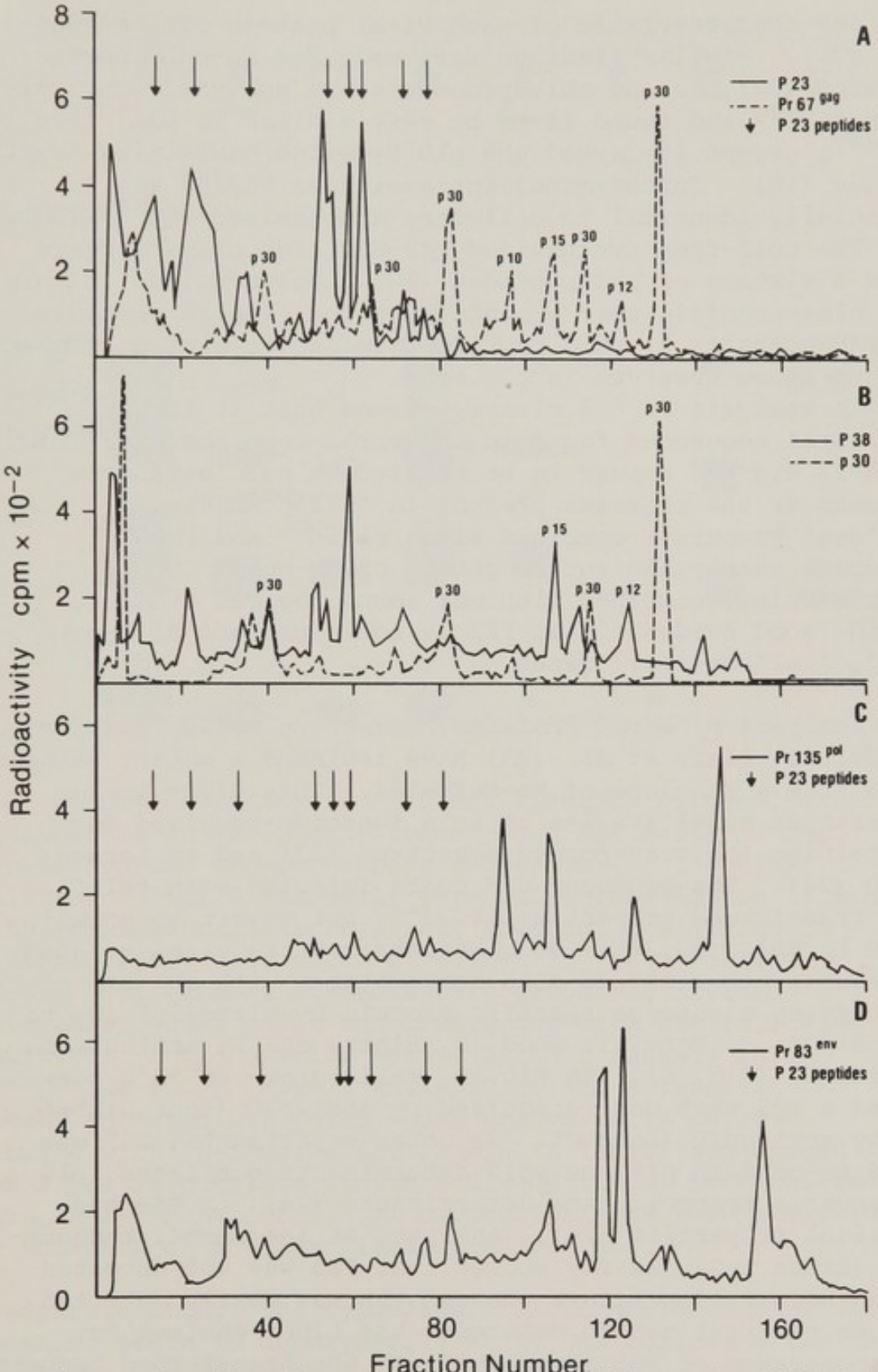

Figure 5. *Tryptic mapping of the ^{3}H-tyrosine-labeled* in vitro-*synthesized P23 and viral precursor proteins.* ^{3}H-tyrosine-labeled *in vitro*-synthesized P23 was digested with TPCK-trypsin and mixed with a similar digest of ^{14}C-tyrosine-labeled authentic polypeptides. The mixture was subjected to ion-exchange chromatography (21).

peptides characteristic of each viral protein can be identified (21). Similar findings were made for Mo-MuLV core proteins (22). Using this procedure, we analyzed intracellular $P63^{gag}$ and found it to be very similar to bona fide $Pr65^{gag}$, except it lacked the p10 tyrosine-containing tryptic peptide (18). Furthermore, intracellular $P63^{gag}$ was essentially identical to cell-free synthesized $P63^{gag}$ (18).

The cell-free synthesized P42-P38 size class appears to be a mixture of polypeptides (20). Polypeptides in this size class contain core protein tyrosine tryptic peptides and P38 contains tryptic peptides similar in elution properties to those observed in P23.

An analysis of P23 clearly showed that it lacked structural sequences found in the viral core proteins (Fig. 5). P23 did not appear to be related to the 'env' gene products or the proteins present in $Pr135^{pol}$ (Fig. 5). The 'gag' precursor combined with $Pr135^{pol}$ and $Pr83^{env}$ encompass nearly the entire genome of Mo-MuLV. Thus, these results, combined with our immunological studies, provide good evidence that P23 is not a product of the 'gag', 'pol' or 'env' genes.

Analysis of Viral Proteins Present in tsl10 Transformed NRK Cells. Blair *et al.* (23) have isolated a mutant sarcoma virus from a subclone of Mo-MuSV-124. This virus has a temperature sensitive lesion in a function required for maintaining the transformed phenotype (23) and is termed tsl10 (24). Non-producer NRK cells infected with tsl10 have transformed properties at 33°C, but revert to normal at 39 to 40°C (23,24). We characterized the viral related proteins synthesized at both temperatures with the aim of identifying a sarcoma specific protein by virtue of the ts lesion (24, J. Horn, T. Wood, D. Blair, and R. Arlinghaus, submitted) (Fig. 6). In Fig. 6, cells grown at 33°C contained a P85 that was recognized by anti-p30 (Lane D) but not by anti-gp70 (Lane C). In other experiments, P85 was found to contain p15 and pp12 determinants but lacked p10 and reverse transcriptase determinants (24). A P58 had identical properties (24). Analyses at the normal temperature (Lanes E-G) clearly showed that P85 was *not* detected at the normal temperature whereas P58 was easily detected.

We next set out to determine the time required for the appearance of P85 after shift to the transformed temperature. Brief pulse-labelings (15 min) at hourly intervals demonstrated that P85 was detectable between two and three hours after shift from 40°C to 33°C (24). Rates of hexose uptake did not begin to change significantly from normal until after 8-12 hrs following the shift to 33° (24). In

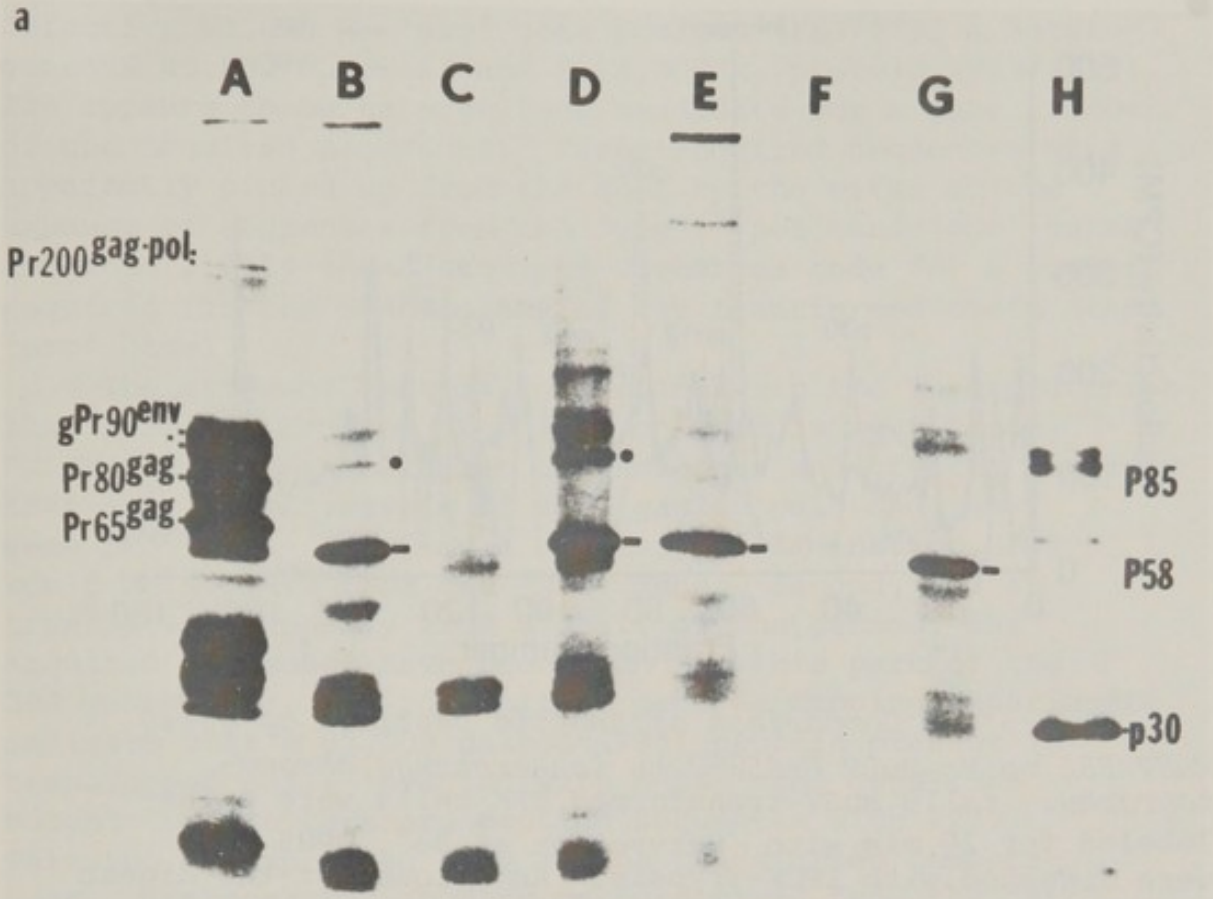

Figure 6. *Analysis of virus-specific polypeptides synthesized in tsl10 MuSV-transformed NRK cells at the permissive and non-permissive temperature.* Identical cultures of tsl10 NRK cells were pulse-labeled for 15 min with ^{3}H-leucine in Earle's salt solution containing 2% dialyzed fetal calf serum at either 33° (B-D) or 40° (E-G). Cytoplasmic extracts were prepared, divided into three equal parts and challenged with the following antisera: anti-MuSV-124 serum (B and E lanes); anti-gp69/71 (C and F lanes); anti-p30 (D and G lanes). R-MuLV infected NIH Swiss mouse embryo cells pulse-labeled with ^{3}H-leucine for 15 min (A lanes) and chased for 2 hrs in complete growth media (H lanes) were reacted with anti-R-MuLV serum. Immunoprecipitates were analyzed by SDS-PAGE on a 6-12% linear gradient gel.

a separate study, the cytoplasmic microtubule complex was shown to begin to depolymerize at 23 hrs following the shift (R. Brown, B. Brinkley, J. Horn and R. Arlinghaus, unpublished results). Thus, the expression of P85 preceeds by several hrs changes usually attributed to the transformed state.

We have determined that P85 contains tryptic peptides found in p15 and p30 (Fig. 7) as well as additional peptides

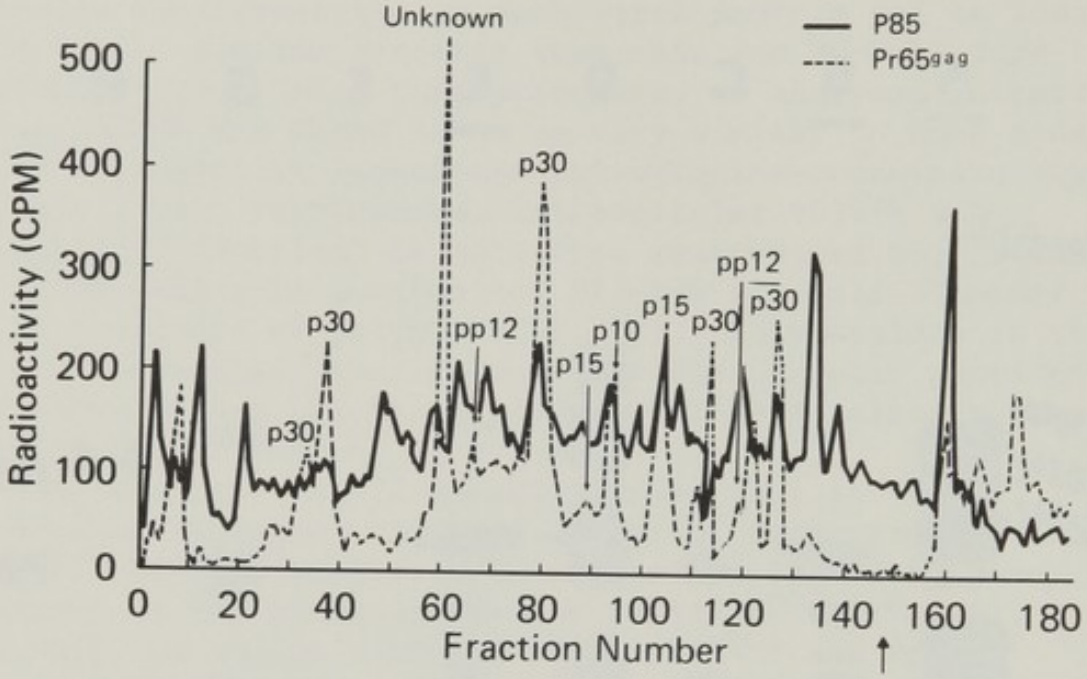

Figure 7. *Comparison of tryptic peptides of ts110 MuSV P85 to Mo-MuLV Pr65gag by ion-exchange chromatography.* ts110 MuSV-transformed NRK cells were pulse-labeled for 20 min with ^{3}H-tyrosine at 33°. P85 were digested with TPCK-Trypsin. An aliquot of the digest was mixed with a digest of ^{14}C-tyrosine-labeled Pr65^{gag} isolated from cl 1 Mo-MuLV infected cells. Samples were analyzed on a Chromobead Type P ion-exchange column (21).

which do not appear to be characteristic of the 'pol' or 'env' gene products. P85 also lacked a p30 tryptic (~fraction 112) and also the p10 tryptic (~fraction 93). In P85 a peak eluted slightly ahead of the p10 peak in this analysis and was absent in a second analysis. In contrast to this, P58 was composed of only viral core protein sequences derived from p15, pp12 and p30. The origin of the additional tryptic peptides in P85 is unknown but some resemblance does exist between those tyrosine-containing tryptics found in *in vitro* synthesized P23 and fractions 10-70 of P85 (compare Fig. 7 to Fig. 5A). However, it remains to be proven that P23 sequences are found in P85. We are currently performing 2-dimensional maps of P23 and P85 to either confirm or disprove this possible relationship.

DISCUSSION

Our goal is to identify and characterize the proteins encoded by the RNA of the 124 strain of Moloney MuSV (18,20, 24). Our studies at this point in time have identified a

defective 63,000 M_r 'gag' gene product ($P63^{gag}$), a heterogeneous 40,000 M_r class and a 23,000 M_r protein (P23) (20). P23 appears to be an excellent candidate for a gene product of the acquired sequences. These acquired sequences were apparently picked up from the host by the virus at the expense of sequences from the 'gag', 'pol' and 'env' genes and most likely these acquired sequences code for a protein required for the maintenance of the transformed state (the 'src' gene).

The evidence that is consistent with the interpretation that P23 is a product of the 'src' gene is as follows. First, P23 lacks antigenic determinants and tryptic peptides that are characteristic of the 'gag', 'pol' and 'env' gene products. Thus, by a process of elimination, P23 would be derived from the 'src' gene. Second, P23 is translated primarily from 3' half of the genome; the acquired sequences have been found in this part of the 30S genome (5). Third, initial peptide mapping experiments indicate that a 85,000 dalton (P85) protein present in transformed cells infected with a ts 'src' gene-like mutant appears to share peptide sequences with P23. This P85 is expressed only at the transformed temperature. Although P23 appears to be derived from the 'src' gene of Mo-MuSV-124, it is clear that more evidence is necessary to establish this interpretation as fact.

Besides P23, $P63^{gag}$ also is derived from the 30S sarcoma genome. Vande Woude and colleagues (25,26) originally determined that a 60,000 M_r 'gag' gene product (P60) was found in pseudotyped clones of ml MuSV (25) as well as in a variety of cells transformed by ml MuSV (26). The $P63^{gag}$ of MuSV-124 appears to be similar to the P60 of ml MuSV. Its role in transformation, if any, is not known.

Regarding P85 in tsl10 MuSV infected cells and its relationship to the transformed state, several points should be discussed. First, P85 was only detected at the transformed temperature (33°) whereas P58 was detected at both transformed and normal (40°) phenotype temperatures (24, J. Horn, T. Wood, D. Blair and R. Arlinghaus, submitted). Second, P85 expression in temperature shift experiments (40°→33°) preceeds by at least 6-8 hrs the increase in rate of deoxyglucose uptake and by 24 to 30 hrs, the depolymerization of the cytoplasmic microtubial complex (24). Both of these changes are believed by many to be characteristic of the transformed state. Thirdly, it is of interest to know whether the tsl10 virus genome in NRK can generate a mRNA that can code for a 'gag-src' protein of the size of P85. No such protein was detectable

in mouse TB cells infected with the parent wild type MuSV-124 virus (Fig. 1, 18). And, analysis of the MuSV-124 genome by heteroduplex mapping (5) clearly indicates that translation of the genome cannot possibly generate a 'gag-src'. We have ourselves proven this point since no protein larger than $P70^{gag}$ is synthesized from 30S MuSV-124 RNA. Thus, it seems clear that neither the wild type MuSV-124 genome or a spliced mRNA derived from the genome yields a 'gag-src' fused protein. It appears that upon subcloning of MuSV-124 and subsequent mutagenesis and further clonal selection, ts110 MuSV may in fact be able to generate a fused 'gag-src' protein.

ACKNOWLEDGEMENTS

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CHARACTERIZATION AND GENETIC ANALYSIS OF RETROVIRUS MATURATION: A ROLE FOR Pr180$^{gag\text{-}pol}$[1]

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INTRODUCTION

In a retrovirus infected cell, the late events of virus maturation occur at the plasma membrane, where viral protein precursors are cleaved to mature proteins and a virus bud forms and develops into a released virion. The relationship between these two facets of maturation--protein cleavage and budding--and the requirements for their successful completion, remain poorly understood. We report here several approaches toward understanding the steps involved in murine retrovirus processing. We have taken advantage of conditional mutants blocked in maturation at high temperature, and have performed both phenotypic characterization and genetic analysis.

METHODS

Wild-type Moloney murine leukemia virus (4) (denoted here as M), M-ts3 (14), wild-type Rauscher virus (denoted R), R-ts24 and R-ts28 (9) were used in these studies. Virus stocks were harvested from single virus/single cell cloned populations. All experiments were performed with NIH/3T3 monolayers grown in DME with 10% calf serum. The XC plaque assay and exogenous reverse transcriptase assay were performed as previously described (6, 7). Radiolabeled lysates of cells and viruses were analyzed by SDS-polyacrylamide gel electrophoresis, often after immune precipitations were performed, as previously described (12). In the protease experiments, 2X crystallized trypsin was applied in the appropriate concentrations (5-20 μg/ml) in DME. Reactions were stopped with soybean trypsin inhibitor. Samples for electron microscopy were fixed in glutaraldehyde with osmium post-fixation; scanning samples were examined after shadowing on a Joel 25;

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[2]American Cancer Society Research Professor.

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transmission samples were stained, sectioned, and examined on a Phillips 201.

RESULTS

M-ts3 and R-ts24 are late mutants, characterized and described elsewhere (9-11, 13, 14). At 39°, cells infected by either mutant do not release virus, and accumulate uncleaved $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ intracellularly. The block is reversible; upon shiftdown to 32° in the presence of cycloheximide, precursors built up at 39° are processed and a burst of mature virus is released.

There has been some debate as to the extent of bud formation on membranes of infected cells maintained at 39°. Early investigators claimed an accumulation of late-stage buds; more recently, however, microscopic evidence suggests that such results were artifacts of fixation procedures or lack of precise temperature control (2, 13, 15, 16, 18). We have repeated transmission and scanning studies and find that 39° cells fixed immediately at 39° have few buds (Table I). As previously described, shiftdown of cultures to 32° usually elicits the appearance of numerous buds and completed virions. However, even slight perturbations of 39° cultures, such as

TABLE I

QUANTITATION OF VIRAL BUDS ON R-ts24 INFECTED CELLS BY SCANNING ELECTRON MICROSCOPY[a]

Treatment of Monolayers	Avg. buds/constant surface area
Decanted and fixed immediately at 39°	26
Decanted, rinsed 3X 39° buffer, fixed 39°	79
Shifted for 5 min to room temperature, washed 3X with room temperature buffer, fixed room temperature	98

[a]NIH/R-ts24 monolayers were grown at 39°. Samples prepared for scanning electron microscopy were examined and photographed at 5,000 X magnification. Using a grid, buds were counted in a constant surface area on multiple photographs. Buds/picture were totaled and averaged among all photographs of the same sample. All counts were obtained in a blind manner.

rinsing with pre-warmed buffer prior to fixation, can cause buds to appear. The time elapsed between seeding and fixation of cultures can also affect these observations. Thus, although tonicity changes and temperature fluctuations can cause rapid bud appearance, we believe that the true mutant phenotype at 39° is of precursor buildup without bud accumulation.

In the process of investigating cultures maintained at 39°, we found that a partial bypass of the mutant phenotype could be effected by exposing the 39° cultures to proteases. Trypsin has been used most extensively, although chymotrypsin and thermolysin are active as well. Other agents which cause rounding and detaching of the fibroblasts (such as cytochalasin or hypotonic buffer) have no effect. Analysis of labeled lysates from trypsin-treated cells shows cleavage of the Pr65^{gag} to characteristic intermediates and to material co-migrating with p30. Pr180$^{gag\text{-}pol}$ becomes cleaved and a species co-migrating with reverse transcriptase can be immune precipitated. p30 can be recovered from the culture supernatant fluid. In addition, supernatant fluid from 39° cultures treated with trypsin contain significant amounts of sedimentable reverse transcriptase activity as compared to 39° controls (Table II).

The finding of sedimentable reverse transcriptase activity after trypsin treatment is especially significant because the precursors of reverse transcriptase have no enzymatic activity (13), and thus the recovery of activity implies that trypsin is inducing accurate Pr180$^{gag\text{-}pol}$ cleavage. As seen by electron microscopy, trypsin treatment causes the appear-

TABLE II
RELEASE OF REVERSE TRANSCRIPTASE ACTIVITY BY R-ts24 INFECTED CULTURES[a]

	Reverse transcriptase (cpm x 10^{-3})			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Maintained at 39°	7.0	0.8	0.4	2.2
Maintained at 39° with 20 µg/ml trypsin	114.1	41.6	5.2	42.0
Shifted to 32°	138.2	94.0	4.6	95.0

[a]*Near-confluent 39° cultures were washed, the medium replaced, and 30 min harvests collected under the above conditions.*

ance of particles indistinguishable from virions. It seems plausible to postulate that the structural gene products themselves may not be defective in these mutants at 39°, but rather that processing functions may be impaired.

Genetic Analysis. To go beyond a phenomenological description, we attempted a genetic analysis of the maturation process. Complementation between various mutant and wild-type viruses, in doubly-infected cells, was therefore assessed. We have found a number of serological and electrophoretic markers that distinguish Moloney and Rauscher polypeptides, and have used them to screen for co-infection and to analyze released progeny.

When clones co-infected with R-ts24 and wild-type Moloney were constructed, the mutant block of the R-ts24 was not dominant: reverse transcriptase released at the high temperature was comparable to that for wild-type Moloney alone. Further, the wild-type virus did complement the *gag* cleavage defect of R-ts24, as evidenced by the release of both Moloney and Rauscher mature *gag* proteins at 39°. We draw two conclusions. First, the Pr65^{gag} itself is not refractory to correct processing at 39°. Secondly, we feel this is genetic evidence for a trans-acting factor, virally encoded, that is involved in *gag* processing.

Our next efforts were aimed at defining the viral regions involved in this complementation. We have previously described a cell line, M23, carrying a deletion mutant of Moloney which expresses only uncleaved Pr65^{gag} (8). We have superinfected this line with M-ts3 and R-ts24, and quantitated the release of enzymatically active reverse transcriptase. By this criterion, there is no complementation of the ts mutants at 39°. However, at low temperature, the co-infecting virus does effect the cleavage and release in virions of M23-derived *gag* proteins. Pr65^{gag} alone, then, is unable to complement the *gag-pol* blockage in these maturation mutants.

To examine whether viral mutants with a defective polymerase function retain the complementing activity, we have also tested the behavior of M-ts3 upon co-infection with R-ts28 (9). We have characterized R-ts28 as having a thermolabile reverse transcriptase which inactivates rapidly at 39° (Traktman and Baltimore, unpublished results). Although R-ts28 infected cells release a normal amount of virions at 39°, these virions are uninfectious and inactive in exogenous reverse transcriptase assays. Such doubly-infected R-ts28/M-ts3 clones do not exhibit complementation as assessed by the inability to detect active reverse transcriptase in 39° culture supernatants. As we had expected

that a processing mutant and a polymerase mutant would be defective in different functions, this result surprised us, and led us to further examine reverse transcriptase maturation and any additional roles for Pr180*gag-pol*.

We have studied a variety of mutants with altered polymerases. R-ts29, as previously described (9, 11, 13), has a temperature-sensitive maturation block as well as a thermolabile reverse transcriptase. In recent work (Goff, Traktman and Baltimore, unpublished results) we have also isolated spontaneous mutants of Moloney virus selected only for thermolability in their reverse transcriptase activity. We have found that several of these display, as unselected markers, a temperature sensitivity for both virion release and *gag* and *gag-pol* processing. As a general rule, in these and previous studies, maturation mutants display this coordinate defect in *gag* and *gag-pol* processing, although the relationship between these pathways is unclear.

The apparent frequency of pleiotropic mutants, bearing defects in polymerase function and virus maturation, taken with the data concerning the lack of complementation between R-ts28 and M-ts3, suggests a role for Pr180*gag-pol* in virus maturation. A consideration of other related systems may be helpful in developing a model for such a maturation scheme. In avian retroviruses, p15 (the most 3' of the 4 *gag* proteins) has been identified as a virally coded protease active in *gag* processing (12). Specific proteolytic activities have been reported in murine retrovirus preparations (17), but whether such activities are of viral origin is unknown. Recent genetic experiments have implicated p10 (positioned analogously to the avian p15) as the site of the lesion in a murine maturation mutant similar to ones described above (1). However, purified murine p10 has no demonstrable protease activity, and it is reasonable to wonder whether adjacent sequences may play a role. The Pr180*gag-pol*, which carries both the *gag* sequence in its N-terminal third (65K) and the reverse transcriptase sequence (85K), contains about 30K of additional uncharacterized protein sequence (5). At least some of this sequence may fall between p10 and the polymerase. Whether this intervening protein sequence has some enzymatic or structural role of its own, or is in some indirect way important remains an unknown and intriguing question.

SUMMARY

We have further characterized the biochemistry and physiology of several temperature-sensitive mutants of murine retroviruses. The arrest of precursor maturation (and associated virion release) in maturation mutants has been

partially bypassed by external protease treatment. In addition, we have shown genetic evidence for a virally coded trans-acting factor that can complement these late mutants. This factor is not provided by Pr65^{gag} alone, and appears to be impaired in reverse transcriptase mutants. We propose that the lesion in the mutants is in fact in the viral processing function, and that the Pr180$^{gag\text{-}pol}$ may play a key role in this pathway.

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THE MOLECULAR BASIS OF REOVIRUS VIRULENCE[1]

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ABSTRACT Recombinants derived from reoviruses type 1, 2 and 3 have been used to study virus-host interactions. We have found that the S1 dsRNA segment encodes the σ1 polypeptide, the viral hemagglutinin. The hemagglutinin is responsible for specificity in humoral and cellular immunity, determines cell tropism in the nervous system as well as binding to cellular microtubules. The M2 dsRNA segment encodes the μ1C polypeptide. The μ1C plays a key role in entry through the gastrointestinal tract and spread in the host. Thus virulence is clearly the result of the interaction between individual viral polypeptides each playing a discrete role in viral host interactions.

MECHANISM OF REOVIRUS VIRULENCE

One of our major goals in studying the genetics of the reovirus serotypes (1, 2 and 3) is to identify the specific role of each viral gene product in the production of viral induced disease. Recent advances in the genetic analysis of the reoviruses have enabled us to identify a crucial role

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for two of the outer capsid polypeptides. In this report we will summarize the status of the genetic map of the mammalian reoviruses as well as aspects of the functions of the σ1 polypeptide (the hemagglutinin - HA) and the μ1C polypeptide.

THE GENETIC MAP

The dsRNA genomes and polypeptides of reovirus serotypes 1, 2 and 3 contain species that migrate with differing electrophoretic mobilities (9). These heterogeneities have been used to correlate genome segments between serotypes 1, 2 and 3 and to identify the dsRNA segments encoding the viral polypeptides (6, 7, 10, 12).

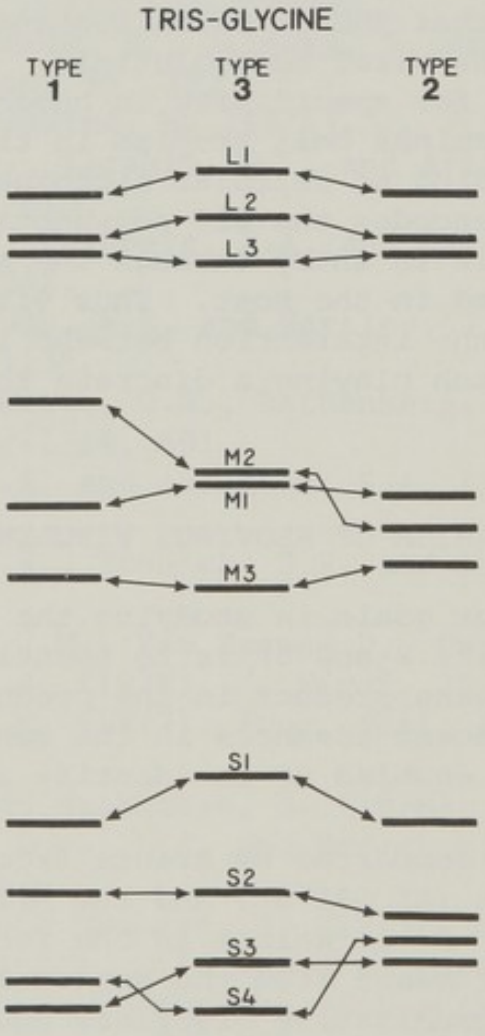

Figure 1. The map of the genome segments of reovirus serotypes 1, 2, and 3 as resolved in Tris-glycine gel-electrophoresis systems (4). Arrows indicate the band of type 1 or 2 that replaced the type 3 band in question to yield a viable hybrid virus. Reprinted from ref. (12) with permission.

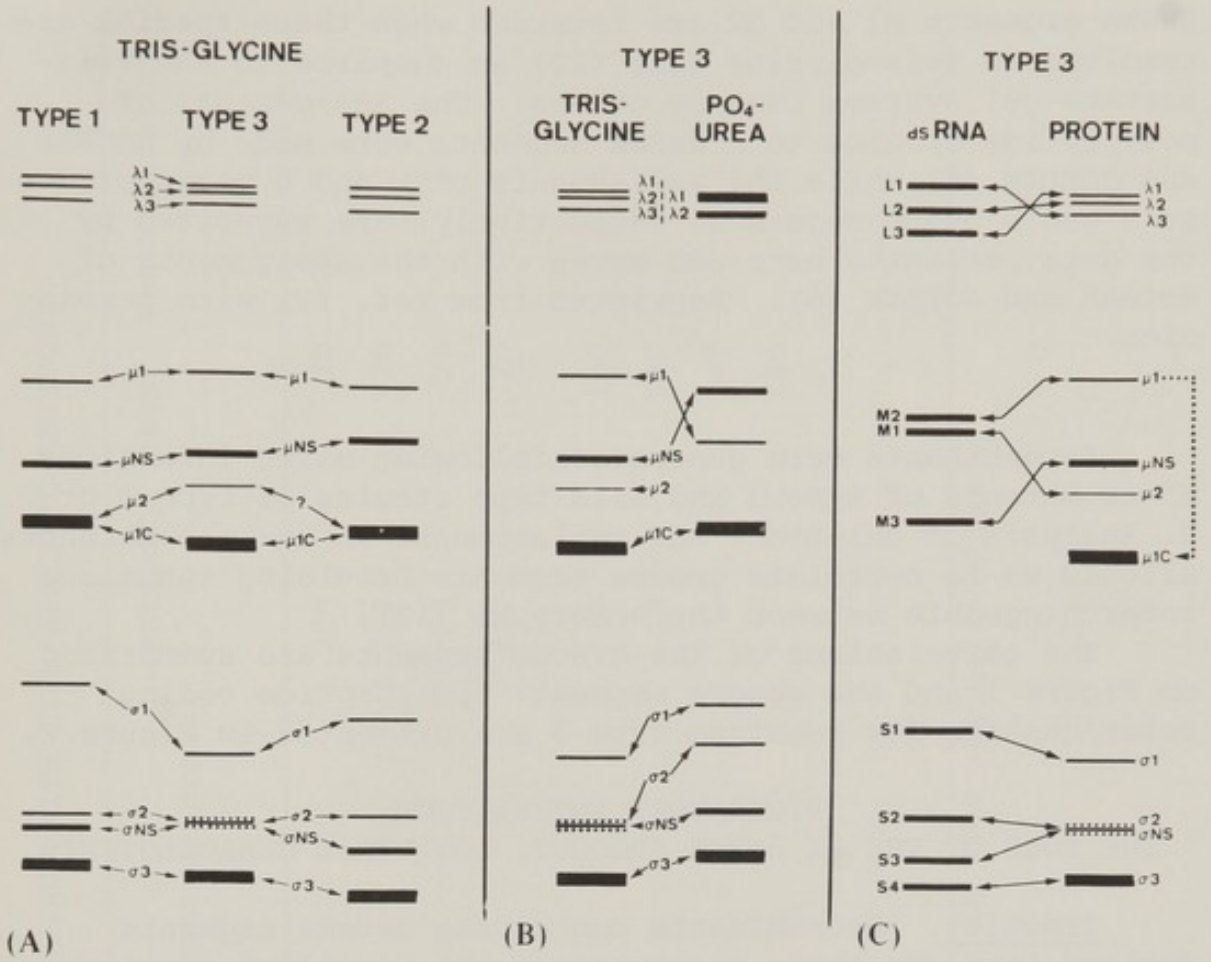

Figure 2: Schematic summary of the corresponding polypeptide species between reovirus serotypes 1, 2, and 3; correlation of type 3 polypeptide species between the Tris-glycine and phosphate-urea gel systems; and coding assignments for all reovirus type 3 dsRNA segments.

A: The correlations of polypeptide species of the μ and σ size classes of reovirus type 1, 2, and 3 as resolved on Tris-glycine gels as descibed by LAEMMLI (4). The dotted line for species σ2 and σNS of type 3 indicates that these species comigrate with σNS appearing as a diffuse band surrounding the more discrete band of σ2. The correlations of the λ polypeptide species between the serotypes has not been determined. B: Correlations of the μ and σ size class polypeptide species or reovirus type 3 as resolved in the Tris-glycine gel system (12) and the phosphate-urea gel system. σ2 and σNS in the Tris-glycine system as above. The relationship of the λ polypeptide species has not been determined. C: Coding assignments relating the polypeptide species of type 3 to the dsRNA segments in which they are encoded as they both are resolved in Tris-glycine gels as described by LAEMMLI (4,12). σ2 and σNS above. The dotted arrow from μ1 to μ1C indicates the primary gene product (μ1) and the cleavage product (μ1C). The relative migration rates of

dsRNA segments M1 and M2 are reversed when these species are resolved in Tris-glycine gels (12) as compared to the Tris-acetate gel systems used by others. The assignments of polypeptide species to L dsRNA segments were made by McCRAE and JOKLIK (6) while the assignments of μ and σ polypeptides to M and S dsRNA segments, respectively, are supported by the data presented here and agree with the assignments of McCRAE and JOKLIK (6). Reprinted from ref. (7) with permission.

Recombinants were generated following mixed infections of ts mutants of type 3 and wild type strains of type 1 or 2. Analysis of deletions and replacements in the recombinants allowed us to correlate genome segments providing functions interchangeable between the serotypes (12).

The correlations of the genome segments are summarized on Figure 1 and the genome segment: polypeptide coding relationships for reovirus type 3 are presented in Figure 2.

VIRUS HOST INTERACTION: THE ROLE OF THE S1 dsRNA PRODUCT, THE VIRAL HEMAGGLUTININ

Immunity. Recombinants containing genome segments derived from the three serotypes of the mammalian reoviruses have been used to study the viral gene(s) responsible for specific interactions with its eucaryotic host. Antibody prepared against type 1 reovirus neutralized type 1 but not type 3 while antibody against type 3 specifically recognizes type 3. This property was found to be linked specifically to the S1 dsRNA segment, identifying it as the neutralization antigen (Table 1; 14). In a similar manner, the S1 segment, encoding the σ1 polypeptide is responsible for hemagglutination. Thus the σ1 polypeptide is the hemagglutinin.

Although reovirus does not mature at cytoplasmic membranes, we were interested in determining whether cellular immunity could be detected following infection with reovirus. We were able to show that cytolytic T lymphocytes (CTL) specifically recognizing one or the other serotypes, could be detected. Using a genetic approach, we were able to show that the viral hemagglutinin is the major determinant of specificity of the CTL (Table 2; 2).

TABLE I

NEUTRALIZATION OF HYBRID CLONES OF REOVIRUS WITH TYPE-SPECIFIC ANTIBODY

Clone	Gene coding for: Outer Capsid			Core					Nonstructural		Antibody titer*			Neutralization type
	M2	S1	S4	L1	L2	L3	M1	S2	M3	S3	1	2	3	
A	1	1	3	3	1	1	1	-	3	3	1,280	-	20	1
B	1	1	1	3	3	3	3	-	3	1	1,280	-	<20	1
C	3	1	3	1	3	1	3	-	3	3	1,280	-	20	1
D	1	1	1	1	3	1	1	-	1	1	1,280	-	20	1
E	1	3	3	3	3	3	1	-	3	3	<20	-	160	3
F	3	3	1	1	3	3	1	-	3	1	20	-	160	3
G	2	3	3	3	3	3	3	2	3	3	-	<20	160	3
H	3	3	2	3	3	3	3	3	3	3	-	<20	160	3
Number of times excluded	3	0	4	3	3	1	4	1	3	3				

* Reciprocal maximum antiserum dilution resulting in >80% plaque reduction.

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TABLE II
IDENTIFICATION OF THE PREDOMINANT VIRAL GENOME SEGMENT DETERMINING CTL SPECIFICITY

CTL*	Uninfected	Targets+ I	$65_{(I)}$	$802_{(I)}$	III	$94_{(III)}$	$204_{(III)}$
C3H I	5	50	ND	38	10	ND	17
C3H III	7	15	ND	14	58	ND	40
C3H $65_{(I)}$	15	63	70	55	30	36	34
C3H $94_{(III)}$	14	27	32	30	65	76	61

*C3H mice were infected with reovirus type I or III or recombinant clone 65 or 94. One week later, spleen cells from infected animals were restimulated *in vitro* with stimulator cells infected with the same virus used for the initial injection. CTLs were assayed at an effector/target ratio of 33:1.

+Targets were syngeneic peritoneal exudate cells infected with reovirus type I or III or various recombinant clones. Subscripts indicate the S1 genome segment - e.g., $65_{(I)}$ is recombinant clone 65 bearing the type I S1 genome segment. Cytolytic activity is expressed as % specific ^{51}Cr release. Spontaneous release ranged from 30 to 40%. ND, not done.

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Cell tropism and neurovirulence. The precise genetic and biochemical basis of tissue tropism and neurovirulence has been poorly understood. Inoculation of type 3 reovirus into newborn mice causes a necrotizing, highly virulent, encephalitis (involving neurons but not ependyma) (5,13). Type 1 infection is generally avirulent, leading to ependymal cell damage (without neuronal necrosis) and hydrocephalus (3). The difference between these two disease patterns is linked to the S1 dsRNA segment (and thus the viral hemagglutinin) (Table 3; 13). These studies suggest that specific interactions between the viral hemagglutinin and cell receptors on neurons (type 3) or ependyma (type 1) is the cause, and primary determinant of, neurovirulence.

Microtubule binding. As part of a series of studies aimed at identifying the specific association of reovirus with various cell organelles (in collaboration with Dr. R. LUFTIG), we have been examining the specific association of reovirus types 1 and 3 with cellular microtubules. The

TABLE 3

PATTERNS OF VIRULENCE, VIRAL TITERS, AND HISTOLOGY FOLLOWING INTRACEREBRAL INOCULATION OF HYBRID REOVIRUS CLONES INTO NEWBORN MICE

	Origin of genome segment[1]												
	Outer Capsid			Core			Non-structural		Uncertain		Pattern of	Viral	
Clone	M2	S1	S4	L1	L2	S2	M3	S3	L3	M1	Disease[2]	titers[3]	Histology[4]
65	1	1	1	3	3	1	3	1	3	3	1	1	Normal & 1
54	1	1	3	3	1	3	3	3	1	1	1	1	Normal & 1
802	3	1	3	1	3	3	3	3	3	3	Intermediate	Intermediate	Normal & 1
80	3	1	3	-	3	1	3	3	1	3	Intermediate	Intermediate	Normal & 1
103	3	1	1	1	3	3	3	3	3	3	Intermediate	1	Normal & 1
204	1	3	1	3	1	1	1	1	1	1	3	3	3
63	1	3	3	3	1	3	1	1	3	1	3	3	3
94	3	3	1	1	3	1	3	1	3	1	3	3	3

[1]Numbers in the table indicate from which type of reovirus the genome segment of the hybrid clone originated. The segments are grouped according to the location of their protein products in the virus.

[2]Pattern of disease. Type 1, infected animals generally survive without overt acute illness. Type 3, infected animals die after an incubation period of 5-10 days with occasional survivors at 3×10^2 PFU/mouse. Intermediate pattern, death usually occurs later than with type 3 and most animals survive at lower doses of viral inoculation (3×10^3 to 3×10^2 PFU/mouse).

[3]Viral titers: Infected brains have revealed three levels of peak virus titers. Type 1 = 10^5-10^6 PFU/ml. Type 3>5×10^9 PFU/ml. Intermediate level = 10^7-10^8 PFU/ml.

