Fundamenta genetica : the revised edition of Mendel's classic paper with a collection of 27 original papers published during the rediscovery era / selection and commentary by Jaroslav Kříženecký ; with introduction by Bohumil Němec.

Nobel Symposia

Medicine and Natural Sciences

Chromosome identification –

technique and applications in biology and medicine

Editors

TORBJÖRN CASPERSSON and LORE ZECH

Administrative editor

VERA RUNNSTRÖM-REIO

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Proceedings of the Twenty-Third Nobel Symposium
held September 25–27, 1972
at the Royal Swedish Academy of Sciences, Stockholm

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Foreword

During the most recent years, our ability to identify single chromosomes in higher animals and plants has improved rather suddenly followed by rapid progress within many sectors of the broad field of genetics.

In such a swiftly developing situation, symposia have an important task to fulfil, since the ordinary lag in publication time in the scientific press makes it difficult to keep abreast of the rapid flow of events. The consequence is unnecessary delays in the organisation of further research and also often duplication of work.

Against this background it seemed especially desirable to gather together, in 1972, a group of investigators in chromosome research for an exchange of ideas and experiences concerning the present situation of chromosome identification methods and their applications. This led to the arranging of the Twenty-third Nobel Symposium which took place September 25–27, 1972.

The present volume records the deliberations of that meeting. The material proved so diverse and wide-ranging that its editing has been a difficult task. The point of departure for the proceedings took the form of a series of quite short, purely technical presentations of the several procedures presently available, described without their historical perspective or their underlying mechanisms. These items are gathered in the chapter "Chromosome Banding Techniques" and which, for logical reasons, are arranged according to the nature of each method and their mutual relationship.

Next are presented the transactions proper. First, applications in the fields of biology and medicine and then the important section on the backgrounds for the different banding techniques. These reports enter so many fields and are of such disparate nature that we saw no other way of presenting them than according to each speaker's name, in alphabetical order.

Because of the diversity of the material, the lively discussions often spanned several lectures simultaneously. For that reason it has proved next to impossible to do full justice in a logical way to all the discussion material which was forthcoming.

The symposium was sponsored by the Nobel Foundation through grants from the Tricentennial Fund of the Bank of Sweden, which is gratefully acknowledged. Thanks are due to the Royal Swedish Academy of Sciences for allowing their premises to be used as venue for the symposium.

A conference of this kind cannot be accomplished without the help of many persons. It is a pleasure to have this opportunity to express our gratitude to all those who assisted in making the Twenty-third Symposium so successful and in particular the personnel from the Institute for Medical Cell Research and Genetics.

For and on behalf of the Symposium Committee
Torbjörn Caspersson

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List of Participants

Bahr, G, Department of the Army, Armed Forces Institute of Pathology, Washington, D.C. 20305, USA

Bootsma, D, Department of Cell Biology & Genetics, Rotterdam Medical Faculty, Rotterdam, The Netherlands

Buckton, K, Clinical & Population Cytogenetics Unit, MRC Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland

Caspersson, T, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Castleman, K, Jet Propulsion Laboratory, California Institute of Technology, 4800 Oak Grove Drive, Pasadena, Calif. 91103, USA

de la Chapelle, A, The Folkhälsan Institute of Genetics, P O Box 819, SF-00101 Helsinki 10, Finland

Comings, D E, Department of Medical Genetics, City of Hope National Medical Center, Duarte, Calif. 91010, USA

Dutrillaux, B, Service de Génétique Fondamentale, Hôpital des Enfants-Malades, 149 Rue de Sèvres, 75 Paris 15, France

Edström, J E, Institute for Histology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Ford, C E, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Fredga, K, Department of Genetics, University of Lund, Sölvegatan 29, S-223 62 Lund, Sweden

Granlund, G, Linköpings Högskola, Department of Electrical Engineering, S-581 83 Linköping, Sweden

Gropp, A, Institut für Pathologie der medizinischen Akademie, 2 400 Lübeck, Kronsforder Allee 71–73, BRD

de Grouchy, J, Laboratoire de Cytogénétique, Hôpital des Enfants-Malades, 149 Rue de Sèvres, 75 Paris 15, France

Hamerton, J L, Department of Genetics, The Children's Hospital of Winnipeg, 685 Bannatyne Avenue, Winnipeg 3, Manitoba, Canada

Hsu, T C, Section of Cell Biology, Texas Medical Center, The University of Texas, M D Anderson Hospital and Tumor Institute, Houston, Tex. 77025, USA

Hultén, M, Department of Clinical Genetics, Karolinska Sjukhuset, S-104 01 Stockholm 60, Sweden

Kihlman, B, Department of Genetics, University of Uppsala, S-752 20 Uppsala, Sweden

Lejeune, J, Service de Génétique Fondamentale, Hôpital des Enfants-Malades, 149 Rue de Sèvres, 75 Paris 15, France

Levan, A, Institute of Genetics, University of Lund, Sölvegatan 29, S-223 62 Lund, Sweden

Lindsten, J, Department of Clinical Genetics, Karolinska Sjukhuset, S-104 01 Stockholm 60, Sweden

Lubs, H A, Department of Pediatrics, University of Colorado Medical Center, John F Kennedy Center, 4200 E Ninth Avenue, Denver, Colo. 80220, USA

Lubs, M L, Department of Pediatrics, University of Colorado Medical Center, John F Kennedy Center, 4200 E Ninth Avenue, Denver, Colo. 80220, USA

Miller, O J, Department of Human Genetics and Development, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, N.Y. 10032, USA

Modest, E J, Children's Cancer Research Foundation Inc., 35 Binney Street, Boston, Mass. 02115, USA

Møller, A, Department of Physiology II, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Ockey, C H, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, UK

Pearson, P L, Department of Human Genetics of the State University of Leiden, Wassenaarseweg 62, Leiden, The Netherlands

Polani, P E, Paediatric Research Unit, Cameron House, Guy's Hospital Medical School, London SE1, UK

Rigler, R, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Ringertz, N R, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Schnedl, W, Histologisch-Embryologisches Institut der Universität Wien, Schwarzspanierstrasse 17, 1090 Vienna, Austria

Therkelsen, A J, Institute for Medical Microbiology, University of Aarhus, 8000 Aarhus, Denmark

Utakoji, T, Pathology Department, Cancer Institute, Toshima-ku, Tokyo 170, Japan

Vosa, C G, Botany School, University of Oxford South Parks Road, Oxford OX1 3RA, UK

Wall, R J, Jet Propulsion Laboratory, California Institute of Technology, 4800 Oak Grove Drive, Pasadena, Calif. 91103, USA

Zech, L, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Zetterberg, A, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Östergren, G, Department of Genetics and Plant Breeding, Agricultural College of Sweden, S-750 07 Uppsala 7, Sweden

Runnström-Reio, V, Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Introductions

Scientific Impact of the Study of Fine Structure of Chromatids

J. Lejeune

Service de Génétique Fondamentale, Hôpital des Enfants-Malades, 75-Paris, France

Since the demonstration of the correct number of chromosomes in man [1] in 1956 and the discovery of the first chromosomal disease in man [2] in 1959, human cytogenetics has developed in an explosive way during the early 'sixties and has since settled down to a steady growth rate.

Since 1965 the number of new syndromes discovered each year has gradually diminished and fears were expressed that cytogenetics had passed direct from infancy to senescence. The reason for this was that whereas the early successes were won easily and rapidly, the techniques remained quite unchanged except for minor refinements.

Even autoradiography, which registered encouraging advances in the study of the X chromosome and, to a lesser degree, in the field of acrocentrics, failed to break new ground in chromatid analysis.

In 1968 a new era was opened when Caspersson et al. [3] observed a peculiar pattern of fluorescence in the chromosomes of *Vicia faba* and *Trillium erectum* following quinacrine mustard staining activated by UV light.

Apparently the importance of the findings was not fully grasped by researchers in human cytogenetics and it was only when Caspersson & Zech [4] revealed the whole human karyotype where every pair was clearly distinguishable that the rush on chromatid fine structure analysis started all over the world.

Although the fluorescence pattern is still the most accurate analytical tool, its technical difficulties prompted research into other procedures more amenable to routine exploitation.

The diversity of tortures inflicted upon chromosomes in order to force them to divulge their inner information is really amazing. Boiling them [5], digesting them [6], burning them with alkali [7] or salts [8], even intoxicating them before fixation [9] — all these maltreatments have been tried in turn.

I do not intend here to review all the available techniques, as I am personally aware of more than 20 variants. I will merely cite some of the more immediate achievements that have resulted from the analysis of the fine structure of chromatids.

Impact on Clinical Cytogenetics

To quote all the improvements in syndrome analysis brought about by the new techniques, whether fluorescence or denaturation, would be an enormous task. I will nevertheless run the risk of missing some of the important improvements by concentrating on only a few of them.

Confirmation of previously established syndromes

New techniques have made refinements in the recognition of chromosomal error in all trisomies and all deletion syndromes.

For instance, in the case of a deletion of the short arm of a group B chromosome, tedious clinical comparisons and painstaking autoradiographic investigation [10] had already led to a distinction between 4p- and 5p- (or "cri du chat") syndromes. With chromatid analysis this distinction was now elucidated and definitely established [11].

A curious feature is that the two clinical syndromes, although clearly distinct, are to some extent apparently related, just as chromosomes 4 and 5 exhibit some similarities. Such a consequence could be extremely significant, since it will be encountered repeatedly.

For the medium-sized chromosome, clinical demonstration of a particular C-trisomy has been

confirmed by fluorescence identification of pair 8 by de Grouchy et al. [12], and now confirmed in at least six cases by our heat denaturation technique [12]. Trisomy 8 is the only C-trisomy hitherto individualized but, as we shall see later, partial trisomy 8 can also be recognized now.

In the case of acrocentrics, chromatid analysis has established the syndrome of trisomy 13 beyond doubt and there are good prospects of individualizing the clinical consequences of trisomy 14 and trisomy 15, together with the partial trisomies for these elements.

For the E group, only trisomy 18 and syndromes 18p- and 18q- have been fully investigated and no new syndromes for the F group have yet been recorded [19, 20].

For the small acrocentrics, highly important data have been gathered. Primarily, the Y chromosome can be detected by its remarkable fluorescence, even in the resting nuclei. This technique possesses the same accuracy for Y diagnosis as does the Barr bodies count for the X chromosome.

For pairs 21 and 22, their first separation by Caspersson & Zech led to unexpected results. The Ph_1 chromosome, marker of granulocytic leukemia, was long supposed to be a deleted 21. The fact that this tiny element looks much more like a deleted 22 was a surprise [13], in view of the long-known association between trisomy 21 and acute childhood leukemia.

This again raises the question: Can chromosomes resembling each other control organs or functions of the same "kinship".

However, it is in the analysis of chromosomal rearrangement that fine structure analysis refined most of the old observations.

Improved analysis of chromosomal rearrangement

Rearrangements between acrocentrics: The detection of each element involved in centric fusion or, more precisely, in reciprocal translocation in the centromeric region of acrocentrics, has confirmed the previously anticipated possibilities.

Hence, in a case of G–G translocation carrier, it is easy to distinguish between a 21–21 and a 21–22 translocation. In view of the fact that 21–21 translocation leads only to trisomy 21 or to unviable monosomy, this recognition is of the utmost importance for genetic counselling.

The same is true of D–G translocation. A demonstrable 22–15 translocation, for example, would be considered a relatively tolerable burden, since neither possible trisomies nor possible monosomies are compatible with late embryonic development, according to our present knowledge.

A systematic study of the D chromosome involved in D-21 translocation is not yet available, yet it could very well be that the malsegregation risk of a carrier is not always the same, depending on whether the D involved is a 13, a 14, or a 15. If one category had a much more stable meiosis than the others, it might explain the curious fact noted by some workers [14] that some D–G translocations seem to be carried by families without apparent damage, whereas other families exhibit the now-classical one-in-five risk if the mother is the carrier, or one-in-fifty if it is the father.

Here again, if a systematic study could possibly relate each type of translocation to a particular meiotic behaviour, possibly different in the two sexes, this would give a hint as to how to prevent malsegregation.

Besides these classical centric fusions, chromosome 21 can suffer ordinary translocation. We have thus observed a mother carrier of a 21q – 18p + translocation in which the terminal seg-

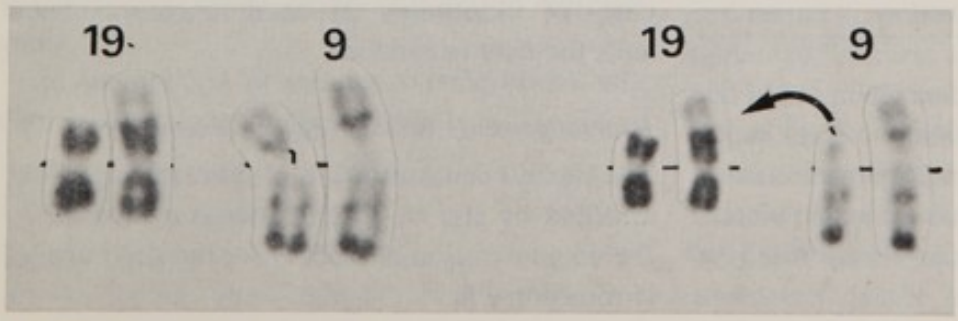

Fig. 1. A case of trisomy for short arm of chromosome 9 (trisomy 9p). (*Right*) the parental translocation: one of the 9 is amputated of its short arm and one of the 19 has received this fragment; (*left*) the child has received two normal 9 and the 19 carrier of the short arm of 9.

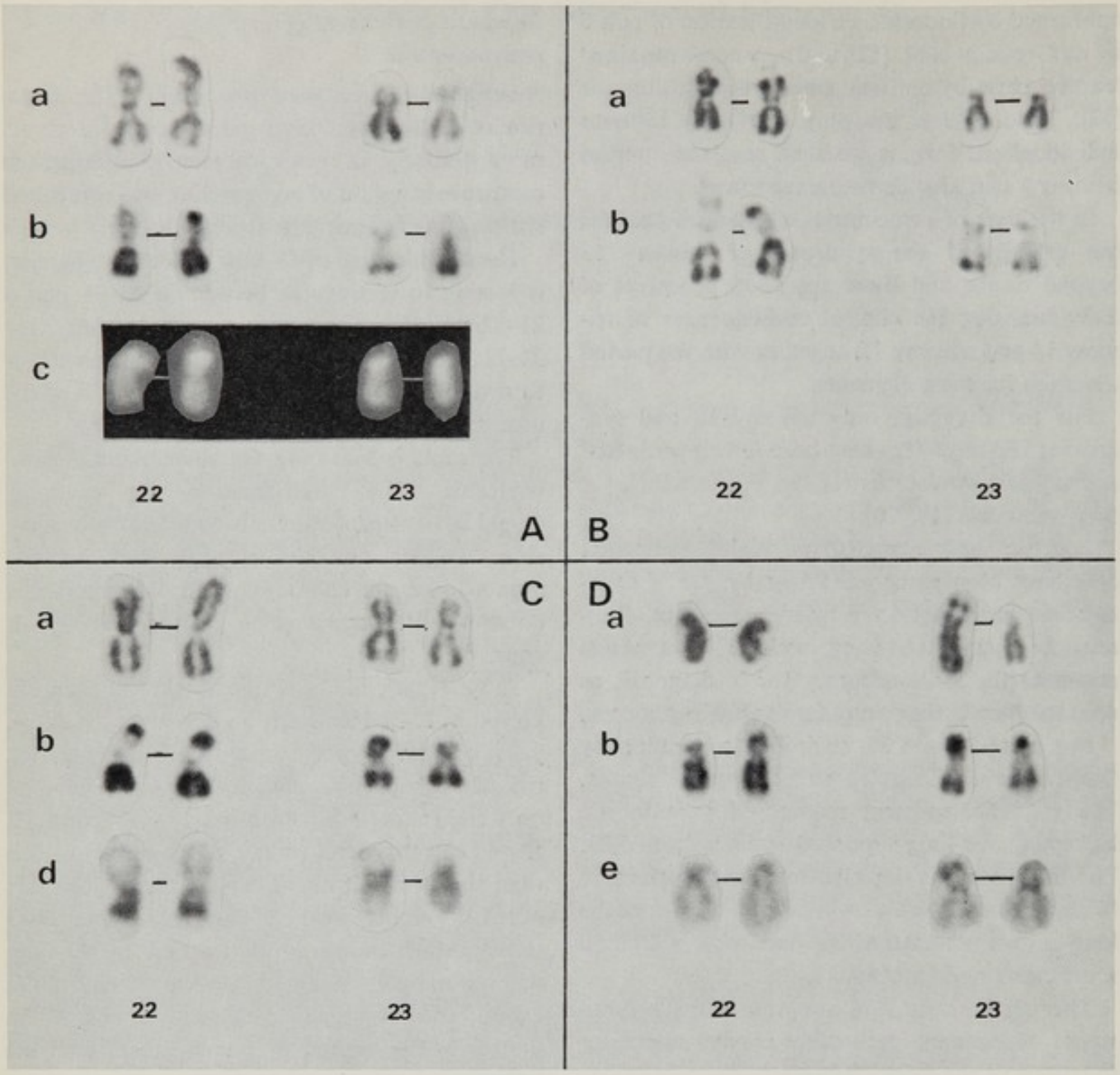

Fig. 2. Analysis of the chromosome 22 of *Pan paniscus* (pigmy Chimpanzee) and *Pan troglodytes* (ordinary chimpanzee). The chromosome 22 has a greatly enlarged short arm in *Pan paniscus* (three different individuals A, B and C compared with the small arm found in *Pan troglodytes* (individual D). This enlarged short arm of 22 in *Pan paniscus* seems rather "euchromatic" by conventional staining (*Aa*, *Ba* and *Ca*). Controlled denaturation by heat demonstrates a heterogeneous structure (*Ab*, *Bb* and *Cb*). Quinacrine fluorescence shows a very brilliant segment (*Ac*). Enzymatic treatment confirms the heterogeneous nature of this short arm (*Cd*).

ment of the 21 could be detected on the top of the short arm of the 18. The 21 trisomic child had a complete trisomy with two no. 21 chromosomes, free and normal, and a third present in two pieces: the deleted 21 and its end part translocated to chromosome 18.

There have been other instances showing partial monosomy of chromosome 21, in particular a patient [15] with 45 chromosomes who apparently lacked a whole 21. Half of the long arm of the 21 (terminal part) was translocated on the short arm of chromosome 9. This type of case was thus a partial monosomy for the juxtacentric portion of 21 and the translocation was undetectable with ordinary staining. Thus all alleged cases of monosomy 21 need to be reanalysed with the new techniques.

Rearrangements between other chromosomes

The clearest demonstration of the resolving power afforded by the new techniques is evidenced by the demonstration of trisomy for the short arm of chromosome 9.

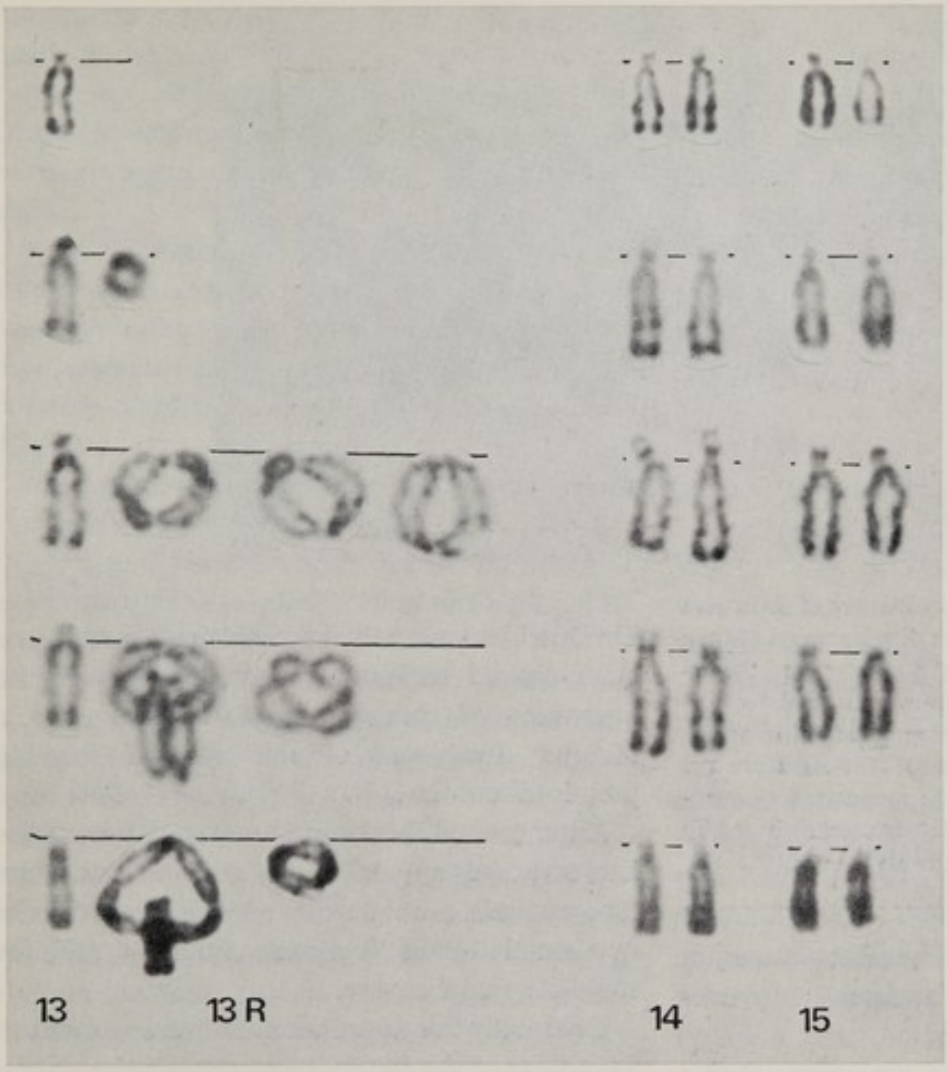

Fig. 3. Some examples of a ring 13. Note that the small ring is restricted to the dark portions of chromosome 13, the juxtacentromeric one and the juxtatelomeric. On double-sized dicentrics the dark juxtacentromeric region remains separated from the dark telomeric region by a a pale region. It can be seen that the sequence of the bands runs in opposite direction in the two chromatids and are opposed by their ends.

The syndrome was isolated at our Institute by M.-O. Rethoré [16], who carefully compared the clinical analyses and chromosomal pictures of four cases of translocation involving the short arm of a C chromosome. Recognition of the donor chromosome as a no. 9 was based on the observation of the secondary constriction which, in some cases, passed over to the carrier chromosome.

As exemplified in various translocations studied by denaturation techniques

9q – 6p+
9p – 22p+
9p – 19p+
9q – 22q+

the short arm of no. 9 remains detectable in each instance and gives definite proof of the clinical entity (fig 1).

In addition to these confirmations, the new techniques have already led to the isolation of new syndromes.

Discovery of new syndromes

Among the expected possibilities, trisomy 22 has not yet been detected by the new techniques. On the other hand, cases with an extra G-like chromosome have turned out to be something else, as already seen in a karyotype of the (9p) trisomy syndrome.

Curiously, we have detected, in two apparently unrelated families, a peculiar translocation which would have escaped detection with the old techniques (and had indeed escaped us).

Here the entire long arm of chromosome 22 is transferred to the long arm of no. 8 and, reciprocally, the terminal portion of chromosome 8 is transferred to the centromeric region of no. 22.

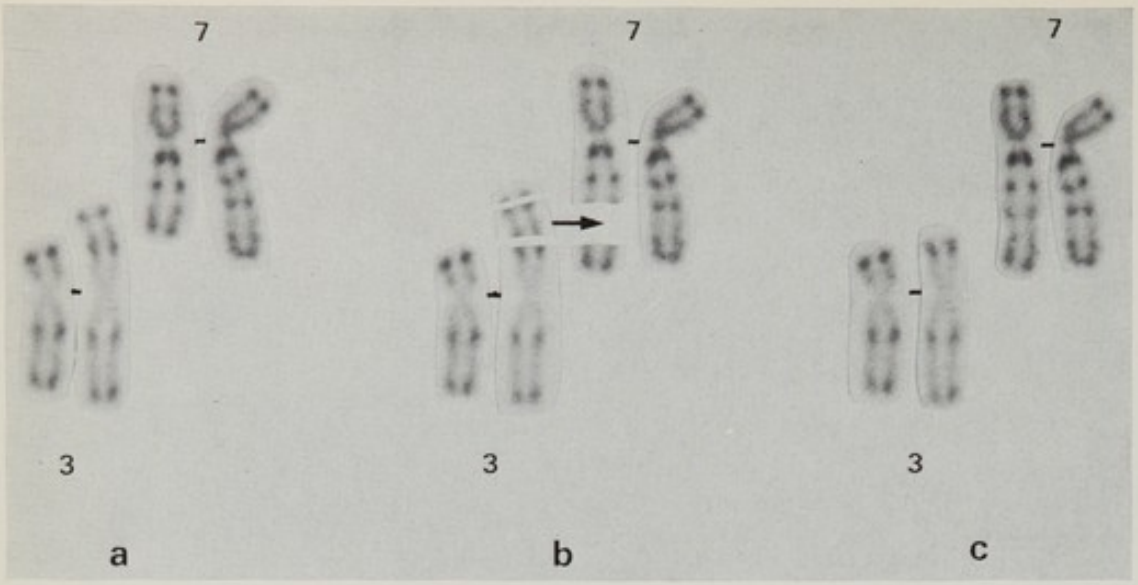

Fig. 4. A case of insertion. The middle part of short arm of chromosome 3 is inserted in the distal portion of long arm of chromsome 7. (*a*) Actual karyotype of the individual; (*b*) cut-out of the chromosomes to show artificially the inserted piece; (*c*) the amputated chromosome 3 at left and the long chromosome 7 at right are the original ones (as shown in *a*). The amputated chromosome 3 at right and the long 7 at left are reconstituted by patching together the cut out fragments seen in (*b*).

The exchanged segments being of equal length, this translocation could not be detected by conventional staining [17].

The children, carriers of an extra G-like chromosome, are trisomics for the distal portion of the long arm of chromosome 8. This type of reciprocal translocation of equal length had been anticipated long ago [18], but awaited new techniques for confirmation.

There is little doubt that other examples will be discovered, leading eventually to the detection of chromosomal imbalance in abnormal children already classified as "normal karyotype" with the old techniques. This implies that every negative chromosomal examination of the last 10 years must be reinvestigated with the new techniques, not to mention all the positive findings.

Analysis of abnormal cells

Although very impressive studies have been devoted to chromosomal changes in neoplastic cells, further cytogenetic analysis was soon blocked because of the impossibility of defining precisely the identity of rearranged chromosomes.