[4]Histology: Type 1, ependymal damage with associated hydrocephalus: Neuronal cells are normal. Type 3, necrotizing encephalitis affecting neurons: Ependymal cells are normal. Normal, no abnormal findings.

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striking result is a strong affinity of type 1 but not type 3 reovirus, for cellular microtubules. Using the recombinant clones, this binding was shown to be determined by the type 1 hemagglutinin (9).

SUMMARY OF FUNCTIONS OF THE VIRAL HA

Thus, the viral hemagglutinin (encoded by the S1 dsRNA segment) plays a critical role in specificity of recognition by both components of host immunity (humoral and cellular) and determines cell tropism in the nervous system; to ependymal cells in the case of type 1, to neuronal cells in the case of type 3. The viral hemagglutinin, by virtue of this recognition of different neural cells, is the prime determinant of the pattern of neurovirulence. Lastly, the hemagglutinin determines the degree of binding to cellular microtubules.

VIRUS HOST INTERACTION: THE ROLE OF THE M2 dsRNA PRODUCT, THE μ1C POLYPEPTIDE

The pattern of neurovirulence following intracerebral inoculation is primarily a property of the viral HA (see above). It was thus surprising that the "virulent" type 3 strain was avirulent when inoculated perorally into suckling mice. Studies using recombinants established the following points (11):

1. The type 3 virus does not grow in intestinal tissue following peroral inoculation of suckling mice;
2. The type 1 virus grows to high titer in intestinal tissue following similar peroral inoculation;
3. The difference in growth in intestinal tissue is not a property of the S1 gene segment but segregates with the M2 RNA segment;
4. The type 3 strain is much more sensitive to digestion by chymotrypsin than the type 1 strain. This property is also a function of the M2 gene product;
5. Recombinants that contain an M2 dsRNA segment from type 1 and S1 dsRNA segment from type 3, are virulent following inoculation of virus into the stomach. In fact such recombinants are now highly virulent.

Thus the M2 dsRNA segment determines the ability of the virus to grow in intestinal tissue and successfully enter the systemic circulation. It remains to be determined if the difference in entry is related to the susceptibility to digestion by enteric enzymes (such as chymotrypsin).

SUMMARY

In conclusion, it is clear that virulence is multigenic. However it is also clear that the specific functions of individual genes (i.e. S1 and M2) play critical roles in the ultimate outcome of a viral infection. Genetic approaches thus provide powerful tools for analysing viral pathogenesis and virulence.

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A GENETIC APPROACH TO CYTOPATHOGENICITY, VIRUS SPREAD, AND VIRULENCE OF NEWCASTLE DISEASE VIRUS (NDV)[1]

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ABSTRACT The noncytopathic (nc) mutants of the virulent AV strain of NDV, selected to form hemadsorbing spots but not plaques on chicken embryo cells (1,2) are restricted in viral RNA synthesis, accumulate less L protein intracellularly, and complement with the Group E ts RNA^- mutant, but not the Group A ts RNA^- mutants of NDV. Plaque-forming revertants of 5 of these mutants show at least partial co-reversion of each of these properties, suggesting that RNA synthesizing capacity and cytopathogenicity are causally related.
At least 3 of the nc mutants (those chosen originally as small hemadsorbing spot-formers) have a second mutation resulting in either the accumulation of an F protein precursor (nc7) or an altered polypeptide (X) related to viral polypeptide p55 (nc4 and nc16). A small plaque-forming revertant of the F protein mutant shows no reversion of the F alteration but reversion for RNA synthesis. A large spot-forming revertant of the X protein mutant shows no increased RNA synthesis, but loss of the X protein. A second step plaque-forming revertant of this spot-size revertant shows increased RNA synthesis. The independent reversion of properties relevant to cytopathogenicity and spread suggest that these are separate properties. Incremental effects of each of these mutations on mean embryo death times suggest that each contribute to virulence.

INTRODUCTION

Using a genetic approach, we have begun to explore the molecular basis of cell killing by NDV, and the relationship of cell killing to virulence in vivo. We selected 6 independent mutants of the Australia-Victoria strain of NDV, which, although unaltered in production of infectious virus (1,2), are less cytopathic than their virulent parent. The selec-

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tion of these noncytopathic (nc) mutants was based on their ability to form hemadsorbing spots, but not plaques, on chicken embryo cells after two days of incubation. In single cycles of growth in cell culture, the mutants produce virus in amounts similar to wild type virus (AV-WT). Under these conditions they are less cytopathic as measured by light microscopy, loss of protein from the culture dishes, and inhibition of total protein accumulation. In embryonated hen's eggs, the nc mutants and AV-WT produce equivalent amounts of virus with similar hemagglutinin/infectivity ratios. Each mutant exhibits an extended mean embryo death time (MDT) -- a correlate of virulence in the adult animal.

We have now characterized the nc mutants and plaque-forming revertants derived from them with respect to viral RNA and protein metabolism.

RESULTS

Figure 1 shows velocity sedimentation patterns of virus-specific RNA synthesized in cells infected with either AV-WT, mutant ncl2, or its plaque-forming revertant, ncl2R1. As previously described for infection by this and the other nc mutants (2), total virus-specific RNA accumulation is considerably less than that in AV-WT infection. Far less 18S mRNA accumulates in cells infected with ncl2 than in those infected with AV-WT; the ratio of 18S to 50S RNA also appears greatly reduced. The plaque-forming revertant, ncl2R1, has regained most of its RNA synthetic capacity and presents a pattern similar to AV-WT. Comparable results have been obtained with the other nc mutants and a series of plaque-forming revertants derived from 5 of them (3). As shown in Table 1, reversion to plaque-forming ability is, in each case, accompanied by increased virus-specific RNA accumulation. Furthermore, revertants forming the clearest plaques usually show the greatest restoration of RNA accumulation capacity, while those which produce less distinct, fuzzy plaques, have only partially reverted for RNA accumulation. Thus, virus-specific RNA accumulation and cytopathogenicity corevert.

In order to learn which gene(s) is altered in the nc mutants to give reduced levels of RNA accumulation, complementation tests were devised to determine whether RNA accumulation is enhanced in cells coinfected with nc mutants and the RNA$^-$ temperature-sensitive mutants previously isolated from the same parental strain (4). Those RNA$^-$ mutants fall into two complementation groups, A and E. Table 2 shows that tsA3 and the tsE1 mutants complement each other for RNA accumulation. This finding is consistent with their complementation for virus production (4). Table 2 also shows that

coinfections of the nc mutants and tsA3 do not result in enhanced RNA accumulation, while coinfections with the Group E mutant consistently result in enhanced accumulation. Coinfection of the nc mutants and other members of ts group A have yielded similar results to those with tsA3 (3). Coinfection of pairs of the nc mutants have also shown no enhancement of RNA accumulation, consistent with their inability to complement for plaque formation (3).

In order to further elucidate the phenotypes of the nc mutants, we next examined the polypeptides accumulating in infected cells. We have shown elsewhere that the nc mutants

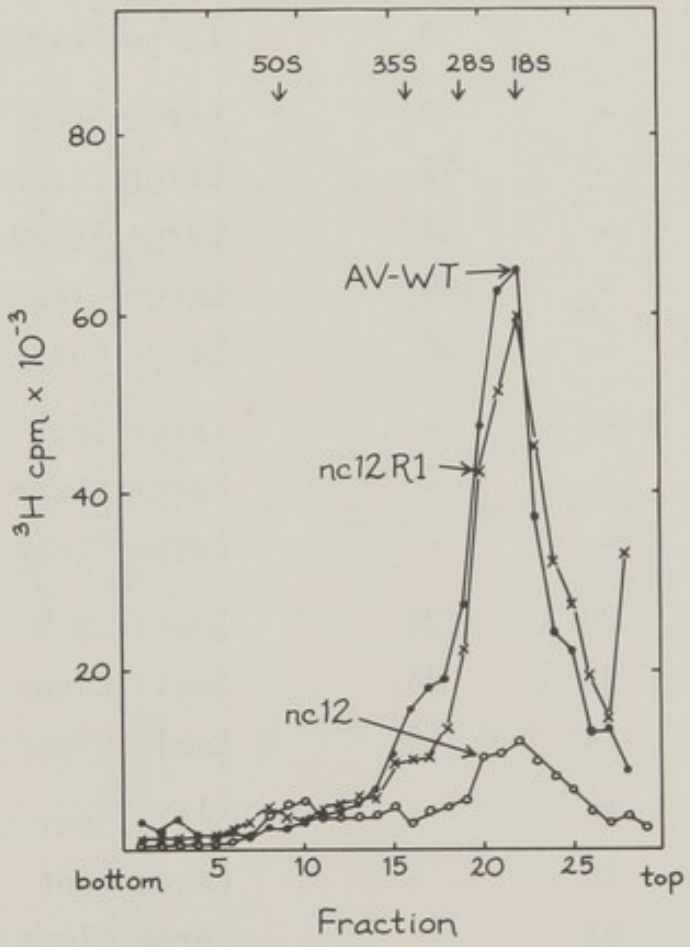

Fig. 1. Velocity sedimentation of virus-specific RNA. Chicken embryo cells infected at an moi of 5 were labeled in the presence of Actinomycin D with ^{3}H (5,6) uridine as described previously (2). RNA was extracted with phenol and run on 15-30% SDS-containing sucrose gradients; gradients were fractionated and the fractions precipitated with TCA, collected on millipore filters, and counted by liquid scintillation.

TABLE I
VIRUS-SPECIFIC RNA ACCUMULATION[a]

Virus	Experiment Number 1	2	3	Plaque or Hemadsorbing Spot Type
AV-WT	100	100	100	Large Clear Plaque
nc 9	20	-	25	Large Spot
nc 9 R1	75	-	62	Large Clear Plaque
nc 9 R2	43	-	65	Large Clear Plaque
nc 9 R3	68	-	-	Large Clear Plaque
nc 9 R4	-	-	34	Large Fuzzy Plaque
nc 12	18	-	4	Large Spot
nc 12 R1	86	-	73	Large Clear Plaque
nc 12 R2	24	-	34	Large Fuzzy Plaque
nc 12 R3	55	-	160	Large Clear Plaque
nc 12 R4	16.5	-	30	Large Fuzzy Plaque
nc 17	15	-	-	Large Spot
nc 17 R1	86	-	-	Large Clear Plaque
nc 17 R2	75	-	-	Large Clear Plaque
nc 7	-	20	24	Small Spot
nc 7 R1	-	58	84	Small Clear Plaque
nc 7 R2	-	-	45	Small Clear Plaque
nc 4	-	30	-	Small Spot
nc 4 S1	-	28	-	Large Spot
nc 4 S1R1	-	93	-	Large Clear Plaque

[a]Values represent the Actinomycin D-resistant incorporation of ^{3}H-uridine into TCA-insoluble material between 4 and 10h post-infection. Values are expressed as a percentage of AV-WT (2).

TABLE II

COMPLEMENTATION FOR RNA ACCUMULATION

Virus	Virus-Specific RNA in Single Infection[b]		Virus-Specific RNA in Double Infection with ts mutant A3[b]	Complementation Index (xA3)[c]	Virus-Specific RNA in Double Infection with ts mutant E1[b]	Complementation Index (xE1)[c]
	37.5°	41.8°	41.8°		41.8°	
AV-WT	78	108	45	0.4	92	0.8
ts A3	100	3	-	-	41	4.6
ts E1	106	6	41	4.6	-	-
nc 9	-	17	13	0.7	52	2.3
nc 12	-	20	11	0.5	88	3.4
nc 7	-	48	30	0.6	86	1.6
nc 17	-	15	8	0.4	95	4.5
nc 4	-	10	0	-	52	3.1
nc 16	-	12	0.4	0.03	52	2.9

a. Experimental procedures were identical to those in Table I. In each case, the total moi was 10.

b. cpm x 10^{-3} with uninfected background of 7.0 x 10^{-3} subtracted.

c. Complementation Index = $\frac{\text{cpm in Double Infection}}{\text{sum of cpm in Single Infections}}$

show less inhibition of total protein synthesis and less conversion to virus-specific protein synthesis than exhibited by AV-WT (1,2,3). Table 3 summarizes the accumulation of ^{35}S-methionine into individual viral polypeptides. Excluding the L polypeptide, cells infected with the nc mutants accumulate less of each of the viral polypeptides (30-63%), than do cells infected with AV-WT. This result is consistent with the reduced mRNA accumulation noted in Figure 1 and Table 1. In contrast, the L polypeptide accumulates to levels only 5-20% of those in AV-WT infection, a result consistent with the possible involvement of the L protein in RNA synthesis and the reduced RNA synthesis exhibited by these nc mutants. In contrast to this considerably reduced intracellular accumulation of the L polypeptide in nc mutant infections, each of the mutants has been shown to accumulate normal amounts of the L polypeptide in virions (2,3). The polypeptides in virions of mutants nc9, ncl2, and ncl7, in fact, show no differences in quantity or migration compared to those of AV-WT. In contrast, however, virions of mutants nc4, nc7, and ncl6, as well as cells infected by these mutants, have revealed several differences (2,3). Figure 2a illustrates the patterns of polypeptides obtained from cells infected by the nc mutants and some of their revertants, as well as from virions of AV-WT and nc7. Mutants nc4 and ncl6 fail to accumulate p55, a newly identified NDV-specific polypeptide (3,6,7) in infected cells as well as in virions (not shown). Instead, they accumulate a polypeptide (X) which has been shown to be related to p55 (2,3) by partial chymotryptic peptide mapping (8)[1]. Thus, mutants nc4 and ncl6, in addition to their reduced accumulation of intracellular RNA, appear to contain a lesion in p55.

Figure 2a also shows that virions of nc7 differ from those of AV-WT and the other nc mutants (2,3) by accumulating a polypeptide (F_7) migrating between HN and F. Partial chymotryptic peptide mapping has shown that F_7 is related to the Fo polypeptide and apparently derived from it by defective

[1]We have previously suggested that the X polypeptide is related to the F polypeptide (2), although peptide mapping in our laboratory indicated a dissimilarity to the Fo polypeptide. Recent studies in several laboratories (3,6,7) have now identified p55, previously assumed to be the F polypeptide, as a new, unique viral polypeptide (3,6,7). The X polypeptide is, in fact, related to p55. Furthermore, under the electrophoresis conditions used to run the polyacrylamide gel in Fig. 2a, p55 migrates more slowly than 55K, its apparent molecular weight under the conditions first employed to identify it.

proteolytic cleavage in nc7-infected cells (2,3). A similar situation has been described for avirulent strains of NDV (2,3,9) which, in certain cells, accumulate a form of Fo in their virions, resulting in an inability to spread from cell to cell, to grow in multiple cycles, and to form plaques.

Like the avirulent strains of NDV (9), mutant nc7 (which characteristically produces infectious virus in amounts only 10% of those observed in AV-WT infection (1,2,3)) can be activated by trypsin to wild-type levels of infectivity and ability to cause fusion from within (3). Trypsin also causes nc7 to form larger hemadsorbing spots (3). Thus, in addition to its reduced intracellular RNA phenotype, mutant nc7 appears to contain a lesion in the Fo polypeptide.

TABLE III

VIRUS-SPECIFIC POLYPEPTIDES IN INFECTED CELL EXTRACTS[a]

	Viral Polypeptides (%AV-WT)					
Virus	L	HN	F_o	F	NP	M
nc 9	5	49	34	37	35	40
nc 7	7.5	44	49	34	39	30
nc 12	5	42	32	52	59	52
nc 17	5	41	40	57	56	63
nc 4	20	55	42	-	59	43
nc 16	7.5	55	43	-	30	37
AV-WT	100	100	100	100	100	100

[a]Data were derived from densitometer tracings of autoradiograms of 7% SDS-polyacrylamide gels (2,3). Each tracing of nc mutant-infected cell extracts, or AV-WT-infected cell extracts was normalized to a tracing of an uninfected cell extract. The level of incorporation of ^{35}S-methionine into each virus-specific polypeptide was then determined by cutting out individual peaks from the normalized tracings and weighing them. The weight of each nc mutant polypeptide from the normalized tracings was then divided by the weight of the corresponding polypeptide in normalized tracings of AV-WT-infected extracts. This procedure represents a variation on the difference analysis used by Hightower and Bratt (5) to quantify virus-specific polypeptides.

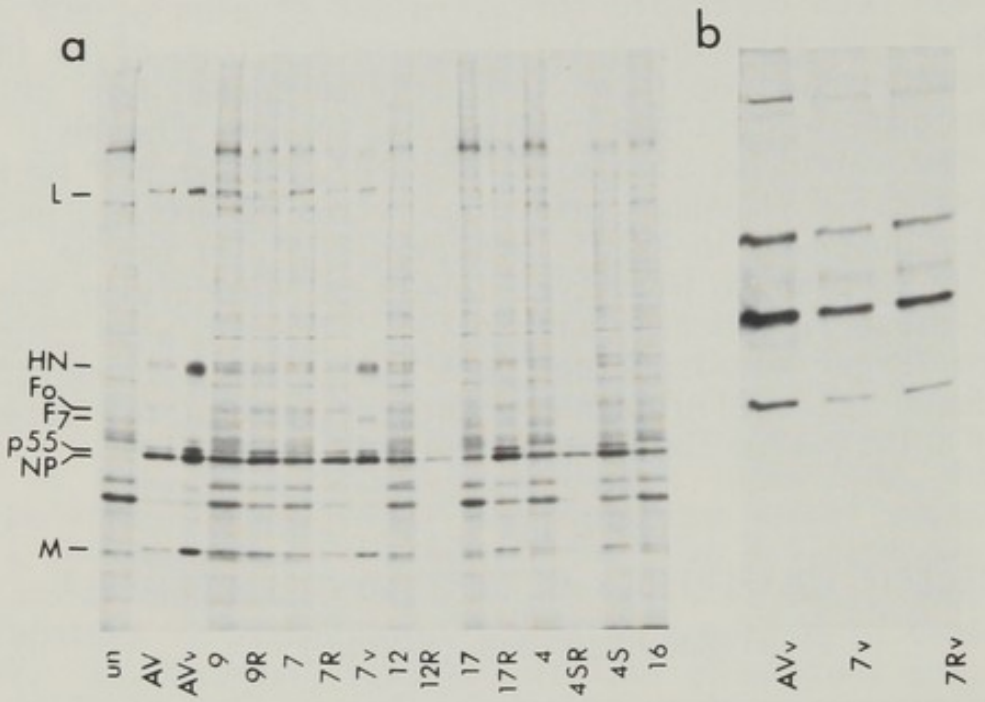

Fig. 2. Polyacrylamide gel electrophoresis of viral polypeptides. a. 10% polyacrylamide gel run at 30mA, constant current; the gel contained no SDS. Samples were extracts of either uninfected (un) or infected chicken embryo cells, except those designated (v), which were of purified virions. b. 10% SDS-polyacrylamide gel run at 30mA, constant current. Samples were extracts of purified virions.

Figure 2a also shows that in cells infected with plaque-forming revertants of the nc mutants, there are greater inhibitions of host cell protein accumulation as well as increased levels of each viral polypeptide, as compared to what is seen in infections by the nc mutants themselves[2]. Thus the increased virus-specific RNA synthetic capacity of the revertants seen in Figure 1 and Table 1 is reflected in the pattern of protein metabolism in infected cells.

[2]The patterns in Fig. 2a represent extracts made of infected cell cultures at 12h post-infection, except for AV-WT-infected cell extracts, which were made at 9h post-infection. At 12h post-infection, the cytopathic effect of AV-WT and the plaque-forming revertants of the nc mutants cause cells to detach from the culture dish. This is the cause of the relatively light autoradiographic patterns for the revertants of the nc mutants.

Mutants nc4, nc7, and nc16 are Double Mutants. Two pieces of evidence suggest that mutants nc4, nc7, and nc16 are double mutants. First, these three mutants form the smallest hemadsorbing spots, suggesting that they may have been selected for lesions in virus spread as well as cytopathogenicity (1). Second, as summarized in Table 4, these nc mutants exhibit the longest MDTs. In order to determine: 1) whether these are indeed double mutants; 2) whether the virus-spread (plaque or spot size) and noncytopathic phenotypes can be genetically separated; and 3) what the relationship of virus spread and cytopathogenicity are to MDT (and, thus to virulence, in vivo), revertants of mutants nc4 and nc7 were analyzed further.

Table 4 shows that a non-plaque-forming, but larger hemadsorption-positive spot-forming revertant (nc4S1) from nc4 accumulates viral RNA at the same reduced levels as nc4. Figure 2a shows that NC4S1 differs from nc4 in that, like AV-WT, it accumulates p55 but not polypeptide X. Thus the viral spread phenotype but not the noncytopathic phenotype, correlates with a lesion in polypeptide p55.

Next we isolated a second step plaque-forming revertant (nc4S1R1) from the size revertant nc4S1. This revertant forms large plaques. Cells infected by this second step revertant accumulate virus-specific RNA at levels similar to those seen in AV-WT infection (Table 1). Inhibition of host protein synthesis, unchanged in nc4S1 infection from that observed with nc4, is also significantly increased in nc4S1R1 infection (Figure 2a). Thus, we have genetically separated the virus spread phenotype from the noncytopathic phenotype which is correlated with reduced RNA synthetic capacity.

Table 4 also shows a reduced MDT for each revertant of the nc phenotype. In addition, mutants nc4S1 and nc4S1R1, show stepwise decreases in their MDT's from 74h for nc4, to 61h for nc4S1, to 44h for nc4S1R1, compared to 42h for AV-WT. Thus, each of the independent lesions affecting spread and cytopathogenicity appear to cause extensions of MDT, suggesting that each contribute to virulence.

Finally, the plaque-forming revertant (nc7R1) of nc7 forms small plaques identical in size to the hemadsorbing spots formed by nc7. Comparison of the virion polypeptides of nc7 and nc7R1 in Figure 2b, shows that this size revertant still accumulates F_1. Thus, once more, the viral spread and noncytopathic phenotypes have been separated genetically. Table 4 shows that the MDT of nc7R1 has decreased from the 74 hours exhibited by nc7, to 63 hours, again supporting the notion that cytopathogenicity contributes to virulence in vivo.

TABLE IV
PROPERTIES OF THE nc MUTANTS AND THEIR REVERTANTS

Virus	Plaques[a]	F_7[b]	X[c]	Relative Spot[d] or Plaque Size	MDT[e]	FFWI[f]
AV-WT	+	no	no		42	8,8
nc 9	-	no	no		63	1,2
nc 9R1	+	-	no		40	1,0
nc 7	-	yes	no		74	0,1
nc 7R1	+	yes	no		63	0,1
nc 12	-	no	no		63	2,2
nc 12R1	+	-	no		40	0,1
nc 17	-	no	no		63	7,8
nc 17R1	+	-	no		48	7,8
nc 4	-	no	yes		74	7,8
nc 4S1	-	-	no		63	3,4
nc 4S1R1	+	-	no		44	6,7
nc 16	-	no	yes		86	8,8
Uninfected	-	-	-		∞	0,1

[a]Plus (+) designates ability, and minus (-) inability, to form plaques at 39.5° after two days of incubation on chicken embryo cell cultures.

[b]"No" expresses the absence, and "yes" the presence, of the F_7 polypeptide in extracts of radiolabeled virions and infected cells.

[c]"No" expresses the absence, and "yes" the presence, of the X polypeptide in extracts of radiolabeled virions and infected cells.

[d]Relative size of hemadsorbing spots • or plaques ° after two days of incubation at 39.5°.

[e]Mean embryo death times determined as described previously (1); average of several experiments.

[f]Cultures infected and fixed and stained (3), were ordered relative to one another with respect to their degree of FFWI on an arbitrary scale of 0-8. All cultures were examined blindly and ranked by at least two individuals. The data represent the results of two separate experiments.

DISCUSSION

We have presented evidence that the six nc mutants of NDV share a lesion in the accumulation of virus-specific RNA. The lower virus-specific RNA accumulation appears to result from a proportionally greater diminution in 18S mRNA accumu-

lation than in other viral RNA species. A pattern of viral RNA accumulation similar to that observed for the nc mutants is also characteristic of the avirulent strains of NDV (2,3, 11,12). These strains are also less cytopathic than virulent strains (13). Other instances where reduced RNA synthetic capacity may be associated with reduced cytopathogenicity, include the persistent infections of NDV and VSV which release temperature-sensitive RNA$^-$ virus (14,15), and the demonstration that clones of mosquito cells more sensitive to a cytopathic effect of Sindbis virus are more permissive for viral RNA synthesis (16).

Our results with the nc mutants make it clear that: 1) the production of infectious virus, per se, need not cause cell death, and, 2) the reduced accumulation of mRNA need not affect the production of infectious virus. We propose that the accumulation of virus-specific mRNA, or some product of it, can modulate cytopathogenicity and that cytopathogenicity is causally related to virulence in vivo. Our MDT results with the nc mutants and their revertants substantiate this hypothesis. In every case, a loss of cytopathogenicity, without a loss in infectious virus production, has caused an extension of MDT. Each revertant exhibits a reduced MDT.

Mutant nc7 shows a further similarity to the avirulent strains of NDV (2,3). In addition to the F protein, this mutant accumulates a form of the Fo polypeptide (F_7) in virions which is presumably related to its reduced ability to spread from cell to cell. The lesion in the Fo polypeptide is genetically separable from the lesion responsible for the noncytopathic phenotype. Our MDT results with this mutant and its plaque-forming revertant suggest that both the virus spread and noncytopathic phenotypes contribute to virulence in vivo.

Mutants nc4 and nc16 share a second lesion resulting in the accumulation of a polypeptide, X, rather than the normal p55. These lesions apparently manifest themselves in reduced virus spread. Our results with the large spot-forming revertant of mutant nc4 and a plaque-forming revertant selected from it, again suggest that virus spread and cytopathogenicity are genetically separable and can both contribute to MDT and thus to virulence in vivo.

Finally, as illustrated in Table 4, we have obtained genetic evidence that the ability to cause fusion from within (FFWI) (15), does not correlate with cytopathogenicity. First of all, mutant nc17, by all evidence thus far obtained, appears to contain a single lesion which affects cytopathogenicity. It is, however, capable of wild-type levels of FFWI. Furthermore, the plaque-forming revertants of mutants nc9 and nc12, are unable to induce FFWI. While it is unclear why

mutants nc9 and nc12, themselves, are unable to cause FFWI (a result suggesting that viral spread and ability to induce FFWI are also not absolutely correlated), it is quite clear that the abilities to cause FFWI and to kill cells, do not go hand in hand.

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IN VITRO SELECTION OF AN ATTENUATED VARIANT OF SINDBIS VIRUS[1]

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ABSTRACT Attenuated virus strains have been derived by an empirical process guided by the observation that blind serial passage in heterologous tissue culture selectively enriches for mutants with decreased pathogenicity for the animal host. We have superimposed a selective pressure for rapid growth in tissue culture upon this process. Sindbis virus (SB) was passaged serially in BHK cells by harvesting only the virus released within the first two hours following the end of the latent period. An attenuated variant (SB-RL) was cloned from the eleventh such passage. We recently have isolated attenuated clones from the fifth rapid growth passage. One hundred percent mortality was observed in litters of one day old mice injected s.c. with 25 or more PFU of SB. In SB-RL infected litters, the survival rate decreased from 60 to 30% with doses ranging from 2.5 to 2.5×10^6 PFU/mouse. The mean day of death in SB-RL infected litters was extended significantly compared to litters inoculated with SB. SB-RL was found to protect mice from challenge with SB. In tissue culture, SB-RL was characterized by a significant reduction in latent period and an increased rate of penetration. These characteristics were expressed only in the BHK cell line used for the original selection, but not in other cell lines. SB-RL also produced higher virus titers and exhibited an increased rate of RNA synthesis. However, the expression of these characteristics was independent of cell type. SB-RL produced small plaques in all cell lines tested but was not temperature sensitive. The RNA's of SB-RL and SB appeared identical in RNase T1 fingerprints (D. W. Trent, personal communication), and the virion polypeptides were indistinguishable by SDS-PAGE. However, SB-RL could be distinguished from SB on the basis of antibody neutralization, suggesting an alteration in the E2 glycoprotein.

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INTRODUCTION

Adaptation of virulent viruses to an alternate host, either animal or tissue culture, has long been recognized as a means of attenuating virulence for the natural host. The molecular mechanism(s) involved in attenuation by blind passage in tissue culture is not understood. One explanation for this phenomenon is that virus replication involves many specific interactions between virus-derived and host-derived products. Selection in a particular host cell for virus mutants which have an increased efficiency in one or more of these virus-host interactions is thought to reduce the efficiency of the analogous interaction in another host cell type. Typically, selection of attenuated strains involves blind passage of virulent virus in a variable number of tissue culture host cells. This is followed by cloning and testing for virulence in animal models. In general, many such passages are required to achieve attenuation, and no particular selective pressure is employed. Attenuated alphavirus strains also have been isolated from persistently infected invertebrate cells after long term culture (1). As persistently infected cultures are maintained by a continuous process of spontaneous curing and reinfection (2), the virus isolated from such cultures would have been subjected to numerous "serial passages." In the case of either blind serial passage or isolation from persistently infected cultures, it has been difficult to examine the process of attenuation experimentally. We reasoned that if adaptation to specific host factors were involved in attenuation, then one could apply a selective pressure to enrich the population for virus mutants which exhibited the most efficient interaction with those factors. Efficient virus-cell interaction should lead to more rapid virus growth. Accordingly, we selected for those individuals in the SB population capable of the most rapid growth in baby hamster kidney (BHK) cells. We found that a stringent selection pressure for rapid growth led to early and significant attenuation of the virus.

RESULTS

Selection for Rapid Growth. The latent period of SB in BHK cells at 37C was 3-4 hrs following a 1 hr period for adsorption. To select for rapidly growing mutants, progeny virus was harvested at 5.5 hrs after the end of the adsorption period and used undiluted as an inoculum for a second passage. The results of continued serial passage under these conditions

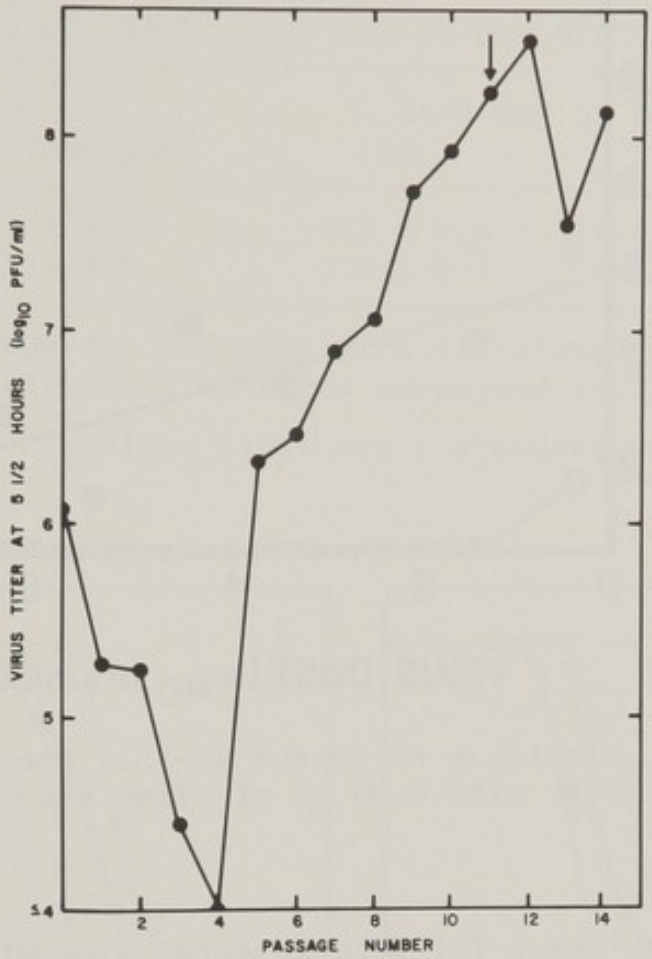

FIGURE 1. Selection for rapid growth. SB (strain AR-339 from the laboratory of H. R. Bose) was passed in BHK cells (ATCC CCL 10) using a 5.5 hr interval for virus growth. SB-RL was cloned from the passage 11 stock by plaque purification.

are shown in Figure 1. After an initial decline in titer through passage 4, the titer rebounded. Given the titer of the inoculum virus, the higher titer in passage 5 represented virus growth rather than survival of parental virions. The titers achieved in subsequent passages continued to increase. These results suggested that an initial rapid growth mutant was selected in the fifth passage and that additional mutations which further accelerated virus growth were selected in the later passages. SB-RL (Sindbis-Reduced Latent Period) was cloned from the eleventh rapid growth passage by plaque purification.

Pathogenesis of SB-RL in Suckling Mice. The virulence of SB-RL for neonatal mice was attenuated significantly compared to the virulence of SB (Figure 2). One-day old

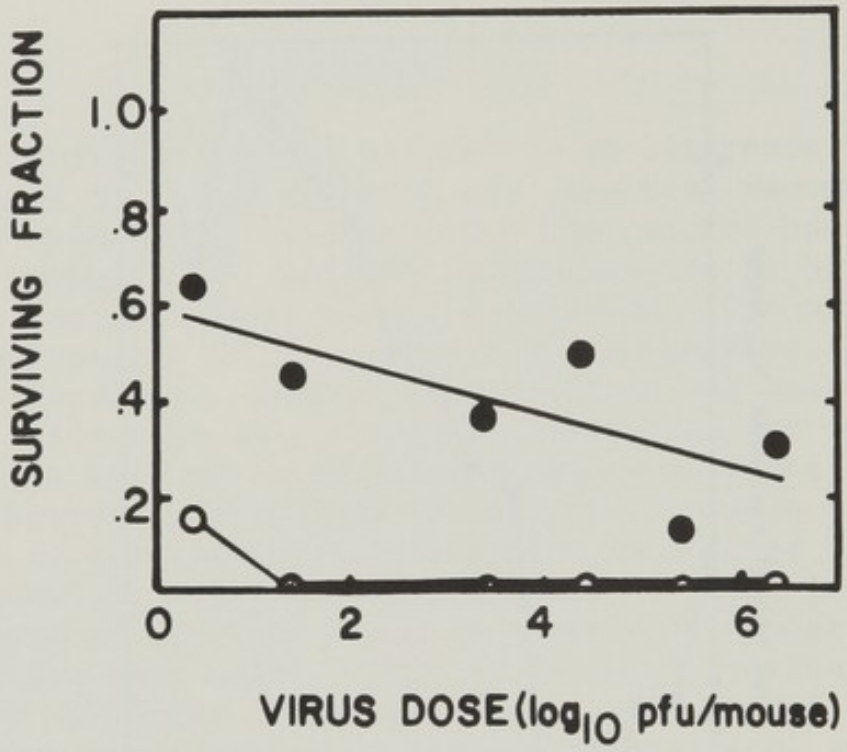

FIGURE 2. Virulence of SB and SB-RL. One-day old mice were inoculated s.c. with 0.05 ml of virus suspended in PBS. ● SB-RL; ○ SB.

litters of ICR-L^+ mice (3) were injected s.c. with doses of SB or SB-RL ranging from 2.5 to 2.5×10^6 PFU/mouse. In the case of SB, all animals died which received doses of 25 PFU or greater. The two mice which survived the lowest dose (2.5 PFU/mouse), had no neutralizing antibody to SB which suggested that they were not infected successfully. In the SB-RL infected litters, the survival rate was reduced from 60 to 30% over a dosage range of 6 logs. Mortality induced by SB-RL appeared to be only marginally dependent on dose, and it was not possible to obtain a classical LD_{50}. As shown in Table 1, the mean day of death in SB-RL infected animals was extended significantly compared to mice inoculated with SB. Viral antigen, as measured by indirect fluorescent antibody staining, was present in localized areas of the brain within one day after s.c. inoculation of SB, and became widely dissiminated by day 5. In contrast, no SB-RL antigen was detected until 3 days post-inoculation, the areas of staining remained localized, and no antigen was detectable after day 11 (Humphries, Kanich, and Johnston, unpublished data).

We also have investigated the ability of SB-RL to protect mice from challenge with SB. Litters of mice 4 days of age were inoculated with either 10^3 PFU/mouse SB-RL, 25

TABLE 1
MEAN DAY OF DEATH IN SB AND SB-RL INFECTED MICE

Virus Inoculum[a]	Mean Day of Death[b] SB	SB-RL
2.5×10^1	4.2 ± 0.4	11.8 ± 0.8
2.5×10^6	3.6 ± 0.5	8.9 ± 2.3

[a]One-day old mice were inoculated s.c. with 0.05 ml virus suspended in PBS.

[b]The values given are ± standard error.

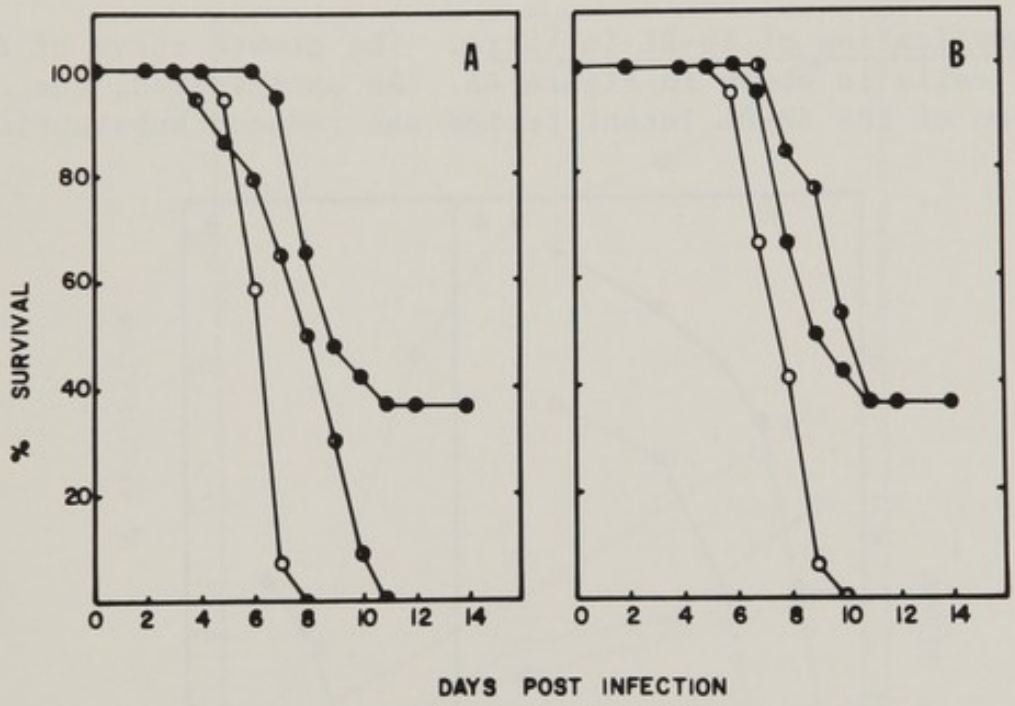

FIGURE 3. Protection of SB-RL-inoculated mice against SB challenge. Panel A: 4 day old mice inoculated with 2.5×10^3 PFU SB-RL●, with 25 PFU SB○, or 2.5×10^3 PFU SB-RL + 25 PFU SB◑; Panel B: mice inoculated with 2.5×10^3 PFU SB-RL at 4 days of age●, 2.5×10^3 PFU SB-RL at 4 days and 25 PFU SB at 5 days◑, or 25 PFU SB at 5 days○.

PFU/mouse SB, or 10^3 PFU/mouse SB-RL+25 PFU/mouse SB. As shown in Figure 3, panel A, simultaneous inoculation of SB-RL and SB did not protect mice from death, although the day of death appeared to be extended somewhat. However, when 24 hours was allowed between inoculation of SB-RL and challenge

with SB, protection was observed (panel B). The ability of SB-RL to limit the lethality of SB may result from a mechanism similar to that responsible for the limited virulence of SB-RL itself. Attainment of a threshold titer of virus in the brain is thought to correlate with expression of disease symptoms and eventual death (4). These preliminary results suggest an auto-interference mechanism which would limit the replication of SB-RL to a level near this hypothetical threshold. Furthermore, the interference would serve to limit the replication of SB administered as a challenge to mice which previously had been inoculated with SB-RL. Although an immune response cannot be ruled out, it seems unlikely that maximal immunological protection would have been established within 24 hrs after SB-RL inoculation. A mechanism involving interferon induction or rapid production of DI particles could account for this result.

Replication of SB-RL In Vitro. The growth curve of SB-RL in BHK cells is shown in Figure 4A. As anticipated, the duration of the SB-RL latent period was reduced substantially

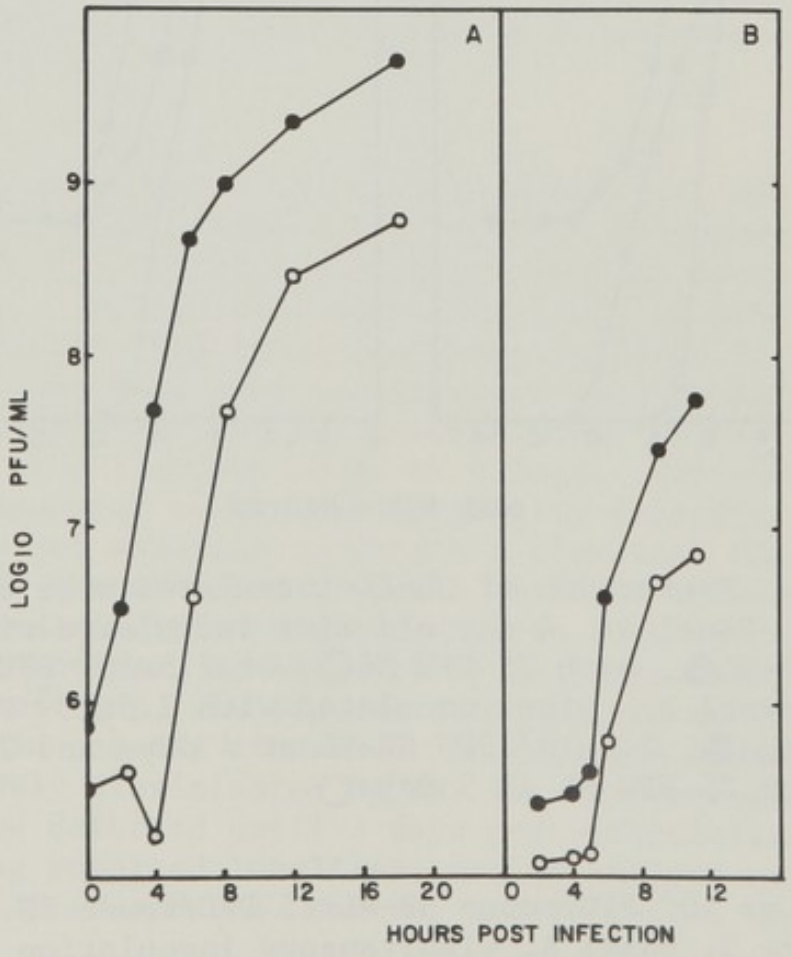

FIGURE 4. Replication of SB and SB-RL in tissue culture. Panel A: growth in BHK cells; Panel B: growth in VSW cells. SB-RL●; SB○.

compared to SB. In addition, the period of logarithmic growth was extended in SB-RL infection, and SB-RL grew to a greater final titer than SB. The reduction in latent period was specific for the ATCC-BHK cells used in the selection of SB-RL in that the SB and SB-RL latent periods were similar in viper spleen cells (VSW, ATCC CCL129) (Figure 4B), in chicken embryo fibroblasts, and in an independently propagated BHK line which had been passaged at least 100 times more than the ATCC-BHK line. However, SB-RL grew to a higher final titer than SB in these cells as well.