Application of the new techniques can radically change this state of affairs and I would like to remark in passing that with Mlle Venuat and Dr Dutrillaux we have been able to observe, in a quasi-diploid malignant strain, a characteristic aberration of chromosome 6, together with a curious streakiness of this element forming pseudo-dicentrics [18].

If the general theory of clonal evolution of the karyotype has any validity, chromatid fine structure analysis should soon provide the evidence by demonstrating "common variants" [19] for some classes of cancer.

Eventually this approach could take immediate advantage of the enormous wealth of information already gathered by cell hybridization techniques [20]. The chromosome maps thus established could give a glimpse of the relationships between gene dosage and metabolic pathways as postulated in the hypothesis of "combinaisons interdites" [21].

No doubt the cytogenetics of tumours will change so rapidly in the next few years that any review of this field is actually impossible.

Impact on Non-human Cytogenetics

Extension of chromatid analysis to animal karyotypes was indeed very attractive and many of us have, fortunately, not resisted that temptation. It is well known that the karyotype of the anthropoid apes orang-utan, gorilla, and chimpanzee, had an "air de famille" and had an obvious resemblance to the human karyotype. A puzzling feature was that although all the elements of the chimpanzee karyotype, for example, shared similarities with the human (apart from the difference in number—48 in the chimpanzee), there was no

full metric agreement between recognisable elements of the two species.

As proposed by de Grouchy & Turleau [22], this curious situation might be explained by a number of rearrangements, especially pericentric inversions.

With Dr Rethoré and Dr Dutrillaux, we have studied two closely related chimpanzees: *Pan troglodytes* (the variety generally seen in zoos) and *Pan paniscus* (the pygmy chimp), thanks to the courtesy of M. Nouvelle (Paris) and M. Vandenbergh (Liège).

The reason for this choice was the earlier finding by Hamerton [23] and by Chiarelli [24] that *P. paniscus* differed from *P. troglodytes* in that a small acrocentric pair in Paniscus was replaced by a small metacentric pair.

Curiously enough, the expected translocation was not found. All pairs are identical in both species but for the no. 22, acrocentric in Troglodytes and metacentric in Paniscus.

By comparing the results of heat denaturation, fluorescence, and enzymatic denaturation, it appears that the metacentric pair is not an isochromosome. The long arm is similar to that of no. 22, whereas the short arm has a very peculiar chromatid structure, highly fluorescent, extremely faint in denaturation but for its telomeric region, and extremely swollen after enzymatic digestion.

Here, it seems that a particular heterochromatic region constitutes the real distinction, as if Paniscus had amplified enormously a short segment of the short arm of no. 22. Hence the difference in the two species seems to be an increase in material—a de novo synthesis of one part of a chromatid (fig. 2).

If these highly fluorescent regions are related to repetitive DNA and act essentially in the regulation process, what we see at work here is the chromosomal picture of a regulatory system.

Besides chromosomal rearrangements, these comparative studies of chimpanzees have also shown how similar some chromatids can be in man and in apes.

If we count only the very typical structures, and reject the less conspicuous chromatids, it seems difficult to avoid the conclusion that at least the long arm of no. 7 as well as of 10 and 11 are identical in both species. A less precise identity is highly probable for 1, 3, 8, X, 16, 19, 20, 21 and 22, but more speculative for others.

Two remarks are apt here:

(1) Some chromatid structures in primates have remained unchanged for 10 million years at least, if we accept morphological similarity as proof of common origin, i.e. if we disregard a possible convergence.

(2) Chromosomal shift by multiple rearrangement is enormous, but reshuffling seems to occur more within a chromosome than between different pairs.

Hence, every heterozygote step must have suffered from a very severe segregational load, a proposition contradictory to the progressive mutational evolution of the neo-Darwinian school. A possible alternative to this paradox is the reduction of the reproductive group, even to the size of a unique couple [25].

It is also possible that the permanence of some chromatids is not fortuitous, but may signify that some combinations are highly preferential, either for positional effect between genes or for regulatory opportunities. For example, it could very well be that an equilibrium exists inside a chromosome, in the sense that a trisomy for one arm could be equivalent to the monosomy of the other, since we have already encountered such a situation in an 18–16 translocation in man [26].

The impact of chromatid analysis on the speciation theory will no doubt prove to be enormous, even if only to exclude some forms from some groups, as is already the case with the Gibbon, which we propose to expel from the primates, for its lack of chromatid structure similarities to man.

Impact on Chromosomal Mechanics

The way chromosomes behave in the intimacy of the cell is quite difficult to understand from fixed preparations but fine structure analysis can be used to test hypothetical explanations. Three simple examples can be chosen.

Ring chromosomes

Following the lead of McClintock [37] I have proposed that the aberrations observed in ring chromosomes could be related to chromatid exchanges before the separation of centromeres [27]. Theoretical models showed that, if this were true, double-sized dicentric rings would have a peculiar structure, in the sense that the sequence

of the genes in the two segments between centromeres would run in the same order but be opposed by their ends. Secondly, the reduction in size would result essentially from loss of the central part of the ring and not from loss of the juxtacentromeric segments. Although this theoretical model was recently challenged by a double breakage hypothesis [28], chromatid analysis revealed that these previsions are indeed confirmed by observations (fig. 3).

For example, as expected, a ring of chromosome 13 showed distinctly the dark juxtacentromeric region and the dark juxtatelomeric region, after rejoining of the short arm and the end of the telomeric region to form a ring. In dicentric rings, the sequence can be observed in both chromatids which are opposed by their ends. Similarly, in small rings the middle segment is lacking.

Selective endoreduplication: This curious chromosomal misdemeanour is exceedingly rare but very interesting. In some cells a distal portion of a chromatid is replicated twice, as we observed nearly 10 years ago. [29]. Curiously, this happens especially often for the terminal segment of chromosomes which exhibits a structural gap at a fixed point. This point is constant for the affected individual and this chromosomal peculiarity can be inherited as a familial dominant trait [30].

Sometimes the distal endo-reduplicated segment has a moniliform appearance and it can be shown that the sequence of these exceptional structures reproduces, with finer details, the denaturation picture. Hence the possibility that the event involves an abnormal replication which, in turn, may be due to the absence of some chemical required to hold together the chromosomal bands.

Remarkably enough, in some exceptional instances, it is not the distal part which is present twice but the other arm and the adjacent centromeric region, as if the real aberration were at the weak point detected on these chromosomes.

The exact significance of these phenomena regarding the fine mechanics of the chromosome is not yet certain.

"*Aneusomie de recombinaison*"

Among the possible origins of chromosome imbalance, the eventuality of "anosomie de recombinaison" [31] still lacks definite demonstration in man. The logic of the argument was that some rearrangement could, at meiosis, produce unbalanced gametes, even without an apparent change of metrics, if only a chromatid exchange had taken place in a particular exchanged segment. The simplest instance is an insertion, for a crossover inside the inserted segment could produce unbalanced chromosomes in meiosis of a heterozygote carrier.

No such type of chromatid exchange has yet been demonstrated, but the existence of insertion is now certain. Six months ago we found the first case, a piece of short arm of chromosome 3 inserted in the distal third of the long arm of chromosome 7.

With ordinary staining, the appearance was that of a reciprocal translocation, but chromatid analysis reveals the insertion beyond any doubt.

Recently, Grace et al. [32] reported another insertion of part of long arm of 7 inside the long arm of 3, and Gray et al. [33] showed an insertion of the short arm of 1 in the long arm of 4.

In other complex rearrangements, pericentric inversion can also be recognised and we have recently analysed one in chromosome 2.

At least the topological conditions for "aneusomie de recombinaison" do exist in man and the eventual occurrence or absence of this aberration would increase our knowledge of meiotic mechanisms.

Direct meiotic studies have not yet fully taken advantage of the new techniques, although, from preliminary observation, Dutrillaux thinks that the position of chiasmata could have a curious correlation with the banding pattern.

Finally, the systematic study of break points in various rearrangements will be statistically feasible with the steady increase of data. In a preliminary study of 28 independent translocations [34], found in different families (with no cases of centric fusion in this sample), we could show that of 56 breaks, 18 were in the centromeric zone, 20 were in the telomeric zone, and only 18 occurred somewhere in between the two.

Although the definitions of centromeric and telomeric zones are quite arbitrary it can be safely assumed that the "in between" is at least ten times longer than both of them together.

Besides this tendency to break at both ends of chromosome arms, no case of translocation

was recorded in which both breaks could be demonstrated to be in the intermediate region in these 28 instances.

Hence it could very well be that breaks themselves are not purely random, but correspond to some peculiarity of chromosomal structure.

This leads us to the last but not the least point of impact of the new techniques. What kind of structure is revealed by the various pictures we can obtain of a chromosome?

Impact on the Knowledge of Chromosome Structure

This last point is of outstanding importance and is to be discussed more fully later. I shall therefore touch upon this subject with great caution.

To comment bluntly on the present state of observation, we can say that all techniques available are consistent, although they do not all show the same picture.

If we take the four categories now studied, we can compare the so-called Q-bands (after treatment with the quinacrine mustard staining of Caspersson et al.), the R-bands (after treatment with the controlled heat denaturation of Dutrillaux & Lejeune), the E-bands (after treatment with the enzymatic digestion of Dutrillaux, de Grouchy & Lejeune), and the G-bands (produced by various Giemsa techniques).

The correlation is as follows:

Q-band	R-band	G-band	E-band
brilliant	faint	dark	swollen
faint	dark	faint	shrunken

Since the sequences obtained for a given chromosome are perfectly comparable we are positive that each technique acts on the same structural peculiarities.

The first impression from the Q-band observation was that quinacrine could attach specifically at some base-pair of DNA and G–C was considered the most likely [35]. Hence, the banding would result from the preferential reaction of quinacrine mustard with portions of DNA especially rich in G–C.

Such an enormous (at the molecular level) concentration of monotonous formula was disconcerting.

With heat denaturation, this DNA-related banding was less obvious, because with the temperature (87°C) and the time used, preferential denaturation of DNA according to its base sequence is not very likely and only immediate renaturation could occur.

But with the demonstration of the enzymatic bands, the pure DNA hypothesis is even less tenable. Actually, Couturier [36] has systematically used many different enzymes and obtained the same pattern with all the proteolytic enzymes, but not with others (especially DNase and RNase). More precisely, he showed that the best conditions for revealing the banding were the exact pH and temperature conditions optimal for the proteolytic activity of each enzyme studied.

Thus we are forced to accept the fact that the enzymatic banding pattern can be related to protein differences along the chromatids.

How do these differences relate to the DNA-specific sequences? What kind of polypeptide sequence do they represent? Why are some of them more readily "digested" than others? All these questions remain to be answered.

But the DNA hypothesis and the acid protein hypothesis are not necessarily conflicting. Of necessity, both DNA *and* protein must be sterically and chemically related to each other so as to build the entity we call a chromosome. With increasing knowledge of the precise mechanism of each staining technique, it could very well appear that all of them reveal a peculiar configuration resulting from DNA/protein interaction and no pure DNA or pure protein properties alone.

In this line of thought, a structural hypothesis for which Dutrillaux is mainly responsible, suggests that chromosomes have special relationships with the nucleoplasm and that banding patterns correspond to these points of interaction between chromatids and nucleoplasm.

In other words, chromosomes would no longer be noodles floating in a bowl of nuclear juice, but would be organelles having definite and localizable molecular interactions, with a structurally non-homogeneous nuclear content. Although it is difficult to interpret such a picture completely, it would be particularly appealing if some meiotic behaviour were found to be related to banding sequences.

Despite all the satisfaction that cytogeneticists have experienced with chromatid fine structure analysis, one disappointment has been keenly felt. A banding pattern is, for a geneticist, immediately

associated with the remarkable "topography" of polytenic chromosomes. Curiously enough, fluorescence studies of giant chromosomes of Dipterae have not yielded any good correlation with the well known maps of these chromosomes, although ordinary mitotic chromosomes of this species have a recognizable fluorescent banding pattern.

Could it be that the two banding types correspond to two separate physiological functions of chromatid segments? This question really is well worth investigating, for the induction of polyteny in cultured cells would be another milestone in cytogenetics.

To conclude these introductory remarks on the scientific impact of chromatid analysis on the recent development of cytogenetics, it can be stated that the discovery of banding patterns not only gave new impetus to research, but has actually rendered all previous findings obsolete.

In view of what we have learned with these techniques during two years, the old preparations seem like a palaeolithic research tool. Yet as scientific progress makes rapidly obsolete the keenest refinements of today, it can be ventured and indeed hoped, that at another meeting such as this, ten years hence, the present developments under discussion at this Symposium will appear to our successors as neolithic technology.

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Cytochemistry in Chromosome Analysis

Some general remarks

T. Caspersson

Institute for Medical Cell Research & Genetics, Medical Nobel Institute,
Karolinska Institutet, S-104 01 Stockholm 60, Sweden

We have witnessed an enormous expansion of our knowledge in cell biology during the last 25 years, first and foremost regarding the mechanisms of cell growth. Cytochemistry has played a very prominent role in this development, most of all in its quantitative form. Early observations of the involvement of nucleic acids in protein synthesis, of the ubiquity of the RNAs and also of the transport of RNA from the nucleus to the cytoplasm were all first made by cytochemical means. Later on other techniques, many involving preparation and isolation of cellular elements in large quantities, opened up ways to unravel the detailed mechanisms of the fundamental growth processes.