The reduction in the SB-RL latent period was at least partially due to an increased rate of penetration in BHK (Figure 5). In VSW cells, SB and SB-RL displayed similar rates of penetration, consistent with the observation that

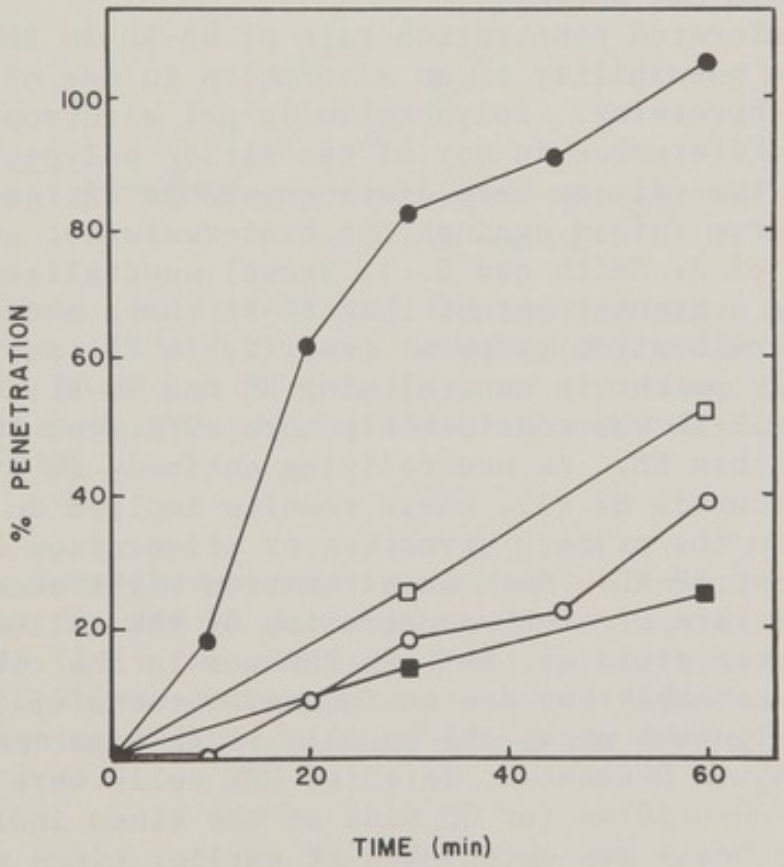

FIGURE 5. Penetration rates of SB and SB-RL. Cell monolayers were infected with SB or SB-RL at 100-200 PFU/monolayer. At the designated times, antiserum prepared against SB or SB-RL was added to neutralize any remaining extracellular infectious virus. After 10 minutes at 37C, the antiserum was removed, and the monolayers were overlayed with MEM containing 1% agarose for development of plaques. The number of plaques obtained after a 60 min adsorption and no antiserum treatment was taken as the 100% value. ● SB-RL in BHK; ○ SB in BHK; ■ SB-RL in VSW; □ SB in VSW.

TABLE 2
NEUTRALIZATION INDEX FOR ANTISERA TO SB AND SB-RL

	SB	SB-RL
Anti SB	1:800[a]	1:1600
Anti SB-RL	<1:100	1:3200

[a]Highest dilution of antiserum which neutralized 50% of the added virus infectivity.

the SB-RL latent period was reduced in BHK exclusively. Adsorption of both SB-RL and SB was essentially complete within 15 min (data not shown).

The accelerated penetration rate of SB-RL in BHK cells suggested the possibility of an alteration in one of the envelope glycoproteins. Polyacrylamide gel electrophoresis revealed no differences in any of the virion polypeptides. However, the two viruses were distinguishable antigenically. Rabbit antiserum raised against the heat-resistant strain of SB (courtesy of J. Smith and D. T. Brown) neutralized SB faster and to a greater extent than SB-RL (data not shown). In a 50% neutralization endpoint test (Table 2), antiserum to SB was equally potent in neutralizing SB and SB-RL; however, antiserum to SB-RL was considerably more efficient in neutralizing SB-RL than SB. As neutralizing antibody is directed exclusively towards E2 (5), these results implied an alteration either in the primary structure or orientation of the E2 glycoprotein of SB-RL. Such an alteration might account for the increased rate of SB-RL penetration of BHK cells.

The greater yield of SB-RL in BHK and in the other cell lines tested probably was due to increased rates of SB-RL RNA synthesis. Figure 6 shows the results of an experiment in which actinomycin D-treated, infected BHK cells were pulse-labeled with ^{3}H-uridine for 30 mins at the times indicated. SB-RL RNA synthesis was detectable at earlier times than SB RNA synthesis in BHK cells (Figure 6) but not in VSW cells (data not shown). However, SB-RL displayed higher maximum rates of RNA synthesis in both cell types.

SB-RL and SB co-sedimented in potassium tartrate gradients, and they appeared morphologically identical in thin sections of infected cells. The genome RNA's of each co-migrated in urea-agarose slab gels. The two RNA's were identical when examined by ribonuclease T_1 oligonucleotide fingerprinting (D. W. Trent, personal communication). SB-RL was capable of interfering with the replication of SB by

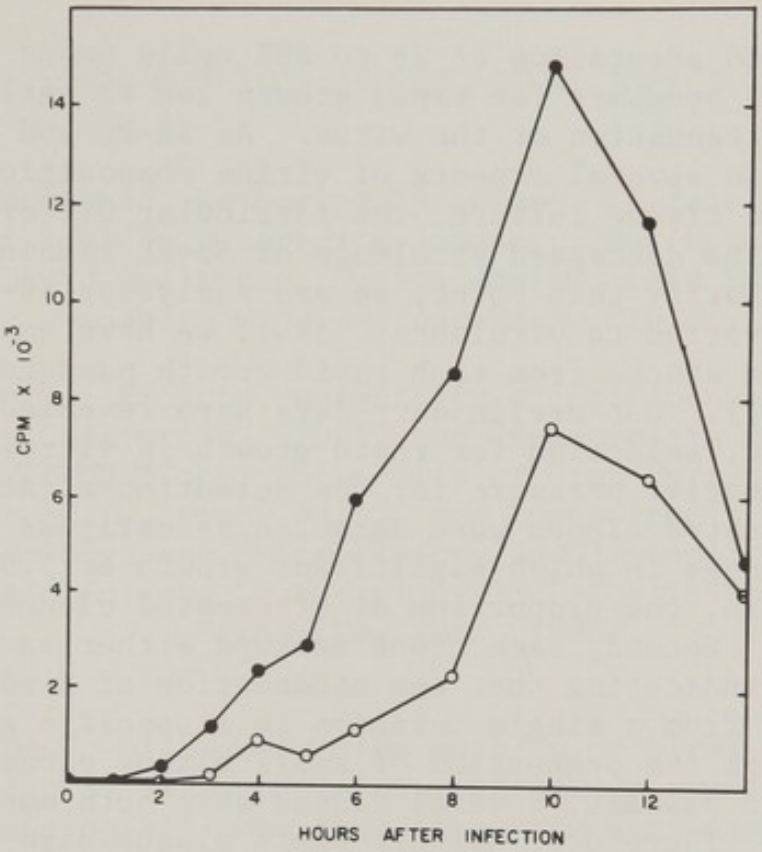

FIGURE 6. Rates of viral RNA synthesis in infected BHK cells. Actinomycin D treated infected BHK cells were pulse-labeled for 30 min with ^{3}H-uridine at the times indicated. Cell-associated, TCA precipitable radioactivity was determined. ●SB-RL; ○ SB.

homologous interference and was sensitive to interference mediated by SB. SB-RL produced smaller plaques on BHK cells than did SB (1.1±.2mm and 2.0±.2mm, respectively). And finally, SB-RL was not temperature sensitive. At 37 and 40.5C, plaquing efficiencies of SB-RL and SB on BHK cells were identical.

DISCUSSION

The features which distinguish the replication of SB-RL from SB fall into two categories: cell-dependent and cell-independent. The reduction in latent period, earlier onset of RNA synthesis, and increased rate of penetration were observed only in BHK cells. The antigenic differences between SB and SB-RL suggested an alteration in glycoprotein E2 which could account for an accelerated penetration rate. Other properties of SB-RL were independent of cell type. The rate of SB-RL RNA synthesis was at least twice that of SB, and the

final yields of SB-RL were greater than SB in all cell lines examined.

The forced adaptation of SB to BHK cells under a stringent selection pressure for rapid growth led to early and significant attenuation of the virus. As SB-RL and SB differ with respect to several aspects of virion composition and replication in tissue culture, the particular difference which accounts for the decreased virulence of SB-RL remains unclear. In order to clarify this point, we are analyzing SB-RL stocks which have reverted to virulence. Also, we have examined several cloned stocks from each rapid growth passage (passages 4-12, Figure 1). Our preliminary data have revealed several points. First, selection for rapid growth in vitro appeared to be the operative pressure for the selection of attenuated virus. Attenuated clones were detected as early as passage 5, the first passage in which significant growth at 5.5 hrs was observed. Also, the proportion of attenuated clones increased with passage. Second, each clone behaved either as attenuated or virulent, indicating that the attenuation of Sindbis could have resulted from a single mutation in a specific gene. Third, although the proportion of small plaque clones also increased with passage, several clones were both small plaque and virulent. Therefore, neither small plaque size nor temperature sensitivity was correlated with attenuation in the case of SB-RL (Olmsted and Johnston, unpublished results).

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VIRAL MUTATION IN PERSISTENT INFECTION[1]

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ABSTRACT Oligonucleotide mapping of viral RNA, peptide mapping of virus proteins, and virion RNA termini sequencing all show that long term persistent infection by Vesicular Stomatitis Virus (VSV) leads to extensive and continuous genome mutation. The DI particles present in persistently infected cells apparently help drive this rapid mutation of infectious virus because serial high multiplicity passages of VSV in acute lytic infections generates more oligonucleotide map changes in virus RNA than does alternating high and low m.o.i. passage, or low m.o.i. passage. Furthermore, virus populations in persistently - infected cultures are selected for resistance to the DI particles present initially in the carrier cultures. The mutant virus clones recovered after many years of persistence contain more than 2% of mutated bases at the 3' end of virion RNA and about 15% at the 5' end. The mutant virus recovered after years of persistence does not rapidly revert, and is as stable as the original input virus clone. A number of biologically interesting mutant phenotypes have been recovered from these persistently infected cells. These include: (1) ts small plaque, avirulent mutants; (2) mutants resistant to DI particles originally present in the persistently infected cells; (3) mutants which give extremely low virus yields on initial isolation and which are able to establish

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persistent infection without added DI particles; (4) mutants selected in vivo which allow persistently infected cells to form tumors and metastases in nude mice (these strongly induce interferon and natural killer cells, but escape the natural killer cell surveillance due to virus mutation); (5) mutants arising spontaneously in the carrier cells in culture which are poor inducers of interferon and of natural killer cells, and which also allow carrier cells to form tumors and metastases due to viral mutations; (6) mutants which show reduced neutralization kinetics with heterologous and homologous antisera; (7) mutants exhibiting apparent smaller size M protein, or a major new virion protein band near the G protein; (8) mutants which have lost all mouse virulence (VSV and rabies virus), and mutants which acquired mouse virulence (rabies HEP Flury vaccine strain). Many of the phenotypes must involve multiple mutations and a number of them may be due to the same mutation(s). Considerable further work will be required to map and understand these phenotypes, but they should provide considerable insight into virus functions. It is clear from this work that RNA virus genomes have an unexpected degree of mutational plasticity, and that viral persistence is a highly competitive, metastable situation which shows no signs of stabilizing as viral genomes and DI particle genomes evolve over many years.

INTRODUCTION

The mechanisms of persistent infection by virulent RNA and DNA viruses have been a subject of considerable investigation recently because of implication for disease mechanisms and for virus natural history and evolution. For a recent overview of this area of virology see reference (1). The mechanisms invoked to explain RNA virus persistence are temperature sensistive mutants (2, 3, 4), small plaque mutants (5), interferon induction and action (6), and interfering (DI) particles (7-16). In some systems (16) a combination of these mechanisms have been invoked. Even among DNA viruses where integration can serve to establish and maintain persistence, DI particles may play a role in many types of persistent infection (17-19).

We recently reported (20) that virus in our long term persistently infected BHK_{21} cells (originally infected with cloned VSV Indiana ts mutant, ts G31, plus DI particles) has been undergoing extensive and continuous mutations in the standard virus RNA genome during more than 5 years of

persistence in cell culture. This was shown by oligonucleotide mapping of cloned virus isolated from carrier cells at various time in the evolution of the persistent infection. Concomitant with the changes in infectious standard virus genome RNA oligonucleotide, the populations of defective interfering virus particles recoverable from these carrier cells have changed frequently in size and oligonucleotide map pattern. We report further observations on this system below.

METHODS

Methods and details are presented in the references cited below. The VSV Indiana carrier culture refered to in this paper is the CAR4 carrier cell in which BHK_{21} cells were originally infected with cloned ts G31 mutant (20) of VSV Indiana along with purified homologous short DI particles to prevent cell death (8). This CAR4 carrier culture has been passaged regularly several times weekly at 37° C. for over 6 years and has grown at normal or near-normal rates despite the fact that 100% or nearly 100% of the cells are continuously infected and expressing virus antigens (22).

The two rabies carriers described below were established in the same manner using rabies virus plus homologous DI particles to infect BHK_{21} cells. One was established using the HEP Flury vaccine strain (30) of rabies virus and its homologous DI particles, and the other using challenge virus standard (CVS) rabies virus and its homologous DI particles.

RESULTS

PEPTIDE MAPPING SHOWS MUTATIONAL CHANGES IN ALL VIRUS PROTEINS AFTER 5 YEARS OF PERSISTENCE

We determined (23) that the extensive genome mutations observed by oligonucleotide mapping were expressed in viral proteins. High temperature, high pressure cation exchange peptide map comparisons of the proteins from cloned virus recovered after 5 years of persistent infection demonstrated that each of the 5 proteins is altered. The membrane proteins G and M and the nucleocapsid protein N exhibited a large number of changes. This was predictable based upon the oligonucleotide map changes (20) and the spot assignments to viral messangers made by Freeman *et al.* (24). The membrane protein changes were not surprising since efficient maturation of virus may not be important for long term persistence of virus intracellularly but the extensive changes in the N

protein were unexpected since this nucleocapsid structural protein is highly conserved in rhabdovirus evolution (25).

VIRION RNA TERMINI SHOW MUTATION DURING PERSISTENT INFECTION

Oligonucleotide map changes after 5 years of persistence indicated that approximately 1% to 2% of the genome RNA sequences were mutated,(20) so it was of interest to examine the rate of change of the 5' and 3' termini of virion RNA. Direct dideoxy substitution sequencing of the 3' terminus of the RNA of 5 year virus showed two base substitutions (and no additions or deletions) in the first 110 residues (23). This approximate 2% mutation level is in accord with the average percentage of changes for the genome as a whole. However indirect RNA sequencing of the 5' end (via DI generation and DI polymerase product sequencing) showed that after 5 years of persistent infection the 46 nucleotides at the extreme 5' end have undergone 7 base substitutions (and no additions or deletions) (26). This very high mutation rate (in excess of 15%) at the 5' end was unexpected since the 3' end of the plus strand RNA is complementary to the 5' end of the minus strand, and 3' ends are important replicase initiation sites. It is possible that these 5' end mutations play a role in reducing virus maturation during persistence (26).

VIRUS RECOVERED AFTER MANY YEARS OF PERSISTENCE IS TEMPERATURE SENSITIVE, SMALL PLAQUE, POORLY-REPLICATING, AND ALTERED IN VIRULENCE.

The most obvious phenotypic alteration in the VSV recovered after 5 or 6 years of persistent infection of BHK_{21} cells <u>in vitro</u> is the tendency to replicate poorly. These mutants are not only temperature sensitive, as was originally observed by Younger and his colleagues (2, 4) and by Fields and Raine (37) but they are debilitated in their replication at any temperature, low or high (27). They replicate optimally at 30° -33° C , and have become increasingly difficult to isolate from the carrier cultures. After 6 years of persistence the carrier cultures are yielding only 10^{-4} p.f.u. per cell per day (or less) although about 100% of the cells are infected and expressing viral antigen and resistance to homologous superinfection. Plaque formation is extremely slow, giving tiny (ca. 1mm) plaques in 3-4 days at

32° C. (whereas wild type virus gives large 2-4 mm plaques in 24 hours at 32° C.). Even after elimination of DI particles by cloning, yields are often variable and low, and cytopathology requires many days to appear (27). One 70 month plaque isolate slowly destroyed a monolayer of 2×10^7 BHK_{21} cells but produced only several p.f.u. (or infectious particles upon dilution). This yield represents approximately one infectious particle per 10,000 affected cells whereas wild type virus produces over 10,000 p.f.u. per cell. This same isolate increased its yield per cell hundreds to thousands fold upon the next few passages (27), as less debilitated mutants apparently were selected in lytic passages. VSV recovered after more than 5 years of persistent infection has lost its neuropathogenicity for mice. Whereas 10 p.f.u. of wild type VSV Indiana injected intracerebrally kills young adult mice within several days (28), 5 year virus does not cause death or obvious disease when a million or more p.f.u. are inoculated intracerebrally (27).

Finally, virus recovered after more than $5\frac{1}{2}$ years has acquired the property of establishing persistent infections in BHK_{21} cells without added DI particles (23). It does so only when infecting at low multiplicities of infection (all cells are eventually killed at high m.o.i.). By the time these infected BHK_{21} cells recover from initial cytopathology and stabilize as carrier cultures, DI are present (23) but their addition was not required at the time of infection. Clearly, the selective forces operating on virus evolution during persistence tend to select variants with lowered cytopathology and lowered virulence. In other systems early-appearing mutants can establish persistence without DI particles (4, 6, 16) but different mechanisms (such as interferon restriction of infection to a small percentage of cells) operate to allow persistence in this type of "steady-state" persistence at the population level.

BHK_{21} CELLS PERSISTENTLY INFECTED WITH RABIES VIRUS MAY GIVE RISE TO VIRULENT OR TO AVIRULENT MUTANTS AT DIFFERENT TIMES.

Rabies virus, unlike VSV, tends to produce disease in vertebrates in which a very long incubation period is followed by a progressive fatal disease process which is not reversed by a late immune response (29). Koprowski and his colleagues (29, 30) prepared a very effective attenuated live virus vaccine (the HEP Flury strain) by a large number of passages in a foreign host (the embryonated chicken egg). We established a carrier state in BHK_{21} cells using this

vaccine strain of rabies (22, 31). Initially this virus caused no disease in young adult mice (the HEP Flury strain kills only newborn mice), but after several years of persistent virus infection recovered mutants caused typical rabies symptoms and death in adult mice (32). However, this characteristic was lost as the carrier state evolved over the next few years. In a similar manner BHK_{21} carrier cells persistently infected with the virulent challenge virus standard strain of rabies virus (CVS strain) shed virulent virus (able to cause rabies in adult mice) during the first year of persistence but not afterwards (32). It appears that mutations in rabies virus persistence are not necessarily always in the direction of reduced virulence, even in non-neural cells *in vitro* where selection for neurovirulence is unlikely to operate. Rabies is a good example of a virus for which there is only one serotype worldwide. Yet it is inappropriate to view all rabies virus strains as being the same virus biologically and chemically because of the extreme mutational plasticity which is now becoming apparent among the RNA viruses. Wiktor and his colleagues have found extensive antigenic divergence and complex strain relationships among different rabies virus isolates by monoclonal antibody typing. As seen in Figure 1, our oligonucleotide map comparisons of the genome RNAs of HEP Flury and CVS rabies virus shows enormous mutational divergence (32), despite their antigenic relatedness and biological relatedness. Obviously RNA viruses have available a very rich repertoire of viable genome sequence arrangements.

VSV MUTANTS WITH ALTERED ABILITY TO INVOKE AND RESPOND TO NATURAL KILLER CELL DEFENSES

We have transplanted CAR4 carrier cells and other carrier cells into athymic nude mice to study *in vivo* selection pressures on the carrier state. Normal BHK_{21} cells and other transformed cells form large tumors in nude mice because they lack T cell rejection (33). Surprisingly, we found that all of our virus carrier cells (VSV-BHK, rabies-BHK, mumps-BHK, influenza-BHK, and measles-HeLa carrier cells) usually do not form tumors in nude mice (34-36). They are either rejected or they form only small nodules greatly restricted in their growth. This nude mouse restriction of persistently infected cells is due to natural killer (NK) cells which are activated by interferon. Irradiation of nude mice (34) or treatment with anti-mouse interferon (36) prevents NK restriction; the carrier cells form tumors (and metastases). The carrier cells are much better NK targets *in vitro* than

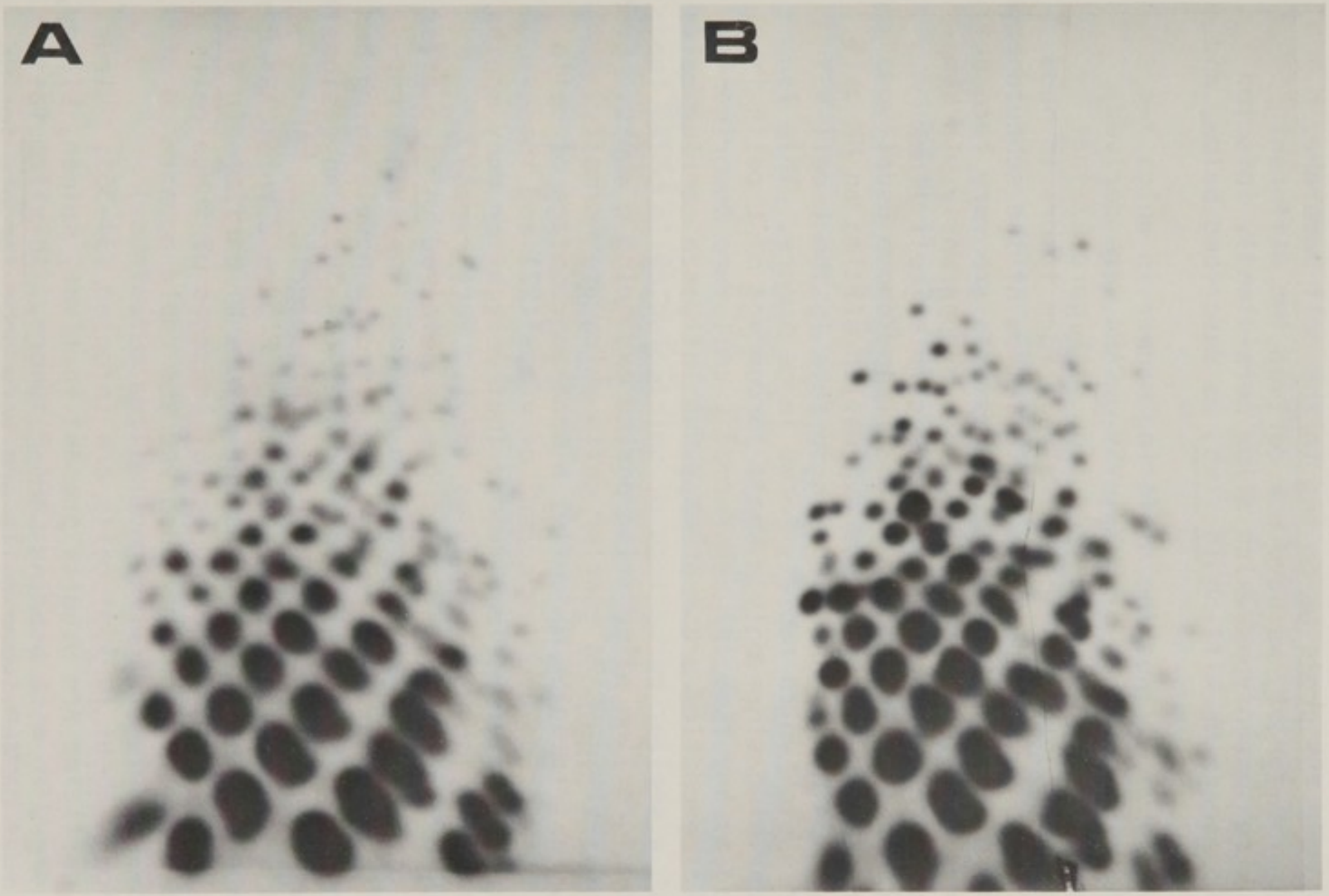

Figure 1: Ti - oligonucleotide map comparison of CVS-11 (A) and HEP-Flury (B) rabies RNA genomes

are the corresponding uninfected tumor cells. The virus induced target for NK killing is not yet known, but the G and M proteins are the most likely targets (either alone or in association with host cell membrane proteins) since they are the only VSV proteins on and in the cell membrane. Whatever the target, we have now derived two different mutant virus phenotypes which allow persistently infected cells to escape NK killing *in vivo*, as described below, and in Figure 2.

Figure 2 outlines the differing manner in which two tumor-forming sublines of BHK_{21}-VSV_{IND}CAR4 were derived. Firstly it should be emphasized (top of Figure 2) that parental uninfected BHK_{21} cells always form tumors in nude mice. These tumors are large solid tumors at the subcutaneous site of infection. Generalized metastases are very rare but sometimes metastases to the lungs occur apparently via hematogenous spread. Secondly, all the carrier cell lines established in this laboratory and listed above usually do not form tumors because virus expression elicits an NK cell response (34, 35). An exception to this is the L cell-VSV carrier system which rapidly forms large tumors. This is not surprising since only a small percentage of L cells is infected at any time (4) and normal L cells are tumorigenic. A partial exception are the BHK_{21}-NWS influenza carrier cells in which a smaller percentage of uninfected cells often leads to tumor formation after a lag period exceeding 6 weeks. Even with BHK_{21}-VSV_{IND}-CAR4 carrier cells which are normally heavily restricted by NK cells, a small percentage of cells in the carrier do become "cured" of virus (22) and these sometimes cause virus-free tumors [but only after many months of NK restriction within nodules (34)]. Very rarely, however, a virus-shedding tumor can be selected *in vivo* after months of NK cell restriction in a nodule. One such virus-producing tumor arose in a nude mouse from a nodule which had been contained by NK cells for 7 months (34-36). We designate this tumorigenic subline CAR4-P (for producer).

As shown on the right hand side of Figure 2 this CAR4-P subline sheds infectious virus and mature DI particle *in vivo* and *in vitro*. Upon repeated serial passages of these cells in nude mice, we regularly observed the rapid appearance of very invasive tumors (with generalized metastases) even though about 100% of cells are infected (34-36). Virus and DI particles are usually present in liver, spleen, kidney, blood, etc., in mice bearing CAR4-P tumors. *In vitro* tests showed that CAR4-P tumor cells are very poor targets for NK cell killing, although they are extremely potent inducers of interferon and of NK cells [probably because they shed virus in large quantities (34-36)]. We isolated and cloned the

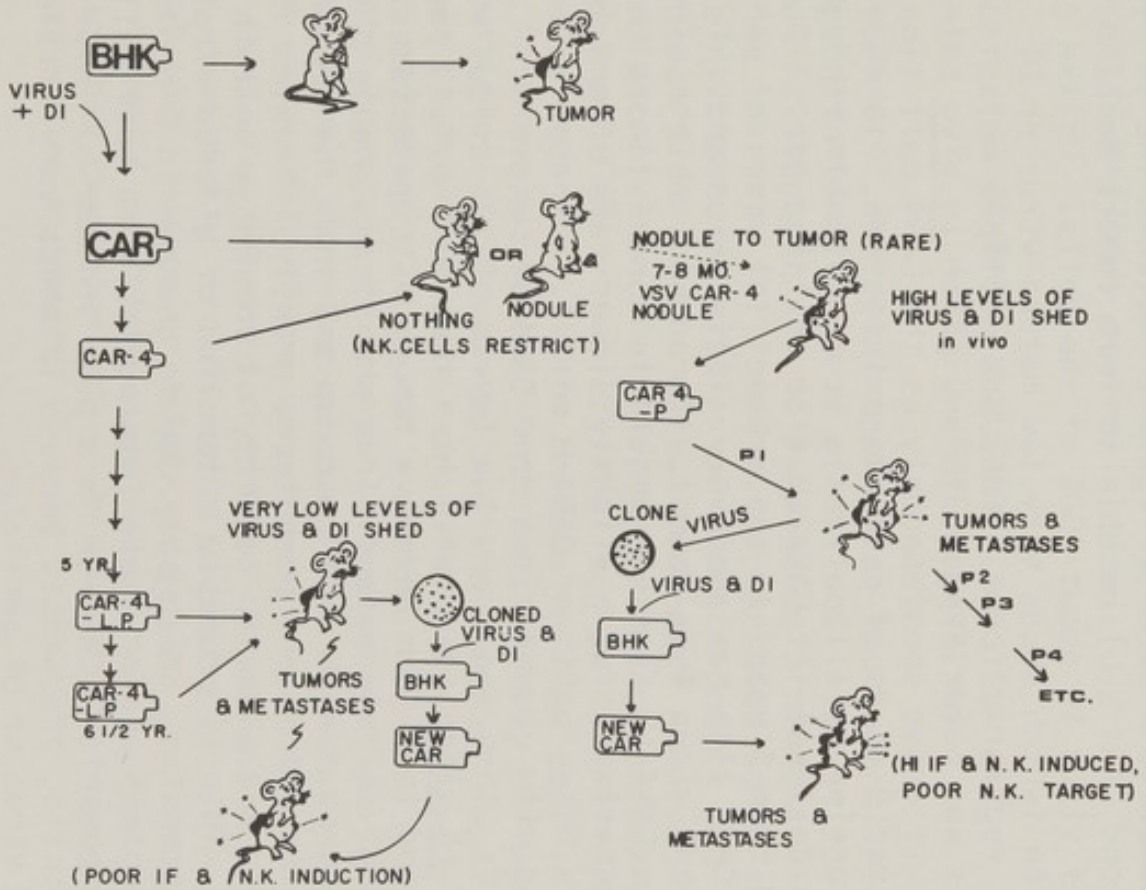

Figure 2: Carrier cell effects on nude mice (see text for details).

virus shed by CAR-P tumors (designated by CAR4-P) and used it (plus homologous VSV-P DI particles) to establish a new carrier state in normal BHK_{21} cells. Surprisingly, we found that the new carriers behaved like CAR4-P, since they too induced invasive tumors and multiple metastases in nude mice (and they too shed large amounts of mature virus and DI particles). Apparently some mutation(s) in the VSV-P virus allows tumor cells carrying this virus to escape NK cell killing.

The lower left side of Figure 2 outlines the derivation of the second CAR4 subline able to form tumors despite the presence of virus in about 100% of the cells. Unlike CAR4-P this subline (which sheds very few mature virus or DI particles) regularly forms tumors and multiple metastases although is was never exposed to any prior *in vivo* selective pressure. It arose spontaneously as the CAR4 cell line has evolved after 5 years of persistent infection. In fact it *is* the CAR4 carrier cell line (not a true subline) although for clarity we designate it CAR4-LP (for low producer). During the period from 5 years to the present ($6\frac{1}{2}$ years of persistence) the CAR4 line has shed very little virus and this is "debilitated" virus (see above). Throughout this recent period this CAR4-LP line has regularly formed tumors and multiple metastases (at a slightly slower rate of growth than occurs with BHK_{21} cells or CAR4-P cells). Carrier cells established with cloned virus from CAR4-LP behave identically: they shed very low levels of infectious virus *in vivo* or *in vitro* (generally less than 10^{-4} p.f.u. per cell per day), and they form invasive tumors with metastases. We conclude that certain mutant virus phenotypes enable BHK_{21} cells to escape the NK cell response and that the presence of the two different mutant phenotypes greatly increases the invasiveness and metastic capacity of the BHK_{21} cell in nude mice. This is probably a function of altered viral surface expression, presumably due to changes in G protein and/or M protein and/or their interaction with host cell surface components. CAR4-LP is a poor inducer of interferon and of NK cells, presumably because it sheds very little infectious virus or DI particles.

CAR 4 MUTANT VIRUS AFTER 5 YEARS SHOWS REDUCED NEUTRALIZATION KINETICS

Since CAR4 virus has obviously been undergoing extensive mutational and phenotypic changes we examined its kinetics of neutralization to determine whether antigenic drift was occurring also. Antibody raised in mice by tsG31 infection

did in fact neutralize 5 year virus significantly more slowly than it neutralized homologous tsG31 virus. Surprisingly however, antibody raised in mice by infection with 5 year virus also neutralized tsG31 significantly more rapidly than it neutralized homologous 5 year virus. Likewise, antibody raised in rabbits to inactive wild type VSV Indiana (Mudd-Summers strain) neutralized homolgous Mudd-Summers virus more rapidly than the Glasgow strain tsG3 mutant, and it neutralized tsG31 significantly more quickly than 5 year virus (38). We conclude that 5 year (VSV-LP) virus is somehow more resistant to antibody neutralization.

SOME CAR 4 MUTANT CLONES EXHIBIT ALTERED PROTEIN PATTERNS

Because of the reduced cellular and humoral reactivity of the above mutants, it is of obvious interest to determine the quality and quantity of expression of mutant virus proteins. We have initiated molecular cloning to aid in rapid RNA sequencing studies of protein-coding and intercistronic regions of the mutant genomes. However, preliminary studies of viron proteins have already demonstrated that 70 month CAR4 virus (VSV-LP) has an "extra" protein in purified virons (38). This protein is identical in molecular weight to non-glycosylated G protein of VSV, but we have not yet identified it as such by peptide mapping, sugar labelling, etc., so it could be a host protein, a pseudotype glycoprotein from an endogenous virus, or a protein from any other source. Since the G protein is present in normal proportions it is hard to explain why a non-glycosylated G protein would be present at about half the concentration of the G protein.

We have also observed that VSV-P is unique among all CAR4 mutants examined so far in exhibiting altered M protein of apparent lower molecular weight on S.D.S. acrylamide gels (38). Attempts to relate such changes in protein patterns to biological characteristics will require a combined genetic and biochemical approach.

DI PARTICLES APPEAR TO INCREASE THE MUTATION RATE OF INFECTIOUS VSV

The unexpectedly high rates of viral genome mutation encountered in persistently infected cells led us to examine whether repeated lytic passages of virus also generated a high mutation rate (20, 23). We did not observe a high mutation rate upon repeated low multiplicity passage of VSV

(20), [although alternating high and low m.o.i. passages did lead to a few oligonucleotide map changes after more than one hundred passages (23)]. Alternating passages at high and low multiplicity allow inter-genome complementation and some interaction with DI particles at every other (high m.o.i.) passage, while preventing the DI cycling interference phenomenon described by Palma and Huang (39). Repeated low m.o.i. passage greatly reduces the opportunity for interactions among a variety of different mutant genomes and among standard virus genomes and DI genomes [which occurs constantly in the cytoplasm of persistently infected cells (22)]. Recently we have observed that repeated undiluted passage of a mixture of VSV and its DI particles leads to rather rapid changes in oligonucleotide maps of infectious virus genome RNA (40). Because of DI particle-virus particle cycling (39) the input m.o.i. of virus and DI particles vary considerably at each undiluted passage, but this type of passage is probably most analogous to the situation in persistently infected cells because many cells remain alive and infected for many days at those passages where the virus-DI particle ratio is optimal for alternation of viral cytopathology. Thus a single passage time may vary from 24 hours to 6 days and in the latter case there is optimal opportunity for prolonged virus-virus and virus-DI genome interactions in the cytoplasm. In any case, we are observing viral genome oligonucleotide map alterations at a rate at least equal to those occurring in persistent infections. The "mutagenic" effect of DI particles is specific for VSV since the rate of mutation of poliovirus is not altered in BHK_{21} cells persistently infected with VSV and its DI particles (40). This effect of DI particles may be due to some mutagenic effect of VSV genomes during replicative competition, and/or to prolonged intracellular genome interactions resulting from DI particle sparing of cell death, and/or to DI particle selective pressures on virus mutant populations. Perhaps all of these (and other) mechanisms are operative.

VIRUS MUTANTS ARE SELECTED FOR RESISTANCE TO POPULATIONS OF DI PARTICLES PRESENT INITIALLY DURING PERSISTENT INFECTION

Kawai and Matsumoto reported that small plaque mutants of rabies virus were resistant to the DI particles originally present in persistent infections initiated with large plaque virus, although these DI particles interfered strongly with the large plaque virus that generated them (41). In contrast the DI particles generated by the small plaque mutants

recovered from persistent infection were able to interfere efficiently with both large plaque and small plaque virus.

We have observed an exactly analogous situation in our persistently infected CAR4 cells (42). Apparently the original input "wild type" DI particles select virus mutants able to escape their interference effects, although the mutants later generate DI particles which interfere with original virus and with mutant virus. We do not yet understand the molecular mechanisms involved but interference ability correlates well with replication ability of each DI particle type when interacting with any mutant standard virus (42). Clearly this type of selective interaction can help drive the evolution of virus populations in persistent infection, and rapid change is clearly involved in persistence.

DISCUSSION

It is now very clear that persistent infection by RNA viruses is a dynamic multifactorial phenomenon that cannot be explained by a ts mutation, by a certain type of DI particle, by interferon induction, or by any other single event or phenomenon. The extent of mutational variability capable of yielding viable virus has been hitherto unsuspected.

It will require extensive genetic and biochemical analysis to determine the basis for the biologically interesting phenotypes described here, but they should provide some important insights into virus functions. The availability of virus mutants able to escape NK cell killing, to promote tumor metastases, to partially escape antibody neutralization, etc., offers some unexpected opportunities since molecular cloning and rapid sequencing techniques will facilitate detailed analysis of these small RNA viruses.

Persistent, slow and recurrent infections *in vivo* in immunologically intact (or debilitated) hosts undoubtedly involve multiple mutational changes in virus akin to, but even more complex than those described here. The adaptational value of antigenic shifts in influenza viruses (43) and strong antigenic variation in visna virus (44) is obvious and widely recognized. The advantages of multiple, but more subtle mutational adaptions may be of equal evolutionary importance for viruses, both in acute and persistent infection.

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ANTIGENIC VARIATION OF VISNA VIRUS

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ABSTRACT Antigenic variants of visna virus have been compared using the genomic RNA and analyzing the large RNase T_1-resistant oligonucleotides. Mutants isolated from a persistently infected sheep contained a small number of changes in their oligonucleotide patterns when compared with parental virus. To determine whether the changes in the nucleotide structure were clustered in one region of the genome, the oligonucleotides of the parental and mutant RNA were ordered along the genome with respect to the 3' polyadenylated end. All but one difference between the parental strain and the antigenic mutant used for mapping were located within 2 kilobases from the 3' terminus. The electrophoretic mobilities of some of the oligonucleotides which differed from the parental to the mutant suggest that they might be derived by simple mutation. To determine whether this was how the mutants arose, the complete nucleotide sequence of several oligonucleotides were determined. These analyses showed that a number of the oligonucleotides which were different between the parental and mutant could be accounted for by single base changes.

INTRODUCTION

Visna virus, an exogenous retrovirus, is the causative agent of a degenerative disease of the central nervous system of sheep (1,2). The disease has a prolonged incubation period of months to years, followed by a subacute progression to death (3). Visna is a classical model of a slow virus infection.

Virus replication occurs slowly in the animal and virus can be recovered from tissue and peripheral blood leukocytes by co-cultivation in culture with sheep cells throughout the infection. However, this lifelong persistent infection produces minimal quantities of cell-free virus. This is in

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contrast to the replication of the virus in sheep cell cultures which occurs with a one step growth cycle of 20 hrs and causes acute cytopathic effects. In the animal an active immune response may be responsible for clearing cell-free virus. Sheep mount both a normal humoral and cell-mediated immune response to the infection; however, the virus persists. This persistence can partially be explained by the presence of proviral DNA in brain cells of infected sheep (4). However, the recurring inflammatory response suggests that there is periodic expression of virus which results in the progressive clinical disease. The evolution of virulent antigenic mutants in sheep during the long infection could account for these periods of active expression of virus.

It has been shown by Narayan et al. (5,6) that visna virus undergoes antigenic variation in the animal. Viruses isolated early after infection from leukocytes of sheep were identical to the inoculum virus. Those isolated one year or longer after infection were antigenically distinct from the virus used in the original inoculum. Early in the infection neutralizing antibody was directed exclusively against the parental virus. However, the sheep subsequently developed neutralizing antibody in their sera to the variant viruses. These viruses are fully virulent when inoculated into sheep causing characteristic lesions in the CNS and eliciting a humoral immune response. In addition, the antigenic variants can be plaque purified and are genetically stable to multiple subcloning.

In order to determine the structural basis of antigenic variation of visna virus, as well as any relationship amongst the antigenic mutants, we have studied the genomic RNA of the parental strain of visna virus and a number of antigenic variants isolated from a single persistently infected sheep. Using the method of RNase T_1 resistant oligonucleotide analysis, the fingerprints of these viruses were compared. To determine if changes in the genome were clustered or randomly distributed, the oligonucleotides of 1514 and LV1-1 were ordered with respect to the 3' polyadenylated end of the genome. The entire nucleotide sequence of a number of the oligonucleotides was determined. From these studies it appears that progressive mutational events in the region coding for the glycoprotein of visna virus provide a mechanism for the variant to evade the humoral immune response. In addition, the successive appearance of antigenic variants may elicit a renewed cellular immune response which may account for the slow progressive nature of visna.

METHODS

Virus Strains. Virus strain 1514 was purified 3 times prior to inoculation into a sheep (7). LV1-1 was isolated from a sheep 2.2 years and LV1-5 and LV1-7, 3.25 years later by co-cultivation of peripheral blood leukocytes with choroid plexus sheep cells (6). The viruses were plaque purified and grown in sheep choroid plexus cells (6), purified by equilibrium centrifugation (8).

RNA Isolation and Fingerprinting. The viral RNA was isolated as previously described (9,10). 0.1-1 µg of RNA from each virus was incubated in the presence of RNase T_1 and bacterial alkaline phosphatase followed by polynucleotide kinase catalyzed transfer of ^{32}P-phosphate from γ-^{32}P labelled ATP to the 5' hydroxyl groups of the RNase T_1 resistant oligonucleotides using the conditions described previously (9,10). The oligonucleotides were separated by two dimensional polyacrylamide gel electrophoresis (9,10). The first dimension (10% acrylamide, pH 3.5) is oriented from left to right, the second dimension (22.8% acrylamide, pH 8.3) is from bottom to top.