Something similar now seems to be happening on the two other main fields of cellular activities, cell differentiation and cell function. One important common aspect of both of these complex fields is the regulation of gene function and here it is evident, at least for the higher organisms, that the very organisation of the gene-carrying elements in the cell nucleus is of crucial importance and also the structural details are so very minute that for their study very advanced cytochemical methods, light-optical and/or electron-optical, are needed.

Because of this the interest in cytochemistry for research on nuclear elements has increased greatly during the most recent years. The study of the metaphase chromosomes has appeared especially promising as in that phase of the cell cycle the gene-carrying material must be assumed to be least cluttered up with non-genic materials. There are many indications that the very arrangement of the genic material in the chromosome plays an important role in the function of the gene system.

If we look back in time there have been many efforts to elicit microchemical information from different parts of metaphase chromosomes. Even in the early 'twenties experiments with dissolving and precipitating agents, chemicals, enzymes, dyes, were carried out. All this work has led to very little. There are two main reasons for that. One is the fact that no methods existed for quantitative cytochemical work, with a result that, for instance, in extraction experiments one has to record the results in some way and refer the data to some chromosome parameter. It was not easy to know even which parameter to refer to. Nowadays it is self-evident to use DNA. It might amuse you that in younger days I have even at one time had to live through quite a fight with a group that claimed that the chromosomes do not contain any nucleic acids at all—this might illustrate how confused the field was. The other factor blocking progress was the extreme difficulty in identifying individual chromosome regions. In order to do any reasonable analytic work one has, of course, to know that exactly the same chromosome region is studied in different experiments. For the most interesting objects in biomedical research, i.e. the mammals, the situation was especially bad, as you all are well aware—even identification of whole chromosomes was often not possible.

This focused the interest of the cytochemists and among them also our own biophysics group in Stockholm on two problems, firstly to elaborate as precise methods as possible for measuring the DNA-amount and distribution in individual chromosomes and chromosome parts and secondly to try to break through the identification block.

A few brief words of our line during the last few years. About 8 years ago ultramicrospectrophotometric techniques were developed to work

up to the very limit of optical resolution. Application to mammalian chromosomes showed that the determination of DNA could be achieved but was too difficult to be of practical use for identification purposes. On the other hand, determination of the DNA-distribution along the individual chromosome could obviously be used for chromosome identification purposes and in addition this could also give the "reference system" for other cytochemical procedures which I indicated above as being desirable. Regrettably, this method was so elaborate and difficult that it must remain a technique for the specialized laboratory.

The fact that DNA-distribution measurements could be used for chromosome identification led us to look around for other parameters than the mere DNA-distribution. As the base distribution in the chromosomal DNA must necessarily for statistical reasons be inhomogeneous along the chromosome it was quite natural to look for base-reacting compounds. For the observation of the reactant we chose fluorescence because of the enormous sensitivity of such methods and, in addition, we had just developed a very well functioning ultramicrofluorimeter. The rest of the story is well known to this audience. Together with an American chemist (Dr Ed Modest, Childrens Cancer Research Foundation, Boston, Mass.) the guanine-binding quinacrine mustard was picked out and proved to give such elaborate fluorescence patterns that they could be used for identification of chromosomes and chromosome regions. The fluorescence pattern proved not to be solely determined by the distribution of the dye as selective enhancement and quenching phenomena occur after binding to the DNA and by an amusing and ironic twist of fate it so happened that the AT-induced enhancement is so strong that the distribution of such groups has greater influence on the fluorescence pattern than has the GC-groups, where QM preferentially binds.

All of this will be discussed later and also the results with a great number of other fluorescent and non-fluorescent compounds, some of them giving more or less different patterns, a most important phenomenon.

It is a most encouraging fact that during the most recent years a whole series of methods have come along, showing banding of different types, produced in different ways. The physicochemical background is in most cases rather unclear, especially for the very important group of Giemsa techniques. The situation where one can produce different types of banding patterns is most encouraging even if somewhat confusing at present —an amusing fact is that one can get somewhat different patterns even by means of different components of the complex Giemsa mixture. All this means, however, that when we have penetrated the chemical background for the different methods we will be in the possession of a great number of new data on the chemical organization of the different parts of the gene-carrying elements of the nucleus.

This opens up entirely new possibilities for the cytochemical analysis of the organization of the gene system and I believe that we can look forward to rapid developments in the very next few years.

In this symposium most attention will be paid to the practical applications of different banding techniques to a variety of problems in biology and medicine. This is the reason why I have used this opportunity to speak at the beginning of our discussions in order to strongly emphasize also the great value of all of these techniques for the fundamental approach to the organization of the gene system and on its mode of work in cell differentiation and cell function. I believe that the new techniques will in the long run make their greatest impact on biology along those lines and that should induce us to look for ever-new banding methods and continually to try to find out what makes them work.

Chromosome Banding Techniques

Surveys of the cytological procedures

Fluorescence Banding Techniques

Lore Zech

Institute for Medical Cell Research and Genetics, Medical Nobel Institute,
Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Certain DNA reacting fluorochromes produce distinct banding patterns on metaphase chromosomes from many species. Work on plant chromosomes showed that those patterns were characteristic for each individual chromosome type and that they could therefore be used to distinguish between chromosomes of similar morphology (figs 1 and 2). Among the about 25 fluorescent compounds tested, the acridine derivatives quinacrine dihydrochloride (Q) and quinacrine mustard (QM) gave the clearest and most stable bands [7, 8, 9].

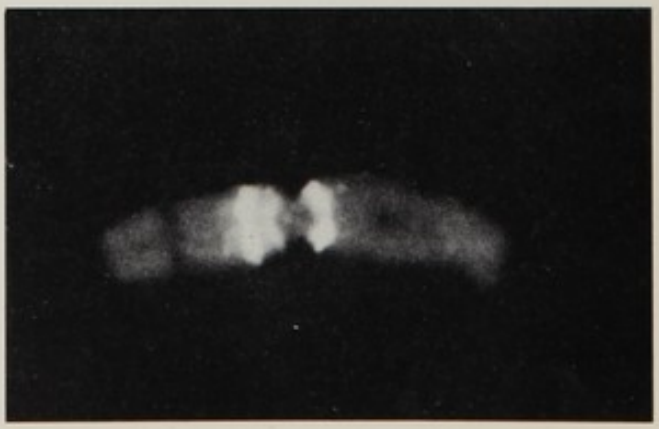
Application of the fluorescence staining method to human chromosomes revealed that they, too, showed distinctive patterns. The fluorescence patterns are different for all 24 human chromosome types and are so clear and reproducible that they can easily be used for chromosome identification [1, 2, 3]. The reproducibility has been proved by analysis of a quite large material of fluorometric measurements (described on p. 50).

The staining procedure, as performed in our laboratory, is the following: Conventionally alcohol-acetic-acid fixed and air dried chromosome preparations are rehydrated in an alcohol series and distilled water, soaked in buffer (McIlvaine's or Sörensen's phosphate buffer) pH 4.1–7.0 and stained either in Q (5 mg/ml) or QM (50 μg/ml) solution for 20 min. After staining, the slides are washed in three changes of buffer of the same pH as used for the staining, mounted in buffer and sealed. The chromosomes are investigated in a fluorescence microscope or—better—photographed in the fluorescence microscope and analysed on the photographic negative or on a print from the negative. Photography is of certain advantage, as under prolonged illumination the fluorescence fades and cannot be restored by restaining.

Fig. 3 shows the fluorescence patterns of all 24 human chromosome types. This arrangement of the karyotype was adopted a year ago by the IV International Conference on Standardization in Human Cytogenetics in Paris [18]. With the exception of a few very short regions of polymorphic character the fluorescence patterns along the chromosomes are identical on homologous chromosomes of the same individual and are also the same for corresponding chromosomes of different persons. So far no tissue-specific banding patterns have been observed either [4]. However, the number of tissues investigated in this respect is still limited because of practical difficulties connected with such studies.

The two X chromosomes of females and of XX-men cannot be distinguished from each other by means of their fluorescent bands [5]. However, the late replicating X chromosome can be distinguished in interphase nuclei of certain cell types, for instance cultured cells from the amniotic fluid, fibroblast cultures from skin and in a small number of cells from the buccal mucosa [14]. It appears as a weakly fluorescent body, larger and

Fig. 1. QM-stained metaphase chromosome from *Vicia faba*. Fluorescence microphotograph. (From ref. [7].)



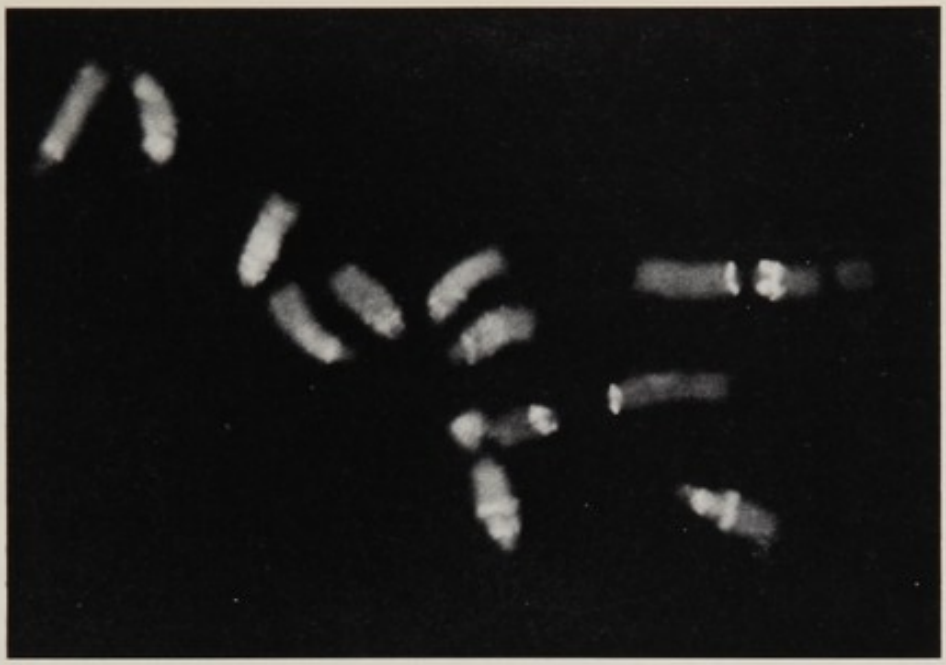
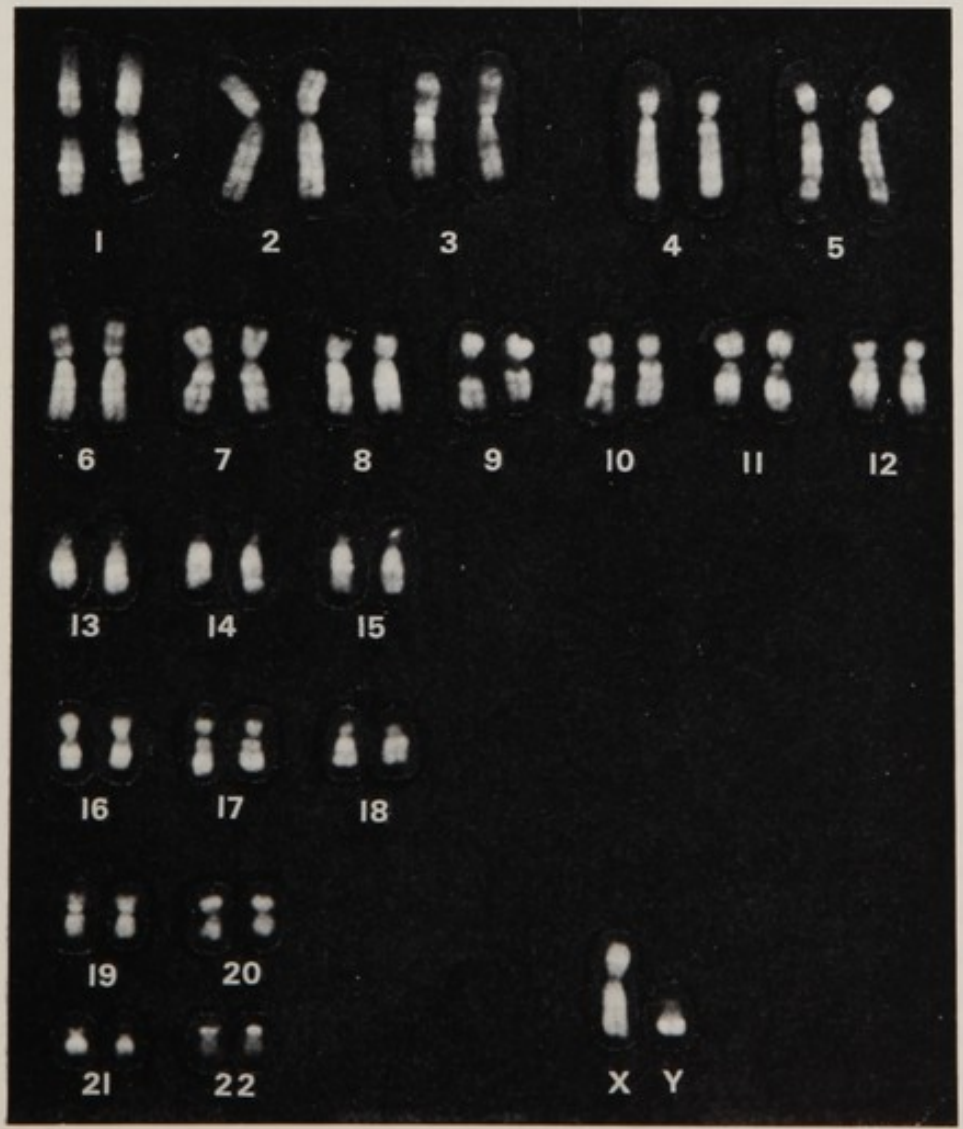


Fig. 2. Metaphase plate of *Vicia faba*. (From ref. [9].)