Sequence Analysis of RNase T_1 Resistant Oligonucleotides. The 5' labelled oligonucleotides were eluted from the second dimension gel and concentrated by ethanol precipitation (9, 10). The sample was dissolved in 35 µl of 20 mM NaCitrate , 1 mM EDTA, 7 M Urea, 0.25 µg/ml of yeast carrier RNA and 0.025% (W/V) of xylene cyanol FF and bromophenol blue adjusted to pH 5.8 with citric acid.

Five µl aliquots were added to microtiter wells with the following additions (10): 1. no addition; 2. 2.5 µg RNase A; 3. 0.125 µg RNase A; 4. 0.25 U of RNase U_2; 5. 0.25 U of RNase U_2; 6. an appropriate amount of Physarum RNase; 7. 0.005 U of RNase T_2, incubated for 1 hour at 50°C. The samples were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea. The sequence deduction was based on the following cleavage specificities of the ribonucleases: RNase U_2 will cleave A-N bonds (11), Phy RNase will cleave A-N and U-N bonds (12), RNase T_2 will cleave all bonds and RNase A has the following range of bond cleavage frequencies: C-A >U-A >>C-C>U-C >>C-U >U-U (13).

The 5' terminal nucleotide was identified by thin layer chromatography on PEI cellulose after complete alkaline hydrolysis of the oligonucleotides as described (10).

Isolation of Subgenomic Fragments of Viral RNAs. Size classes of subgenomic poly (A) containing RNA fractions were

derived from 70S RNA containing partially fragmented RNA. Approximately 100 µg of 70S RNA was denatured by heating for 5 min at 95°C in 1 ml of 5 mM EDTA, pH 7.0, the poly (A) containing RNA molecules were isolated by chromatography on an oligo (dT) cellulose column (9,10) and separated according to size by velocity sedimentation through linear gradients of 0.44-0.88 M sucrose in 50 mM Tris HCl pH 7.5, 100 ml NaCl 1 mM EDTA, and 2 g/l of Na-dodecyl sulfate, centrifuged for 4.5 hrs at 37,000 rpm at 20°C in an SW 41 rotor. A parallel gradient contained sedimentation markers, ^{3}H labelled rigosomal RNA 28S, 18S and 4S. The RNA from pooled fractions of the gradient was concentrated by chromatography on an oligo (dT) cellulose column followed by ethanol precipitations (9,10). The RNA samples were analyzed by two dimensional mapping of RNase T_1 resistant oligonucleotides as described above.

Ordering of the Oligonucleotides. The location of the oligonucleotides near the 5' end relative to the poly (A) sequence was determined by visual inspection of RNase T_1 fingerprints of subgenomic RNA fractions isolated as described. Detailed ordering was done with the oligonucleotides in the 3' region of the genome of virus strain LV1-1. The relative content of RNase T_1 resistant oligonucleotides in two short poly (A) containing subgenomic fractions of RNA from virus strains LV1-1 and 1514 was determined by measurement of Cherenkov radiation of 5' labelled oligonucleotides of second dimension gels. The values were normalized to the amount of radioactivity in the corresponding oligonucleotides derived from analysis of 70S RNA.

RESULTS

We have utilized several viruses isolated from a sheep inoculated with visna virus strain 1514 to study the structural changes that occur (6) in the viral RNA during antigenic variation. The viruses were isolated by co-cultivation of peripheral blood leukocytes with sheep cells in cultures, thus we denote them as leukocyte viruses (LV) from a particular sheep (#1) with the order of isolation from that animal (6). LV1-1 was isolated eighteen months after sheep #1 had been inoculated with strain 1514, while LV1-5 and LV1-7 were isolated 3.25 years after inoculation. None of these isolates were neutralized by the serum obtained from sheep #1 early (up to 2 years) after infection, although the animal subsequently developed neutralizing antibody to each of the variants. The three variants comprised 2 serological groups based on the time after infection that neutralizing antibody

was detected and the level of antibody produced by the animal (6). LV1-1 and LV1-5 were similar in that no antibody was detected until 3.5 years after infection and only low levels were found. The animals sera contained neutralizing antibody against LV1-7 at 2.5 years after infection and the level was twice the level of that directed against LV1-1. All three viruses retained their resistance to neutralization after multiple subcloning (6). In addition, these viruses were indistinguishable from the parent in their virulence in tissue culture or in sheep.

RNase T_1 Fingerprints of the Viral RNAs. RNA isolated from 1514, LV1-1, LV1-5 and LV1-7 was examined by mapping of T_1 resistant oligonucleotides. Oligonucleotides produced by digestion of viral 70S RNA with RNase T_1 were radioactively labelled at the 5' end with ^{32}P, the mixture was fractionated by two dimensional gel electrophoresis and the oligonucleotide pattern analyzed by autoradiography using the methods described elsewhere (9,10). Figure 1, panels a,b,d, and e show the autoradiograms (termed RNase T_1 fingerprints) of oligonucleotides derived from 1514, LV1-1, LV1-7 and LV1-5 respectively. Approximately 80 large oligonucleotides of each virus are resolved.

The fingerprint patterns of the four viral RNAs are strikingly similar (see Fig. 1, panels a,b,d,and e). To determine whether the majority of the oligonucleotides did have the same electrophoretic mobilities, samples of the labelled oligonucleotides from 1514 and LV1-1 (1c) were mixed and analyzed on the same gel. Figure 1c, schematically diagrammed in Figure 1f, shows that the majority of the oligonucleotides co-migrate in the two dimensional system. Similar mixing experiments have been done with LV1-5 and LV1-7 and again the majority of the oligonucleotides migrate together. This indicates that the genomes of these viruses have very similar nucleotide sequences. However, a number of oligonucleotides unique to 1514 and LV1-1 could be identified, while LV1-5 and LV1-7 contained a subset of these oligonucleotides. Oligonucleotide spot numbers 2,21 and 24 are present in the parental strain 1514 and are absent from LV1-1. Spot numbers 101, 102, 103, 104, 105 and 106 represent oligonucleotide sequences which are found in LV1-1 but not in 1514. LV1-7 contained oligonucleotides 2 and 24 common to 1514 but lacked 21 and contained oligonucleotide 104 found also in LV1-1. The fingerprint of LV1-5 lacked oligonucleotides 21 and 24 found in 1514 but contained two oligonucleotides 103 and 104, common to LV1-1. Oligonucleotide 105 is not completely resolved from the neighboring oligonucleotide. Therefore, in LV1-1 it could not be established from the

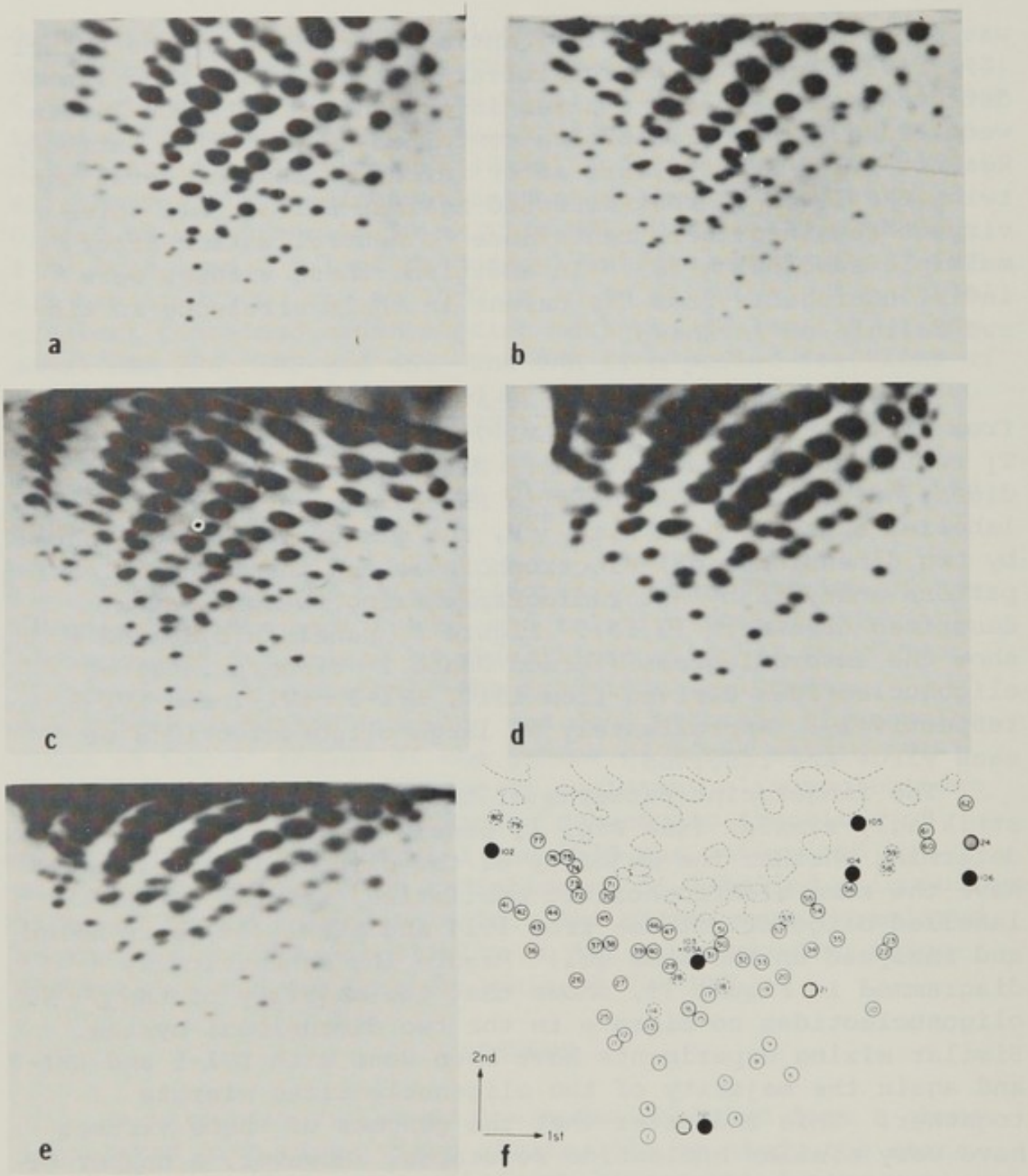

FIGURE 1. RNase T_1 fingerprint of 70S RNA of visna virus strain 1514 (a); and LV1-1 (b); LV1-7 (d); LV1-5 (e). Analysis of a mixture of the two viral RNA of 1514 and LV1-1 is shown in (c) and the number used in identification of the oligonucleotide is given in the diagram (f) using the following symbols: ○ Single oligonucleotides common to the two strains; ◎ Single oligonucleotides unique to strain 1514; ● Single oligonucleotides unique to strain LV1-1. The spots represented by broken lines contain oligonucleotides in more than unimolar amounts. Some of these sponts containing only a few oligonucleotides have been assigned a number.

established from the fingerprint of the 70S RNAs whether or not this oligonucleotide was missing in 1514. However, studies with subgenomic fragments of the viral RNA of 1514 and LV1-1 (see below) demonstrate that oligonucleotide 105 of LV1-1 is absent from the fingerprint of 1514 RNA.

Nucleotide Sequence Analysis of Oligonucleotides. What is the relationship amongst the oligonucleotides of the viruses that do not co-migrate? Since 1514 and LV1-1 contained all the oligonucleotides found in the four viruses, these two were used for oligonucleotide sequence analysis. The electrophoretic mobilities of some of the oligonucleotides in the LV1-1 fingerprint suggests that they might be derived by simple mutations from oligonucleotides in the parental strain (10). For example, the relative mobilities of oligonucleotide 2 (of 1514) and 101 (of LV1-1) suggest that these oligonucleotides may be related by a single base change of either an A or a C to U. The mobility of 106 (of LV1-1) suggests that it may be different from 24 by addition of a single U residue. Therefore, we decided to obtain the complete nucleotide sequence of the oligonucleotides unique to each virus. The 5' labelled oligonucleotides were isolated from the second dimension gel and their nucleotide sequences determined by mapping of cleavage sites for sequence specific ribonucleases (11).

The sequence of these oligonucleotides is given in Table 1. The sequence of 106 differs from that of 24 only by an additional U. The sequence of 101 is different from that of 2 only in the 5' terminal oligonucleotide, a change from a cytidine to a uridine. The ' terminal sequence of

TABLE 1

SEQUENCES OF THE OLIGONUCLEOTIDES OF 1514 AND LV1-1

Virus	Spot No.	Nucleotide Sequence
1514	2	C A U C C C A A A U A U C U U A A A A C U A A C G
LV1-1	101	U A U C C C A A A U A U C U U A A A A C U A A C G
1514	24	C U U U U U U G
LV1-1	106	C U U U U U U U G
1514	21	U U U U A U C C A A A U A U G
LV1-1	104	U U U U A U C C A G
LV1-1	105	U A A U U U A G
LV1-1	102	A A C C C C C A A G

oligonucleotide 104 is the same as that of the first eight nucleotides of oligonucleotide number 21 of 1514. Thus, oligonucleotide 104 of LV1-1 could be derived from oligonucleotide 21 of 1514 by a mutation from an adenine to a guanine located three bases from the 5' end of the oligonucleotide. Such a change would provide a new cleavage site for ribonuclease T_1. In addition, both LV1-5 and LV1-7 lack oligonucleotide 21 while having an oligonucleotide at a mobility identical to spot 104.

Sequences of oligonucleotides 102 and 105 that were found only in the fingerprint of LV1-1 were determined. No sequences similar to these were apparent in the sequences of the oligonucleotides of 1514. Sequence analysis of the material corresponding to spot number 103 of LV1-1 did not lead to the determination of an unambiguous nucleotide sequence. This is most likely due to the presence of more than one oligonucleotide in this sample. As we were unable to separate individual components of this material by gel electrophoresis, we took a different approach in the analysis of this sample.

The oligonucleotide material corresponding to spot number 103 was divided into three aliquots, which were digested to completion with RNase U_2, RNase A and RNase T_2 respectively. The radioactive cleavage products derived from the oligonucleotide 5' ends were then analyzed by electrophoresis on DEAE paper at pH 3.5 (14). A single product, identified as pAp was detected in the aliquots treated with RNase U_2 and T_2. The complete RNase A digestion, however, produced two products. These two products were found to contain an equal amount of radioactivity (experimental value 1.01). Therefore, we conclude that spot number 103 of LV1-1 represents two unique oligonucleotides which are not present in the fingerprint of 1514.

Ordering of the Unique Oligonucleotides of 1514 and LV1-1. In order to evaluate the biological significance of the difference in nucleotide sequence between RNA of the four viral strains, we wished to determine the position on the genome of the unique oligonucleotides. Previous studies have shown that the genome of visna is not permuted relative to the 3' poly (A) containing terminus (15,16,17). Therefore, it should be possible to construct a physical oligonucleotide map of the genome of visna virus by an analysis of the relative content of the large RNase T_1 resistant oligonucleotides in different subgenomic size classes of poly (A) containing RNA (18). Since strain LV1-1 contained all the altered oligonucleotides found in the other two variants, it and 1514 were used for the mapping studies. For this purpose

70S RNA was denatured by heating and the poly (A) containing molecules isolated by chromatography on oligo (dT) cellulose. The poly(A)containing RNA was fractionated according to size by velocity sedimentation in sucrose gradients. RNA samples isolated from different parts of the gradient were analyzed by RNase T_1 fingerprinting. Examples of such fingerprints of LV1-1 are shown in Fig. 2. For each RNA fraction, the relative content of the large RNase T_1 resistant oligonucleotides was determined by visual insepction of the autoradiogram and by measurement of Cerenkov radiation of gel pieces containing individual oligonucleotides.

This analysis permitted us to construct a precise oligonucleotide map of the 3' region. Oligonucleotides derived from other parts of the genome, where the mapping technique is less accurate, were divided into four groups according to their location relative to the poly (A) sequence as shown in the oligonucleotide map of Fig. 3. The analysis of the oligonucleotides in the simplest fingerprints (see Fig. 2) also permitted the identification of oligonucleotide 105 as unique to LV1-1 but did not reveal any differences between RNA of the two strains which had not been detected by the analysis of the complete genome.

Figure 3 presents the combined results of analysis of 1514 and LV1-1, as we found that co-migrating oligonucleotides derived from the two viruses were located in the same region of the genome. We also found that oligonucleotides from these two strains that have related but not identical nucleotide sequences were derived from the same region of the genome, indicating that such oligonucleotides (2 and 101, 21 and 104, 24 and 106) are allelic.

The oligonucleotide map shows that all but one of the differences between the parental strain and LV1-1 are located in the 3' region of the genome. Oligonucleotides 24 and 21 of the 1514 strain are located in this region of the parental strain. Oligonucleotides 102, 104, 105 and 106 of LV1-1 virus are located in the 3' region of this strain. Of the oligonucleotides that differ between the two isolates, only oligonucleotides 2 and 101 map in the 5' half of the genome. The identity of the oligonucleotides 24, 21, 104, 105 and 106 in the subgenomic fragments was confirmed by sequence analysis (Table 1).

Spot number 103, which represents more than one oligonucleotide in the complete genome of LV1-1, is also present in the fingerprints of the short poly(A)selected RNA. Further analysis of this material by complete digestion by ribonuclease U_2, A and T_2 indicated that the short subgenomic RNA fraction contained oligonucleotides 103A and 103B in equal amounts. Oligonucleotides 103A and 103B must therefore

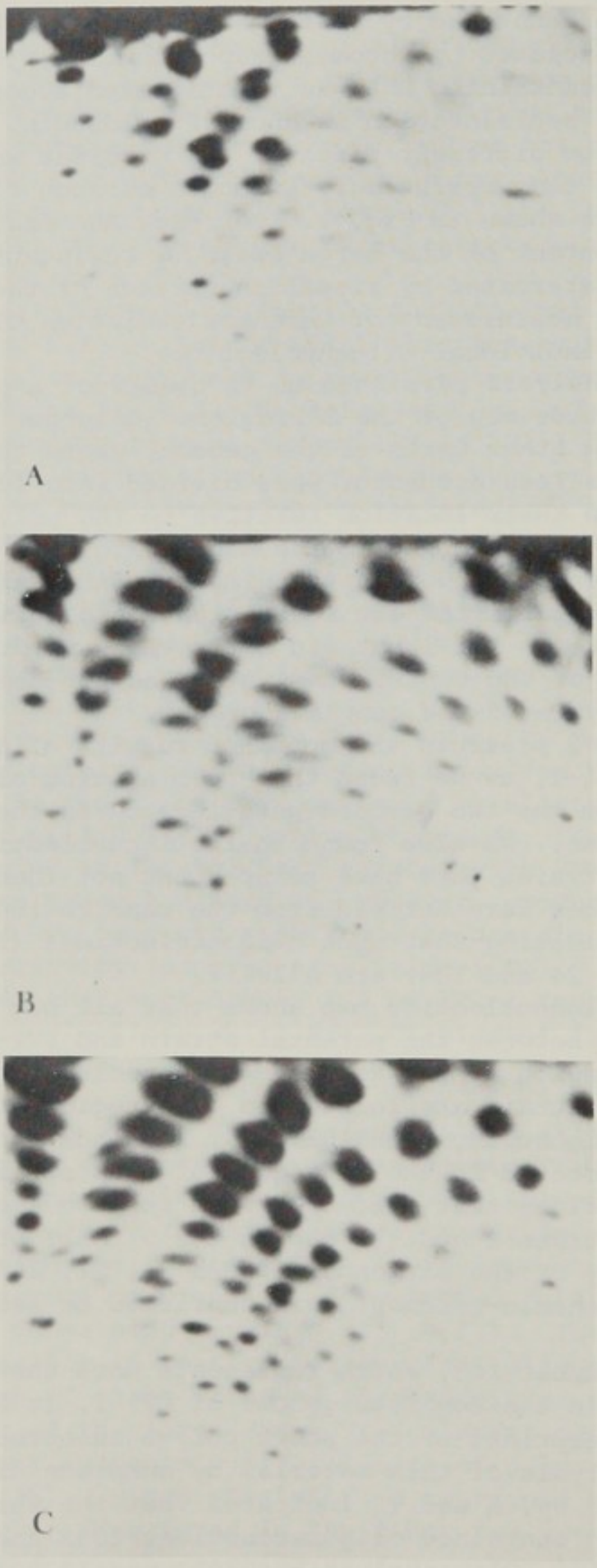

FIG. 2.

be derived from the same part of the 3' region of the genome of LV1-1.

Inspection of the fingerprints of the viral RNAs of the variants LV1-5 and LV1-7 suggested that the differences in the oligonucleotides of these strains from the parental strain 1514 were a subset of the changes observed between 1514 and LV1-1. To determine which of the RNase T_1 oligonucleotides of the variants co-migrated with the oligonucleotides of 1514, samples of the labelled oligonucleotides of LV1-5 or LV1-7 were mixed with those of 1514 and analyzed on the same gel (data not shown). The fingerprint of LV1-7 differs from that of 1514 only by the absence of oligonucleotide 21 and the presence of oligonucleotide 104. The mutation from an adenosine to a guanosine in oligonucleotide 21 observed in LV1-1 (Table 1) could account for this difference. The fingerprint of LV1-5 differs from that of 1514 by the

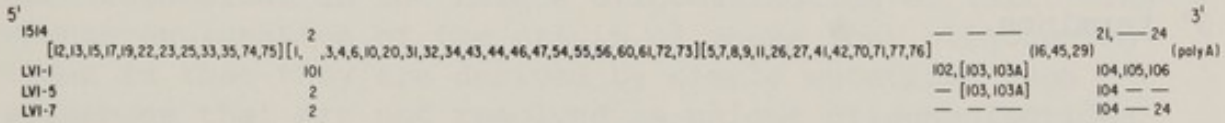

FIGURE 3. Ordering of RNase T_1 resistant oligonucleotides along the genome of visna virus LV1-1. The central line of numbers represents oligonucleotides common to the viruses. The numbers above the central line represent oligonucleotides unique to strain 1514 and the numbers below the central line represent oligonucleotides unique to strain LV1-1, LV1-5 and LV1-7. The numbers on the vertical axis indicate allelic oligonucleotides of the variants. No order of the oligonucleotides within the brackets is implied. The map presented is based upon the analysis of 6 size classes of LV1-1 RNA and 4 size classes of 1514 RNA.

FIGURE 2. RNase T_1 fingerprints of short subgenomic fractions of poly (A) containing RNA isolated from the virus LV1-1. The RNA samples analyzed have a sedimentation coefficient of less than 28S and were prepared as described in Methods. Panel A contains the shortest fragments of viral RNA, pooled gradient fractions of ~8-10S, B contains pooled fractions of ~10-12S, C contains pooled fractions of ~12-18S.

absence of oligonucleotides 21 and 24 and the presence of new spots 103 and 104. All the new oligonucleotides found in LV1-5 and LV1-7 were also present in LV1-1. Therefore, the changes observed in these variants are probably subsets of the changes observed in LV1-1 . We have not ordered the T_1 resistant oligonucleotides of LV1-5 or LV1-7 relative to the 3' terminus as was done for 1514 and LV1-1. However, it is reasonable to assume that they occupy the same relative positions on the genome as they do in 1514 and LV1-1.

The mapping analysis shows that six out of seven alterations in the genome of the antigenic variant LV1-1 are located among the oligonucleotides closest to the poly (A) sequence. In addition, all the changes of LV1-5 and LV1-7 appear to be a subset of the changes which occurred in the 3' terminus. This would place them within 2 kilobases from the 3' end, assuming an even distribution of the marker oligonucleotides along the genome. The altered oligonucleotides are located in at least two distinct groups within the region. The oligonucleotides 104, 105 and 106 are located very near to the poly (A) tail and oligonucleotides 102, 103A and 103B are derived from a sequence further from the 3' terminus.

DISCUSSION

Analysis of the RNase T_1 oligonucleotide fingerprints of the RNAs of naturally occurring antigenic variants of visna virus has revealed that genomic changes are clustered in the 3' terminus of the genome. The variants differ from the parental strain by only a small number of alterations in their nucleotide structure. When 80 unique oligonucleotides from each of the four variants were compared, LV1-1 differed the most from the parental virus with 7 oligonucleotides with altered mobilities. Variant LV1-7 contained only one such altered oligonucleotide and this oligonucleotide is common to LV1-1. Variant LV1-5 contained two altered oligonucleotides which are also present in LV1-1. Thus, it appears that alterations in the nucleotide sequence of virus 1514 occurred progressively giving rise to variant LV1-7 then LV1-5 and finally LV1-1. It is interesting to note that the order of isolation of these variants from the animal does not seem to reflect when the virus arose. However, the appearance of neutralizing antibody in the animal serum correlates well with the results obtained from the RNase T_1 fingerprints. Neutralizing antibody directed against variant LV1-7 could be detected in the serum of sheep #1 one year prior to that against LV1-5 or LV1-1. Thus, there appears to be sequential

mutation of visna virus in a persistently infected animal which can be correlated with the animal developing a specific humoral immune response to each antigenic variant.

A minimum of seven single base mutations could account for the differences observed between LV1-1 and 1514. However, the unique ribonuclease T_1 resistant oligonucleotides comprise only 15% of the total sequence (80 oligonucleotides an average length of 20 nucleotide $\cong$ 1,600 nucleotides). Therefore, the total number of differences between these two virus strains and the other variants is probably much higher than the seven differences observed here. Although the unique oligonucleotides represent a portion of the nucleotide structure of the viruses, it is likely that they are representative of the changes that are occurring throughout the genome.

Sequence analysis demonstrates that three of the seven changes observed in LV1-1 can be attributed to single base changes in the primary nucleotide sequence. The other four alterations which are in the 3' region are detected as unique oliognucleotides in LV1-1 for which no related sequences have been identified in the unique oligonucleotide of 1514. The simple explanation of the origin of these four oligonucleotides is that they are derived by simple mutations from sequences that are not resolved as unique oligonucleotides in the fingerprint of the genome of 1514.

The clustering of the differences between these viral strains in the 3' region of the genome suggests that changes in this region are a result of selection of variants that are not neutralized by the immune responses of the animal. Therefore, we would expect the viral antigen that elicits neutralizing antibody to be coded for by this region of the genome. Although the genetic organization of visna virus is not presently known, by analogy with other retroviruses, the 3' terminal region would be expected to contain the genetic information of the envelope glycoprotein. The major envelope glycoprotein is known to be the antigenic target for neutralizing antibodies in other retroviruses (19,20). It has recently been shown that antibody against the envelope glycoprotein of visna virus will neutralize the virus (21). In addition, data obtained by B. Nexo (22) in our laboratory has demonstrated by partial protease digestion of the glycoproteins of 1514 and LV1-1 that there are multiple structural differences between these proteins. For these reasons it is likely that some of the changes in the structure of the genomic RNA in the parental and variant strains are responsible for changes in the amino acid sequence of the envelope glycoprotein.

Changes in a single amino acid can change the recognition

of a protein by an antibody. This has been shown to occur in influenza virus (23,24). Recent studies have shown that alterations of a single amino acid in the hemagglutinin protein of influenza changed the neutralization properties of the virus when tested with a monoclonal antibody (23,24). The change was not detected by animal antisera. It seems likely that multiple changes in protein sequence are required to evade neutralization by a polyvalent antibody response. Thus, the multiple changes that have been detected in the visna virus genome in LV1-1, LV1-5 and LV1-7 naturally occurring antigenic mutants, probably represent a number of amino acid changes in the envelope glycoprotein. The changes in the viral antigen of LV1-1, LV1-5 and LV1-7 were biologically significant since there was sequential development of antibody in the sheep sera to each of these variants.

Antigenic variation of visna virus in a persistently infected animal can be explained by the accumulation of multiple mutations in the viral genome. This is supported by the seemingly progressive appearance of changes in the 3' end of the genome of antigenic variants LV1-7, LV1-5 and LV1-1.

The sequential replication of each new antigenic variant provides a mechanism for development of new pathological lesions in the chronically infected animal. This explanation is consistent with the presence of both acute and chronic inflammatory lesions in the brains of animals with visna. The protracted course of the disease could be explained by the time required to accumulate a significant number of mutations required to evade the immune reaction to the predecessor virus.

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POSSIBLE MECHANISM OF ROTAVIRUS PERSISTENCE

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ABSTRACT Infection of BSC-1 cells with bovine rotaviruses usually results in a cytolytic infection. This communication describes virus:cell interactions in cells persistently infected with a bovine rotavirus. Viral persistence does not adversely affect these cells (RP-BSC-1 cells) which grow at a rate comparable to that of uninfected cells. In addition, RP-BSC-1 cells continuously produce Rotavirus and are immune to super-infection with related viruses. To further characterize virus:cell interactions during persistent infections, viral expression was examined in synchronized RP-BSC-1 cells. Viral expression was limited to mid and late G1 when 80% of cells exhibited viral antigens detectable by immunofluorescence and PAGE. Entry into S-phase caused a degradation of viral proteins and a sharp decline in viral expression. Degradation was probably brought about by factors synthesized in S-phase as exponentially growing BSC-1 cells yielded predominantly incomplete virus particles. The results suggest that some strains of rotavirus are unable to shut-down host macromolecular synthesis and therefore can only replicate in certain permissive phases of the cell cycle. As the infected cell traverses from 'G1' into the 'S' phase of the cell cycle, viral replication and viral gene expression ceases, only to resume when the cells re-enter a permissive phase.

INTRODUCTION

Rotaviruses are one of the etiological agents of neonnatal diarrhea in man (1,2)and other animals (3,4,5). Infection generally results in virus replication in the epithelial cells at the tips of the intestinal villi, with subsequent denudation and diarrhea due to maladsorption. In-vitro they exhibit a wide variety of virus:cell interactions ranging from cytolytic to low grade inapparent infections. Cytolytic infections are manifested, in tissue-culture, by a rapid cessa-

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TABLE 1
EFFECT OF ANTI-ROTAVIRUS ANTIBODY ON VIRUS YIELD AND INFECTIOUS VIRUS PRODUCTION BY RPSBC-1 CELLS

Treatment	Infectious Centers / Cells Plated	Virus Yield (PFU/ml)
10% FBS		
passage 1	ND	8×10^5
3	$4 \times 10^5/5.5 \times 10^5$	7×10^4
5	ND	9×10^5
6	$3 \times 10^5/4 \times 10^5$	4×10^5
Antirotavirus serum		
passage 1	ND	<100
3	$6 \times 10^5/8 \times 10^5$	<100
Rotavirus Ab removed		
passage 5	ND	3×10^4
6	$3 \times 10^5/6 \times 10^5$	8×10^5

TABLE 2
RESTRICTION PATTERN OF VIRUS REPLICATION IN RP-BSC-1 CELLS[a]

Virus	MOI	VIRUS YIELDS (PFU/ml) BSC-1	RP-BSC-1	Ratio
HSV-1	1.00	3.0×10^5	2.8×10^5	1.07
VSV	1.00	1.2×10^8	2.0×10^7	6.00
REO Type 3	1.00	1.4×10^5	<10	$>10^7$
(Dearing Strain)	0.10	1.8×10^7	<10	$>10^7$

[a]Cultures were infected with various viruses and harvested 48 hours post-infection. Virus yields were assayed on GBK cells by a standard plaque assay.

tion of host macromolecular synthesis, virus replication and degradation of host DNA followed by lysis of the infected cell and release of progeny virus (6).

Despite their cytolytic nature, some bovine rotaviruses are thought to persist in at least a few infected individuals thus insuring continued infection of young susceptibles and perpetuation of the disease. Although the mechanism by which the viruses persist in animals is not known, its elucidation is of interest since it may eventually enable us to better understand the epidemiology of rotavirus infections and perhaps that of other persistent viral diseases.

Since it is more difficult to study persistence in animals we have attempted to establish such an infection in-vitro. This report involves the characterization of BSC-1 cells persistently infected by the bovine rotavirus isolate 2352.

RESULTS

Infection of BSC-1 cells at a multiplicity of infection of 1 with the 2352 isolate of bovine rotavirus resulted in the development of viral induced cytopathology in at least 60% of the cells and destruction of about 10% of cells, by 24 hrs post-infection. However by 48-72 hrs P.I. the remaining 90% of cells reverted to a normal phenotype, continued to grow and formed a confluent monolayer two to three days after infection. Although these cells (RP-BSC-1) appeared similar in morphology to uninfected BSC-1 cells, they continued to produce infectious virus (Table 1) and about 20 to 30% of the cells fluoresced when stained with fluorescein conjugated anti-bovine rotavirus antisera.

RP-BSC-1 cells have now been passaged at weekly intervals for more than six months and they continue to produce virus and express viral antigens. The cells can also be frozen in liquid nitrogen and recovered with ease. The low level of cell death at each cell passage was not sufficient to alter the growth of rotavirus persistently infected cells. Thus regardless of the original plating density, uninfected BSC-1 and RP-BSC-1 cells grew at the same rate and reached the same plating density (Fig. 1).

To determine the effect of rotavirus persistence on the outcome of other viral infections in RP-BSC-1 cells we compared the ability of RP-BSC-1 and BSC-1 cells to support the growth of Herpes simplex virus-I, Vesicular stomatitis virus, and Reovirus type III (Table 2). Persistence appeared to have no effect on the replication of HSV-I and VSV whereas Reovirus, a virus very similar to rotavirus, was drastically reduced.

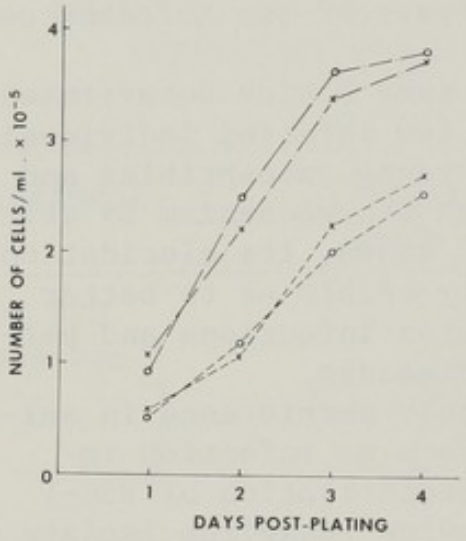

FIGURE 1. Growth rates of BSC-1 and RP-BSC-1 cells. Cells were dispensed into tissue culture dishes at a starting density of either 0.5 X 10^5 cells/ml (----) or 1 X 10^5 cells/ml (-·-). Cell numbers of BSC-1 cells (0) and RP-BSC-1 cells (X) are shown for each initial plating density.

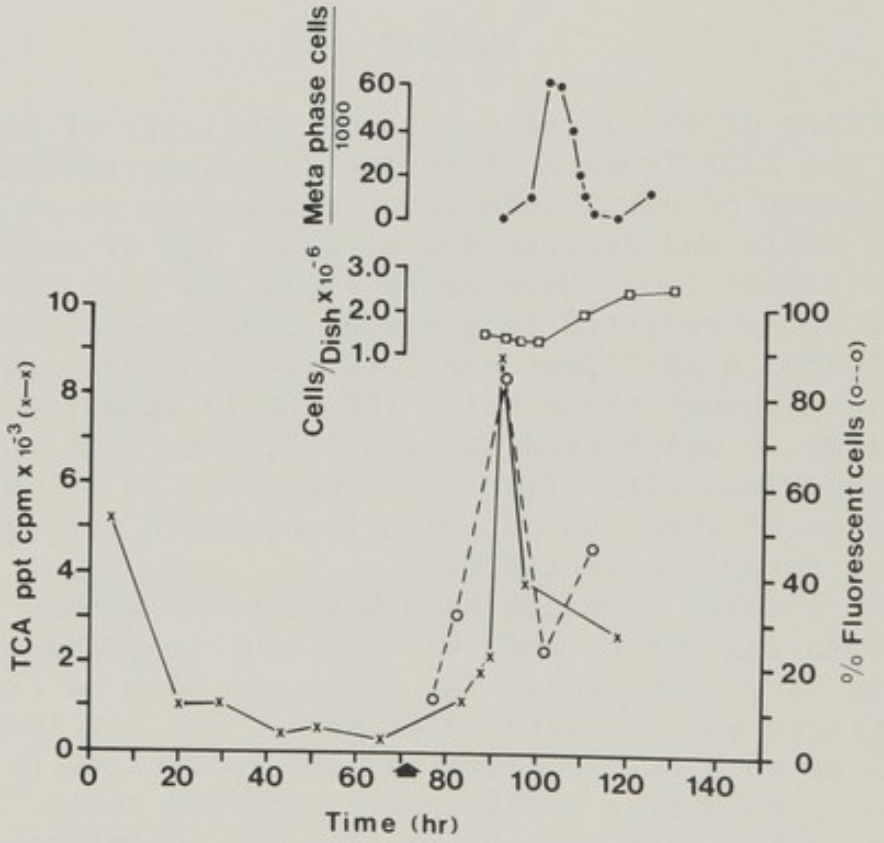

FIGURE 2. Expression of rotavirus antigens in synchronized RP-BSC-1 cells. Arrow indicates time of synchronization. Rate of DNA synthesis is expressed as the amount of ^{3}HTdR incorporated into acid unsoluble material during a 1 hr pulse with 10 µCi ^{3}HTdR (X). At various times after activation cells were fixed with acetone and stained with fluorescein labelled anti-rotavirus antisera (---°--). Inserts indicate time of cell division (- -) and mitosis (-·-).

Virus may persist in a culture if a small number of cells are infected at any particular time. These infected cells could conceivably die and release virus to infect a few more cells thus establishing an equilibrium between infection and cell death with concurrent cell growth. To test this possibility as the reason for viral persistence, RP-BSC-1 cells were grown for five passages in the presence of 10% antirotavirus antisera that had a neutralizing titre of 1/1024 against rotavirus strain 2352. Under the above conditions, neutralization of infectious virus in the extracellular fluid should have greatly reduced the number of infected cells in the culture and led to eventual 'curing' of the culture. However, after removal of antirotavirus antisera following the fifth passage, the cells continued to produce infectious virus and express viral antigens, indicating that rotavirus viral genomes persisted within the cells (Table 1).

To determine the fraction of RP-BSC-1 cells that contained rotavirus genomes, cultures of RP-BSC-1 cells were harvested by trypsinization, washed extensively and assayed as infectious centres (IC) on MA104 cells (7). The IC to total cell ratio was found to be close to 1 (Table I) indicating that most if not all RP-BSC-1 cells contained at least 1 complete viral genome. Furthermore the IC to total cell ratio remained close to one even after cultivation of cells for three passages in the presence of anti-rotavirus antisera (Table I) confirming that persistence was not maintained due to the continued infection of uninfected cells by virus in the growth supernatent. These results, which suggested that all cells were infected, appeared to be in contradiction to the observation that only 20 to 30% of cells in a randomly growing culture exhibited viral antigens when stained with fluorescein conjugated anti-rotavirus antisera. This led us to speculate that perhaps viral information in RP-BSC-1 cells was only expressed during certain phases of the cell-cycle, when the host cells were permissive to viral expression. To test this hypothesis RP-BSC-1 cells were synchronized by serum activation following arrest in the 'Go' phase due to nutrient deprivation (8). RP-BSC-1 cells proved very amenable to synchronization by this technique. Following serum-activation the cells underwent at least one cycle of synchronous growth. Twenty to twenty-two hours after serum activation the cells were in mid 'S' phase, and the culture contained a maximum number of metaphase cells 10 hrs later. A doubling of cell numbers takes place between 30 and 48 hrs after serum activation (Fig. 2). The lengths of the various phases of the cell cycle were the same for uninfected BSC-1 cells (data not shown). Synchronized RP-BSC-1 cells grown in Lab tech chamber slides were fixed in acetone at various times after serum

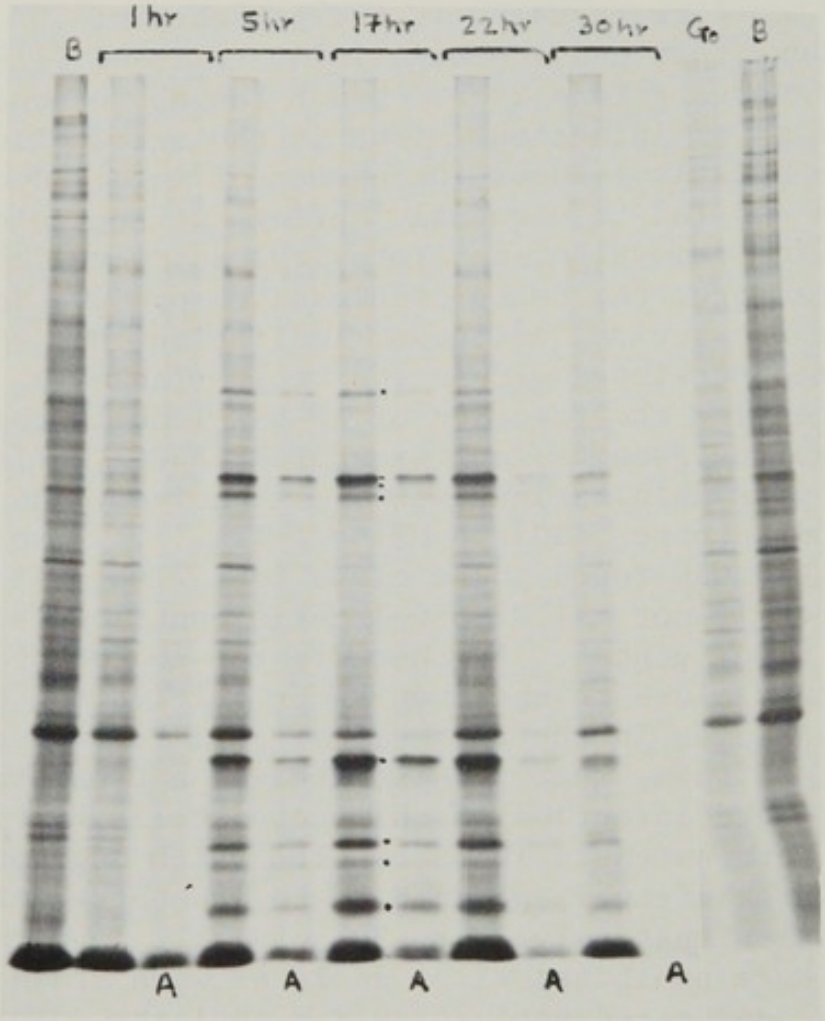

FIGURE 3. Viral protein synthesis in synchronized RP-BSC-1 cells. At 1, 5, 17, 22, and 30 hrs after activation synchronized cells were labelled with ^{35}S-methionine in the absence or presence (A) of 5 µg actinomycin D/ml of low methionine medium. After a two hour labelling period cells were harvested and analyzed by polyamylamide gel electrophoresis. Go-arrested RP-BSC-1 cells and uninfected BSC-1 cells (B), were labelled and analyzed in the same manner.