Fig. 3. The human karyotype stained with QM.



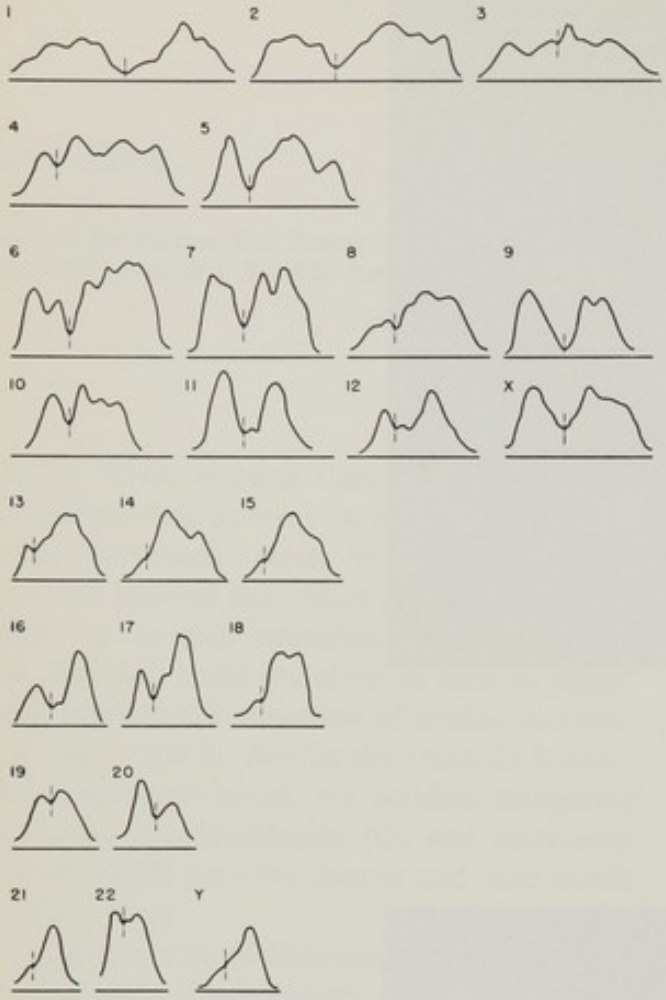


Fig. 4. Photometrically determined QM fluorescence patterns for the 24 types of human metaphase chromosomes. (From ref. [3].)

much less distinct than the Y chromosome in male cells. In lymphocytes from the blood, however, it cannot be seen at all after staining with fluorescent dyes.

In addition to the reproducible pattern of cross striation along all chromosomes, certain short polymorphic regions often exhibit an especially intense fluorescence. The most conspicuous chromosome in this respect is the Y chromosome with a very brightly fluorescent segment on the distal part of its long arm [17]. This region can vary considerably in length from individual to individual. It persists during interphase as a fluorescent spot which is easy to observe [16] in the nucleus and can therefore be helpful for sex determination in antenatal diagnosis and also, for instance, in screening studies for detecting subjects with an XYY chromosome condition.

In addition to the distal part of the Y chromosome, other—usually sma.ler—chromosome regions of polymorphic character are found close to the centromere of chromosomes 3, 4, 13, 14, 15, 21 and 22 [1, 2, 3]. All these regions together do not amount to more than 1–2% of the total metaphase chromosome length and do by no means complicate the identification work. The fact that they are heritable makes them very valuable for tracing the origin of some chromosomes, for instance in forensic studies, as paternity determinations or in cases of chromosomal abnormalities especially triploidy. Work of this type has already been published [11, 12, 13].

The distribution of fluorescence along Q- or QM-stained human metaphase chromosomes can, with a rather simple technique, be easily measured photoelectrically on photographic negatives or on positives [1, 2, 3]. If such measurements are recorded, the curves obtained represent an objective distribution pattern of fluorescence along the chromosomes (fig. 4) and, if so desired, these curves can be treated statistically [6]. At our Institute, about 30 000 curves from normal chromosomes have hitherto been measured by this method and the evaluation of this material, performed partly by computer, confirms that the chromosomal fluorescence patterns are precisely reproducible from one individual to another (except for the very short polymorphic regions described above) [15]. Therefore these curves give a reliable background for studies of chromosomal aberrations in clinical material or in material treated with external mutagens, as well as for the analysis of chromosomes in somatic cell hybrids between human and other species. The measuring technique is described more in detail by Caspersson (pp. 50–55).

In addition to Q and QM, a considerable number of other fluorochromes, first of all acridine derivatives, have been tested for their ability to give banding patterns on metaphase chromosomes. Most of these substances were found to be unsuitable for chromosome identification because their overall fluorescence was too poor and faded rapidly. Some of these fluorochromes, however, are of interest because they produced patterns which differ in small details from those obtained by Q and QM. For instance, proflavine and acriflavine, which both give distinct fluorescent bands on plant chromosomes [8], produce fluorescence patterns also on human chromosomes, although much less clear than those obtained with Q and QM. It is of special interest,

however, that the polymorphic regions of the autosomes as well as the distal part of the male Y chromosome do not display the bright fluorescence which is so conspicuous in Q- or QM-stained preparations.

On certain plant chromosomes ethidium bromide produces a banding pattern which is the reverse of that obtained by Q and QM [8]. In human metaphases, however, the pattern is not reversed but comparable to that obtained by proflavine and acriflavine, including the lack of the bright fluorescence in the polymorphic regions.

A fluorochrome which has been widely used for very different types of cytochemical work is acridine orange (AO). For investigation of metaphase chromosomes the fixation and isolation procedure, which affects the secondary structure of the DNP complex, strongly interferes with the AO-binding capacity of the chromosomes and the fluorescence patterns obtained are weak and not always reproducible. They become more pronounced if the chromosomes are treated prior to AO staining in one way or another. In some plant tissues, for instance, cold treatment before fixation produces a more distinct fluorescence pattern along AO-stained chromosomes. In human chromosomes partial denaturation of the DNA followed by renaturation treatment results in very characteristic changes of the stainability of certain chromosome regions with AO [10], thus indicating interesting differences in the molecular configuration of the chromosomal material.

In summary, it can be said that Q and QM are the best fluorochromes for chromosome identification found as yet. They produce banding patterns which are distinct, reproducible, and relatively stable during UV-irradiation. Photoelectrical measurements of fluorescence patterns are very easy to perform and have given a solid statistical basis for the reproducibility of the patterns. DNA-binding fluorochromes such as proflavine, acriflavine, ethidium bromide and acridine orange also produce bands on metaphase chromosomes which, however, are of little use for chromosome identification. Nevertheless, comparison between the mode of binding of such different substances can be expected to give valuable information about the chemical and physicochemical organisation of the metaphase chromosome.

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Constitutive Heterochromatin (C-Band) Technique

T. C. Hsu

Department of Biology, The University of Texas at Houston,
M. D. Anderson Hospital and Tumor Institute, Houston, Tex. 77025, USA

Since some cytologists still object to the term heterochromatin, the noncommittal term C-band suggested by the Paris Chromosome Conference [6] will be adopted here. Actually our definition will be very arbitrary, viz, C-bands are the chromosomes or chromosome segments which would consistently show deep Giemsa staining in response to the treatments originally proposed by Pardue & Gall [5], Arrighi & Hsu [1] and Yunis et al. [8]. It so happened that C-bands of *Drosophila melanogaster* coincide precisely with the heterochromatic segments well known among geneticists [3].

The C-band technique was discovered as a byproduct of the in situ DNA/RNA hybridization procedure. As far as revealing C-bands is concerned, some of the original steps were conceivably unnecessary, and some may be changed either to improve the quality or to adapt to special materials where the standard procedure yields poor results. Since the procedures are available in the literature, we shall refrain from presenting the detailed steps here but shall discuss only the points which may help the cytologists in obtaining C-bands in animal chromosomes.

Slide Preparation

Most laboratories use air-dried or flame-dried slides. A few laboratories still practice the classic squash technique. In our experience, squash preparations made with "subbed" slides give most reliable results, but air-dried preparations are acceptable. We do not prefer flame-dried preparations mainly because the G-bands also would show, to some extent at least, thus confusing the results.

We found that slides heated (60°C) for 15 min or so give more consistent results. Since most laboratories performing C-band staining are equipped with an oven set at 60–65°C, the heat-drying of slides should offer no additional hardship. We now use heat-dried preparations for all banding procedures.

Treatments

Stefos & Arrighi [7] modified the original procedure for revealing C-banding of avian chromosomes by deleting both the HCl and the RNase treatments, and by preparing the NaOH solution in SSC (1 × SSC, pH 12). Hsu [4] used their formula and obtained good results with *Drosophila* metaphases. In general, either the original (omitting HCl and RNase) or the Stefos & Arrighi system works well. Most investigators feel that NaOH at 0.07 N for 2 min is too strong and call for the reduction of normality or the shortening of treatment time. It is interesting to note that the standard treatment time was insufficient for certain carnivores (p. 104) and hedgehogs [2]. These authors used the original NaOH concentration and treated the slides for 5 min for best results.

Another modification, made by Lubs' group [4], is also noteworthy. It is to completely avoid the use of alkaline. Instead, the slides are treated in HCl (0.2 N) for about 15 to 30 min at room temperature (25°C) and incubated in SSC as usual. This method may be meritorious.

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Lubs: The amount of heat applied to the slide at the time of preparation is one important variable; if more heat is utilized in preparation, less rigorous treatment is subsequently necessary for C-banding. With flame-drying the NaOH treatment can be omitted and C-banding obtained.

Discussion

Ford: As one who had difficulties with the word "heterochromatin" many years ago and after the publication of the letter by Baker & Callan in Nature (166 (1950) 227) managed to get by without it, may I congratulate you on your objectivity. Of course we have need of a general term indicative of differential behaviour of chromosome segments. The trouble with the common use of "heterochromatin" is that so many differential properties have been attributed to it and it now has such strong overtones of identity that if one differential property is observed, it is tacitly assumed that other properties would also be exhibited. As Baker & Callan pointed out, there is less objection to the adjectival form "heterochromatic" which, though literally it means "differentially coloured", might be employed simply to mean "differential".

Pearson: What is the theoretical difference between flame-dried and squashed chromosome preparations as regards the production of both C-banding and G-banding?

Hsu: Probably the ways the cells are fixed. For squash preparations one uses 45–50% acetic acid and for air-dried or flame-dried preparations we use alcohol-acetic acid as fixative.

Hsu: (in response to a question by Pearson): There is a considerable amount of evidence to show that C-bands contain a large number of repeated DNA sequences. I would like to think that the differences in response to the treatments are reflections of the composition of the DNA (base composition, number of repeats, arrangement of sequences, etc) contained in these segments. I do not know whether the hypothesis can be extended to interpret the G-bands.

Giemsa Banding Techniques

W. Schnedl

Histologisch-Embryologisches Institut der Universität Wien, 1090 Vienna, Austria

The first demonstration of a differential staining of chromosomes was made possible by the quinacrine fluorescence analysis introduced by Caspersson et al. (see for instance [4, 5]). Later the so-called Giemsa banding techniques became available which were developed independently by several investigators (for instance [6, 7, 10, 16, 17, 18, 22]). With these techniques banding patterns can be seen in human chromosomes which, in general, agree with those visualized by the quinacrine fluorescence method. Several of the original Giemsa banding methods are derived from the Arrighi & Hsu method [1] and from the technique of Yunis et al. [25]. The use of proteolytic enzymes for demonstrating banding patterns in chromosomes was introduced by Dutrillaux et al. [7] and this method was modified later by several authors [21, 24]. Recently, still other techniques have become available, one of which uses potassium permanganate [23]. The interpretation of all these different methods which produce the same banding patterns in the chromosomes is rather complicated.

Material and Methods

Chromosome preparations obtained from blood cultures or fibroblast cultures can be used. Preparations made directly from bone marrow will also give good results if the chromosomes are not overcontracted. Our own material is prepared by a simple air-drying procedure. Some authors use flame-dried preparations. Such flame-dried preparations may require a modified treatment in order to obtain banding patterns. As mentioned above, several methods can be used for the demonstration of banding patterns.

In our laboratory the following method has proved to be most useful. First the slides are treated with NaOH. Several concentrations have been tried: 0.002, 0.007 and 0.07 N. The optimal concentration has to be tested for every new batch of preparations. Various treatment times should also be tested (between 30 and 120 sec). After washing in ethanol and drying, the slides are incubated for about 12–18 h in Sörensen's buffer (M/15 KH_2PO_4/Na_2HPO_4) at pH 6.8 and 59°C. Sometimes an incubation in buffer without an NaOH pretreatment will be optimal.

Besides Sörensen's buffer 2×SSC can be used as an alternative [22]. 2×SSC has a faster effect than Sörensen's buffer. The incubation time with 2×SSC must therefore be shortened. In our material, 6 h is optimal in most cases.

If banding patterns are prepared by using proteolytic enzymes, various concentrations of the enzyme (pronase or trypsin) should be tested. For descriptions see Dutrillaux et al. [7], Seabright [21] or Wang & Fedoroff [24].