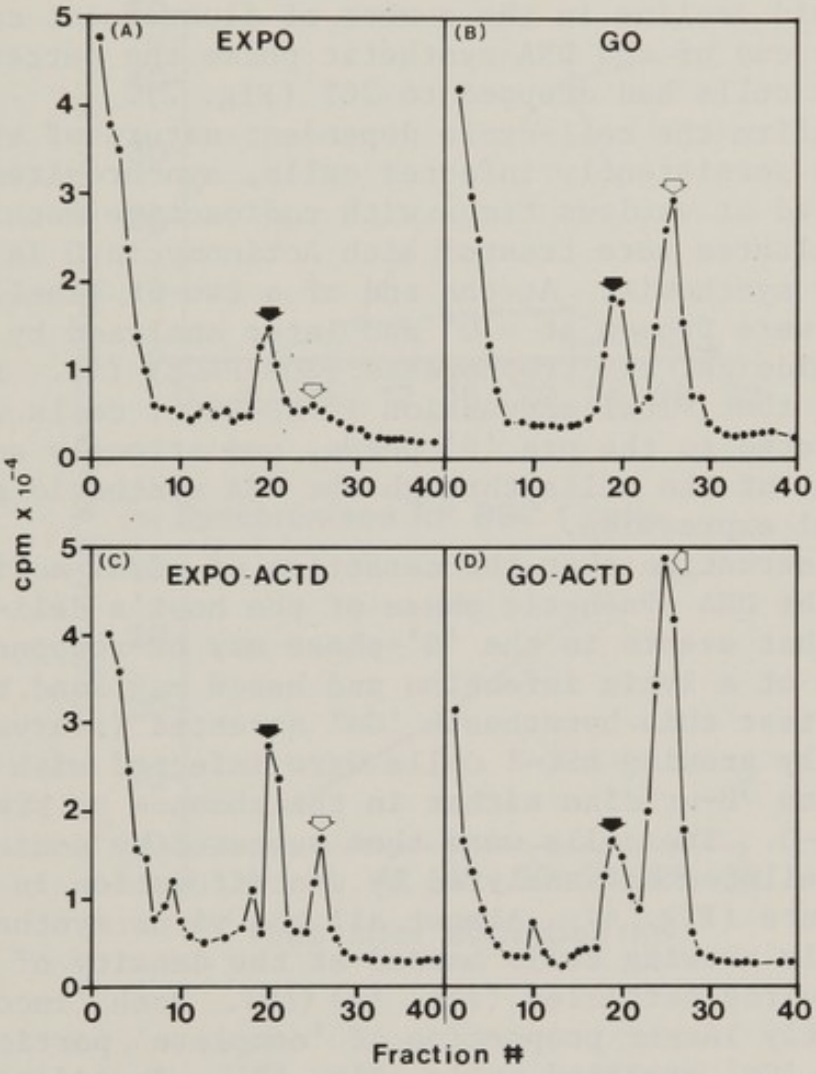

FIGURE 4. Analysis of rotavirus 2352 grown in Go arrested and exponentially growing BSC-1 cells on CsCl gradients. Go arrested or exponentially growing BSC-1 cells were infected with 2352 and overlayed with growth medium or medium containing 5 µg actinomycin D/ml. Go cells received spent medium. After infection cells were labelled with 10 µCi ^{3}HUR/ml of medium. Following incubation for 24 hrs cells were disrupted by sonication, the virus pelleted and analyzed on CsCl gradients. Solid arrows mark the density of 'incomplete' particles while open arrows denote the position of 'complete' particles.

activation and stained with fluorescein conjugated anti-rotavirus antisera. At least a thousand total and fluorescent cells were counted for each timepoint in order to determine the fraction of cells exhibiting viral antigens. The percentage of such cells increased sharply as the cells entered 'S' phase till a time coincident with mid 'S' phase when more than 80% of cells exhibited viral antigens. This was followed by a rapid decline in the number of fluorescent cells such that by the end of the DNA synthetic phase the percentage of fluorescent cells had dropped to 20% (Fig. 2).

To confirm the cell-cycle dependent nature of viral expression in persistently infected cells, synchronized cells were labelled at various times with radioactive methionine. Parallel cultures were treated with Actinomycin D in order to reduce host synthesis. At the end of a two hr labelling period, cells were frozen at -70° and later analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (9). Fig. 3 illustrates that viral expression in RP-BSC-1 cells was indeed restricted to the pre 'S' phase, and strongly suggests that passage of the cells through the DNA synthetic phase curtailed viral expression.

The observation that the cessation of viral activity was linked to the DNA synthetic phase of the host's cell-cycle suggested that events in the 'S'-phase may be responsible for suppression of a lytic infection and hence may lead to persistence. To test this hypothesis 'Go' arrested (starved) and exponentially growing BSC-1 cells were infected with 2352 and labelled with ^{3}H-uridine either in the absence or presence of actinomycin-D. The cells were then ruptured by sonication, the virus pelleted and analyzed by centrifugation in isopycnic CsCl gradients (Fig. 4). Almost all the virus synthesized in exponentially growing cells banded at the density of 'incomplete' rotavirus particles (Fig. 4A) (10). Both 'incompletes' and a slightly larger proportion of 'complete' particles were observed in 'Go' arrested cells (Fig. 4B). In addition, treatment with actinomycin-D, at concentrations which greatly decreases host cell expression (6), significantly increased the amount of complete particles produced in both 'Go'-arrested and exponentially growing RP-BSC-1 cells (Fig. 4C, D).

Infection of BSC-1 cells with cytolytic rotaviruses leads to a cessation of host cell expression (6). The inability of 2352 to continue replication through all phases of the host's cell-cycle may stem from the inability of this virus to shutdown host synthesis consequently making it more sensitive to interfering host-factors synthesized during certain phases of cell growth. To evaluate the ability of 2352 to affect host expression we compared the activity of the 'S'-phase enzyme thymidine kinase (TK) in synchronized RP-BSC-1 cells and in synchronized BSC-1 cells that were infected with a cytolytic

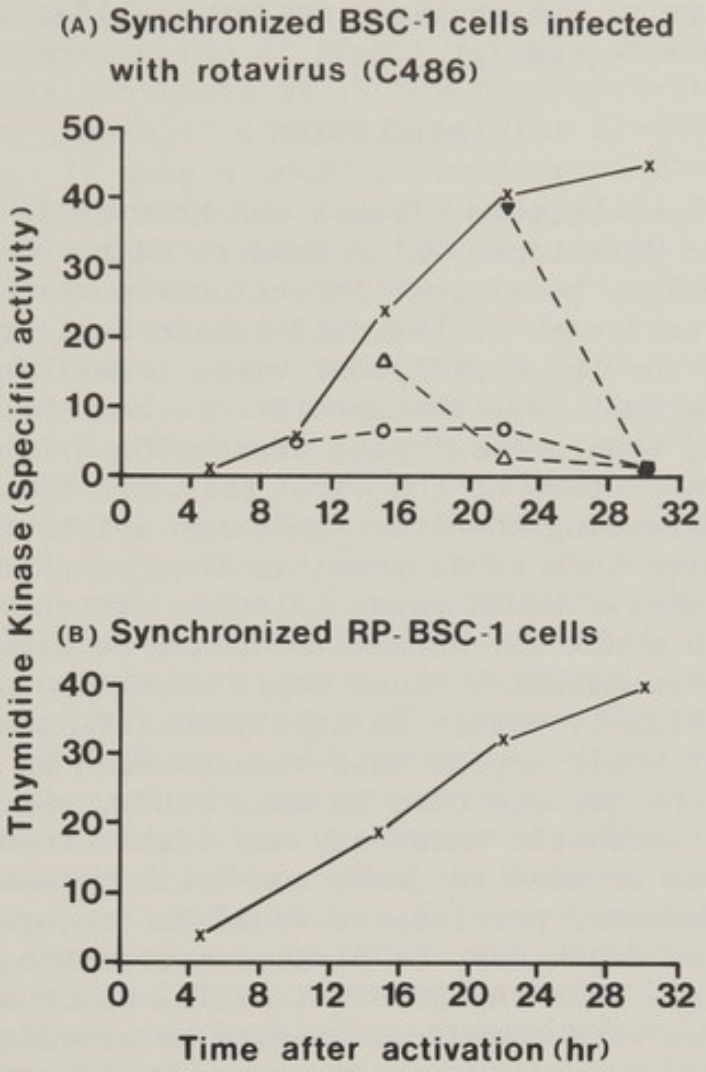

FIGURE 5. Thymidine kinase activity in synchronized BSC-1 and RP-BSC-1 cells (11).

A) Synchronized BSC-1 cells were either left uninfected (X) or were infected at 5 hr (0), 10 hr (Δ) or 20 hr (▼) after activation. At various times after activation cells were harvested and assayed for thymidine kinase.
Thymidine kinase specific activity is expressed as picomoles of ^{3}HTdR phosphorylated per 45 minutes per mg protein X 10.

strain of rotavirus (C486) (11). Infection of BSC-1 cells before the onset of 'S'-phase completely prevented the synthesis of this enzyme. Infection during the 'S'-phase also led to an almost immediate cessation of TK activity (Fig. 5A). The synthesis of TK in synchronized RP-BSC-1 cells, on the other hand, was not affected (Fig. 5B).

DISCUSSION

Interactions between viruses and host cells can range from cytolytic infections which lead to death of the cell to relatively benign, persistent infections in which the basic growth characteristics of the cell remain unaltered. The exact reasons for these different virus : cell interactions is probably varied. For instance it has been proposed that interferon may reduce viral gene expression or the host cell may prevent maturation of the virus (12). If the host cell is capable of expressing specific functions which could alter virus expression then it is possible that viruses which are extremely cytolytic would cause cellular destruction before the cell could mount any defensive attack to virus replication. Thus viruses which cause rapid cessation of host cell functions invariably result in cytolytic infections. On the other hand, it would appear that viruses such as rotavirus strain 2352, the isolate used in this study, which do not shut down the host synthetic machinery may either result in abortive infections or tend to cause non-lytic persistent infections. Furthermore, persistence would be favoured if viral gene expression could only be limited to certain phases of the cell cycle. Thus the RP-BSC-1 cells, which are persistently infected with rotavirus appears to provide an excellent model to study events involved in virus replication and persistence.

Our results suggest that in addition to cellular events present in the pre 'S' phase of the cell cycle other physiological changes, which can be induced by membrane alteration as a result of trypsinization, are also required for virus reactivation. Thus serum activation alone without cell passage or disloding of cells with a rubber policeman followed by passage stimulated the cells to enter the cell-cycle but did not cause virus reactivation. However, if trypsin was used to dislodge the cells, virus reactivation occurred with maximum viral expression occurring about 17 hr after reactivation, when the cells were in early 'S' phase. Although trypsin has been shown to activate numerous viruses (13, 14) as well as rotavirus (15)we do not feel that trypsin was responsible for maintaining the virus in a state of equilibrium by reinfection at each passage level for a number of reasons. Firstly, passage in the presence of anti-rotavirus serum, which neutra-

lized extracellular virus, did not affect the number of cells expressing rotavirus antigen or the cells ability to produce infectious centers (Table I). Secondly, primary infection with rotaviruses, generally results in polypeptide synthesis within 1 hr post-infection and peaks at 6-7 hrs post-infection whereas reactivation of RP-BSC-1 cells demonstrated maximum polypeptide synthesis at 17 hrs post-activation. Thirdly, it was not possible to superinfect the RP-BSC-1 cells with Reovirus. If only a small percentage of the cells were infected then the uninfected cells should still synthesize Reovirus. Thus our data suggest that every cell is infected but only expresses viral antigens at specific stages of the cell cycle once activated by trypsinization.

We have infected a calf with strain 2352. After an initial diarrhea the calf recovered but continued to excrete rotavirus for at least 5 weeks (last time tested). In contrast calves infected with C486, a cytolytic strain of rotavirus, stop shedding approximately 1 week after diarrhea subsides (unpublished results). Thus our present in-vitro model may have in-vivo relevance. Since rotavirus strain 2352 produced more complete particles in 'Go' arrested cells and since apical villous epithelial cells, where rotaviruses grow, are presumably in a 'Go' state it would ensure the production of complete particles which could spread to other animals. Furthermore, the proteolytic enzymes present in the intestinal lumen may activate the virus to insure reinfection of other intestinal epithelial cells. Thus we propose that complete virus particles are produced in 'Go' arrested intestinal epithelial cells with subsequent death and denudation. The extracellular particles are activated by proteolytic enzymes and the virus can then reinfect other intestinal epithelial cells. However, since the cells in the crypts are actively cycling they do not produce virus. When these cells migrate to the tips of the villous they reach 'Go' and are in contact with proteolytic enzymes. This combination results in virus activation and release of infectious virus. Such a procedure can continue indefinitely until the local defense mechanisms such as IgA are sufficient to neutralize all the virus prior to reinfection.

Attempts to isolate and passage isolates such as 2352 may prove difficult to do using conventional methods, where the cells are generally exponentially growing, since incomplete non-infectious particles would be the predominantly produced. This may explain why many rotaviruses are difficult to isolate and passage in vitro. Our present results suggest that the use of Actinomycin D in the cultures may improve the chances of virus isolation of such viruses. We are presently attempting to see the frequency of such isolates in nature in

an attempt to try and determine the role they may play in the epidemiology of rotavirus infections. The recent report of Begin et al. (16) which showed that a large proportion of rotavirus isolates were noncultivatable by conventional methods suggest that the frequency of such isolates may be high. In fact such isolates may be favoured in nature because they would represent a continuous source of virus for reinfection.

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NEUROVIRULENCE AND PERSISTENCY OF MOUSE HEPATITIS VIRUSES IN RATS[1]

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ABSTRACT The murine coronavirus JHM induces in weanling rats different types of central nervous diseases ranging from an acute pan-encephalitis to a late demyelinating encephalo-myelitis. The occurrence and rate of these different disease types is associated with the virus variant used for inoculation. Except for MHV 2, neurovirulence was not observed in four other murine coronavirus strains. The relationship of these coronaviruses to JHM was investigated by cross neutralization and oligonucleotide maps of their genomic RNA.

INTRODUCTION

Murine coronaviruses induce in mice a variety of diseases, ranging from acute hepatitis, enteritis and encephalomyelitis to inapparent infections. Of particular interest are the central nervous system (CNS) diseases associated with this virus group, especially the mouse hepatitis virus strain JHM reveals a distinct neurovirulence for mice and rats (1,2,3,4). In rats different disease processes accompanied by demyelination are observed (5,6,7), providing the opportunity to investigate the virus host relationships which lead to myelin destruction.

In the present communication the neurovirulence of four murine coronavirus strains (MHV 1, MHV 2, MHV 3 and MHV A59) for rats is compared to the JHM virus.

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RESULTS

Neurovirulence of MHV-JHM. Infection of suckling rats with JHM-virus regardless of virus history resulted in an acute encephalomyelitis characterized by necrotic lesions in all parts of the CNS followed by hepatitis and rapid death within eight days after infection. Intracerebral inoculation of weanling rats however, led to three different courses of a central nervous disease (5,6,7) as summarized in table 1.

The Acute Panencephalitis (APE) is characterized by a rapid onset within 12 days post infection (p.i.), severe necrosis and acute inflammations in most parts of the brain.

The Subacute Demyelinating Encephalomyelitis (SDE) does not occur before 12 days p.i. and shows a more protracted clinical course. Neuropathologically, inflammatory changes are present consisting mainly of mononuclear lymphocytes, plasmacells and macrophages without lesions of necrosis. In contrast to the APE neuronal involvement is minimal or absent. Moreover, a primary demyelination in restricted areas of the brain and spinal cord is noticed which can easily be detected by histological staining procedures.

The Late Demyelinating Encephalomyelitis (LDE) develops after an incubation period of 2 - 8 months. The main histological lesions of the CNS consist of demyelinating areas present in the spinal cord and cerebrum. Demyelination is accompanied by remyelination of the CNS and PNS type. Inflammatory infiltrations can be detected in the neighborhood of demyelinating plaques. The attempts to isolate infectious virus from diseased brain material were successful in all animals tested, regardless of the different CNS disease process. It is noteworthy, that even in the animals with LDE infectious virus was present in brain, indicating JHM virus persistency during the incubation period.

The occurrence and rate of the different types of CNS disease induced by JHM virus is associated with the properties of the virus preparation used as inoculum as summarized in table 2. Uncloned wild type JHM virus induced in weanling rats mainly APE and SDE but only occasionally LDE. This finding suggests that uncloned JHM consists of a heterologous virus population with different biological

TABLE 1

DIFFERENT TYPES OF CNS DISEASE INDUCED BY JHM VIRUS IN WEANLING RATS
(STRAIN CHBB/THOM)

	ACUTE PANENCEPHALITIS	SUBACUTE DEMYELINATING ENCEPHALOMYELITIS	LATE DEMYELINATING ENCEPHALITIS
INCUBATION TIME	7 - 12 DAYS	14 - 30 DAYS	2 - 8 MONTHS
CLINICAL SIGNS	MOTIONLESS, FORELEGS OFTEN PALSY	HINDLEGS PALSY OR TETRAPLEGIA	
LOCALISATION OF LESIONS	DISSEMINATED MAINLY IN THE GREY MATTER	DISSEMINATED IN THE WHITE MATTER	
NATURE OF LESIONS	OFTEN NECROTIZING	DEMYELINATING, PRESERVATION OF AXONS AND NEURONS	
CELL INFILTRATIONS	GRANULOCYTES, LYMPHOCYTES, MACROPHAGES, GLIAL NODULES, MULTINUCLEATED GIANT CELLS	LYMPHOCYTES, PLASMA CELLS AND MACROPHAGES	
REMYELINATION	−	−	+
VIRUS ANTIGENS (FA)	NEURONS AND GLIA	GLIAL CELLS	
VIRUS PARTICLES (EM)	DEGENERATING OLIGODENDROGLIA	DEGENERATING OLIGODENDROGLIA	
NEUTR. ANTIBODIES	−	+	+

TABLE 2

CORONAVIRUS JHM INFECTION IN WEANLING RATS

VIRUS PREPARATION	ACUTE PANENCEPHALITIS 4 - 12 DAYS P.I.	SUBACUTE DEMYELINATING ENCEPHALOMYELITIS 14 - 24 DAYS P.I.	LATE DEMYELINATING ENCEPHALOMYELITIS MONTHS P.I.
UNCLONED WILDTYPE	10 - 15 %	15 - 30 %	< 1 %
CELL ADAPTED SAC-JHM CLONED	70 - 95 %	< 1 %	–
TS 42	–	–	5 - 15 %
TS 42-1027 REISOLATE 3 MONTHS P.I.	< 1 %	35 - 45 %	< 1 %
TS 43	–	10 - 15 %	10 - 20 %
TS 43 - 4313 REISOLATE 4 MONTHS P.I.	–	10 - 15 %	–

properties. This is supported by the observation that cloned tissue culture adapted JHM virus always caused in weanling rats an APE, whereas certain temperature sensitive mutants derived from that JHM virus clone after mutagenization with fluoruracil caused SDE or LDE. No acute disease process could be induced with these ts-mutants. Reisolated mutants from diseased animals with LDE did not always maintain their temperature sensitivity but were different from revertants by the type of neurovirulence. Moreover, some characteristics in tissue culture were similar to the ts-mutants originally inoculated. The reisolate ts 42-1027 is an example for this phenomenon. This variant was reisolated from a diseased animal 3 months after infection with ts 42. The reisolate is much less temperature sensitive than the original mutant. It had an efficiency of plating of $1{,}6 \times 10^{-1}$ compared to $1{,}4 \times 10^{-4}$ for ts 42. In animals, this reisolate caused predominantely a high rate of SDE in contrast to its parental virus, which induced only few cases of LDE.

Neurovirulence of other MHV-strains. The virulence of cloned MHV-strains (MHV 1, MHV 2, MHV 3 and MHV A59) was compared to JHM virus after intracerebral inoculation of defined virus doses into weanling rats (Table 3). With the highest virus dose used, only MHV 2 induced one case of an acute encephalomyelitis. No clinical disease was observed in the other animals injected with MHV 1, MHV 3 and MHV A59. Pathological investigations carried out 24 days after virus inoculation revealed only mild inflammations in liver tissue without detectable neuropathological changes. No infectious virus was recovered from CNS tissue 4 - 8 days p.i. All the inoculated animals developed a humoral immune response against the different murine coronaviruses except for MHV 1. This virus strain grows in tissue culture lines of murine origin but appears not to be infectious for rats.

Cross Neutralization among MHV-strains. Antigenic comparisons among the different cloned murine coronavirus strains were carried out by cross neutralization. The results are summarized in table 4 and indicate that each virus strain is antigenically distinct in the structural protein(s) which induced neutralizing antibodies. However, some cross neutralization among the murine coronaviruses can be noted. MHV JHM and MHV 2 demonstrated a significant bilateral cross reactivity. MHV A59 and MHV 1 cross reacted only in one direction, since anti MHV 1 serum hardly neutralized MHV A59 virus.

Relative oligonucleotide homology between strains of murine coronaviruses. The genomic RNA of the cloned MHV strains was labeled by growth in presence of ^{32}phosphorus. The genome from the purified viruses was extracted and further purified as described previously (8,9,10). After RNAse digestion oligonucleotides were analysed in a two dimensional gel electrophoresis (11). The position of the large oligonucleotides within the fingerprint was compared. The result of this study (manuscript in preparation) is summarized in table 5 and indicates distinct differences between JHM virus and the other murine coronavirus strains. MHV 2, MHV 3 and MHV A59 are more closely related to each other than with MHV JHM, underlining the unique properties of this virus.

TABLE 3

CNS LESIONS INDUCED BY MHV-VIRUSES IN WEANLING RATS (STRAIN CHBB/THOM)

VIRUS	DOSE $TCID_{50}$/RAT I.C.	CNS LESIONS WITHIN 24 DAYS P.I.	LIVER INFLAMM. LESIONS 24 DAYS P.I.	NEUTR. ANTI-BODY TITERS 24 DAYS P.I.	CNS VIRUS ISOLATION 4 - 8 DAYS P.I.
MHV JHM (WT SAC)	4×10^4	25/30	0/5	1:56	8/8
MHV 1	4×10^4	0/13	0/5	< 1:8	0/8
MHV 2	4×10^4 $4{,}8 \times 10^6$	0/15 1/10	2/5	1:70	0/8
MHV 3	4×10^4 $3{,}4 \times 10^6$	0/15 0/10	1/5	1:52	0/8
MHV A59	4×10^4 $6{,}4 \times 10^6$	0/15 0/10	3/5	1:76	0/8

TABLE 4

CROSS NEUTRALIZATION

ANTISERUM

VIRUS	MHV 1	MHV 3	MHV A59	MHV JHM	MHV 2
MHV 1	++++	+	++	-	-
MHV 3	-	++++	+	+	+
MHV A59	+	+	++++	+	-
MHV JHM	-	+	-	++++	++
MHV 2	-	+	-	++	++++

++++ - HOMOLOGOUS TITER
++ - SIGNIFICANT CROSS REACTION, HETEROLOGOUS TITER WITHIN 4 X OF HOMOLOGOUS TITER
+ - WEAK CROSS REACTION, HETEROLOGOUS TITER WITHIN 16 X OF HOMOLOGOUS TITER
- - NO CROSS REACTION

TABLE 5

RELATIVE OLIGONUCLEOTIDE HOMOLOGY
BETWEEN STRAINS OF MURINE CORONAVIRUSES

	A59	MHV3	MHV2	JHM
A59	++++	+++	++	+
MHV3		++++	++	+
MHV2			++++	+
JHM				++++

++++ IDENTICAL*
+++ CLOSELY RELATED
++ SOME COMMON SEQUENCES
+ VERY FEW COMMON SEQUENCES

*Represents the comparison of T_1 maps from different RNA-preparations of the same strain.

COMMENTS

From the five coronaviruses tested, only JHM revealed significant neurovirulence for weanling rats. The other murine coronaviruses induce beside liver inflammations no CNS lesion in general even after intracerebral inoculation. JHM virus infection resulted in three different CNS processes depending on the property of the inoculated virus preparation. This association provides a basis to investigate the mechanisms by which the different pattern of diseases are induced. It is conceivable that biochemical analysis of different mutants might unravel a specific marker which contributes to neurovirulence. The observation that JHM virus persists in weanling rats for months before LDE is recognized suggests that specific events, either host or virus derived, prevent a rapid spread of virus infection.

The temperature sensitivity of clones provides only a marker for a genetic lesion, because this lesion might be independent from the regions influencing the type of neurotropism which develops after inoculation in rats. This is suggested by the reisolation of mutants with reduced temperature sensitivity which nevertheless retain a specific neurovirulence.

At present the selective neurotropism of different JHM mutants cannot be directly correlated to biological or biochemical properties of this virus. However, the preliminary data obtained from the comparison of oligonucleotide patterns indicates that JHM contains unique RNA sequences which are not found among the other murine coronaviruses. Further studies will define the genetic basis which contributes to neurovirulence, virus persistency and virus host interactions which lead to a demyelinating CNS disease.

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PERSISTENT INFECTIONS OF BUNYAVIRUSES IN *AEDES ALBOPICTUS*[1]

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ABSTRACT *Persistent infections have been established in* Aedes albopictus *cell cultures with Inkoo virus or Uukuneimi virus. Both of these cultures are resistant to homologous infection but are susceptible to heterologous infection. Both persistently infected cell populations show no CPE and are producing low levels of virus or viral particles after more than 20 cell passages. Co-infections of* Aedes albopictus *cells with standard virus and virus from Inkoo persistently infected cells show a decrease in the initial production of plaque forming units. Similar experiments in BHK (WI-2) cells show no such decrease. RNA purified from either virus particles or infected cell cytoplasms obtained from persistently infected cells reveals several small RNA species not found during infection of vertebrate cells. The possible significance of these findings to the horizontal transmission of arboviruses in nature is discussed.*

INTRODUCTION

The family Bunyaviridae represents the largest grouping of viruses currently known, with over 200 members described. These agents are transmitted by either ticks or mosquitos in their natural settings. We have used two members of this group; Inkoo virus, originally isolated from the mosquito *Aedes communis* (1) and Uukuneimi virus, found in the tick *Ixodes ricinus* (2). Buynaviruses are negative strained RNA viruses, consisting of a lipoprotein outer membrane

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surrounding a helical ribonucleoprotein core (3). The single-stranded genome is composed of three unique RNA segments; L (2.4×10^6 D), M (1.1×10^6 D) and S (0.5×10^6 D) (4). During lytic infection of vertebrate (BHK) cells, virus grows in the cytoplasm and matures by budding from smooth membrane (Golgi) regions (3).

One possible model for the invertebrate vector phase of the transmission of these and other arboviruses proposes that the viruses establish a persistent infection in the tissues of their insect host. It is known that mosquitos and ticks contain infectious virus at low levels. However the insect hosts are not affected. These features are characteristic of viral persistence are that cells surviving the initial infection (in some cases this is the entire population) display no CPE and that persistently infected cells continuously produce virus or viral particles and antigen. Such persistent infections have been established in BHK cells using combinations of *ts* mutants and DI particles of VSV (5). Persistent infections in insect cell culture have been achieved with Sindbis virus (6), Semliki Forest virus (7) and Dengue virus (8), using standard virus alone. We report here the establishment of persistent infections with two Bunyaviruses, Inkoo virus and Uukuneimi virus.

METHODS

Cells and virus; growth and plaque assay. The vertebrate cells were either BHK (WI-2) (obtained from Dr. M. Brinton, Wistar Institute, Philadelphia, Pa.) or primary chicken embryo (CE) cells prepared by trypsinization of 11-12 day embryos from a defined flock kept locally at the University Poultry Research Center. Vertebrate cells were grown at 37°C in Dulbecco's Modified MEM (DME) supplemented with 10% calf serum. *Aedes albopictus (A. alb.)* cells were supplied by Dr. S. Buckley (Yale Univ., New Haven, Conn.) and were maintained at 28°C in M&M/VP12 medium containing 20% fetal bovine serum. Uukuneimi virus (S23) was from the original stocks of Dr. Ralf Petterson (University of Helsinki, Finland). Inkoo virus was supplied by Dr. N. Karabatsos (CDC, Ft. Collins, Col.). A vaccine strain (TC-83) of Venezuelan Equine Encephalitis (VEE) virus was supplied by Dr. M. Parker (Colorado State University, Ft. Collins, Col.).

Virus growth on vertebrate cells was carried out as described elsewhere (9). Similar methods were used for infection of *A. alb.* cells in culture. All plaque assays were

carried out in BHK (WI-2) cells under agarose overlays essentially as described by Pettersson and Kaariainen (9).

Virus Purification. Media from infected cells was clarified by low speed centrifugation and virus particles were pelleted by centrifugation in a SW27 rotor at 24,000 rpm for 2 hours at 4°C. Virus particles were resuspended and banded in potassium tartrate/glycerol or Renografin gradients (10) in the SW27 rotor at 24,000 rpm for 6 to 12 hours at 4°C.

Radiolabeling and Purification of Viral RNA Species. Viral particles were purified from media from infected cells grown in the presence of carrier-free $H_3{}^{32}PO_4$ (100 to 600 uCi/ml) in DME. Intracellular virus specific RNAs were obtained from infected cells treated with Actinomycin D (2.5ug/ml) and grown in phosphate-free DME during the period of labeling with ^{32}P-orthophosphate.

Virion RNA was prepared by extraction of purified particles with phenol/chloroform/isoamyl alcohol (50:48:2) as described previously (12), followed by ethanol precipitation at -20° C. Intracellular virus specific RNA was prepared from cytoplasmic extracts of infected cells. Cells were lysed in RSB (0.1M NaCl, 0.01 M Tris-HCl, pH 7.4) containing 1% Nonidet P40. Nuclei and debris were removed by low speed centrifugation and the resulting cytoplasmic extracts were phenol extracted as described above and the resulting total nucleic acid recovered by ethanol precipitation.

Denaturing Agarose Gel Electrophoresis. RNA species were electrophoresed through 1.5% agarose gels containing 5mM methyl mercuric hydroxide (13). Mercury was inactivated by treatment of the gel with mercaptoethanol and the gels were dried and autoradiographs produced using Kodak Royal X-Omat film.

RESULTS

When *A. alb.* cells were infected with either Inkoo virus or Uukuneimi virus, no CPE was observed. Cells were subcultured after three to five days and have been carried in this manner for more than 20 passages. Culture fluid from the persistently infected cells was examined for virus by plaque assay. Cells persistently infected with Inkoo virus were releasing virus into the media to give a titer of about 10^4 pfu/ml. Cells persistently infected with Uukuneimi virus were releasing no detectable plaque forming virus. However, these cells were producing material which would label and purify as viral particles (see below).

Persistently Infected Cells are Resistant to Homologous Infection. Persistently infected *A. alb.* cells were super-infected with homologous virus or with a heterologous virus (VEE). The data in Figure 1 show that cells persistently infected with Inkoo virus (A. alb/INK) are resistent to super-infection by standard Inkoo virus. The initial infection of *A. alb.* cells with this virus produces more than 10^{10} pfu/ml, whereas the super-infection of A.alb/INK cells shows only the level of virus being expressed by these cells (about 10^4 pfu/ml). VEE virus, on the other hand, grows quite well in A.alb/INK cells, yielding about 10^8 pfu/ml.

Similar results were obtained with cells persistently infected with Uukunemi virus (A.alb/UUKU), Figure 2. In this case, Uukuneimi virus grows poorly in primary infection of *A. alb.* cells, yielding only about 10^5 pfu/ml. Super-infection of A.alb/UUKU with Uukuneimi virus results in *no* plaque forming units detectable in the culture medium, just as with the A.alb/UUKU cells alone. However, super-infection with VEE virus results in about 10^9 pfu/ml. In addition,

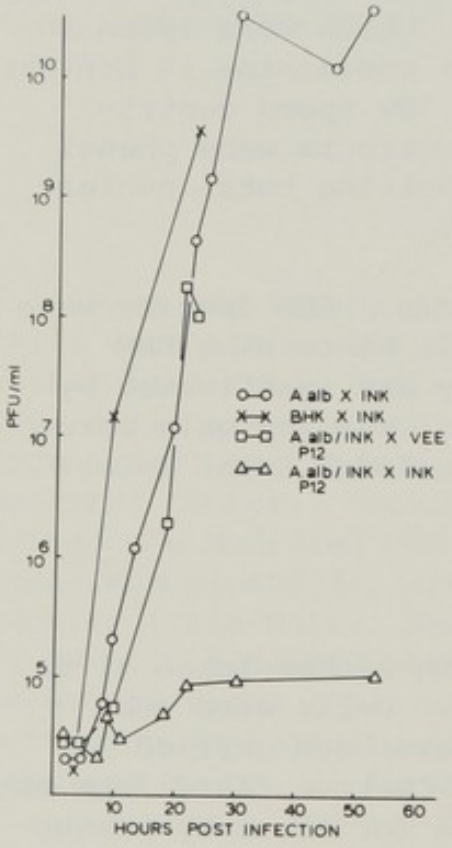

Fig. 1. Homologous and heterologous infection of A.alb/INK.

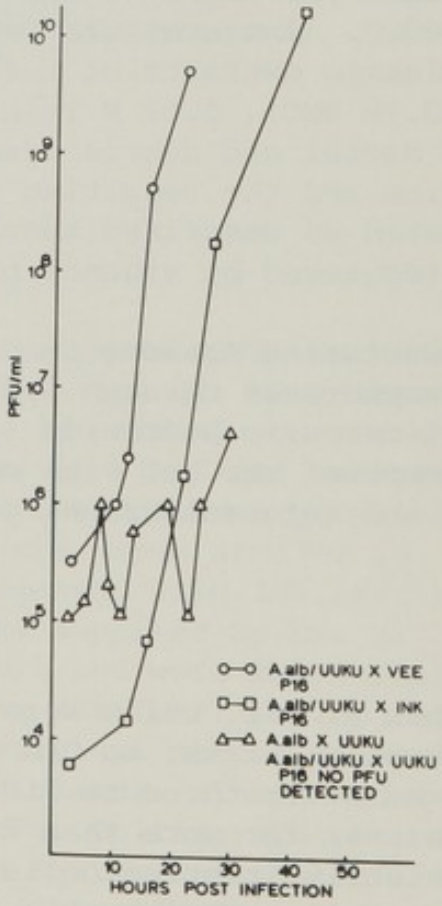

Fig. 2. Homologous and heterologous infection of A.alb/UUKU.

super-infection of A.alb/UUKU with Inkoo virus appears essentially normal, resulting in about 10^{10} pfu/ml. We have not examined the fate of these doubly infected cells.

Virus Released from Persistently Infected Cells May Cause Interference. Uninfected *A. alb.* cells were co-infected with standard virus and with dilutions of virus released from persistently infected cells. These experiments were performed with A.alb/INK because the population of particles released from these cells contained plaque forming virus. Figure 3 shows that, while primary infection of *A. alb.* cells with Inkoo virus results in about 10^{10} pfu/ml, co-infection with various dilutions of the virus preparation from A.alb/INK decreases the initial yield of virus. Whether the final yield of virus is affected is difficult to determine. Uninfected *A. alb.* cells will establish new persistent infections under these conditions and the end point of these infections is not easy to define. Our data suggest that at least early rates of virus production are decreased. This may be interference or may reflect other phenomena.

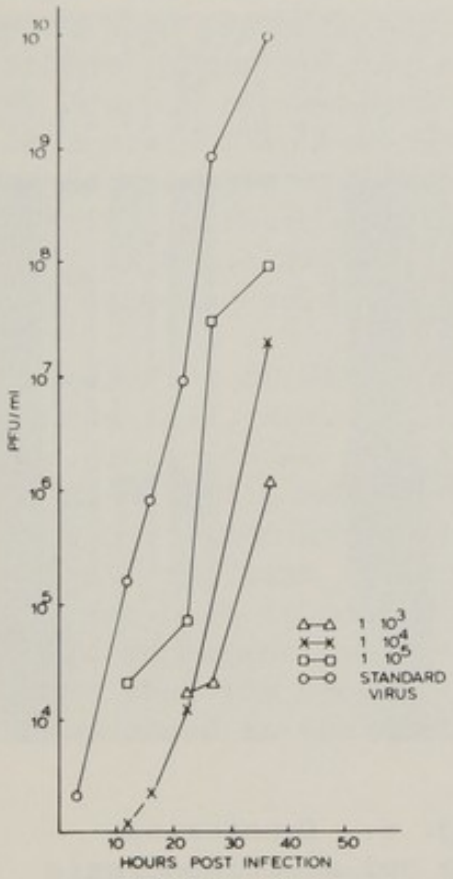

Fig. 3. Co-infection of A. alb. with standard Inkoo virus and virus from A.alb/INK.

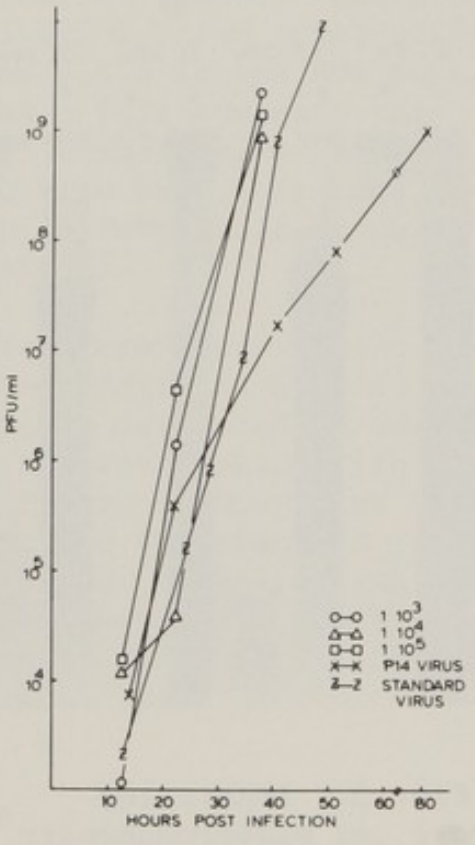

Fig. 4. Co-infection of BHK(WI-2) with standard Inkoo virus and virus from A.alb/INK.

In contrast to these data, infection of BHK (WI-2) cells with mixtures of standard Inkoo virus and virus from A.alb/INK cells results in no decrease in yield (Figure 4). These data suggest that the results of co-infection experiments are host dependent. If the decreased yields of standard virus observed in mosquito cells is due to interference (perhaps by DI particles), these data also suggest that the generation and maintenance of the persistent state depends upon some features of both the host and viral systems.

Particles Released from Persistently Infected Cells have Altered RNA Segments. Viral particles from A. alb. cells infected with standard Inkoo virus were labeled in the presence of ^{32}P-orthophosphate and purified as described. RNA species from these particles were separated on denaturing agarose gels. Autoradiographs (Figure 5, lanes labeld "V")

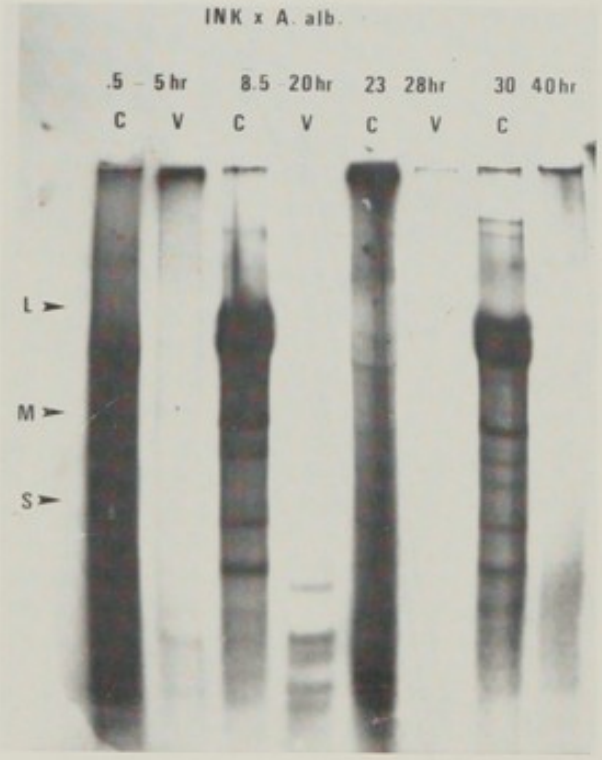

Fig. 5. Denaturing agarose gel electrophoresis of intracellular and virion RNA during initial infection of A. alb. Cells with Inkoo virus.

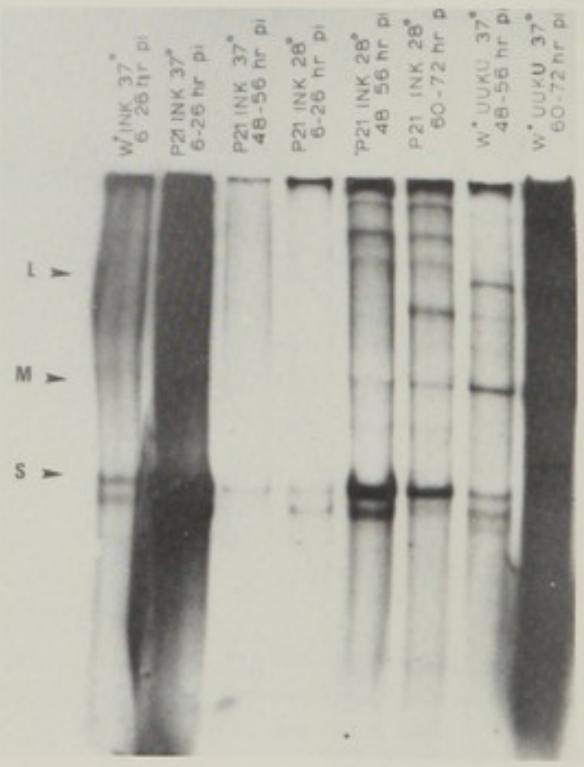

Fig. 6. Denaturing agarose gel electrophoresis of intracellular RNA from BHK cells after infection with virus from A.alb/INK cells.

reveal, in addition to the expected three RNA species (L, M and S) a series of small RNA's, migrating faster than the S RNA segment. These RNA species can be found in particles released during early times after the initial infection of cells with the virus. We have not yet been able to detect any heterogeneity in the particle population during sedimentation in potassium tartrate/glycerol or Renografin gradients.

Intracellular Viral RNA from Persistently Infected Cells Also Have Altered RNA Segments. *A. alb.* cells were labeled for the indicated times after initial infection with Inkoo virus. Intracellular virus-specific RNA (Actinomycin D resistant synthesis) was separated on denaturing gels. Again, small RNA species are apparent (Figure 5, lanes labeled "C") in addition to the expected genome size specied. These gels also reveal that the intracellular RNA species of Inkoo virus infected cells is more complex than the pattern appearing in mature virions. Other data (Parker and Hewlett, unpublished observations) suggest that Inkoo virus-infected BHK cells contain more than three RNA species, perhaps as many as six. The present data reveals many RNA species in the region of the genome size segments and at sizes smaller than S. While some of these larger species are extensively labeled intracellularly, they are not represented at the same ratio in the RNA population contained in released particles. This suggests that some form of preferential packaging of small viral RNA may be occurring in these cells. These results have also been obtained with A.alb/UUKU, even though no detectable plaquing virus is being released (data not shown).

Infection of BHK Cells with Virus from Persistently Infected Cells Results in Loss of the Small RNA Species. Virus preparations from A.alb/INK cells were used to infect BHK (WI-2) cells and the resulting RNA was labeled with ^{32}P at various times after infection. Figure 6 shows that RNA synthesis in these cells has returned to the standard pattern, shown for both Inkoo and Uukuneimi virus, revealing RNA species representing the three genome segments. These data suggest that the synthesis of the small aberrant RNA species is suppressed in the vertebrate cells.

DISCUSSION

The maintenance of buynaviruses during horizontal transmission through invertebrate hosts in nature may be related to the establishment of a persistent infection in tissues of the host insect. We have established a persistent infection in cell culture in order to examine the molecular

aspects of this process. Our results indicate that the replication cycle of Inkoo virus is altered during the establishment of persistence in *A. alb*. The presence of small RNA's, possibly representing sub-genomic species, in virus particles being released from infected cells suggests the occurrence of defective-interferring (DI) particles. If the decreased initial yield of standard virus in a co-infection experiment (Figure 3) is truly interference, then the persistent state may be generated and maintained by DI particles. Huang and Baltimore (13) have suggested just this role for DI particles in the course of natural infections. However, since we have not clearly demonstrated that interference is occurring and since we have not observed particles which can be classed as DI, this model must remain only one of several possible explanations for these phenomena. It may be, for example, that the temperature of growth of the host organism plays a role in the course of infection. We have examined the population of particles released from A.alb/INK by plaque morphology at the higher temperature, suggesting the presence of *ts* mutants in this population. In other experiments, however, we have found that the lack of "interference" observed during co-infection of BHK cells with standard virus and virus from A.alb/INK (Figure 4) does *not* depend on the temperature. Therefore it is not clear at present what the mechanism might be of this altered pattern of virus growth in the mosquito.

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EXPERIMENTAL RELAPSING MYELITIS IN HAMSTERS ASSOCIATED WITH A VARIANT OF MEASLES VIRUS[1]

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ABSTRACT Chronic relapsing myelitis was induced in hamsters by intracerebral inoculation of measles virus when the animals were less than one day old. Disease appeared from two months to one year after infection and was characterized pathologically by mononuclear cell infiltration, gliosis, and demyelination. Usually the disease was slowly progressive with prominent myoclonus of the hind limbs. In some animals the disease occurred as episodic paresis of the hind limbs with near total recovery between periods of paralysis. Virus could not be isolated by cocultivation of cord with monkey kidney cells. The strain of virus used was distinctive in that it contained high levels of a naturally occurring viral variant, differing from typical measles in several ways. In cultures of monkey kidney cells the variant induces cell rounding and swelling rather than the usual measles syncytia formation. In acutely infected neonate hamster brain the variant grows less readily than does the syncytiagenic form of the virus and is less encephalitogenic. The variant has a decreased buoyant density in potassium tartrate gradients compared to typical measles, yet it expresses the same major structural polypeptides. However, the relative amounts of the two viral envelope glycoproteins are altered in the variant. The variant can be cloned and titered independently of the syncytiagenic type of virus and is therefore unlikely to be a defective form of typical measles virus. This experimental system will be of value in the study of the immunological and pathogenetic aspects of chronic neurologic diseases associated with unusual or aberrant viral infections.

INTRODUCTION

Virus or virus-like agents have been implicated in several chronic diseases of the human central nervous system (CNS) (1-6). These diseases, while associated with widely

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varying virus groups and showing diverse clinical patterns, bear several important similarities. In most cases the associated virus is either a non-conventional agent (1,2) or a common virus with unusual properties (3,5,7). In virtually all of the diseases, onset of neurological signs is delayed months or years after initial viral infection, and the clinical course is prolonged (1,2,7,8,9).

The identification of these chronic CNS virus diseases has led to the suggestion that multiple sclerosis (MS) may be related to a chronic virus infection (10,11). Epidemiologic studies have suggested that MS is associated with an infection early in life, with delayed disease onset (12,13). Measles virus has been specifically implicated in the pathogenesis of MS by serologic studies (14-16), and local CNS synthesis of anti-measles antibodies has been demonstrated (11,17). Numerous attempts to isolate virus from CNS tissues of MS patients have been unsuccessful (10).

Since common viruses with unusual properties have been shown to induce chronic CNS disease and since measles virus has been implicated in the pathogenesis of MS, the study of variants of measles virus in terms of CNS disease is of importance. A variant of measles has been identified, partially characterized, and investigated as a CNS pathogen in the golden Syrian hamster. This animal system was chosen since it has been found to model the established CNS complications of measles infection including acute encephalitis (18,19), subacute sclerosing panencephalitis (SSPE) (20,21), and retinitis (22).

MATERIALS AND METHODS

Animals and Virus. Pregnant outbred golden Syrian hamsters were housed prior to and after delivery in Labline isolation cubicles. Adult animals were housed together in numerically coded cages. LEC strain of measles virus, originally isolated from the brain of a child with SSPE (23), was obtained from Dr. Erling Norrby and was enriched in this laboratory for the viral variant. Details of stock virus preparation and the procedure developed for titering the viral variants have been described (24). In short, the virus stocks were obtained by low multiplicity of infection passage of the parent LEC virus in Vero cells; and the viral variants were titered by virtue of their different cytopathic effects (CPE) as $TCID_{50}$ per ml.(see RESULTS). The titers of the syncytiagenic variant are designated S_{50}/ml, and the titers of the non-syncytiagenic variant are termed RC_{50}/ml. All virus stocks contained both viral variants, but the relative amounts of the two variants differed among the

stocks used.

Other Techniques and Procedures. The procedures used in these investigations, including viral purification in density gradients, viral hemagglutinin (HA) assay using African green monkey erythrocytes, SDS-polyacrylamide gel electrophoresis, and viral isolation from CNS tissue by cocultivation with Vero cells have been described in detail elsewhere (19,24,25).

RESULTS

Identification of the Round Cell Variant of Measles Virus. In Vero cultures infected with the LEC measles stocks used in these studies, two discrete types of CPE were seen with the proportion of each varying both with the stock used and with the conditions of infection. One type of CPE was syncytia formation (Figure 1A), the kind most commonly associated with measles virus (26). The second type of CPE was that of cell rounding and swelling with increased refractility (Figure 1B). The viral variant responsible for this second type of CPE was termed the "round cell variant".

By a method of limiting dilution, separate Vero cultures were infected with single infectious units of each variant. The time from infection to the appearance of CPE and the rate of viral progeny (as HA activity) production were determined. Marked differences were noted. Syncytial CPE appeared within two days of infection, while eight days elapsed before

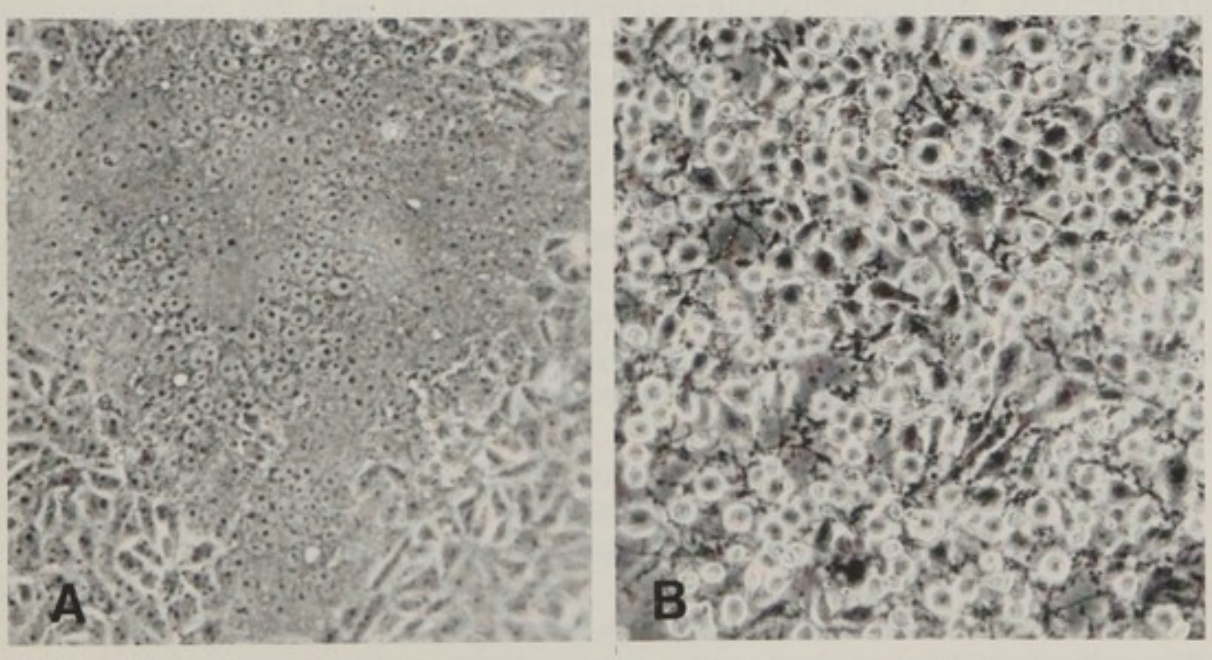

FIGURE 1. Two forms of LEC measles virus CPE in Vero cells. A. Syncytial CPE: a syncytium surrounded by normal cells. B. Round cell CPE: induced by a viral variant.

TABLE I

GROWTH CHARACTERISTICS AND PHYSICAL PROPERTIES OF VIRUS RELEASED FROM CULTURES INFECTED WITH VIRAL VARIANTS[a]

Viral Variant	Time from infection to CPE appearance	Peak cell-free HA titer released[b]	Buoyant density of released virus[c]
Round cell	8 days	196	1.19
Syncytial	1-2 days	6	1.22

[a]Vero cultures were infected with single infectious units of each variant

[b]Proportional to infectious virus titer

[c]g/cm^3 in potassium tartrate

the appearance of the round cell CPE. Further, viral progeny were produced at much higher levels in the round cell variant infected culture. When the virus particles released from separate cultures showing syncytial and round cell CPE were purified and analyzed, their equilibrium buoyant densities in potassium tartrate were found to be significantly different with the syncytial CPE derived virions being more dense (Table I).

Radiolabelling with ^{125}I of intact virions from cultures showing the two types of CPE followed by analysis using SDS polyacrylamide gel electrophoresis revealed the relative proportions of the surface polypeptides (H and F1) present in the virion populations (Figure 2). Gel conditions were chosen so that the F1 polypeptide was present in approximately equal amounts in the syncytial and round cell CPE derived samples. However, it was found that the amount of the H polypeptide varied considerably between the two samples, with a larger amount being present in the round cell CPE derived virus. This relative enrichment of the syncytial CPE derived virions for the F1 polypeptide is consistent with the cell fusion seen with the syncytiagenic variant since the F1 viral polypeptide is associated with cell fusion (27).

Following the elucidation of these differences between the syncytiagenic and round cell variants *in vitro*, it was of interest to determine their comparative properties *in vivo*.

The relative neurovirulence of several stocks of LEC virus containing various proportions of the variants was determined in neonate hamsters. From these data the lethal dose 50% (LD_{50}) for each stock was determined and compared with the relative proportions of the syncytiagenic and round

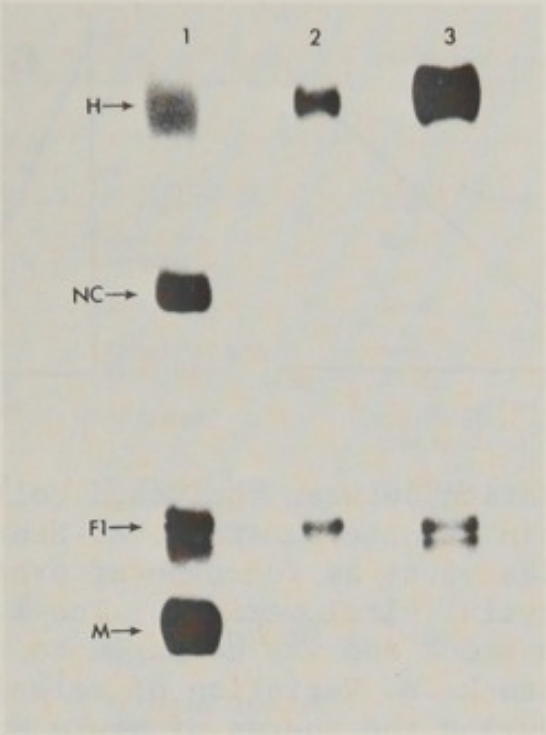

FIGURE 2. An autoradiogram of SDS-polyacrylamide gel analyzing the structural polypeptides of round cell CPE derived virions (lane 1) and the viral membrane associated proteins of syncytial CPE (lane 2) and round cell CPE (lane 3) derived virions. Under the conditions used, the viral P polypeptide does not significantly label with ^{125}I. The band immediately below the Fl polypeptide is a proteolytic breakdown product of the H polypeptide. Arrows indicate the positions and designations of the polypeptides of the reference Edmonston strain of measles which was run in parallel.

cell viral variants (expressed as RC_{50}/S_{50} ratio) in the stock (Figure 3A). An inverse relationship was found between the proportion of the round cell variant in a stock and its neurovirulence. This finding is consistent with the high neurovirulence and syncytiagenic nature of most newly isolated SSPE strains and neuroadapted strains of measles (5,6,8,9,18,23).

To explore more fully the low neurovirulence of the round cell viral variant, newborn hamsters were intracerebrally (i.c.) inoculated with a dose of a LEC virus stock of known RC_{50}/S_{50} ratio. At times after infection animals were taken and the ratio of the two variants in the cell-free virus present in the encephalitic brains was determined. No differences were found between clinically ill and well animals in either the amount of virus present or in the RC_{50}/S_{50} ratio of the virus. The ratio of the original virus stock

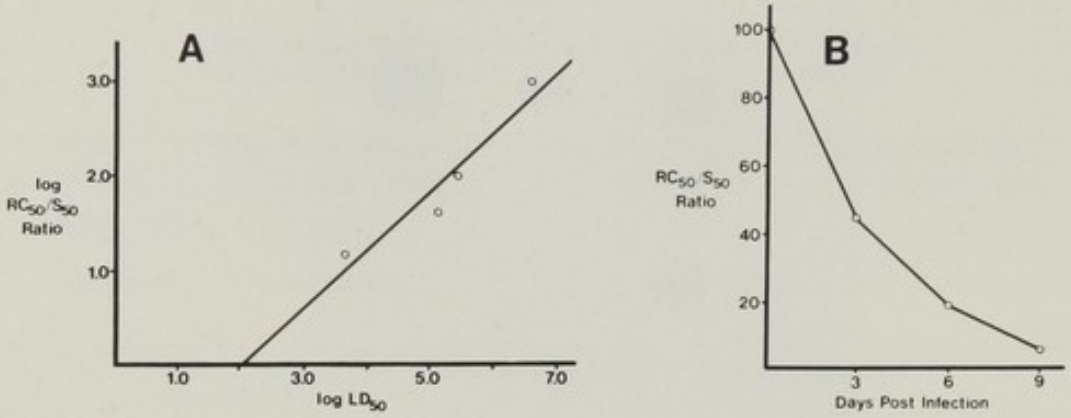

FIGURE 3. Relation between the round cell variant and acute encephalitis in neonate hamsters. A. Neurovirulence of stocks of LEC measles virus as function of proportion of round cell and syncytial viral variants. The i.c. LD_{50} was determined for each stock and was compared to the RC_{50}/S_{50} ratio of the same stock. B. Variation of relative proportions of viral variants during the course of acute encephalitis. Animals were i.c. inoculated with a dose of virus that gives a mortality of approximately 90%. At times after infection animals were randomly selected, killed, and homogenates of their brains were titered for the two variants. Each point represents the mean value of at least four individual animal determinations. No cell free virus could be isolated at times after nine days.

was taken as the day 0 value. Results are shown in Figure 3B. By the ninth day after infection the RC_{50}/S_{50} ratio had fallen more than ten-fold, indicating a replicative disadvantage of the round cell variant during the infection.

The poor replication of the round cell variant in the CNS raised the question of the long term effects in animals infected in their CNS with such a nonproductive virus. Newborn animals were i.c. inoculated with a virus stock containing the round cell variant at one thousand-fold excess over the syncytiagenic virus. The surviving animals (approximately 75%), none of which showed any sign of acute illness, were observed for late onset neurologic disease. Approximately 20% (7/36) of these animals developed such disease from two months to one year after the initial infection ($p<0.01$ compared to sham infected controls, none of which showed any disease). Representative clinical courses of two affected animals are shown in Table II.

Most of the animals showed progressive disease courses as illustrated by animal #6793-2; however, some animals had

TABLE II

CLINICAL COURSES OF TWO[a] ANIMALS THAT DEVELOPED DELAYED ONSET[b] CNS DISEASE AFTER NEONATAL INFECTION WITH MEASLES VIRUS[c]

Time after infection (weeks)	Animal #6345-2	Animal #6793-2
24	Right hind limb paresis	Normal
26	Normal	Normal
28	Paresis of both hind limbs	Normal
30	No change	Hypokinesis; right side myoclonus
32	Right hind limb normal; paresis of left hind limb	No change
34	No change	"
36	Left side myoclonus; paresis of left hind limb	"
38	No change; taken for study	"
40	-	Abnormal gait; right side myoclonus
42-46	-	No change
48	-	No change; taken for study

[a] Disease occurred in 19% (7/36) of the animals ($P < 0.01$ relative to sham injected controls)

[b] No acute disease was seen in any animal which developed delayed onset disease

[c] Virus used was enriched one thousand-fold for the round cell variant over the syncytiagenic variant, and the input dose was approximately 1.4×10^6 RC_{50}

courses in which periods of abrupt illness were separated by intervals of spontaneous remission. One such animal, designated #6345-2 in the table, had three acute disease exacerbations separated by periods of at least partial remission ranging from two to eight weeks.

When these animals were killed for study, extensive attempts at virus isolation from CNS tissue by cocultivation with Vero cells were unsuccessful. Pathologic changes were largely restricted to the spinal cord. An example of a typical lesion is shown in Figure 4. In general, lesions were restricted to the dorsal root entry zones and consisted of demyelination, necrosis, and mononuclear cell inflammation. A dramatic gliosis was present, and numerous plasma cells could be identified throughout the lesions.

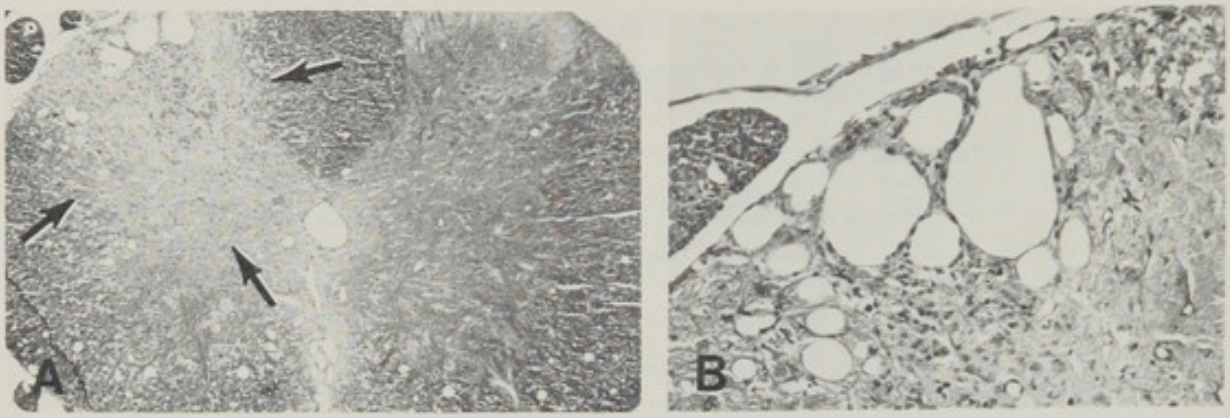

FIGURE 4. Histopathology of a lesion in the spinal cord of animal #6345-2 in Table II. A. Cross section of the cervical cord. A demyelinating lesion can be seen in the vicinity of the left dorsal root entry zone (arrows). Luxol fast blue stain for myelin. B. Higher magnification view of the lesion seen in A . Of note are the marked gliotic changes, the diffuse mononuclear cell inflammatory infiltrate, and the lipid-laden macrophages in the posterior column. Hematoxylin-eosin stain.

DISCUSSION

A variant of measles virus, termed the "round cell" variant, has been identified and characterized. Its identity as measles virus was proven by its neutralization with measles specific antisera (data not presented) and by the identity between the structural polypeptides of Edmonston measles virions and those of virions released from cells infected with the round cell variant (Figure 2). The viral variant differs from common strains of measles in several respects. First, it induces cell rounding, swelling, and increased refractility as its predominant CPE in cultured cells rather than the cell fusion usually identified with the virus (26). Second, the round cell variant appears to grow slowly with up to 8 days being required for CPE to appear after infection. This contrasts sharply with the one to two days required for syncytial CPE to appear (Table I). Third, cultures infected with the round cell variant produce significantly higher levels of viral progeny than do those infected with the syncytiagenic form of the virus (Table I). Fourth, virions released from cells showing the round cell type of CPE have a higher buoyant density in potassium tartrate than do virions released from syncytia (Table I). Fifth, round cell CPE derived virions are enriched for the H glycoprotein with respect to the F glycoprotein when compared

to syncytial CPE derived virions (Figure 2). Sixth, the round cell variant is less neurovirulent in newborn hamsters since higher doses are required to induce encephalitis (Figure 3A), and the round cell variant replicates poorly in brain compared to the syncytiagenic virus (Figure 3B).

The effects of infection of brain with the round cell variant in terms of delayed onset CNS disease was invesigated by inoculating newborn animals with a large i.c. dose of the virus. Approximately 75% of the animals survived the acute infection and of these survivors approximately 20% developed a delayed onset CNS disease. In most cases disease was slowly progressive; however, disease courses of exacerbation and remission were also noted. Pathology was largely restricted to the spinal cord and consisted of mononuclear cell inflammatory infiltration, demyelination, gliosis, and necrosis.

This chronic myelitis in hamsters bears some resemblance to certain forms of MS. Measles virus has been serologically implicated in the pathogenesis of MS with onset of CNS disease being delayed after acute viral infection (10,11,14). The lesions of both the hamster myelitis (Figure 4 and data not presented) and MS (27,28) appear to begin as mononuclear cell infiltration followed by demyelination and tissue necrosis. The terminal state of lesions in both diseases is replacement of neural tissue with glial scar (Figure 4,27,28). Further, the clinical course of MS can be either one of continuous progression or one of intermittent disease with complete or partial recovery between exacerbations (30). A similar disease pattern was found in the clinically affected animals of these studies (Table II, RESULTS). The similarities between this experimental disease in hamsters and human MS raise new possibilities concerning the etiology of MS since the experimental disease is associated with a variant of measles virus that has not previously been used in experimental pathogenesis studies.

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ANTIBODY-INDUCED MODULATION OF VIRAL ANTIGENS FROM INFECTED CELLS: BIOLOGICAL AND MOLECULAR STUDIES OF MEASLES VIRUS INFECTION AND IMPLICATIONS FOR UNDERSTANDING VIRUS PERSISTENCE AND RECEPTOR DISEASES[1]

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ABSTRACT. Measles virus infected cells express two virus specific antigens on their surface. These are the hemagglutinin (HA) and fusion (F_1) polypeptides. Their turnover (half life) from the plasma membrane is 9 to 10 hours but in the presence of antibody, there is an initial rapid loss of F_1 and HA followed by a slower phase in which T 1/2 is 12 to 15 hours. Reduced numbers of these surface viral antigens enable the cell to resist lysis by immune reagents. In addition, the loss of F_1 molecules during antibody induced antigenic modulation accounts for the lack of both cell-cell fusion and giant cell formation by infected cells cultured with antibody. Expression of the internal phosphoprotein (P) and of the matrix or membrane (M) protein is also altered by antibody. These changes in P and M viral polypeptides are specific, since antibodies directed against measles virus antigens expressed on the cell's surface cause such effects but antibodies directed against nonviral antigens expressed on the surfaces of infected cells do not. Functionally, the P protein and its analogs in other viral systems appear to be associated with the transcriptive complex, whereas the M protein is associated with nucleocapsid recognition and alignment at the plasma membrane. Therefore, alterations in these polypeptides would lead to those aberrations in measles virus synthesis and maturation that are the observed hallmarks of persistent measles virus infection.

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INTRODUCTION

In its original sense antigenic modulation referred to a cell's resistence to lysis by antibody and complement. Working with thymus leukemia (TL) antigens expressed on normal thymus cells in certain mouse strains and on leukemia cells, Boyse, Old and associates (1-4), who first observed antigenic modulation, noted that the phenotypic expression of TL was suppressed by TL antibodies both *in vivo* and *in vitro*. For this decreased expression of TL to occur, active metabolism of the affected cells was necessary. Several other retroviruses perform comparably (5-9). In general, the model systems used to study these events have involved an infecting agent that does not *per se* shut off host protein synthesis sufficiently to kill the cell.

Among virus infections studied in this respect, the antibody induced modulation of measles virus antigens on the surfaces of infected HeLa cells has been examined intensely (10, 11). When measles virus induced cytopathology occurs during infection, it is usually related to a membrane event such as fusion. During antigenic modulation, antibodies can function to remove from the cell's surface viral antigens that are responsible for fusion, thus minimizing or preventing cell-to-cell fusion and allowing both cell survival and viral persistence. Modulation continues as long as sufficient antibody is in the cultured medium to bind to the cell's surface and strip off viral antigens. Once the initial phase has begun, infected cells can be cultured in serum containing specific antibodies and a functional complement source without being lysed. Such modulation diminishes the amount of viral antigen expressed on the cell's surface to a point that binding of antibody and complement or immune lymphocytes is inadequate for lysis. Nevertheless, infected cells cultured with the specifically reactive antibody grow at a similar rate to uninfected cells (10).

For some experiments, it is important to segregate the affects of acute from long term modulation. During acute modulation of measles virus infected cells, defined as less than 72 hours of exposure to antibody, if one removes antibody, viral antigens return to near normal levels on the cell's surface within 18 to 24 hours. But, the longer the cells are cultured in antibody, the more time they need to re-express measles virus antigen after antibody has been removed (10).

We believe antibody induced antigenic modulation to be an important mechanism in the initiation and also helpful in the maintenence of virus persistence. In this article we will review this process as it occurs with retroviruses and then

provide experimental details concerning antibody induced measles virus persistence. In order to appreciate the molecular mechanism(s) involved we will also use this report to describe the appearance, turnover and relationship(s) of the various measles virus polypeptides to each other on the cell's surface.

TL Antigens and Retrovirus Models. Early experiments with modulation (1,3,4) involve the TL differentiation antigen of murine leukemia cells. Mice immunized against TL+ leukemia cells generated high titers of cytotoxic antibodies. Later, when these mice were challenged with TL+ leukemia cells, instead of the expected tumor rejection, the leukemia cells multiplied inspite of high titers of antibody. Study of these TL leukemia cells showed that they have lost the ability to absorb TL antibody (phenotype TL-) and to undergo lysis by TL antibody and complement. Culturing TL+ cells with antibodies to TL rendered the cells resistant to antibody and complement-mediated lysis and caused the loss of TL phenotype. When antibody was removed from the *in vitro* or *in vivo* milieu, these cells became TL+ and regained their sensitivity to lysis. TL+ thymocytes from nonleukemic mice could also be modulated in a similar manner (2).

Next, Boyse, Old and colleagues noted (12) that modulation did not require the whole immunoglobulin molecule, but that the Fab fragment by itself was sufficient to induce modulation. Hence, although capping required divalent antibody, modulation could proceed with univalent antibody. Nevertheless, modulation also occurred efficiently and effectively at physiologic conditions with multivalent antibody. Stackpole *et al*. (13) demonstrated TL antibody redistributed antigen into aggregates, then caps. Such caps remained on the cell's surface and only small amounts were pinocytosed, as confirmed by others (14-16). Stackpole *et al*. (17) further noted that this modulating activity was enhanced by complement and that the third component of complement seemed to play an important role in this process.

Aoki and Johnson (5) studied Gross virus-induced leukemia E♂ G2 transplanted into immunized syngeneic C57BL/6 mice. In this situation, amounts of specific cell surface antigen (GCSAa) decreased although those of other antigens GLSAa negative were transplanted into recipients lacking the specific antibodies, GCSAa reappeared on tumor cell surfaces in normal quantities. Electron microscopy confirmed the modulation of GCSAa. Ioachim and Sabbath (6) extended these findings *in vitro* in cells capped and modulated using immunofluorescence. Loss of GCSAa from the surfaces of tumor cells required 24 hrs of cell culture with antibody, but 48 hrs after antibody was

removed from the medium, GCSAa reappeared and resumed control levels.

The findings were similar when Genovesi et al. (7) cultured Friend leukemia virus (FLV) infected cells with immune sera. These cells lost their surface expression of FLV and their susceptibility to lysis by antibody and complement. When antibody was removed from the culture medium, within 48 hrs the cells reexpressed FLV antigens and were again susceptible to immune lysis. Actively growing cells were more easily modulated than resting cells. Soon afterwards, Bubbers and Lilly (18) showed that treatment of leukemia cells with antibody prepared against whole virus also caused resistance to immune lysis. Concomitantly, the cells were less susceptible to lysis by antisera against certain H-2 haplotypes suggesting that antibody to FLV reduced the number of FLV surface antigens and of certain H-2 molecules. However, because of possible H-2 contamination in the FLV preparation used to raise the antiserum, interpretation of the findings is difficult. Other investigators (8,19) showed that modulation of FLV occurred in vivo. As leukemia progressed, they noted that the surfaces of leukemia cells expressed fewer FLV antigens, and the cells became resistant to lysis. Loss of surface determinants was FLV-specific, considering that quantitative absorption with H-2 alloantisera caused no alteration in H-2 antigen levels. Leukemia cells reexpressed normal levels of FLV when transferred to nonirradiated immune recipients. Through the use of recombinant mice, the disease was linked to the RFV-3 genetic locus, and the production of FLV antibodies was associated with this locus.

In addition, Calafat et al. (9) showed that murine mammary tumor virus antigens could undergo redistribution and modulation. With a different murine tumor cell line, Yagi et al. (20) showed similar results. Tumor cells implanted into immunized mice showed a significant decrease in synthesis of viral antigens and, by electron microscopy, decreased expression of viral particles. Removal of these tumor cells and passage in vitro, resulted in cells reexpressing antigen. When tumor specific antibody was added to the cultures, the polypeptide profile of their mammary tumor virus antigens changed.

Measles Virus. In nature, certain infections are associated with virus persistence in the presence of specific antiviral antibody. For example, chronic measles virus infection persists in the face of a vigorous host immune response. Measles virus infection in humans usually follows an acute, self-limiting course as the patient mounts an immune response that clears the virus from his tissues. Conva-

lescence is associated with low titers of antibody to measles virus and immune lymphocytes that remain throughout life. In contrast, in the unusual instance of chronic measles virus infection, subacute sclerosing panencephalitis (SSPE), patients have extraordinarily high titers of antimeasles virus antibodies, yet virus persists. Although virus can be isolated from the central nervous system and lymphoid tissues (21), antiviral antibody titers are noted to be 10-fold or higher above normal immune individuals in sera and other body fluids (10,21); the complement system is functionally active (10), and cytotoxic immune lymphocytes are present in the peripheral blood (22,23).

To explain the events that occur in SSPE, we have suggested that antibody to measles virus modulates or strips viral antigens off surfaces of virus infected cells (10,24). This suggestion is based on experimental evidence and clinical observations in patients with SSPE. First, specific measles virus antibodies added to cultured cells infected with measles virus modulate viral antigens off the cells' surfaces (10). Because the cells then express less virus antigen on their surfaces, they avoid immune lysis by humoral or cell mediated immunologic mechanisms (10,25). Hence, cells denuded of viral antigens escape immune assault but retain viral genetic information. Second, during antibody modulation in vitro, viral nucleocapsids accumulated inside the cell dramatically increase in numbers and are positioned randomly, a picture closely resembling the distribution of nucleocapsids in cells obtained by biopsy from patients with SSPE (26,21, 27).

We envision the modulation scheme to proceed as follows in the measles virus system (Fig. 1). Virus infected cells express measles virus antigens at the surface. In the presence of antibody, antigens and antibodies complex on the membrane than aggregate near one pole of the cell. After further incubation in the presence of antibody, these antigen-antibody complexes shed into the supernatant fluids. Such cells denuded of virus antigens on their surfaces are now resistant to lysis by virus specific antibody and complement or immune lymphocytes (10,25). Infected modulated cells, when recultured in the absence of specific antibody, reexpress viral antigens and become completely susceptible to lysis by immune reagents.

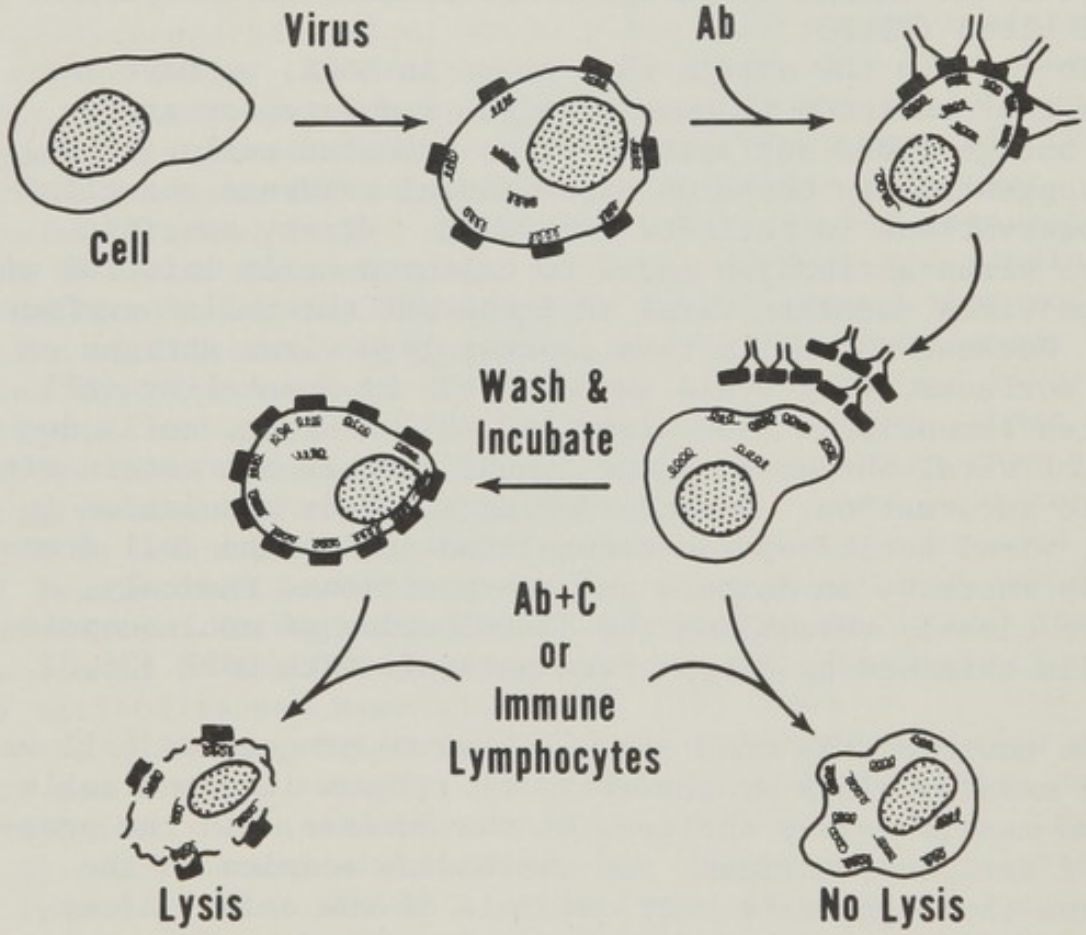
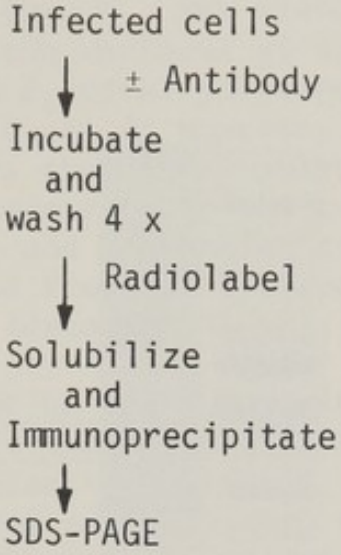


Fig. 1. Experimental procedure for studying measles virus infected cells and the expected outcome from experimental manipulations.

MATERIALS AND METHODS

A detailed description of the methods employed may be found elsewhere (10,11,28). Schematically, the basic modulation and labeling procedure is shown here.



Briefly, HeLa cells, in suspension, were infected with measles virus at an MOI of 1 and cultured overnight. The cells were then incubated in the presence or absence of media containing anti-measles virus antibody. After various intervals, the cells were washed 4 times and labeled internally with ^{35}S-methionine or ^{32}P, or surface iodinated (^{125}I). The labeled cells were solubilized in detergent (NP-40), and the virus specific polypeptides were immunoprecipitated first using a polyvalent antibody to measles virus and then using Staphylococcus aureus (S. aureus). These precipitated polypeptides were then analyzed by SDS polyacrylamide gels (PAGE).

RESULTS

Expression of Measles Virus Polypeptides in Cytosols and on the Surfaces of Infected HeLa Cells. Under the conditions used, six distinct measles virus polypeptides are found in ^{35}S-labeled, immunoprecipitated cells (Fig. 2, left lane).

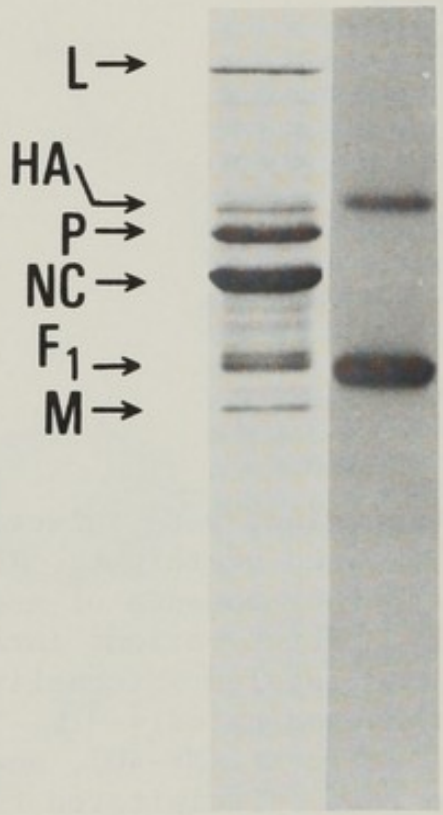


Fig. 2. Autoradiogram of measles virus polypeptides. Left lane represents ^{35}S-methionine labeled measles virus polypeptides immunoprecipitated from infected HeLa cells. Six major structural viral polypeptides are seen. Right lane represents surface radioiodinated labeled measles virus hemagglutinin (HA) and fusion (F_1) polypeptides expressed on the cell's surface.

These polypeptides are: large (L) protein, hemagglutinin (HA); phosphoprotein (P); nucleocapsid (NC); fusion protein or hemolysin (F_1); and membrane or matrix (M) protein. The apparent molecular weights determined electrophoretically by comparison to several protein standards with known molecular weights are: 160,000, L; 80,000, HA; 70,000, P; 60,000, NC; 42,000, F_1; and 36,000, M. The right lane depicts viral proteins expressed on the surfaces of infected cells labeled with ^{125}I . Both HA and F_1 viral polypeptides are located on the surfaces of infected cells; however, the absence of P, NC and M proteins indicates that these polypeptides are confined inside of the plasma membrane or the cells' cytoplasm. Nevertheless, the P, NC, and M proteins can be labeled with ^{125}I if they are obtained from solubilized purified virions or cytosol preparations (data not shown).

The Turnover of HA and F_1 Measles Virus Polypeptides on the Surfaces of Infected Cells. Studies on the turnover of HA and F_1 from infected cells and the binding of antibodies to cell surface viral antigens were done in collaboration with J.G. Patrick Sissons. Twenty-four hrs after infection with measles virus at an MOI of 1, HeLa cells were labeled with ^{125}I by the lactoperoxidase method, washed extensively and returned to culture. At intervals, equivalent volumes were harvested, washed, solubilized and evaluated for cell viability, which exceeded 97%. Analysis by PAGE for ^{125}I radiolabeled polypeptide followed, after which bands corresponding to the HA and F_1 polypeptides were cut from the gel, counted for radioactivity and quantitated for HA and F_1 content. The T 1/2 times of HA and F_1 were calculated by linear regression analysis from several time points. As one sees in Fig. 3, these T 1/2 are 10 and 9 hrs, respectively, for HA and F_1.

The Turnover of HA and F_1 Measles Virus Polypeptides on the Surfaces of Infected Cells in the Presence of Antibody. Cells were treated as above except that antimeasles virus antibody was added to the cultures. The initial loss of both HA and F_1 (Fig. 4) was calculated from ^{125}I counts as 35 to 40% of both HA and F_1 polypeptides. After this initial loss, a slower but continued release of viral polypeptides followed, and TCA precipitable counts increased in the supernatant fluid. The calculated T 1/2 times for the HA and F_1 polypeptides on antibody treated cells after the initial loss of surface antigens were 15 and 12 hrs, respectively.