Results and Discussion

With the Giemsa banding methods, characteristic banding patterns can be obtained in animal and plant chromosomes. Fig. 1 (*a*, *b*) shows karyotypes prepared from normal human males. Although the appearance of the chromosomes is somewhat different, both the incubation procedure (fig. 1*a*) and the trypsin method (fig. 1*b*) give the same banding pattern. The banding patterns in the chromatids are very similar to those which can be seen by the quinacrine fluorescence method. Additionally, most of the regions that can be stained by the Arrighi & Hsu method [1] are stained here too. Thus the secondary constrictions in chromosomes 1 and 16 are strongly stained. However, the secondary constriction in chromosome 9 remains unstained by the so-

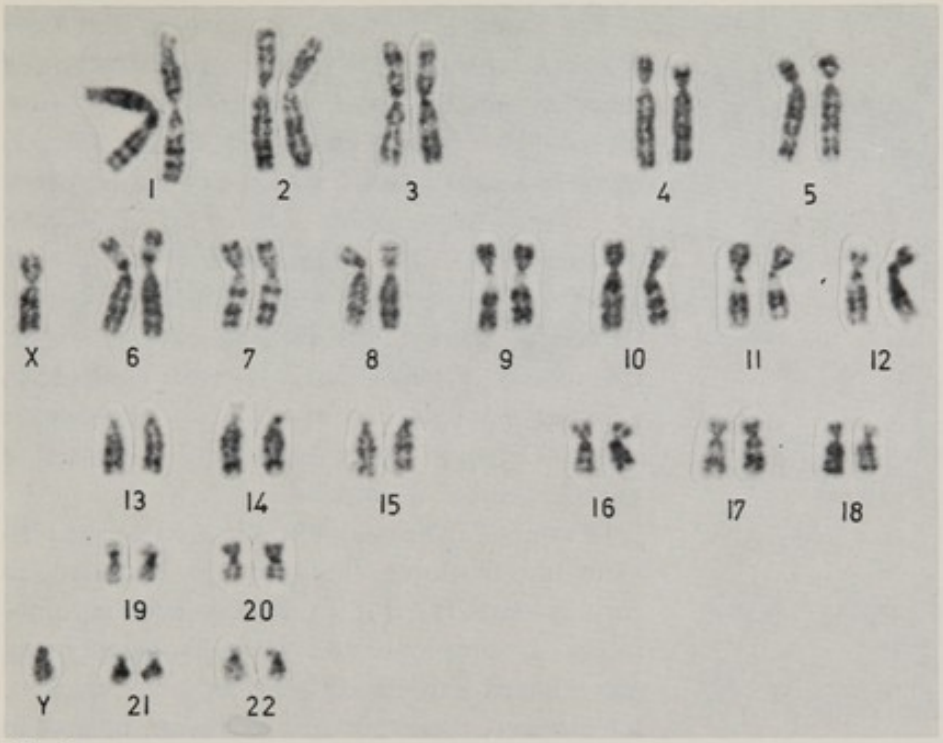


Fig. 1 a

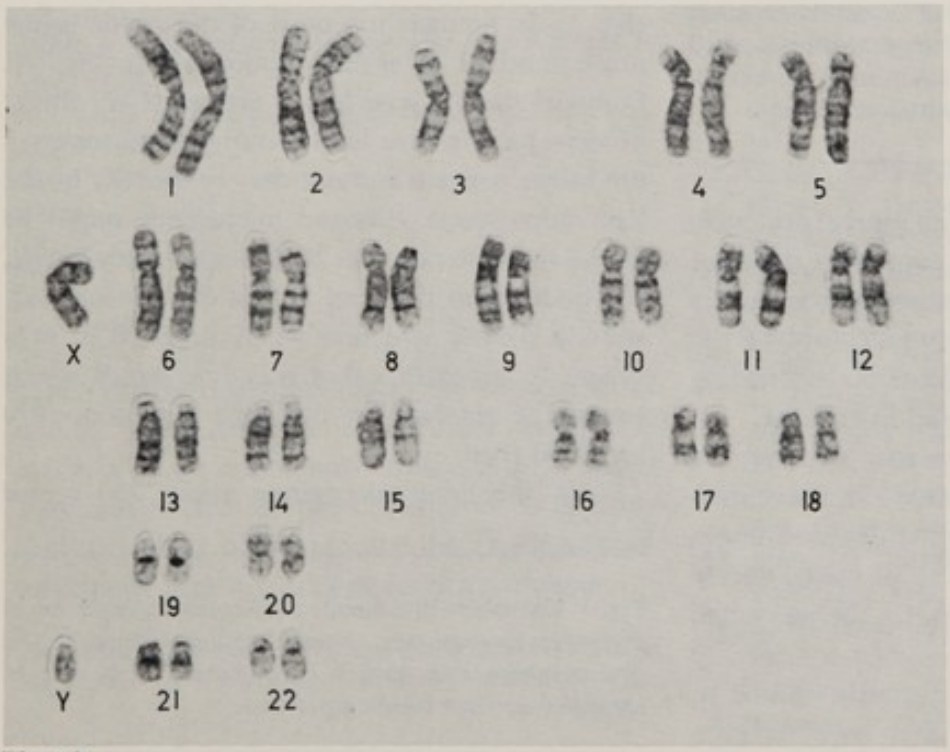


Fig. 1 b

Fig. 1. Normal human chromosomes, male. (*a*) Banding patterns obtained by the incubation procedure used in this laboratory; (*b*) banding patterns obtained by a treatment with trypsin or pronase [7].

called Giemsa banding methods, whereas it can be differentiated by the Arrighi & Hsu method. This region can be selectively stained by special methods [2, 9]. Also, in the Y chromosome, an inconsistent behaviour is observed.

In mouse chromosomes the Giemsa banding methods also disclose the quinacrine bands [8, 15] and the centromeric heterochromatin which can be detected at once by the Arrighi & Hsu method [3, 14, 19]. Therefore it is likely that in the Giemsa staining technique two mechanisms are superimposed.

In many mammals densely stained regions at the centromeres can be visualized when using the Arrighi & Hsu method, as has been extensively described by Hsu & Arrighi [13] and Arrighi &

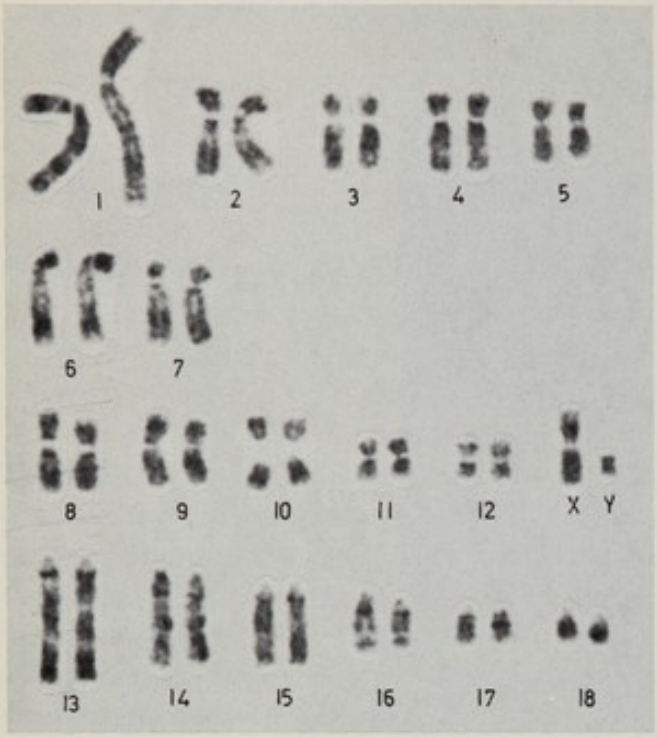


Fig. 2. Karyotype of the pig, male; Giemsa banding patterns. The centromeric regions of several chromosome pairs are occupied in material which remains unstained by the Giemsa banding method. However, in most cases, dark bands lie adjacent to these unstained regions.

Hsu [1]. Since this seemed to be a general principle, we were astonished to find a somewhat different situation in cattle [20]. The centromeric regions in cattle autosomes remain nearly unstained by the Giemsa banding methods, a situation contrasting the observations in humans and in rodents.

Negatively stained centromeric regions are found not only in cattle; in sheep, too, the centromeres remain unstained by the usual Giemsa staining methods and again, as in cattle, darkly stained material lies adjacent to the unstained centromeric regions.

The pig is a species more distantly related to the cow. Yet here too, several chromosomes contain unstained material at their centromeres. Fig. 2 shows a karyotype of the pig arranged according to the quinacrine banded karyotype published by Gustavsson et al. [12]. In pig too, darkly stained regions can be seen neighbouring the unstained centromeric regions.

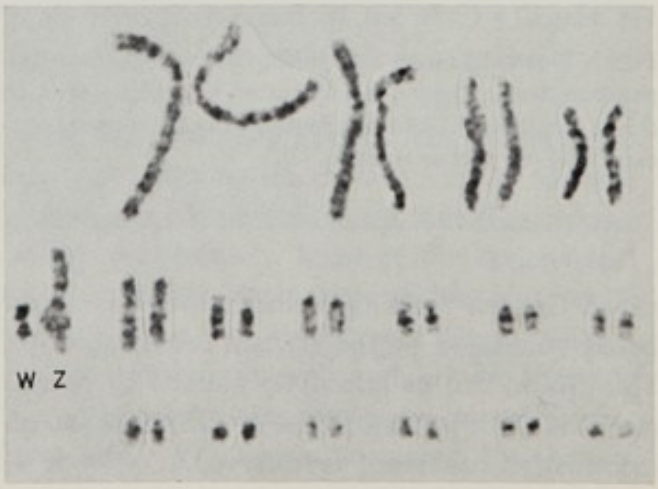
Material that remains unstained by the Giemsa staining procedures is not only found at the centromeric regions of chromosomes. In the hedgehog [11] similar material is localized in the distal parts of some chromosomes. A similar situation holds true in some carnivora (p. 104).

The banding patterns obtained in the autosomes are characteristic of all species. In cattle and sheep at least some of the chromosomes show identical banding patterns. However, also in other species, the total number of major bands (the bands seen in the contracted metaphase chromosomes) is of the same order of size. Table 1 lists the number of major bands in some species. Of course, such counts are subjective; however, the number of major bands is rather constant in different mammals. In man and in artiodactyla about 80 major bands are found. In mouse a smaller number is counted.

Of course, these major bands are not the ultimate substructures that can be observed in chromosomes [17, 18]. In the elongated chromosomes at prophase and prometaphase, many more bands can be differentiated. In chicken, for instance, these "minor" bands can be used to differentiate some of the microchromosomes (fig. 3). In humans too most of the major bands are composed of several minor bands (fig. 4). Some of these minor bands are relatively thick; others—for instance in B group chromosomes—are rather delicate and not easy to identify by the light microscope. Electron microscopy might be useful in these cases (p. 280). Some major bands, for instance in the long arm of chromosome 11, show a diffuse structure when analysed in prophase. It is possible that very fine bands which cannot be resolved by the light microscope are localized there.

With the light microscope about 250 minor

Fig. 3. Karyotype of a female chicken, including some of the microchromosomes; Giemsa banding method. In this prometaphase cell several microchromosomes can be identified by their banding patterns.



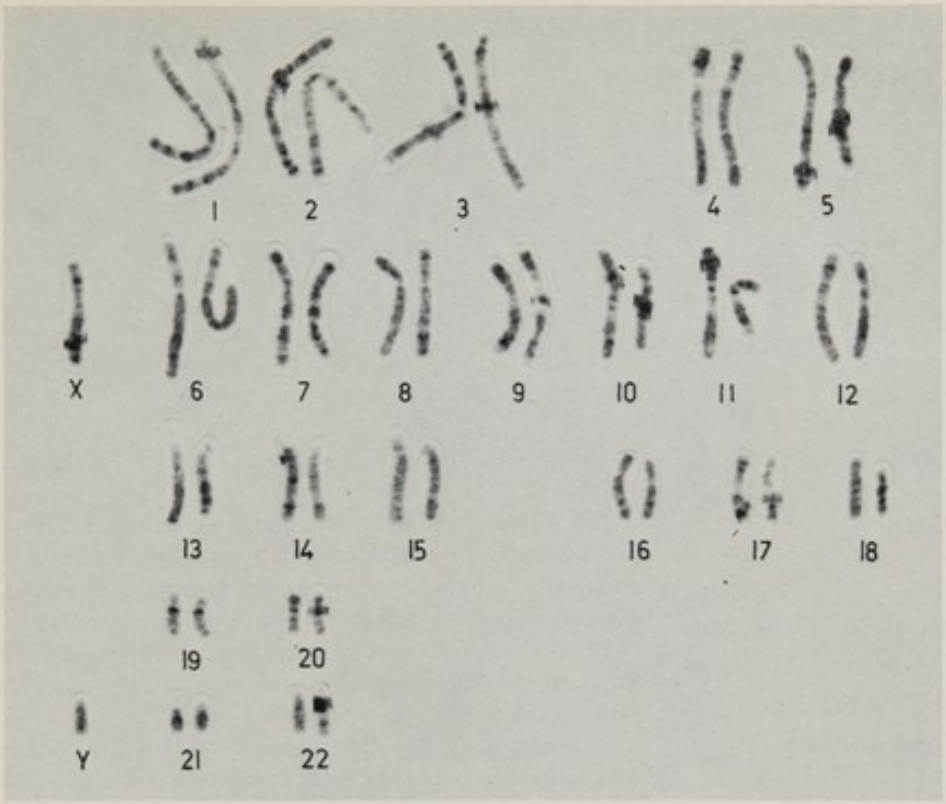


Fig. 4. Prometaphase of a human male, karyotype. Giemsa banding method. About 250 minor bands per haploid set can be identified in prophase cells of human.

Table 1. *Approximate numbers of major bands in some species*

man	82
cattle	81
sheep	83
pig	72
mouse	56
rat	66

bands can be differentiated in the haploid set of human prophase cells. If a rough estimate is made, a single minor band would include about 3 million nucleotide pairs. However, it is likely that the minor bands are not the smallest structural units that are present in chromosomes.

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Application to the Normal Karyotype of R-band and G-band Techniques involving Proteolytic Digestion

B. Dutrillaux

Institut de Progenèse, 750 06 Paris, France

Controlled Heating Denaturation: R-band Technique

After the description in April 1971 of the controlled heating denaturation technique [3] showing bands on chromosomes, other methods have been proposed by various authors [7, 10, 12].

However, this original denaturation technique has remained the only way of producing a banding reverse [2] to that of the fluorescence technique.

At the Paris Conference on Standardization in Human Cytogenetics, the bands revealed by this method were termed R-bands (as distinct from fluorescent bands, termed quinacrine or Q-bands) while the structures revealed by other denaturation techniques were called G-bands (revealed by Giemsa staining).

The R-band method has been routinely applied, with success, to more than 1 000 patients referred to this laboratory.

Technique

Since its introduction, this technique has been slightly modified and codified and gives, in effect, very constant results.

Following routine cell culture and the usual cytologic treatments [13], the slides are left to air dry spontaneously for several days. The best results are generally obtained after 8 days. Then the slides are treated with Earle's medium, settled at pH 6.5 for 10 to 20 min at 87°C, rinsed with tap water, and stained with Giemsa.

After an experience of one year, with phosphate buffer as denaturation medium, we finally preferred Earle's medium, which regularly gives the best results [1].

The slides previously stained by the ordinary technique may be treated directly without removing the stain [9], so that one can obtain standard staining and denaturation of the same metaphase. Different stages of denaturation can be observed. If the treatment is prolonged, chromosomes become unstainable.

The slides may react differently to treatment from one laboratory to another and it may be necessary to adapt the technique to each case. Generally speaking, the only step to be adjusted is the duration of heating.

Thus, if after 20 min of treatment, the slides do not show any modification upon staining, it is necessary to prolong the treatment.

Conversely, if the slides appear quite unstained, it will be necessary to shorten the treatment, even to a few minutes.

A good treatment causes a reduction of the general stainability and it may be useful, for the taking of microphotographs, to use phase contrast and an orange filter.

Results

As can be seen in fig. 1, the bands are located at identical points on the two chromatids of a chromosome showing the same pattern in the two homologues. All chromosome pairs are clearly identifiable. Centromeric zones and secondary constrictions are unstained. The ends of the chromosome arms (telomeric regions) are vividly stained, in contrast with the results obtained by other banding techniques. A different stainability can be observed in the region of the short arms of acrocentrics. This polymorphism is generally more evident after denaturation than with the conventional staining.

Proteolytic Digestion: G-band Technique

A few months after the description of the first denaturation technique, we had the fortune to discover the role of proteolytic enzymes in chromosome banding.

Following the initial description of the role of pronase in August 1971 [5] we made complementary reports about the use of other proteolytic enzymes, such as trypsin and α-chromotrypsin,

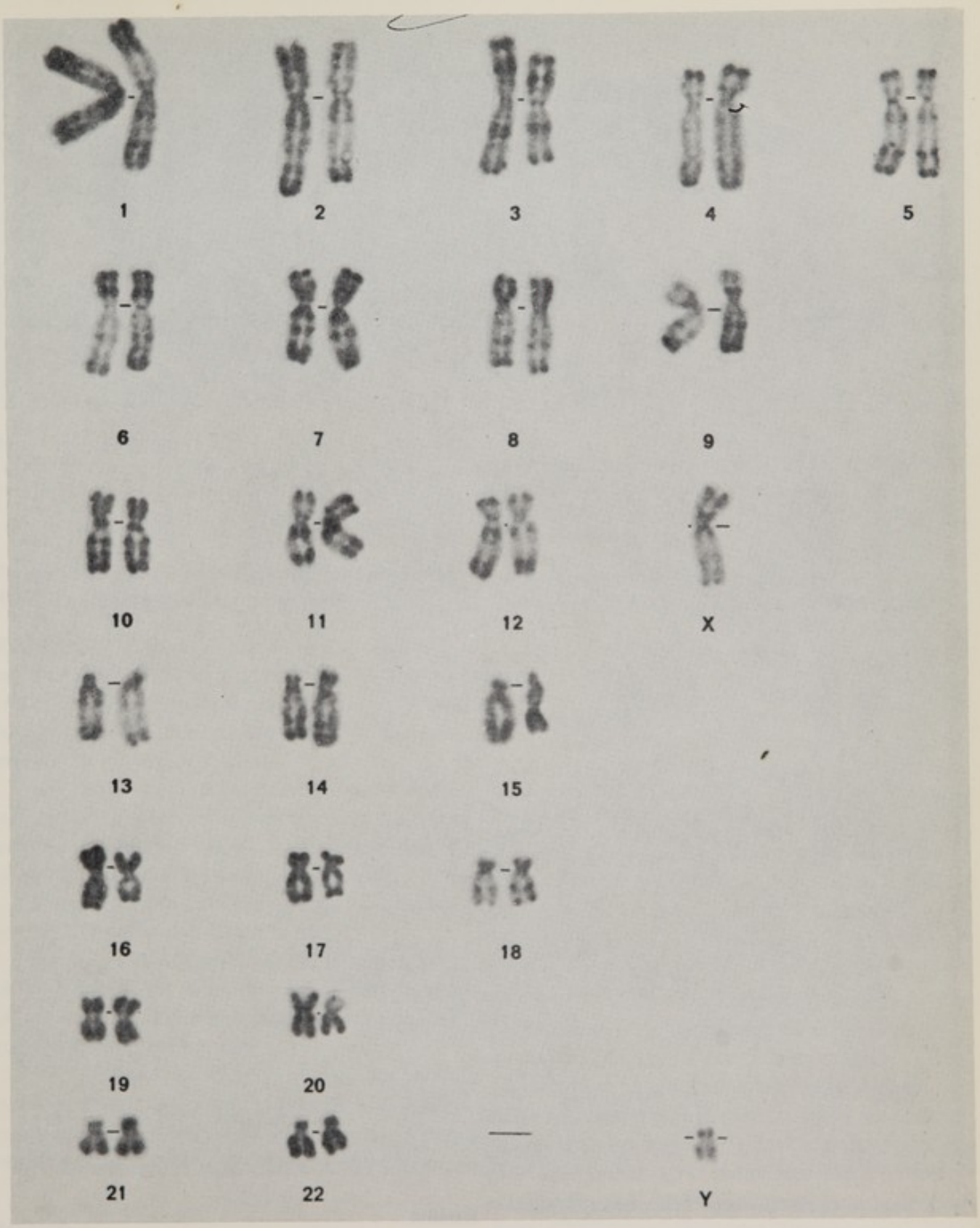

Fig. 1. Human male karyotype after controlled heating denaturation.

at the Paris Conference [4]. Subsequently, minor modifications were developed by several authors [8, 11, 14].

Technique

Proteolytic enzymes such as pronase, trypsin, α-chymotrypsin and protease, are generally used at a concentration of 0.05 mg/ml of buffered solution. For each enzyme, the pH will be chosen corresponding to the maximal activity. The incubation is made at room temperature or at 37°C. The duration of the treatment varies from some dozens of seconds to a few minutes.

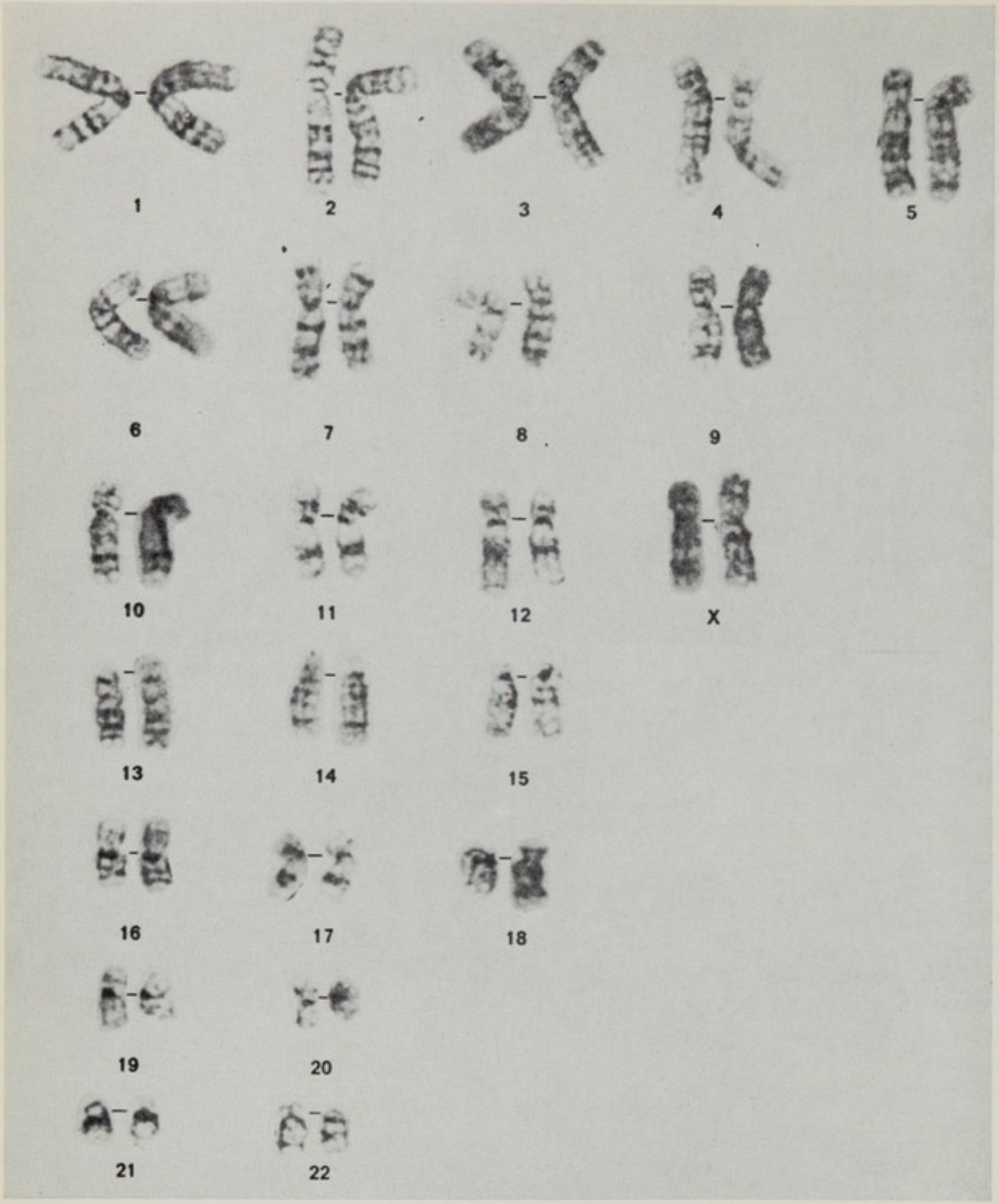

Fig. 2. Human female karyotype after proteolytic digestion.

With any of the various proteolytic enzymes which can be used, the results are (qualitatively) exactly comparable. Any difference depends much more on the good conservation of the enzymes used and on the quality of the preparations than on the nature of the enzymes themselves.

Results

In fig. 2 the chromosomes appear generally swollen; some parts appear as clear bubbles, others as dark bands. The localization of the dark bands corresponds to that of the G-bands obtained by denaturation techniques. Centromeric regions are vividly stained. Satellites are small and dark and satellite stalks swollen and clear.

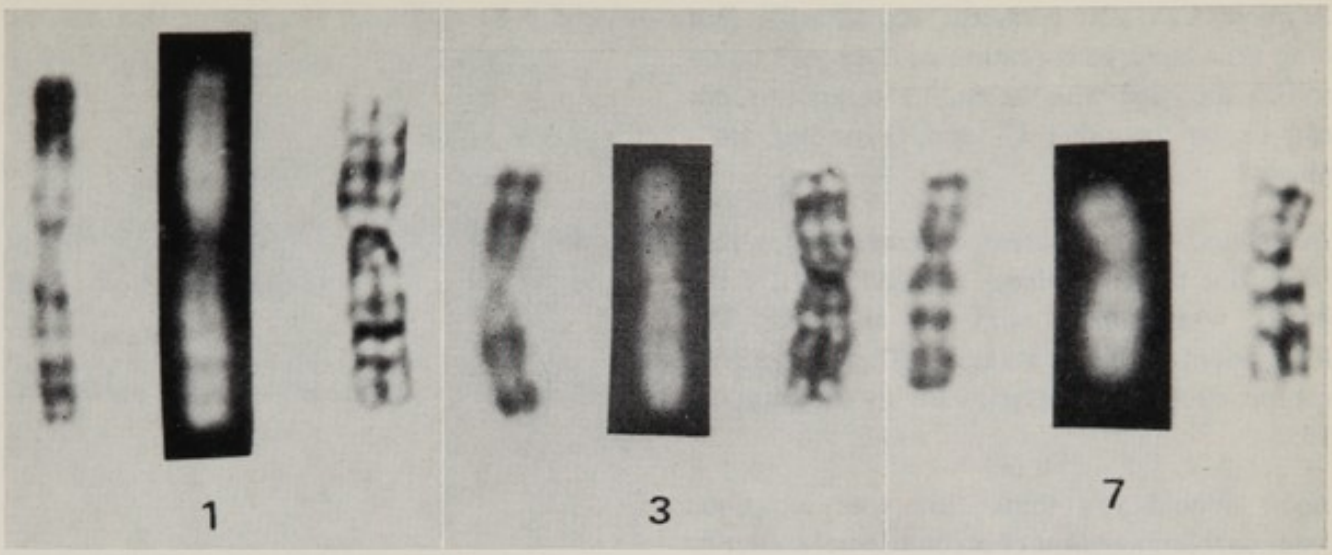

Fig. 3. Comparison of banding patterns obtained with controlled heating denaturation, fluorescence and proteolytic digestion: chromosomes 1, 3 and 7.