Accessibility to Antibody of Viral Surface Glycoproteins on Infected Cells. At 40 hrs after infection, HeLa cells

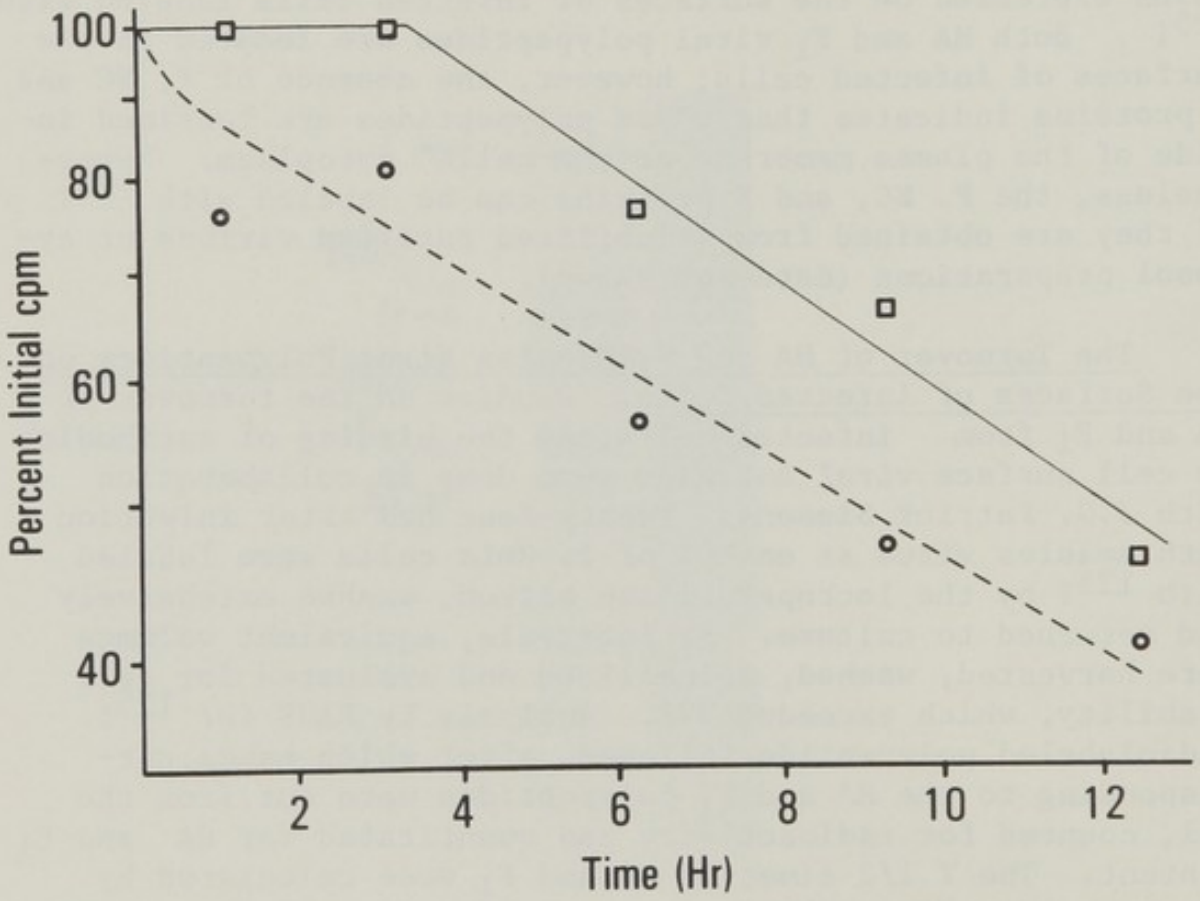


Fig. 3. Turnover of the HA and F_1 polypeptides from the surfaces of measles virus infected HeLa cells. HA is represented by 0---0 and F_1 by □—□. The T 1/2 for the HA and F_1 is 9 and 10 hours.

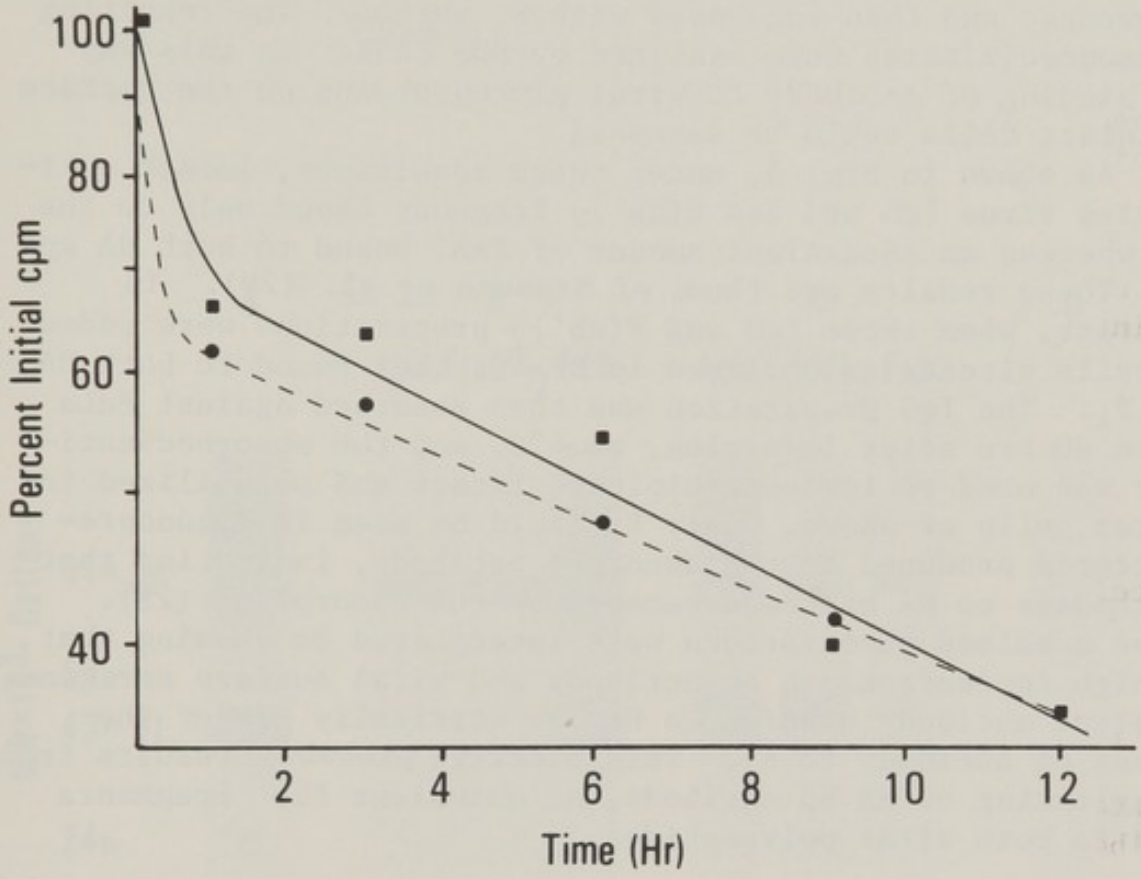


Fig. 4. Turnover of the HA and F_1 polypeptides from the surfaces of measles virus infected HeLa cells cultured in the presence of measles virus antibody. HA is represented by ●----● and F_1 by ■——■ . After the initial rapid loss of antigens, the T 1/2 for the HA and F_1 is 12 and 15 hrs., respectively.

were surface labeled with ^{125}I. These cells retained more than 90% viability after labeling. The surface radiolabeled, infected cells were then incubated for 30 min at 4°C with whole IgG purified from the serum of a patient with SSPE or with the $F(ab')_2$ or Fab' fragments. The cells were then washed in cold medium, solubilized in 2% NP-40, incubated with rabbit antibody to human IgG in the case of $F(ab')_2$ and Fab' fragments, and then incubated with S. aureus. The resulting immunoprecipitates were analyzed by SDS PAGE. In this way the binding of antibody to viral glycoproteins on the surface of intact cells could be assessed.

As shown in Fig. 5, under these conditions, intact antimeasles virus IgG and its $F(ab')_2$ fragment bound only to the HA, whereas an equivalent amount of Fab' bound to both HA and F_1. These results are those of Sissons et al. (28). In contrast, when these IgG and $F(ab')_2$ preparations were added to cells already solubilized in NP-40, they bound to both HA and F_1. The IgG preparation was then absorbed against HeLa cells 40 hrs after infection, at 4°C, and the absorbed antibody was used to immunoprecipitate intact and solubilized infected cells as above. Only F_1 could be seen in immunoprecipitates produced by the absorbed antibody, indicating that antibodies to HA had been removed by the absorption (28). These combined observations were interpreted as showing that, at high concentrations of antibody and viral surface antigens, divalent antibody binding to HA can sterically hinder the access of antibody to F_1. This blockage probably results from crosslinking of HA by antibody, as univalent Fab' fragments bind to both viral polypeptides.

Redistribution of HA and Exposure of F_1 on the Surfaces of Infected Cells. HeLa cells were infected with measles virus at an MOI of 1. Twenty-four hrs later when they expressed antigens on their surfaces, cells were labeled with ^{125}I and then cultured with antimeasles antibody. At varying intervals after antibody was added, the cells were washed, solubilized and combined with S. aureus in order to determine which of the viral polypeptides were accessible to bind to immunoglobulin on the cells' surface. Hence, only the antibody, which was complexed to the cell surface viral antigens could be precipitated under these conditions. As shown in Fig. 6, 1 and 2 hrs after addition of antibody, antibody bound only to the HA and not to the F_1. Not until 4 hrs after the addition of antibody was F_1 immunoprecipitated indicating a new distribution or exposure of F_1.

^{35}S Labeled Measles Virus Polypeptides in the Cytosols of Infected Cells Cultured with Antibodies to Measles Virus.

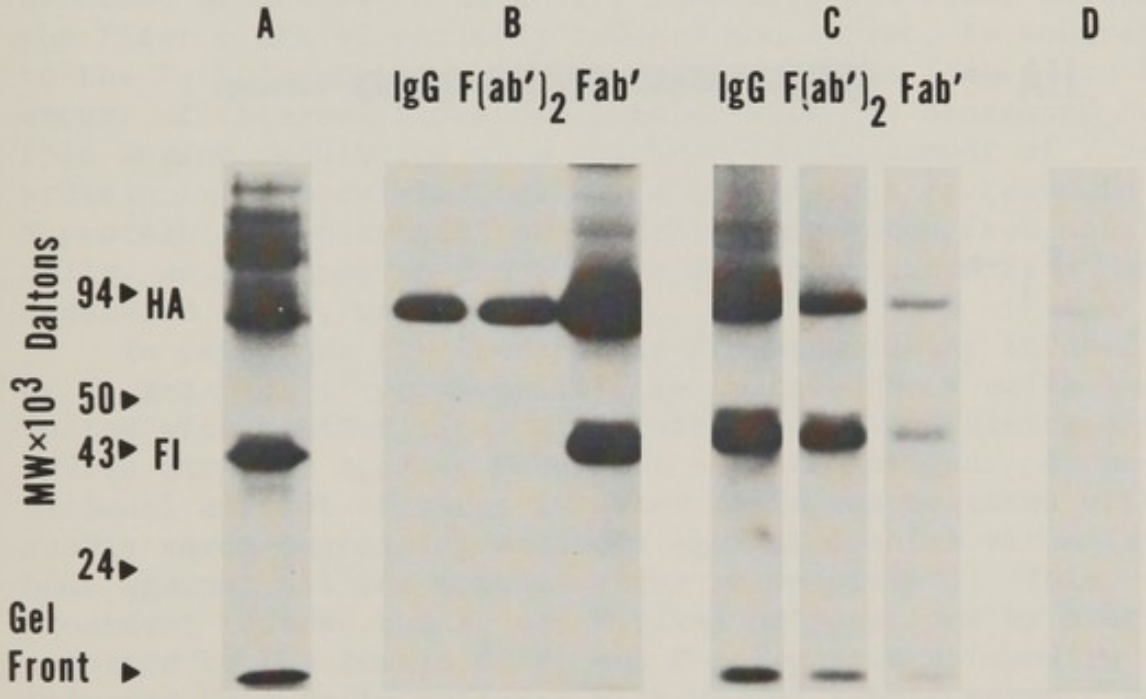


Fig. 5. Binding sites of IgG antibody and its fragments on surface of measles virus infected HeLa cells. Autoradiogram of SDS PAGE of immunoprecipitates of surface radiodinated HeLa cells infected with measles virus. A, detergent solubilized cells--not immunoprecipitated. B, 100 µl IgG, $F(ab')_2$ or Fab' (containing 2 mg protein) added to intact cells before detergent solubilization. C, 15 µl IgG, $F(ab')_2$ or Fab' added to detergent solubilized cells. D, control-solubilized cells with rabbit anti-human IgG only. HA and F_1 refer to the measles virus hemagglutinin and fusion polypeptides, respectively.

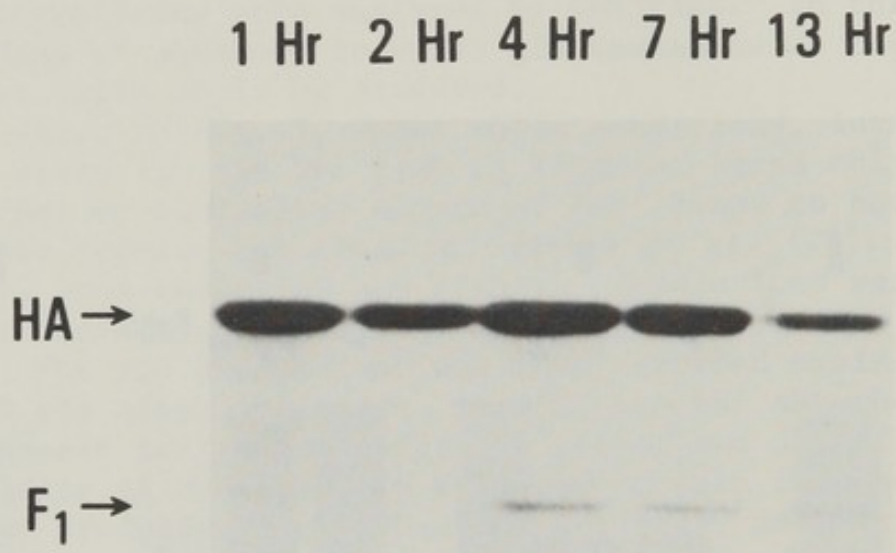


Fig. 6. Presence of HA and F_1 on the surface of measles virus infected HeLa cells upon addition of anti measles virus antibody to culture fluids. Autoradiogram of immunoprecipitates of surface radioiodinated HeLa cells infected with measles virus. At the time of radiolabeling viral antigens were maximally expressed. Thereafter, HeLa cells were incubated in the presence of antibody for various intervals. At the times shown cells were harvested and complexes immunoprecipitated.

HeLa cells were infected at an MOI of 1, and 20 to 24 hrs later were washed and incubated with or without medium containing human antibody to measles virus. In separate experiments, infected cells were cultured with antibody for 6, 12, 18, or 24 hrs, all of which produced equal results so that 6 hrs incubation was used thereafter. We then analyzed immunoprecipitated cytosol fractions from modulated and nonmodulated infected cells and uninfected controls. The NC peaks from modulated and nonmodulated cells were adjusted to equal values for internal consistency to allow quantitative analysis of the other viral polypeptides. Comparison of the HA and the F_1 viral polypeptides, which are eventually expressed on the cell's surface, indicated that the HA remained relatively unaffected or decreased slightly, less than 1.3-fold, during the first 6 hrs of antibody induced modulation, in contrast to the F_1 polypeptide which diminished 4-fold (Fig. 7). The amount of internal P protein labeled with ^{35}S decreased 8.6-fold during modulation when compared to the amount of ^{35}S-P protein in nonmodulated cells. A significant decrease in the M protein in the cytosol preparations harvested from modulated cells, as compared to the reciprocal preparations from nonmodulated cells, was also noted.

To determine the specificity of the antibody induced loss of F_1 and P viral polypeptides, we cultured HeLa cells infected with measles virus with rabbit serum containing antibodies directed against HeLa cell surface antigen. An additional aliquot of virus infected cells was cultured with rabbit serum containing antibody against measles virus (antibody against all the measles virus polypeptides). This treatment reduced the F_1 and P viral polypeptides by over 50% compared to the levels of F_1 and P polypeptides found in infected HeLa cells cultured with normal rabbit serum. For these experiments we determined that ^{125}I labeled IgG rabbit antibody to the HeLa cell surface bound as many molecules per infected cell (4×10^6) as did ^{125}I IgG rabbit antibodies to measles virus (27). Despite the binding of large numbers of nonviral antibodies to the surfaces of virus infected cells, none of the measles virus polypeptides (HA, P, NC, F_1 or M) decreased in quantity. In contrast, binding of rabbit antibodies to measles virus antigens on the cell surface caused significant loss of F_1 and P polypeptides. Hence, simple perturbation of the membrane does not cause antibody induced modulation, rather the process shows unique specificity.

^{32}P-Labeled Measles Virus Polypeptides Expressed in Cytosol Fractions from Cells Cultured with Human Antibodies to Measles Virus. We next confirmed the experimental results from labeling with ^{35}S-methionine by using a different

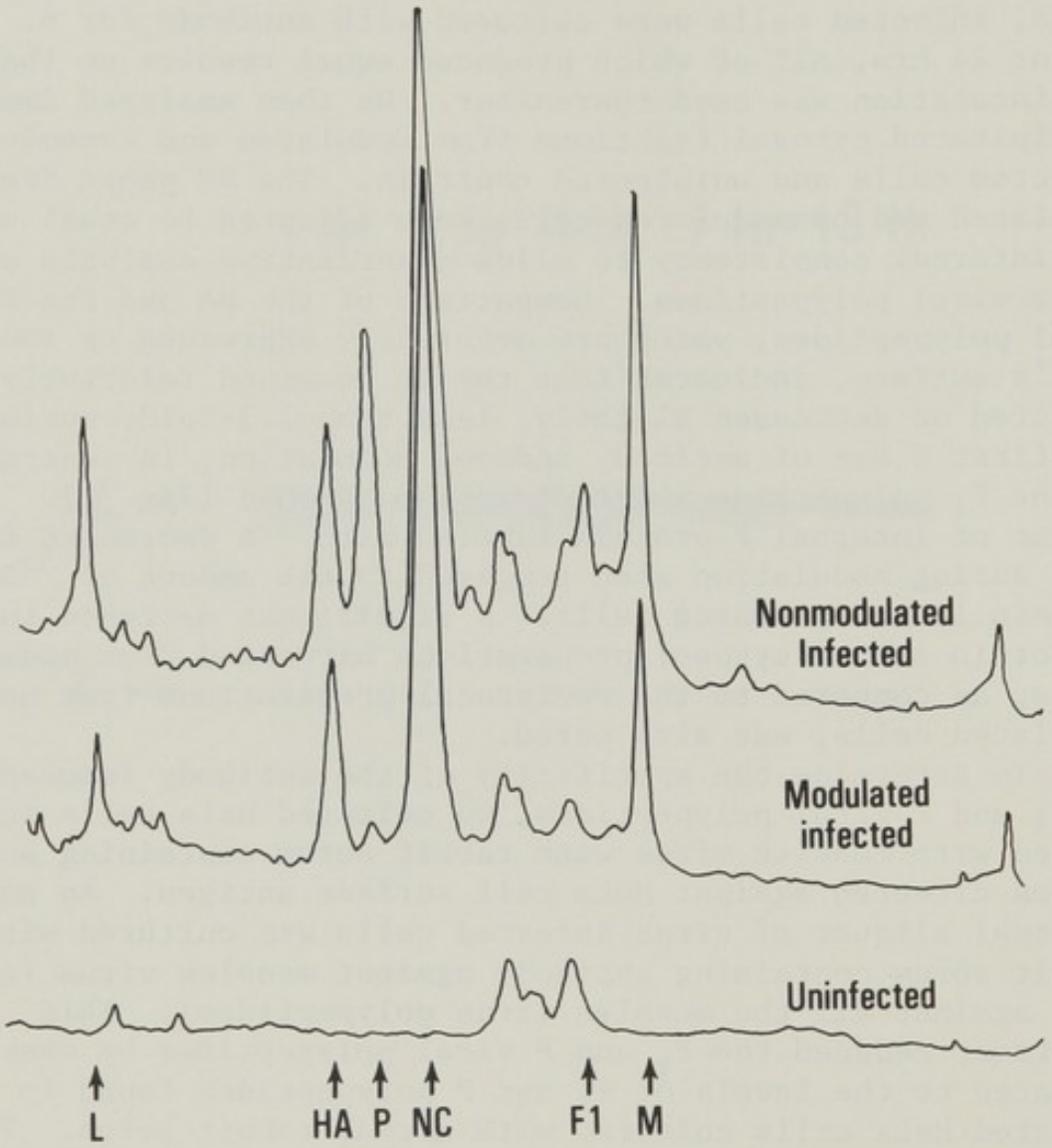


Fig. 7. Profile of measles virus polypeptides in HeLa cells infected with measles virus and cultured with (modulated) or without (nonmodulated) measles virus antibody. Representation of a densitometer scan of cells pulse labeled with ^{35}S for 2 hrs. Prior to labeling, cells were incubated with or without measles virus antibody for 6 hrs. P protein and F_1 are reduced in antibody treated cells. The peak found in uninfected cells migrating just before F_1 likely represents cellular actin.

internal label and determined which measles virus phosphoprotein decreased during antibody induced modulation. HeLa cells infected with measles virus were pulse-labeled with ^{32}P and cultured in the presence or absence of antimeasles virus antibody. The NC peaks found in the cytosol fraction from infected cells were equated and the area under the P protein and M protein measured and expressed as a percent of the NC peak. As seen in Fig. 8, the P protein from nonmodulated cells represented 97% of the NC, whereas the P protein from antibody treated modulated cells was only 39%. Thus, cells cultured with measles virus specific antibody lose P protein containing ^{32}P residues. The M protein increased in phosphorylation from 17% to 72% in nonmodulated versus modulated cells, respectively. The decrease in ^{32}P in P protein and the increase in ^{32}P in M protein within cytosols of cells treated with antiviral antibody compared to those from cells not treated with antibody was consistently observed in three independent experiments.

DISCUSSION

In this paper we initially reviewed antibody induced modulation of cells infected with viruses and then documented two new observations concerning antibody induced modulation of measles virus antigens on infected cells. First, we clearly establish that some, but not all, measles virus polypeptides are altered in cells confronted with measles virus antibody. Second, we find that the alteration of measles virus polypeptides is specific in that it occurs only with antibodies directed against measles virus antigens and not with antibodies directed against nonviral antigens expressed on the surfaces of these infected cells.

Experiments were done to identify and trace the turnover rate of the HA and F_1 polypeptides expressed on the surfaces of measles virus infected cells. We used ^{125}I to label tyrosine residued accessible on the plasma membranes of infected HeLa cells. The turnover of HA and F_1 represents a biphasic response in the presence of antibody. There is an initial rapid loss of antigen followed by a slow release.

In addition, we characterized the phosphoproteins of this virus in infected cells. P is a phosphorylated protein whose incorporation of ^{32}P is significantly reduced during antibody modulation. As recorded in Fig. 8, in unmodulated cells, P, NC and M polypeptides all incorporated ^{32}P, indicating that these three structural proteins of measles virus are phosphorylated within infected cells. Comparatively, in modulated cells, only trace amounts of ^{32}P were incorporated into P polypeptides, yet ^{32}P incorporation into M protein was

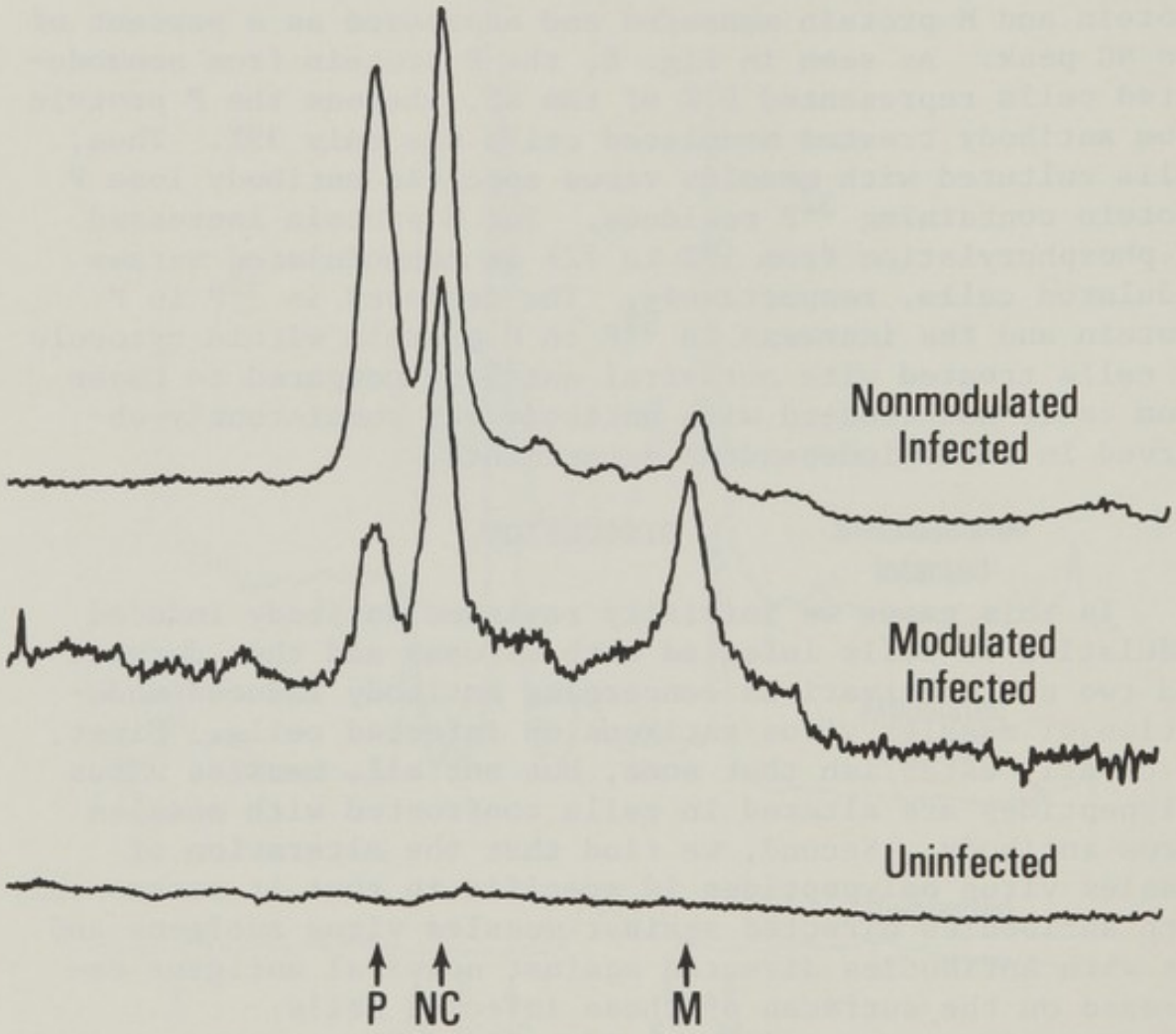


Fig. 8. Densitometer scan of the profile of ^{32}P labeled measles virus polypeptides in HeLa cells infected with measles virus and cultured with (modulated) or without (nonmodulated) measles virus antibody.

enhanced. Although the biological consequences of these findings in relation to P and M are unclear, changes in the phosphorylation of M protein might alter recognition of nucleocapsids and its alignment at the plasma membrane, thereby inhibiting viral maturation. Alterations in phosphorylation could change the migrational characteristics of M protein on PAGE and might partially explain the differences in migration of M protein reported by other investigators in experiments with SSPE material (29-31). Because P protein seems to form complexes with NC (32,33), possibly P is a rate limiting protein for the regulation of measles virus synthesis. Whether the events described reflect a transcriptional or translational block is unknown. Studies directed at the transcriptional complex are ongoing and should provide answers for these events.

The molecular events occurring during antibody induced antigenic modulation of measles virus polypeptides could account for several biological observations previously reported. Measles virus infection itself does not effectively shut off host protein synthesis (34). Rather, death from measles virus is in part caused by membrane damage as cells fuse and syncytia form. Cell fusion is a property of the F_1 polypeptide of measles virus (35,36). Hence, when antibody decreased the numbers of F_1 molecules on the surface of infected cells, the result is diminished fusion between neighboring cells so that fewer infected cells die. This sequence is the molecular correlate of the biological observation that infected cells cultured in the presence of antibody are normal in cytomorphology (10,37). If no F_1 molecules were present, no interaction could take place between them, antibody to F_1 and a complement source, and hence no immune lysis would occur with virus infected cells. HeLa cells infected with measles virus and cultured in the presence of antibody show a decrease of HA on the cell's surface and resist lysis mediated by antibody and complement (10). How aberrations in P and M might favor viral persistence is less clear, but undoubtedly relates to lessened manufacture and assembly of viral polypeptides inside the cell and a subsequent decrease in viral budding.

In patients with SSPE, both cytotoxic antibodies and sensitized lymphocytes directed against measles virus antigens are found in the circulation. Biopsied cells from these patients express few or no viral antigens at the surface but contain abnormally large numbers of nucleocapsids arranged at random in the cytoplasm instead of their usual linear conformation beneath the plasma membrane. Towards understanding these events, we now demonstrated that antibodies acting on the plasma membrane of an infected cell specifically

alter viral antigens expressed on the cell's surface or within its cytoplasm. Such an alteration favors the survival of virus infected cells by reducing the number of F_1 molecules on the cell's surface, thereby allowing this cell to escape fusion and death. Further, the decreased expression of antigen enables such infected cells to escape host immune surveillance mechanisms, while the continued replication of virus encourages generation of mutants which themselves favor virus persistence (38). The number of antibody molecules needed to modulate virus antigens off an infected cell is less than that required to participate in immune lysis of measles virus infected cells (24,25). Moreover, antibody induced alteration of viral polypeptides inside the cell and changes in P or M viral polypeptides might account for the accumulation of nucleocapsids inside the cell and the failure of nucleocapsids to align under the plasma membrane. There is evidence in several systems (reviewed in 39) that antigenic modulation occurs in vivo. Prolonged measles virus infection is best induced in monkeys and hamsters when the host is immune or passively receives antibodies before or along with the inoculation of virus (40,41). Hence, in addition to protecting a host from virus infections, antibody can aid in the initiation of viral persistence. Ultimately, the molecular basis underlying acute and chronic antigenic modulation should provide a means of understanding both virus persistence and those disorders in which virus coexist with a vigorous host immune response.

In conclusion, the observations with a variety of viruses (AKR virus, Friend leukemia virus, mammary tumor virus, measles virus) and the cells they infect suggest that these observations on antibody induced modulation may play a role in other viral infections and herald important membrane phenomena. For instance, the persistent infections of man associated with herpes viruses (simplex, cytomegalo) and rubella virus occurs in the presence of a vigorous antibody response. Further membrane receptor diseases like some instances of diabetes, myasthenia gravis and thyroid illness also occur in the presence of antibody to the insulin, acetylcholine and thyroid hormone receptors, respectively. In all these instances, both viral and receptor diseases, antibody binds to unique antigens on the cell surface and clustering, aggregation or shedding occurs. Thus, our observations with measles virus antigens, infected cells, and antibodies to measles virus may well shed light on a variety of poorly understood but important observations associated with human diseases.

ACKNOWLEDGMENTS

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WORKSHOP SUMMARY: HERPES AND POX VIRUS

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GENOME STRUCTURE: The molecular organization of herpesvirus genomes was summarized by George Bornkamm. To date, herpesvirus genomes can be divided into four classes based upon their molecular organization. In one class, exemplified by herpes simplex virus (HSV) and cytomegalovirus (CMV), two unique DNA sequences, one long and one short, are bounded by terminal, inverted repeat sequences. Short direct repeats are found at the termini of molecules and at the joint between the long and short regions. This unusual structure permits the unique regions to invert with respect to one another giving rise to four types of molecules within the virus population. A second class of genome structure is exemplified by pseudorabies and equine abortion viruses. The DNAs of these viruses also consist of long and short unique sequences. However, only the short unique sequence is bounded by terminal, inverted repeats. Such a repeat is present only at one end of the long sequence. Thus, in contrast to HSV and CMV, only the short unique region of PRV and EAV can invert. In Epstein-Barr virus (EBV), which exhibits a third kind of genome structure, there is no evidence for such inverted repeats in the genome. EBV contains from 4 to 12 internal tandem repeats which are flanked by a long and a short unique region. At the termini of the molecule are direct terminal repeats. The fourth class of herpesvirus genome is exemplified by herpesvirus saimiri (HVS) and herpesvirus ateles (HVA). These viruses have a single, long unique region flanked by direct repeats which are present in multiple copies at both ends of the molecule.

Like the herpesviruses, vaccinia virus possesses terminal tandem repeats at both ends of the genome. In contrast to the herpes and pox viruses, the genome of bacculovirus, an insect virus, is circular with no evidence for direct or inverted repeats.

HERPES SIMPLEX VIRUS GENETICS: A brief description of the physical and genetic maps of HSV-1 and HSV-2 was presented by Priscilla Schaffer. The mapping of viral transcripts, polypeptides, functional markers (including viral thymidine kinase. DNA polymerase, phosphonoacetic acid and acycloguanosine resistance and four syncytial loci) and temperature-sensitive mutants was described. This information is available in

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Genetic Maps, Vol. 1, 1980 from Stephen J. O'Brien, Laboratory of Viral Carcinogenesis, NIH.

The order of markers in the L region of the genome on the genetic and physical maps is roughly colinear. Moreover, comparison of the order of mutants on the genetic and physical maps suggests that all four forms of the genome are infectious and participate in the recombination process.

Two problems which have served to confuse the interpretation of genetic and phenotypic data were mentioned. These include a) inconsistencies in polypeptide nomenclature and b) the use of nonisogenic materials in physical mapping and phenotypic studies of ts mutants. In the latter case, the use of multiple virus strains which exhibit differing restriction enzyme cleavage patterns, possess significant deletions, and/or carry spontaneous mutations, can lead to mismapping and erroneous phenotypic data.

THE PROBLEM OF INVERSION IN HERPESVIRUS GENOMES: The possible mechanisms by which inversions may occur in the genome of HSV was discussed by Michael Wagner. The simplest mechanism (originally proposed by Sheldrick and Berthelot) is based upon the presence of inverted internal and terminal repeats and involves homologous intra- or intermolecular recombination. In contrast, Bernard Roizman and his colleagues proposed a copying mechanism whereby the terminal repeated sequences were copied from sequences at the joint. This mechanism was based on observations with HSV-1 x HSV-2 recombinants in which substituted heterologous repeats at the ends of S were found, after replication, at the opposite end of S, implying that the identity of the repeats bounding S is obligatory.

Neil Wilkie has argued that one need not invoke a copying mechanism in order to obtain identity of the terminal repeats of L and S. In studies of intertypic recombinants in which L could not invert, whereas S could invert freely. Wilkie noted that 80% of the recombinant molecules were "frozen" in this configuration and 20% had inverted. He therefore proposed that inversion occurred through segment inversion by a recombination mechanism and that those molecules which were able to invert have a selective growth advantage. Such a recombination mechanism would obviate the need for copying. In order to explain the high frequency of inversion, Wilkie further proposed that inversion occurs by a site-specific or specialized mechanism involving the "a" sequences in the joint region and at molecular termini. Thus, current thinking on the problem of inversion includes not only the mechanism responsible for inversion (i.e., a copying mechanism or a recombinational mechanism) but also whether inversion is a site-specific or generalized phenomenon.

With regard to the significance of inversion, the availability of recombinants which are "frozen" in one segment means that inversion is not an essential replicative function. Do these studies tell us anything about which of the molecular arrangements of the genome can replicate? Unfortunately, the inability to separate a single isomer free of all others precludes our ability to assess the ability of individual molecules to replicate. Genetic analysis of HSV has also failed to determine which isomers are active in that, to date, the genetic map does not reflect the expected order of markers on any one genomic isomer.

REGULATION OF TRANSCRIPTION AND TRANSLATION: Bob Millette described the kinetics of transcription and translation of immediate early, delayed early and late virus genes as first outlined by Honess and Roizman (1973-74). Immediate early transcripts hybridize to 10-12% to viral DNA, delayed early transcripts to 30% of the genome, and late transcripts to 40-48% of the genome. In calculating the quantities of transcripts present in infected cells, one must also consider the half-life of the mRNA species in question. Hans Wolff, for example, has shown that late mRNAs have short half-lives. Importantly, although the onset of DNA synthesis roughly separates early protein synthesis from late protein synthesis, two classes of late proteins exist: those which have an absolute requirement for DNA synthesis (only 2 or 3 of these proteins have been identified) and those which do not (most late proteins).

HSV-SPECIFIC THYMIDINE KINASE (TK): Current knowledge of viral TKs was summarized by Bill Summers. Viral TKs are specified by HSV-1 and -2, equine herpesvirus, murine but not human CMV, pseudorabies virus and vaccinia. In the case of herpesvirus TK, Wildy and Tenser demonstrated independently that virus virulence is a function of the presence or absence of viral TK. In cell culture, however, no differences in the ability of the virus to replicate have been noted.

Problems currently being investigated include the biochemical nature of the enzyme and the physical and genetic structure of the TK locus. In the case of HSV-1, the active enzyme has been purified and is a dimer of a 42K protein. The substrate specificity of HSV TK (most deoxypyrimidines) has enabled the biochemical distinction to be made between viral and cellular TKs. Don Coen's work with TK and acycloguanosine suggests that the enzyme possesses purine kinase activity as well.

The gene has been mapped physically by three-factor crosses by Jim Smiley using chain termination mutations in TK and the PAA^r marker. Reyes and Hayward have transferred TK activity with the fragment generated by Eco RI cleavage of the

3.5 Kb Bam HI fragment. Ken Kramer and Neil Wilkie have translated the TK polypeptide from gradient purified mRNAs. Mike Wagner's sequence studies indicate that the start of the TK message is between the Eco RI and Bgl II sites in the left-hand half of the 3.5 Kb Bam HI fragment and that the termination is near the Sma I site to the right.

HSV GLYCOPROTEINS: The properties, map positions and known functions of the five viral glycoproteins were described by Sheila Little. The molecular weights of glycoproteins gA, gB and gC range from 118,000 to 128,000, gD is approximately 55,000 and gE, 85,000. gA and gB map from 0.30-0.41 on the physical map of HSV-1, gC from 0.55-0.65 and gD from 0.90-0.95; the map location of gE is not known. With the exception of gC, the map locations of these glycoproteins are the same on the HSV-2 genome; here, however, gC maps from 0.65-0.70. gB provides an essential replicative function at the level of penetration of the virion into the cell and gE exhibits Fc binding activity. The functions of gA, gC and gD are not known; however, gC is not essential for replication since viable variants lacking gC have been described.

The identification of at least four syncytial (or syn) loci in the HSV system argues against the use of the syncytial marker in genetic studies unless the investigator is sure of the specific identity of the syn locus (or loci) with which he is working.

Two new techniques have been exploited to produce useful new information. Utilizing monoclonal antibodies specific for antigenic determinants of individual HSV-1 and HSV-2 glycoproteins, Lenor Pereira has identified a type-specific determinant on what was originally thought to be a type-common glycoprotein, glycoprotein gD.

Using glycoprotein-specific antisera in the immune cytolysis test, Bette Pancake has developed a procedure for selecting mutants which are defective in the expression of individual viral glycoproteins.

VIRAL DNA IN TRANSFORMED CELLS: Harold zur Hausen pointed out problems associated with nonspecific binding of viral DNA probes in in situ hybridization studies.

Workshop Summary: Adenovirus/SV40/Polyoma I.

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The workshop focused upon type 5 adenovirus, polyoma and SV40 mutants and early proteins involved in cellular transformation. A listing of these virus coded proteins, mutants and assigned functions is presented in table 1. Several general points can be derived from a comparison of these data. First, some of these viral gene products (Py-large T antigen, Ad5ts36, Ad5ts125) may have a dramatic effect upon the frequency of viral transformation, but may not be required for a transformation event. The Polyoma tsA and Ad5ts36 mutants do not transform cells in culture under the nonpermissive conditions (Fried, 1965; Williams, Young and Austin, 1974) yet the minimal genomic DNA restriction enzyme fragments required to obtain transformation can eliminate these gene products and still transform cells in culture at a low frequency (Graham et al., 1974) or produce tumors in animals (Israel et al., 1979). The Ad5ts125 mutant transforms cells at higher frequencies than wild type virus but is similarly not required for a transformation event (Ginsberg et al., 1974; Graham et al., 1974). Thus several viral gene products can have an effect upon the frequency of transformation but are not absolutely required for the event. Indeed in at least one case the presence of an intact viral genome and Py-large T antigen results in autonomous (not integrated) replicating copies of viral DNA (Basilico et al., 1979) and spontaneous virus production. These data indicate that free viral DNA may be produced from an integrated copy via a replication, rather than excision, mechanism (requires T-antigen) as first postulated by Botchan et al. (1979). Thus to produce a transformed cell line that is not producing virus may require either loss or alteration of the T antigen genetic information (Basilico et al., 1979). Alternatively the viral DNA could have an altered origin of replication and a functional large T-antigen (Y. Gluzman, et al., 1979).

A second set of viral gene products, Py-middle and small t antigen, SV40 large and small t antigens and Ad5 region 1A and B, appear to be required to maintain transformation at least in some circumstances (Benjamin et al., 1980; Tegtmeyer, 1975, Sleigh et al., 1978; Graham et al., 1974). One of the common features of these proteins, reviewed in table 1, is the large number of viral gene products involved in DNA replication. It does not appear likely that normal viral DNA replication is required for transformation, since SV40 DNA lacking the origin of DNA replication can still transform cells in

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Table 1
Adenovirus, Polyoma and SV40 Early Proteins
Involved in Transformation

Virus	Proteins(MW)	Mutants	Function-Biochemical Activities
Polyoma	large T-antigen(88K)	tsA	DNA Replication, Establish Transformation, Nuclear Location
	middle T-antigen(56K)	hr-t	Protein Kinase Assoc. (Mid-T) Membrane Assoc. (Mid-T) Transformation
	small t-antigen(22K)	hr-t	lytic cycle-assembly of virions
SV40	large T-antigen(94K)	tsA	DNA Replication, modulate transcripts, Transformation, Nuclear location, Membrane Assoc.Antigen ATPase, Protein Kinase Assoc.
	small t-antigen(17K)	del	Transformation, cytoplasmic location
Adenovirus (Type 5)	region 1A (25K, 53K, 47K, 41K, 35K)	hr-t, hr-del	Transcription and/or RNA processing,Transformation, nuclear location
	region 1B (58K, 15K)	hr-t hr-del	DNA Replication, modulates regions 2, 3, Transformation, Nuclear location
	region 5 (?)	ts 36, 149	DNA Replication, decreased transformation
	region 2 (72K)	ts125	DNA Replication, DNA-Binding Protein,modulates early proteins Nuclear location, increased Transformation Frequency

culture (Y. Gluzman et al., 1979). This brings up the possibility that an abnormal form of viral DNA replication (not utilizing the viral DNA origin) could generate an essential intermediate in the transformation process or these viral gene products act upon another template (cellular DNA in the transformed cell) (Levine and Burger, 1972). Many of these viral gene products are also involved in transcriptional regulation (SV40 large T, Polyoma large T, Ad5ts125, Ad5 region 1B) (Tegtmeyer, 1974; Alwine et al., 1977; Carter and Blanton, 1978, Ross et al., 1980) or RNA processing (Ad5 region 1A) (Jones and Shenk, 1979) and this adds a second dimension to the level of viral control of transformation. Some of these viral proteins have a nuclear location in the cell (Py-large T, SV40 large T, Ad5 regions 1A, B, 2) some a cytoplasmic location (Py and SV40 small t) and others are plasma membrane associated (Py-middle T, SV40 large T antigenic determinants) (Ito et al., 1977; Chang et al., 1979). With this kind of multiplicity of function and cellular location, it is not surprising that the transformed phenotype would be composed of many different properties.

The study of the biochemical activities of these tumor antigens has begun. Polyoma middle T antigen has a protein kinase activity associated with it while SV40 large T antigen has an ATPase and possibly a protein kinase activity in purified preparations of this protein (Griffin et al., 1979; Tjian and Robbins, 1979; Smith et al., 1979; Eckhart et al., 1979; Schaffhausen and Benjamin, 1979). The role of these enzymatic activities in transformation remains to be elucidated. A study of the interactions of these viral tumor antigens with cellular proteins is also under way. The SV40 large T antigen is complexed with a 54,000 MW cellular protein in virus infected and transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). The SV40 small t antigen appears to interact with two cellular proteins (56,000 MW and 36,000 MW) in the cytoplasm of virus infected cells (Rundell, 1980). These types of experimental approaches should begin to disect the role of viral tumor antigens in altering a normal cell to the transformed phenotype.

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WORKSHOP #3 SUMMARY: ADENOVIRUSES/SV40/POLYOMA II

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Study of the molecular biology of small and intermediate size DNA tumor viruses has contributed to our understanding of the biochemistry of gene expression in mammalian cells. Perhaps the most novel stage of the process of transmission of information from DNA to protein in mammalian cells is the synthesis and modification of mRNAs. The complexity of this stage reflects the fact that in eucaryotic cells, DNA is partitioned into the nucleus isolated from the cytoplasmic protein synthesis machinery. The structure of mRNAs expressed from genomes of DNA tumor viruses is also complex as their DNAs are transcribed in the nuclei of host cells.

This workshop will cover some of the topics presented in the poster session this afternoon. Most of the experiments discussed were concerned either with the mapping and sequence content of viral mRNAs or regulation of expression of viral mRNAs. At the end, it will become clear that this type of information can be used to express novel proteins in mammalian cells utilizing SV40 vectors.

SV40-associated small RNA (SAS-RNA)

Two mRNAs are thought to be transcribed from the early region of SV40. The sequence content of these two mRNAs is shown in Figure 1; the largest mRNA encodes the 17,000 dalton small T antigen while the other mRNA translates into the 94,000 large T antigen. The latter protein is thought to be intimately involved in transformation of cells. Both mRNAs are expressed before the amplification of viral DNA and continue to be synthesized during the late stage of infection. Until recently, these were the only RNAs from the early region thought to accumulate in cells during lytic infection. However, last year, Mark and Berg (1) reported evidence for accumulation of a small RNA complementary to the early mRNA of SV40 near position 21 map units. Dr. Alwine has made significant progress in defining this entity (2,3). First by a combination of hybridization and nuclease techniques, this small RNA was precisely positioned as being complementary to the L strand of the virus between positions 2760 and 2830.

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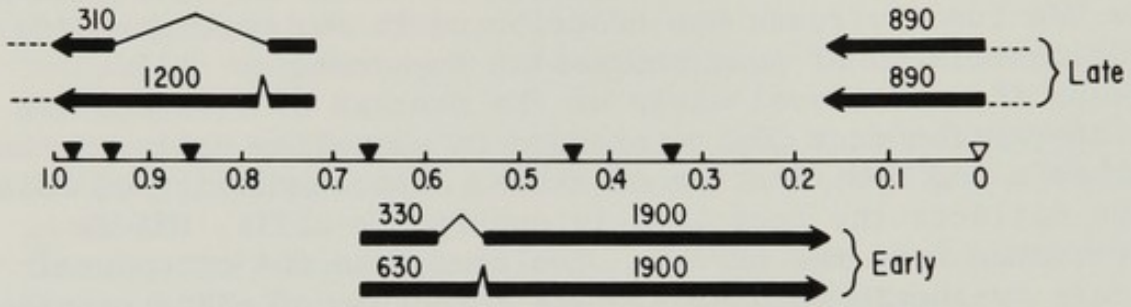

FIGURE 1. *Transcription map at SV40. Heavy lines represent the nucleotide sequences included in the early and late mRNAs and indicate the direction of transcription. Numbers above the heavy lines represent length in nucleotides. Numbers below the narrow lines indicate SV40 genome map units. The cleavage sites of the Eco R1 and Hind III endonuclease are indicated as* ▼ *and* ▽, *respectively.*

Thus, the RNA is approximately 70 nucleotides long. Synthesis of this RNA only appeared during the late stage of virus infection and was dependent on the A gene product. During the late stage, the SAS-RNA accumulated to levels comparable to late mRNAs and since both are transcribed from the L strand, it is likely that synthesis of this short RNA is dependent on initiation at late promoters. To confirm the viral origin of the small RNA and determine its function, Dr. Alwine has examined several deletion mutants of SV40. All mutants with

deletions of SV40 sequences between 2760-2830 failed to stimulate synthesis of the small RNA. Many of these mutants are non-defective for growth in monkey cells; hence, this small RNA is not required for virus replication.

At the moment, the transformation potential of SV40 mutants that fail to synthesize the SAS-RNA has not been tested. This would be the most direct test for any role of SAS-RNA in viral transformation. In this vein, Dr. Alwine has attempted to detect the SAS-RNA in SV40 transformed cells without success. Thus, the existence of the small RNA does not provide an obvious explanation for the differences between the suspected transforming proteins of SV40 and polyoma virus (4). The transforming potential of polyoma is thought to be encoded by a middle-size T antigen of 55,000 daltons. SV40 does not code for an equivalent protein and many suspect that a large T antigen of SV40 possesses the transforming function of polyoma's middle T antigen. Investigators have speculated that there might be more than one type of 94,000 dalton T antigen synthesized in SV40 transformed cells. One of these could have functions equivalent to those of the polyoma middle-size antigen. However, definitive evidence for heterogeneity in the amino acid sequence of SV40 large T antigen polypeptides has not been obtained. Whether expression of SAS-RNA could affect synthesis of different forms of early polypeptides is unclear.

Effect of deletions on late mRNA structure

Late in the lytic cycle of SV40, three viral polypeptides are translated from two-size classes of mRNAs: VP1 translates from a 16S RNA while 19S RNA yields both VP2 and VP3. All 19S mRNAs can have more than one set of leader sequences. Shown in Figure 2 is a schematic of principal splice sites utilized for the synthesis of 19S RNA in cells infected with w.t. SV40 (5). Note that all spliced 19S RNAs have a leader joined to the acceptor site at nucleotide 476; any one of the three sequences, positions 211, 291, or 444, can be joined to this site. In addition, a small amount of unspliced 19S RNA is also present in the cytoplasm of w.t.-infected cells. A number of viable mutants of SV40 have been isolated with deletions in sequence complementary to these leaders. Piatak et al. (5) have shown that some of these deletions shift the distribution of sites found as 5' termini of late mRNAs, the frequency of splicing at various sites, and the amount of unspliced RNA found in the cytoplasm (see Fig. 2).

For example, dl 1613, a mutant which is missing sequences to within a few nucleotides of position 444, still stimulate the synthesis of all forms of 19S RNA. In particular, the

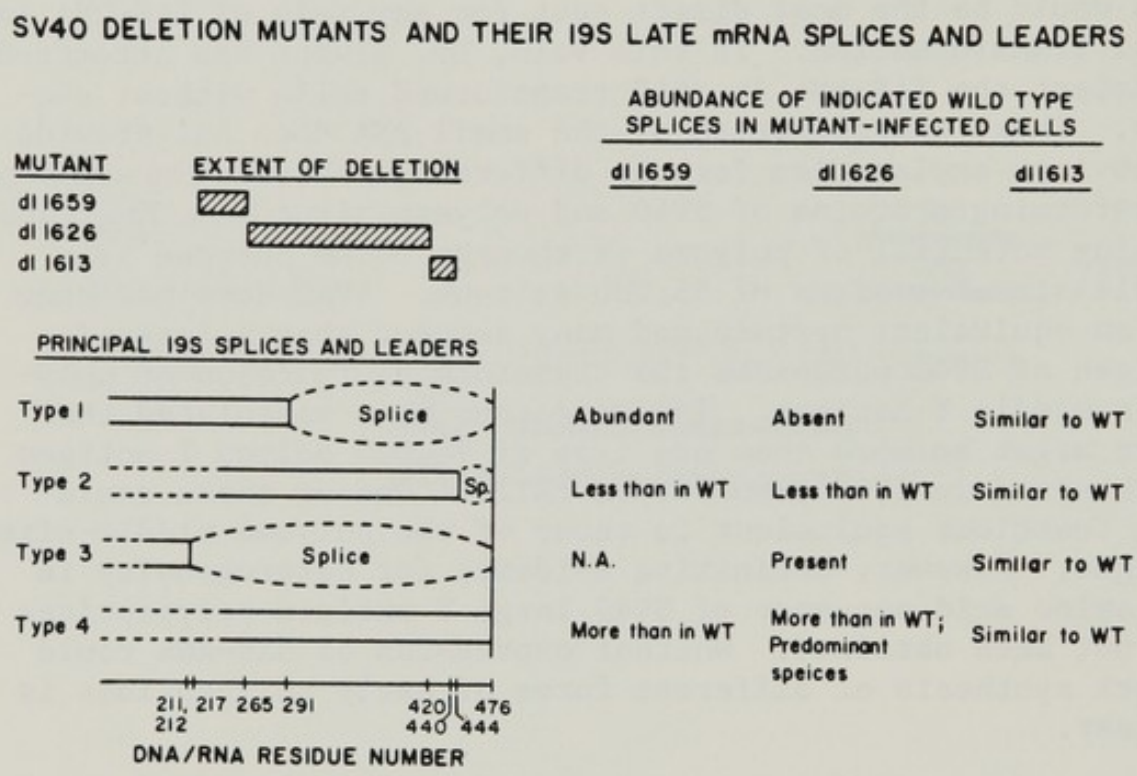

FIGURE 2. *Effects of deletions on structure of 19S RNA. Type 4 is a cytoplasmic RNA without a splice in the 200-476 region. The structure of RNAs from mutant-infected cells was determined by reverse transcription of mRNA and sequencing of products.*

splice joining positions 444 and 476 was consummated. The two other deletions, dl 1659 and dl 1626, affected the distribution of mRNAs in a more drastic manner. Both mutants have lost sequences spanning one splice boundary and obviously cannot stimulate the synthesis of that spliced mRNA. In addition, both deletion mutants yielded increased amounts of colinear 19S cytoplasmic RNA. In fact, cytoplasmic RNA without a splice in the 200-476 region was the most prominent RNA in cells infected with SV40 dl 1626. Whether this means that unspliced 19S RNA can be transported to the cytoplasm of

cells or that these 19S RNAs have undergone splicing in another segment of the RNA, i.e. downstream from 476, is not clear. However, if the former is the case, it would suggest that the conclusion drawn from expression of other mutants of SV40, i.e. that an RNA must be processed by splicing to appear in the cytoplasm, may not be a general rule. The obvious problem of how to control extraction procedures to avoid cross contamination of nuclear and cytoplasmic fractions always arises in these kinds of studies. Dr. L. Villarreal described in the poster session experiments which established conditions that almost exclusively partitioned spliced and unspliced late RNAs from w.t. SV40-infected cells into cytoplasmic and nuclear fractions, respectively.

Expression of rat insulin gene from SV40 vector

Perhaps the most direct use of our understanding of SV40 molecular biology has been the use of this virus as a vector for expression of genes in mammalian cells. This has been a focus of Paul Berg's laboratory at Stanford University and he summarized these studies in a keynote address (6). To date, the genes that have been expressed from an SV40 vector are: cellular and cDNA globin genes, drosophila histone genes, cDNA copy of the mouse gene for dihydrofolate reductase, and E. coli guanine phosphoribosyl transferase. Mammalian cells can be cultured under conditions that select for expression of the latter two genes. For example, expression of guanine phosphoribosyl transferase can be selected for by requiring growth in the presence of Xanthine while inhibiting endogenous synthesis (7). Hopefully, expression of these genes will permit the development of SV40-derived vectors that autonomously replicate in a plasmid state in mammalian cells.

There are a number of potentially useful applications of SV40 cloning vectors for synthesis of proteins in mammalian cells. These possibilities were vividly demonstrated by the reports of Gruss and Khoury (8) and Nguyen-Huu (9) that a cellular gene for rat preproinsulin I cloned in the late region of SV40 was expressed after infection of permissive cells. In both cases, the 1.5 kb rat insulin I gene (10,11) was found to be expressed as several forms of spliced mRNAs. It was unclear whether these mRNAs were initiated in SV40 sequences or in the inserted cellular sequences. Both laboratories agree that the mRNAs were polyadenylated at sequences in the cellular insert. Perhaps, most interesting is the observation that cells, infected with the SV40 recombinants, secreted proinsulin

into the media. One could infer from these results that pre-proinsulin was synthesized in the infected cell and matured by cleavage during transport to the cell's exterior. This process seemed to be quite efficient as ^{35}S-labeled proinsulin was easily detected in the media. Thus, secreted hormones can readily be expressed in mammalian cells by the use of SV40 vectors.

Adenovirus regulation

Regulation of the lytic cycle of human adenovirus is complex and offers the possibility of study of a number of examples of one viral gene product controlling the synthesis of other viral products. One example of this type of regulation was the recognition that a viral gene product from the E1A region enhanced transcription of four other early regions of the genome. This class of gene products has been referred to as a pre-early class and was thought to be the first gene products expressed after infection of cells (12, 13). Mathews et al. (14) have examined the synthesis of viral mRNAs in cells where protein synthesis was inhibited by anisomycin. This inhibitor can be used in various concentrations up to 500 μM. At this concentration, the inhibitor will reduce protein synthesis to less than 1% of the untreated control. When infected cells were treated with levels of anisomycin which inhibit protein synthesis by only 90% or so, the level of early viral mRNAs was increased by 5-20 fold. This effect has been characterized previously in studies of cycloheximide inhibition and seems to be due to both inhibition of synthesis of a repressor type activity and increases in half-lives of RNA species (15,16). When higher levels of anisomycin were used, synthesis of mRNAs from regions of the genome dependent on expression of a pre-early protein product was inhibited. Most novel is the finding that inhibition of protein synthesis by very high levels of anisomycin inhibited the synthesis of two pre-early mRNAs from the E1A region, but still permitted synthesis of other viral mRNAs. At levels of anisomycin of 100 μM, a concentration which inhibited synthesis of mRNA from early region E1A some 20 fold, RNAs for polypeptides of molecular weights 13.5K and 52,55K from regions 17-21.5 map units and 29-41.0 map units, respectively, continue to be expressed. Little is known about the structure for the mRNA for the 13.5K protein. However, the mRNA for the 52,55K polypeptide is initiated at the late promoter and has the standard three leader segments. The fact that an mRNA for the 52,55K polypeptide is processed from the late transcription unit in the absence of other late mRNAs suggests regulation of expression at post-initiation stages in the late transcriptional

unit. In addition, the synthesis of RNAs from the 16-39 map unit region of the genome in the absence of mRNAs from other early regions could be interpreted to mean that expression of these polypeptides is required for the synthesis of other mRNAs. A major problem with this hypothesis is the synthesis of mRNAs from E1A and E1B in transformed cells which do not contain sequences from 11.4 to 39 map units. An alternative possibility would be that nearly complete inhibition of protein synthesis interferes with the normal signals of viral regulation and induces aberrant synthesis of mRNAs. Be that as it may, the identification of new polypeptides encoded in the 16-34 map unit region of the genome is important. During the course of these protein inhibition studies, Mathews et al. (14) have mapped four additional polypeptides to this region: polypeptides of 11.5K, 17K, 87K, and 13.6K molecular weights. The functions of these gene products are unknown; however, Ad5 ts36 and Ad5 ts149 both map in this region.

Functions of 72K polypeptide

Although adenovirus codes for some 35 or so polypeptides, one early gene product seems to function in DNA replication, transcription control, and RNA processing; this is the 72K polypeptide from early region II. At the non-permissive temperature, cells infected with mutants with a thermolabile 72K protein, Ad5 ts125 or Ad5 ts107, fail to replicate viral DNA and fail to suppress transcription of early RNAs (17). More recently, it was recognized that mutants of adenovirus which replicate to high titers in monkey cells have alterations in the region of the genome encoding the 72K polypeptide (18,19). Normally, infection of monkey cells by Ad2 results in a deficiency of mRNA for fiber (IV) while the host-range mutants which replicate permissively in monkey cells stimulate a w.t. level of fiber mRNAs. Thus the 72K polypeptide may be involved in control of post-transcriptional processing of late RNAs.

Another set of data suggesting that the 72K polypeptide is involved in post-transcriptional events in mRNA synthesis comes from the studies of Ad5 enhancement of AAV reproduction. AAV (adeno-associated virus) depends on coinfection by adenovirus to replicate. Adenovirus 5 mutations in early regions 1A and 1B, complementation groups I and II, respectively, of host-range deletion mutants (13), fail to complement AAV DNA replication after infection of non-permissive KB cells (20). Thus gene products from E1B or E1B and E1A are required for complementation of AAV. The situation is different after co-infection with Ad5 ts125. In this case, AAV DNA replication

occurs and some AAV mRNAs are produced; however, no AAV polypeptides are synthesized (20). The AAV mRNAs made have an altered spectrum of spliced structure suggesting the defect in AAV polypeptide synthesis might be post-transcriptional. How a single strand DNA binding protein can have this multitude of functions is not at all clear. However, given the availability of purified 72K protein and the previously developed genetics of this polypeptide, these riddles should not go unsolved for long.

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WORKSHOP ON PICORNAVIRUSES/TOGAVIRUSES/HEPATITIS/ CORONAVIRUSES

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A number of different viruses were discussed in this workshop. It was clear that cloning techniques (Hepatitis) and sequencing of RNA (polio) were having an enormous impact on genetic studies with previously refractory agents. The following points were among the highlights.

1. Hepatitis B virus is now being cloned and sequenced in a number of laboratories. In addition, various cultured liver cells are becoming available for study and may soon allow better studies on synthesis and expression of hepatitis B virus antigens.

2. Formal genetic studies of Togaviruses containing positive sense single strand RNA have been somewhat limited due to lack of detectable recombination. Studies on structure and modifications of the glycoproteins are providing important insights into the precise patterns of assembly and insertion into the plasma membrane. For example, it appears that covalently attached palmitate molecules are clustered in hydrophobic, membrane associated regions of the glycoproteins. Studies on cells presently infected with sindbis and semliki forest virus suggest a role for DI particles in initiation of the persistent infection and also indicate that there is a high frequency of mutations during persistence. The relationship of the various types of mutations to the structure of glycoproteins (and other structural proteins) is an area of considerable interest. In other discussions , it was noted that attenuation of venezuela equine encephalitis virus (an alphavirus), occurs by

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mutation in one of the two surface glycoproteins.

3. A sequence analysis of the 3'end of poliovirus genome has revealed a large number of non-translated nucleotides. In addition, a sequence corresponding to the amino acid sequence of VpG has been detected. Other studies of the poliovirus genome have revealed evidence for three separate ribosomal binding sites on the poliovirus genome.

4. RNA species have been detected in cells infected with the infectious bronchitis coronaviruses. Tl Fingerprinting is providing specific details of the relationship of subgenomic RNA species to genome RNA. A single host gene controls resistance to MHV induced hepatitis. Different strains of JHV induce different types of nervous system pathology with varying degrees of demyelination.

WORKSHOP ON RHABDOVIRUSES AND PARAMYXOVIRUSES

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About 100 people convened for this workshop; among these 10% were actively engaged in discussion. The discussion was focused on only 2 aspects and much of the information came from the VSV system.

The first topic concerned genome organization and functional sequences. Jack Rose summarized what is currently known about VSV RNA sequences. At the beginning of each coding region there are 5 nucleotides in common followed by 2 differing ones and then another 3 in common (3' UUGUC--UAG 5'). At the end of each coding region, there are 4 nucleotides in common (3' AUAC 5') followed by 7 U's. In between the coding regions there are 2 untranscribed nucleotides (3' CA 5'). At the ends of the genome of VSV there are untranscribed controlling regions which account for approximately 50 nucleotides at each end. The functional significance of these specific terminal sequences is still relatively unknown. It is clear that considerable future work will relate what is known about these sequences and other sequences to interactions with specific controlling elements.

A discussion of whether the plus-strand, genome-sized RNA contained poly(A) at its 3' end remained unresolved. Although poly(A) fragments larger than 20 nucleotides long have not been isolated from plus-strand of DI RNAs, annealing studies with RNA from standard virions show that the plus strand anneals to poly(U). It is possible that this annealing is due to the 5 stretches of A7 which would be derived from the termination of each coding region.

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John J. Holland mentioned that variations in the genome of VSV are considerable and occur throughout the genome. With one persistently infected culture, variations at the 5' end were 15% compared to an overall 2% mutation rate in the remainder of the genome. Since mutations at the 5' end are postulated to affect the synthesis of the genome of VSV, as well as the genomes of DI particles, John surmised that such sequences were of prime importance during interference by DI particles. In relation to interference and terminal sequences, Alice Huang presented evidence for 2 different DI particles derived from the same parental type. They differ in their ability to compete with one another during genome replication and yet, by hybridization analysis, appear to have the same 3' and 5' end sequences. This appeared to mitigate the suggestion that the primary sequence at the termini determines interference.

In relation to genetic variability, Craig Pringle mentioned his results on the New Jersey serotype of VSV, in particular group D ts mutants, in which multiple mutations were reflected by differential mobility of several proteins even though the temperature-sensitive lesion mapped within only one complementation group. He cautioned against the gene assignment studies which were determined by migration differences between proteins.

Jack Keene, in an attempt to define the structural relationship between the RNA binding N protein and the genome, reported on the methylation of nucleocapsids with dimethylsulfate, indicating that the RNA within the nucleocapsid was reactable. Although this suggests that the bases are free in the nucleocapsid structure and that the phosphodiester backbone is protected by the RNA binding protein, no one was able to report convincing hybridization of messenger RNA to nucleocapsids.

The second half of the discussion was focused on mechanisms of transcription and replication. Alice Huang opened the discussion by presenting kinetic studies on each of the RNA species synthesized during VSV growth. By studying both accumulation and rate of synthesis, she was able to conclude that templates for replication were determined prior to 2 hr after infection and that the amount of these templates remained constant throughout the rest of the growth cycle. In addition, it was determined that the small RNA, found in cells

co-infected by standard virus and DI particles, was synthesized by transcriptive enzymes and had a rate of synthesis which increased during the growth cycle. Therefore, in order to study controls during replication, especially those factors involved in competition between DI and standard virus RNA synthesis, it appears necessary to examine infected cells rather early after infection.

In order to resolve the issue of whether transcription of individual messenger RNA species resulted from prior cleavage of a large precursor plus strand or was individually initiated, Amiya Banerjee presented evidence concerning the incorporation of ATP analogs during in vitro polymerase assays. Although these experiments indicate that the analog blocked initiation, he wished to interpret these results in relation to phosphorylation or dephosphorylation of putative controlling proteins. It was felt from recent experiments published on fish rhabdoviruses that initiation of individual messenger RNAs was indeed the mechanism of transcription. Since small RNA and leader RNA can no longer be considered abortive replication products, but rather authentic products of transcription, their function was questioned. Experiments designed to irradiate DI particles and reduce subsequent RNA synthesis, but allowing small RNA to be synthesized in cells, led to a lack of interference by these DI particles. These experiments are consistent with the extraordinary UV-sensitivity of the interference capability of DI particles and may indicate that small RNA interferes only in conjunction with a genome of DI particles which is capable of being replicated.

WORKSHOP SUMMARY: Segmented RNA Viruses, Dr. W. K. Joklik, Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Discussion focussed on the following topics:

1. The role of persistent infections as sources for the generation of virus variants. When viruses are passaged at low multiplicity, strong selective pressures are generated that prevent the emergence of variants. By contrast, persistently infected cells, in which the milieu in which the virus multiplies is not a major limiting factor, provide not only conditions in which many types of virus variants that arise can multiply at a substantial rate but also every opportunity for complementation; as a result variants persist that would be at a profound disadvantage under conditions of passage at low multiplicity. Several speakers reported the isolation of numerous types of mutants from persistently infected cells. These include deletion mutants and temperature-sensitive mutants, as well as mutants in numerous viral genes. Thus persistent viral infections provide unrivalled conditions for the spontaneous emergence of viral variants: all that is necessary for the isolation of specific variants is the application of selective pressures. However, it was also pointed out that whereas persistently infected cells provide a very favorable medium for the emergence and persistence of viral variants, they are not a completely neutral medium; for the nature of the cells does indeed influence the nature of the variants that emerge. The most interesting example along these lines is provided by insect cells in which the emergence of DI particles is often greatly suppressed. Examples of other similar systems were reported.

2. It was pointed out that whereas numerous viral variants emerge readily in persistently infected cells, some portions of the viral genome seem to be conserved much more strongly than others. There was considerable discussion concerning which portions of viral nucleic acids and proteins tended to be conserved during evolution. For example, not only are numerous examples of strong evolutionary conservation of sequences at the termini of viral nucleic acids known, but portions of the coding sequences of viral genomes also tend to be conserved. The best examples of this type are provided by antigenic determinants on virus-coded proteins. Thus the antigenic determinants on most proteins coded by the three reovirus serotypes tend to be highly conserved, and only a few

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proteins are "type-specific"; this is unexpected because the genetic relatedness between reovirus serotype 2 on the one hand and serotypes 1 and 3 on the other is so low that their coding sequences can share few if any similarities or homologies. Another example is provided by the measles-canine distemper-rinderpest group of viruses. Many of the proteins coded by these three viruses possess similar antigenic determinants in spite of possessing almost no amino acid sequence homology.

3. The phenomenon of extragenic suppression, best studied among the reoviruses, was discussed. It was pointed out that careful analysis of the gene suppression patterns should yield valuable information concerning the ability of the corresponding proteins to interact either physically or functionally.

4. The nature of the viral RNA polymerases was discussed. In most cases the RNA polymerases seem to be complex: almost always the enzymes appear to consist of several subunits, some of which may be host-coded. Careful study of viral variants should elucidate the nature of these RNA polymerases.

5. Discussion touched the subject of protein modification, including glycosylation, phosphorylation, methylation, polyadenylation and ADP-ribosylation (the latter two recently observed with respect to reovirus-coded proteins); and the function of the S1 gene of reovirus, which is responsible for eliciting the most active neutralizing antibodies, is responsible for reovirus type-specificity, is the reovirus-cell attachment protein and the hemagglutinin, and specifies virulence, tissue tropism and certain aspects of cytopathogenicity such as association with microtubules.

WORKSHOP SUMMARY: CELL GENES AND VIRAL TRANSFORMATION

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This gathering of the faithful discussed the oft-discussed problem of how RNA tumor viruses transform cells. The focus was on those viruses that carry out *in vitro* "transformation." Ignored till later discussion were those viruses that cause cancer in animals but do not change the growth properties of cells in culture. The only exception was the spleen focus forming component of the Friend murine leukemia virus complex (SFFV), which was included because it causes a rapid effect of erythroid differentiation even though it cannot cause transformation in culture.

First the roll was taken of all those systems in which a cellular gene is combined with a retrovirus skeleton to generate a transforming virus. The leading system is still Rous sarcoma virus where the *src* gene is the prototype of all transforming genes. Its origin from cellular genetic information is well established but the organization and function of the cell gene is still open to question. A number of newly characterized avian sarcoma viruses were discussed. Two such agents, Fujinami virus and PRCII virus, have recently been shown to contain transforming information that has no cross reaction with the Rous sarcoma virus *src* gene. Four other avian transforming agents also have been characterized: prototypes of these systems are avian erythroblastosis virus, MC29, avian myeloblastosis virus, and reticuloendotheliosis transforming virus. These seven avian transforming viruses all have, as far as is known, non-cross reactive cellular genetic information as a crucial component of their structure. Thus at least seven genes of the avian genome can participate as crucial genetic elements in the causation of tumors by viruses.

The roll was continued by turning to mammalian viruses. Non-cross reactive genes have been found in murine sarcoma virus, Abelson murine leukemia virus, and feline sarcoma virus. Recently it has been shown that in the Harvey and Kirsten strains of rat-derived sarcoma virus there is a segment of normal rat cell genetic information as well as a long stretch of sequence of a virus-like structure endogenous to rat cells. Thus four more systems have been identified in which normal cellular information plays a role in viral trans-

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formation. Other systems that have not yet been investigated include the simian sarcoma virus. It is not clear whether SSFV contains a crucial cellular component or is rather a virus-virus recombinant.

The mechanism by which cellular genes--when incorporated into viral genomes--are able to cause transformation is a topic of active investigation. Two different paradigms have been uncovered: a protein kinase that phosphorylates tyrosine residues in itself and in other proteins and a GTP binding protein. Protein kinase systems include the transforming proteins of Rous sarcoma virus, Fujinami sarcoma virus, Abelson murine leukemia virus and probably feline sarcoma virus. The GTP binding activity has only been found thus far in the Kirsten and Harvey strains of sarcoma virus where a surprisingly small protein, one of 21,000 daltons, appears to be responsible for transformation.

These transforming proteins are either made as fusion products with virus proteins or are made as independent proteins. In both Rous sarcoma virus and Kirsten/Harvey strains, the proteins that are made are exactly the same size as normal cellular proteins. In all of the other cases, the transforming cellular information is linked to the gag gene precursor and is synthesized as a readthrough product of the gag protein.

Although these cell-derived proteins are generally considered to be responsible for viral transformation, hard evidence for their involvement exists in only the Rous sarcoma virus system. There, temperature sensitive mutants have been mapped to the src gene and temperature sensitive protein kinase activity has been uncovered. Recently, transformation defective mutants of Abelson virus have been isolated and shown to lack protein kinase activity although they can act as acceptors in a trans-kinase reaction. Furthermore, mapping studies with fragments of the viral genome have located the murine sarcoma virus transforming activity in the cellular insert and similar evidence for Kirsten sarcoma virus has been gathered. Thus it seems increasingly evident that the putative transforming proteins are in fact responsible for changing the growth properties of infected cells.

One of the more remarkable aspects of the transforming proteins is their specificity of action. Although Rous sarcoma virus can transform a great variety of adherent cells, it generally causes rhabdomyosarcomas in animals and it has never caused transformation of hematopoietic cells. By contrast a variety of viruses of both avian and murine species do transform hematopoietic cells that they affect. Both SFFV and avian erythroblastosis virus are quite specific

for erythroid cells while Abelson virus is quite specific for early cells in the B lymphocyte pathway. There is no information about how the target specificity of these viruses relates to the type of transforming protein that they make.

A question that is widely debated but still unsettled is whether the transforming proteins act in a quantitative or qualitative fashion. One could imagine that simply as a consequence of being put under the control of a viral promotor, the enormously increased amount of gene product made from normal cellular genes would derange cellular growth properties. Such a quantitative model is favored by many workers. It is important to recognize, however, that in no case is it clear that the transforming protein is an unmodified version of a normal cellular protein. In those cases where the protein is linked to the gag gene product, it is evident that the protein is not truly normal and in all cases it is possible that small mutational events have crucially changed the properties of the protein.

There was general agreement that those transforming proteins that have been immunologically identified are localized in the plasma membrane of the cell. For most, there is no evidence that they penetrate to the outside surface of the plasma membrane but exact mapping of the topological relationship between the membrane and the protein has still to be performed. This localization suggests a number of possible ways by which the virus could transform cells. The old notion that transport of low molecular weight molecules might be involved gains support from this localization as do notions of cytoskeleton derangement, and hormone receptor functions. Which if any of these cellular activities are involved in viral transformation remains to be discovered.

At the end, the participants could not resist suggesting mechanisms by which non-defective viruses could cause cancer. Some thought that important genes might be hidden in the virus, possibly at the 3'-end. Others thought that the promotor function inherent in the terminal repeat structure might catalyze downstream transcription and thus activate to high production genes that are normally regulated to low levels. Finally the notion that the envelope gene is important was emphasized by the variety of results showing that viruses with hybrid envelope proteins are often present in tumor cells.

Comparison of the defective and non-defective cancer-inducing viruses shows that the variety of conceivable mechanisms of carcinogenesis has been increased by the discovery of the multiplicity of types of cancer-inducing retroviruses. Given the variety of mechanisms that must be involved in normal cellular growth control, it would be

expected that a wide variety of transformation mechanisms would underlie the multiplicity of cancers evident in animals.

WORKSHOP SUMMARY: Immunogenetics of Animal Viruses, Irving L. Weissman, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305.

Although this workshop was entitled "Immunogenetics of Animal Viruses" the focus of discussion was somewhat broader. There were three main topics covered in the workshop: 1) Biological and molecular-genetic characterization of the slowly transforming leukemogenic retroviruses: the major model system was the spontaneously arising AKR thymoma; 2) Non-immunological host response or restriction mechanisms preventing retrovirus infection, transmission and/or tumorigenesis; 3) Immunological restrictions of retrovirus tumorigenesis: the major model system cited was that of natural killer (NK) cells.

In the AKR system production of viral representatives of endogenous Akv-1 and Akv-2 viral genes occurs early in life, and spread of these apparently nontransforming viruses from cell to cell occurs throughout late gestation and early adulthood. Productive infection of the target organ for leukemogenesis (the thymus) by these viruses is a late event in this process. Following productive infection of the thymus by N-ecotropic endogenous virus, the animal enters what is known as a "preleukemic" state. This is characterized by the appearance of virus infected lymphoid cells in bone marrow and thymus which bear surface differentiation antigens (such as H-2 and Thy-1) at levels expressed only by a relatively infrequent subpopulation of lymphocytes in the normal thymus. Furthermore, there appears to be some increased (but non-neoplastic) proliferation of these cells in the thymus. These cells express AKR envelope and glycosolated gag gene products on their cell surface. The preleukemic state lasts until approximately 6 to 9 months of age, and is often followed by a stage wherein the thymus involutes and most virus infected lymphocytes disappear. At some point during this involutional stage small foci of lymphoblastoid cells appear in the thymic cortex, enlarging to give rise to thymic lymphomas. These lymphomas may then spread from the thymus, homing to thymus dependent regions of lymphoid tissues (specified by surface receptors for high endothelial venules in those tissues), and eventually give rise to blood born leukemia. The early lymphoma cells also express high concentrations of surface gag and env gene products, but some lymphomas may be completely different from cells predominating in the pre-leukemic period in terms of surface expression of differentiation antigens such as H-2 and Thy-1.

In terms of virological events, AKR xenotropic virions appear in the blood in high titers just prior to the appear-

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ance of leukemic cells. Concomitant with the appearance of the thymic lymphoma cells several unique types of viruses are found. Cloned T leukemia cells from these thymuses express these virions, but do not express detectable levels of N-ecotropic or xenotropic virions. The lymphoma-produced retrovirus particles that appear have been incompletely characterized, and a major portion of the discussion in this workshop centered on their characterization.

Rein first discussed the point that at least two types of virus particles can be isolated according to their biological activity: the well known duotropic MCF viruses first reported by Rowe *et al.*, which may grow in (but not transform) mouse and mink fibroblasts, and fibroblast replication-defective viruses. Coffin pointed out that the origin of the MCF-viruses was unsure, but that they contained gag and pol genomes representative of the N-ecotropic parent, and invariably had changed sequences in the env and adjoining C region. Although he reported that Nancy Hopkins' analysis of the oligonucleotides from this region showed variations throughout the env region, there appeared to be some clustering of these changes within subregions of env and C. Cloyd (working with Rowe) showed that these MCF viruses were capable of accelerating leukemogenesis in AKR mice. Rein then characterized the second type of virus present in T lymphoma cell cultures - a virus which was defective in replication in certain target cell lines (S^+L^-), and which could be rescued by and coreplicate with ecotropic virions. He felt that these virions make up at least a part of what Nowinski and Hays called SL virions, and he predicted that these virions too might be quite active in accelerating AKR leukemogenesis. Weissman pointed out that a cloned T lymphoma line (KKT-2) may contain at least three antigenically distinct subsets of virus-determined glycosylated cell-surface gag molecules (mw gp85/gp95). It seems likely that these unique gag determinants are markers for simultaneous infection of KKT-2 cells by several "SL" viruses, although other explanations for these antigenically distinct subsets are possible. The genetic map of replication defective and other SL virions has not been characterized sufficiently to know which virogenes are present, and what is their possible origin.

Which genes in the thymotropic, slowly-transforming retroviruses cause neoplastic transformation of their target cells? Several hypotheses were proposed and discussed: The receptor-mediated leukemogenesis hypothesis (Weissman, McGrath, Baird) proposes that the inducing virus provides envelope antigens for recognition by (and infection of) subsets of (anti-env) antigen reactive T lymphocytes in the thymus. This hypothesis states that neoplastic proliferation by these cells

and their clonal progeny would be due to their continued production of the virus (antigen) for which they have a receptor-mitogen complex. In this autostimulation model, the viral oncogene would be env, and genetic alteration in the env region would serve to create new antigenic determinants for recognition by T cells with receptors specific for them.

Next, several models were proposed which involved site specific integration of the MuLV genome into the target (thymocyte) chromosomal DNA. In the Jaenisch model the important integration site would be at or near promoter sequences involved in expression of T cell differentiation markers. This would result in expression of these viruses only in cells expressing T cell differentiation markers. In the Coffin-Robinson model the C region itself is a promoter, acting at the 5' end for viral messages, and at the 3' end for cellular messages important in the uncontrolled growth of the target cells. In the Cantor model, the integration event would obliterate a negative regulator of T cell proliferation. Each of these hypotheses still must explain the inability of any of the groups to demonstrate any common integration site(s) for any particular T-MuLV and its' induced T lymphoma. However, it is possible that each functional subclass of T cells might have unique genetic regions controlling cellular proliferation; and therefore one might expect some common integration sites only within a particular T cell functional subclass.

Other hypotheses were presented which compare T-MuLV's to acute transforming viruses which contain within them gene sequences which code directly for the transformed state (e.g. src, erb, Abelson sequences). Three models appear to be possible: 1) The viruses could contain as yet undetected leukemia genes, expressed at low levels and located in but not yet mapped to the gag-pol-env-C interstices. 2) The major infectious virus could act as a helper to rescue endogenous transforming gene sequences. In this case, the two types of viral genes are not carried in the same coding sequence and must be re-rescued with each succeeding viral transfer of leukemia. For example, recent work by Simone Brutlag in Henry Kaplan's lab suggests there is a recurring 30S sequence in RadLV transformed cells; and Rein's demonstration of replication defective viruses in AKR lymphoma cells coinfected with MCF's might suggest other candidates. 3) Finally, Benjamin suggested that the transforming sequences might be present in the viral genome and translated in another reading frame.

The rest of the workshop was concerned with host responses to MuLV infection. We first considered restrictions of viral

spread from cell to cell. One of the most important gene loci present in all tested strains of mice is called Fv-1; it restricts the spread of virus infection from cell to cell in a quantitative fashion. Years ago Jolicoeur and Baltimore demonstrated that Fv-1 could act in at least one viral system by preventing viral RNA from providing a DNA copy which integrates into chromosomal DNA. While these studies were only carried out in fibroblasts (not the target tissue for MuLV transformation), restrictive systems which act as dominant host range determinants have now been shown to act at the level of circularization of the linear DNA copies of the virus to their proviral preintegration form (Jolicoeur). How such a system could work was left open, although it seems likely that some as yet unidentified host cell protein might be responsible. While the Fv-1 locus had been divided into two alleles - $Fv\text{-}1^n$ and $Fv\text{-}1^b$ - two striking experiments were reported at the workshop that questioned this limited polymorphism. Cloyd showed that MCF virus grows well in only a few mouse strains, and that resistance in this cell maps in the region of Fv-1 and Gpd. However, both susceptible (AKR) and resistant (C58) mouse strains are $Fv\text{-}1^n$ (by Friend virus typing techniques). While Cloyd proposed that this might reveal an even greater polymorphism (more than two alleles) at the Fv-1 locus, it seems just as likely that the Fv-1 region may consist of two or more tandemly placed restriction genes, some restricting some virus types, others restricting other virus types. Thus it shall be important in the near future to test whether Fv-1 restriction of MCF is allelic with Fv-1 restriction of Friend virus, or whether these are pseudoalleles. It shall also be important to test whether the Fv-1 linked restriction of MCF viruses acts by preventing integration at the linear → circular step.

Gardner then described an even more potent (and dominant) restriction gene found in wild mice which restricts absolutely AKR viremia and T cell lymphomagenesis. It is interesting that this gene does not affect wild mouse amphotropic viremia and subsequent appearance of non-T cell leukemias. Murray made the important point that most labs have studied the rather limited gene pool provided by inbred mouse strains, whice are in turn derived from an even lower number of founder stocks. Sampling of wild mice not only assays conditions as they may have existed as driving forces during evolution, but also may provide many more examples of genetic restriction of T-MuLV expression and/or spread.

Mak reported experiments from his laboratory and Axelrad's laboratory indicating that the Fv-2 locus acts at the level of hematopoietic target cells. He quoted two observations:

Fv-2 resistance could largely be overcome by bringing injected bone marrow (target) cells into a regenerative phase following irradiation; and SFFV sequences could be found in cytoplasmic RNA of hematopoietic cells from FV-2^s mice, but are absent from Fv-2^r strains. Axelrad's experiments indicate that Fv-2 acts at the level of target cells and proposed the target cells are cycling cells in the erythroid lineage. Fv-2^r strains would contain mainly noncycling target cells (except during postirradiation regeneration), while Fv-2^s strains would contain mainly cycling target cells. It is also possible that the Fv-2 locus acts to restrict or prevent expression of SFFV related sequences in hematopoietic cells, whether of endogenous origin or provided by an injected Friend virus complex.

In a final portion of the session we reviewed the effector elements of the immune system which act to neutralize or remove virions in the extracellular space (the humoral immune system) and virion-infected cells (the cell-mediated immune system). It is well known that the cytotoxic T cell component of the cellular immune system recognizes viral-infected target cells by some complex system involving co-recognition of viral components on the cell membrane and host cell major histocompatibility complex determinants (H-2 in mice). However, the T cell system is not the only system of cell mediated immunosurveillance of virus infected cells: thymus-deprived mice contained in their bone marrow and other hematopoietic tissues a class of cells that may directly lyse MuLV infected target cells, and these are called natural killers (NK). These NK cells are capable of recognizing and lysing MuLV expressing lymphoma cells and normal lymphoid cells expressing the MuLV-related antigens; preliminary evidence indicates that at least some NK cells may recognize *env* gene products. As a population, they appear not to be H-2 restricted in target killing. Two groups have demonstrated that some NK cells express markers found only on T lymphocytes in the immune system (Thy 1 - Herberman; Hollander, Pillemer, and Weissman). Thus it is possible that these represent precursors of T cells which are already committed in their function to recognize and lyse MuLV envelope bearing cells, but that they are not yet restricted to recognize such targets in the context of MHC determinants. (The use of Thy-1 as a marker for T cell lineages is not entirely proper, as several nonlymphoid cells may express it.) Cantor then spoke of his work on NK cell clones, showing that they recognize and lyse MuLV bearing cells in a non-H-2 restricted fashion, a finding that confirms that NK populations which lack H-2 restriction of recognition are also unrestricted at the level of individual cells. Cantor also demonstrated that

NK cells can recognize and lyse mitogen-activated B lymphocytes, and proposed that this may represent the residue of a primitive recognition system that was later to evolve into a receptor mediated recognition of B lymphocytes by another subclass of T cells - the helper T cells. These results recall the experiments of Wecker and of Moroni which demonstrate that normal B lymphocytes are gp70 negative while antigen-activated B lymphoblasts express envelope gp 70 molecules on their surface. It is tempting to propose that these are the components recognized by NK cells on activated B lymphocytes, and that these NK cells may normally serve some regulatory function in determining the magnitude of antibody responses. It is clearly an issue of great importance to settle if NK cells recognize gp70 determinants, how their function is induced, and what is their response to infection by the very viruses they recognize.

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