Comparison of Banding Patterns Obtained with these Two Methods and Fluorescence

The vividly stained bands observed after controlled denaturation correspond to the weakly fluorescing bands of the QM technique [2] as well as to the unstained but swollen bands revealed by proteolytic digestion [6].

Conversely, the lightly stained bands of the controlled denaturation correspond to the brightly fluorescent bands and to the deeply stained bands of the proteolytic digestion.

Particularly clear banding patterns of chromosomes 1, 3, and 7 are shown in fig. 3.

Finally, by using R-band denaturation and G-band proteolytic digestion, it has been found that the R-band technique gives the more regular and the more precise results.

This technique is actually applied for early metaphasic and prophasic chromosomes, giving an appreciable increase in information. These results will be reported in the near future.

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Discussion

Lejeune: With the same reagent, changing time or temperature, it is possible to produce at will G-, R- and C-bands. Hence the different varieties of treatment produce minor changes in the proteins which result in specific staining properties. By comparing these reactions it seems very likely that the amino acid content of these proteins could differ only slightly and nevertheless produce quite different staining under different conditions.

Lubs: We have observed the same sequence in response to over- or undertreatment.

Dutrillaux: It appears that the more physiological the denaturation medium is, the more precise is the banding. We use Earle's medium, which gives the finest bands.

Pearson: Can you comment on why the first long arm band in chromosome 2 cannot be resolved too well with the enzyme techniques, but can be by the other G- and Q-banding techniques?

Dutrillaux: This apparent contradiction is very likely due to the tendency to a diffusion of the bands, when treated with this technique. The locations of the bands are stable. The same phenomenon could be observed also in chromosome 15.

Bahr: Would you think that your technique leads to rearrangement of chromosomal matter or do you believe that there is any chemical specificity involved? I am intrigued by the fact that both in your technique and in electron microscopy the chromatid ends are most prominent.

Dutrillaux: We surely destroy, or modify the chromosome, and also all the material which surrounds it. During the very first steps of the technique of R-banding, we can observe a retraction, and probably a dissolution of the cytoplasm. This elimination of cytoplasm is slowed down at the place of the nucleus. Then the chromosomes appear "nude". They are, however, surrounded by some material which retracts and also is dissolved and removed. This may correspond to the "nucleoplasm".

As the nucleoplasm retracts, it first uncovers the centromeric regions of all chromosomes. Then this retraction continues around the chromosomes. This material stays closely related to the regions where the bands will appear.

We do not understand the significance of this strange mechanism, but it indicates that the chromosome cannot be considered as a simple molecule of DNA, but as a complex organelle with intimate relations to its surroundings, and that any treatment primarily directed against chromosomal DNA may also attack many other cellular components.

Immunofluorescent Studies of Chromosome Banding with Anti-nucleoside Antibodies

O. J. Miller, R. R. Schreck, S. M. Beiser and B. F. Erlanger

Departments of Human Genetics and Development, Microbiology, and Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032, USA

In 1966, we decided to investigate the possibility that immunofluorescent techniques could be used to probe the chemical structure of chromosomes if one used antibodies to DNA that were specific for a given purine or pyrimidine base, and studies were initiated the following year by M. V. R. Freeman, a postdoctoral fellow. Two years earlier, Erlanger & Beiser [6] had shown that it was possible to obtain DNA-reactive antibodies of the desired base specificity by immunizing rabbits to suitable hapten-protein conjugates. These were prepared by treating ribonucleosides or ribonucleotides with a slight excess of periodate to oxidize the 2′ and 3′ vicinal hydroxyl groups to aldehyde groups, coupling these derivatives to bovine serum albumin (BSA) at pH 9–9.5, and stabilizing the product by reduction with $NaBH_4$ to the tertiary amine. This procedure can conjugate up to 30 hapten groups per albumin molecule. Antisera to more than 20 nucleoside and nucleotide determinant groups, including the five major purine and pyrimidine bases and several dinucleotides have been produced in this way in the laboratories of Erlanger & Beiser [7].

The specificity of the antisera has been tested by a variety of techniques, including complement fixation, microquantitative precipitation and hapten inhibition of precipitin or complement fixation reactions. The antisera are highly specific, and it has thus been possible to obtain essentially monospecific anti-purine or anti-pyrimidine sera either directly from the animal or after appropriate procedures. These antisera react not only with the corresponding hapten-protein conjugate, but also with single-stranded, denatured or partially denatured DNA (table 1). They have the same specificity on reacting with DNA as with the hapten-protein conjugate, showing no cross reactions with any of the other bases in DNA [9].

Reaction with Denatured Chromosomes

These anti-nucleoside antibodies do not react with native DNA. In order to employ them in chromosome studies, it is first necessary to generate regions of single-stranded DNA within the chromosomes [8]. We initially used NaOH for this. The fluorescence appeared to be uneven along the chromosomes, and the centromeric regions did not fluoresce. However, the chromosomes were usually so distorted that distinctive banding patterns could not be seen. We obtained much better results when, at the suggestion of A. S. Henderson, we began denaturing the chromosomal DNA by a 30 min exposure at 65°C to a 95% solution of formamide in SSC (0.15 M sodium chloride and 0.015 M trisodium citrate), with or without 0.25% formaldehyde present to prevent renaturation of single-stranded DNA. We used an indirect immunofluorescent method, treating the slides first with an antinucleoside antiserum prepared in rabbits and, after a buffered saline rinse, with fluoresceinated anti-rabbit globulin produced in sheep.

Anti-A antisera produced detailed and consistent fluorescent banding along human chromosomes. Each chromosome could be identified by its characteristic banding pattern (fig. 1). These patterns were strikingly similar to those produced by quinacrine mustard ([2] and fig. 2) or by Giemsa or other basic dye staining after treatment of the chromosomes with trypsin ([15]

Table 1. *Specificity of anti-nucleoside antibodies*

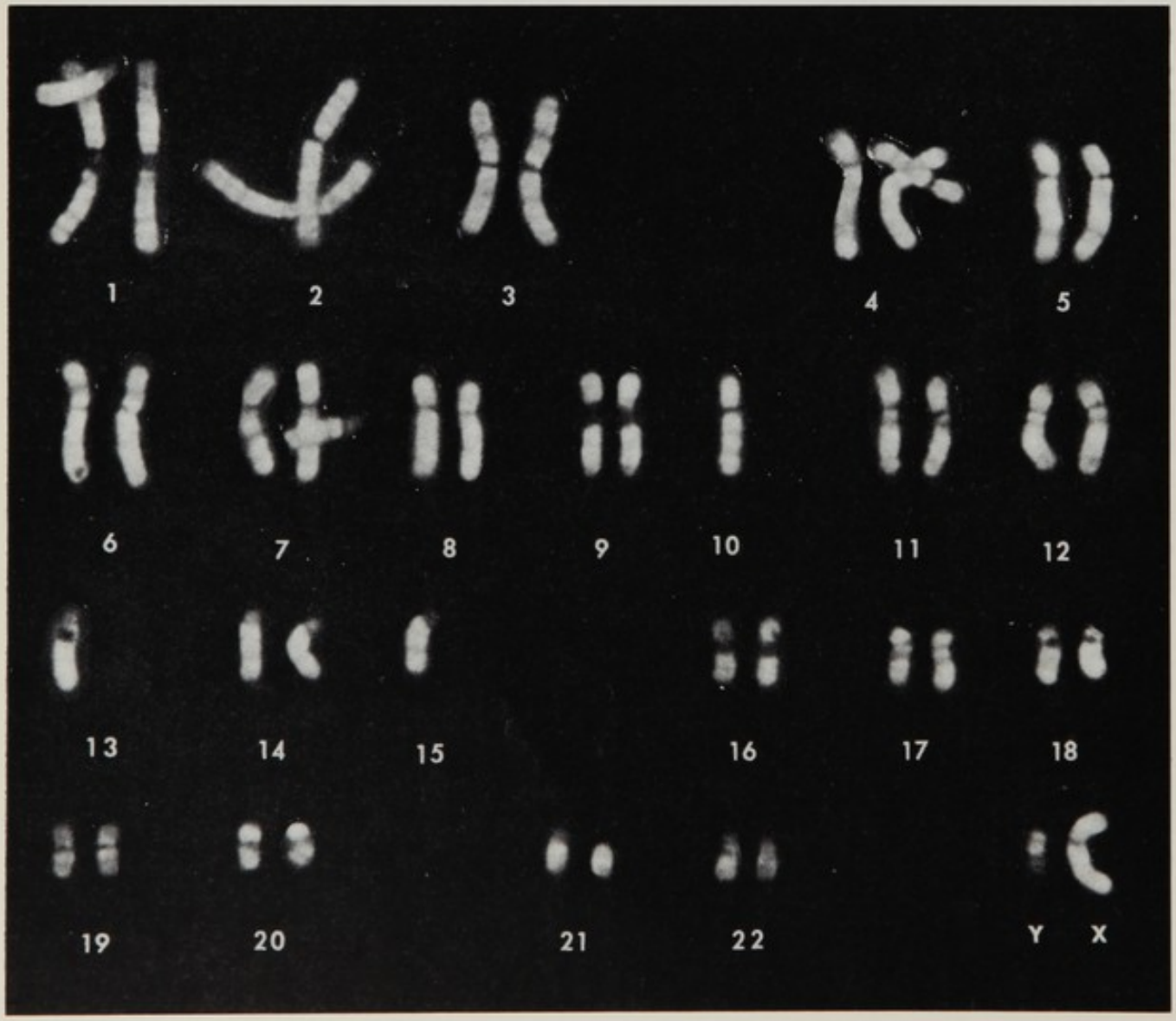
Antisera	Reactivity with A	T	C	G
Anti-A	+	−	−	−
Anti-T	−	+	−	−
Anti-C	−	−	+	−
Anti-G	−	−	−	+

A, adenine, or
adenine in single-stranded DNA, or
adenine conjugated to bovine serum albumin.

and fig. 2) or 2 × SSC at 60–65°C [17]. It should be noted that after the trypsin or SSC treatment, the chromosomes did not bind anti-adenosine as would have been expected if these treatments produced single-stranded DNA. Differential denaturation or renaturation is therefore unlikely to be responsible for the G-banding patterns. The marked similarities between the quinacrine and anti-A banding provide support for the suggestion of Pachmann & Rigler [12] and Weisblum & de Haseth [19] that bright quinacrine fluorescence occurs in AT-rich regions, since we would expect anti-A binding to be most pronounced in these areas. However, there is one striking discrepancy between the anti-A and Q-banding patterns: that of the distal part of the Y. Either this region is not AT-rich, or else it is AT-rich but the bases are not available for antibody binding, perhaps because of a lack of denaturation or rapid reassociation of DNA in this region.

The results obtained using antisera of different base specificity are relevant here. Anti-guanosine produced chromosome banding which was similar to that produced by anti-A but not as distinctive

Fig. 1. Immunofluorescent karyotype showing differential binding of anti-adenosine to formamide-denatured human chromosomes [4].



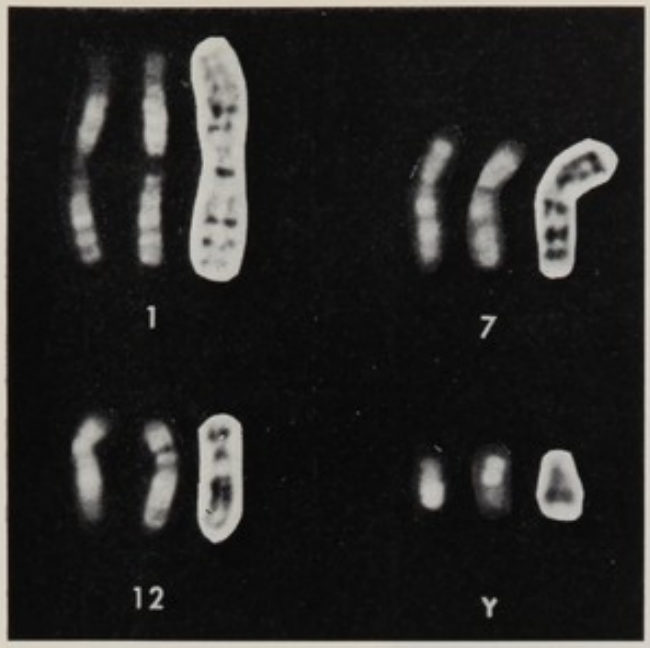


Fig. 2. Composite partial karyotype of four human chromosomes showing the close similarity of the banding patterns produced by quinacrine (*left*), anti-A (*center*) or the trypsin-Giemsa technique (*right*) [4].

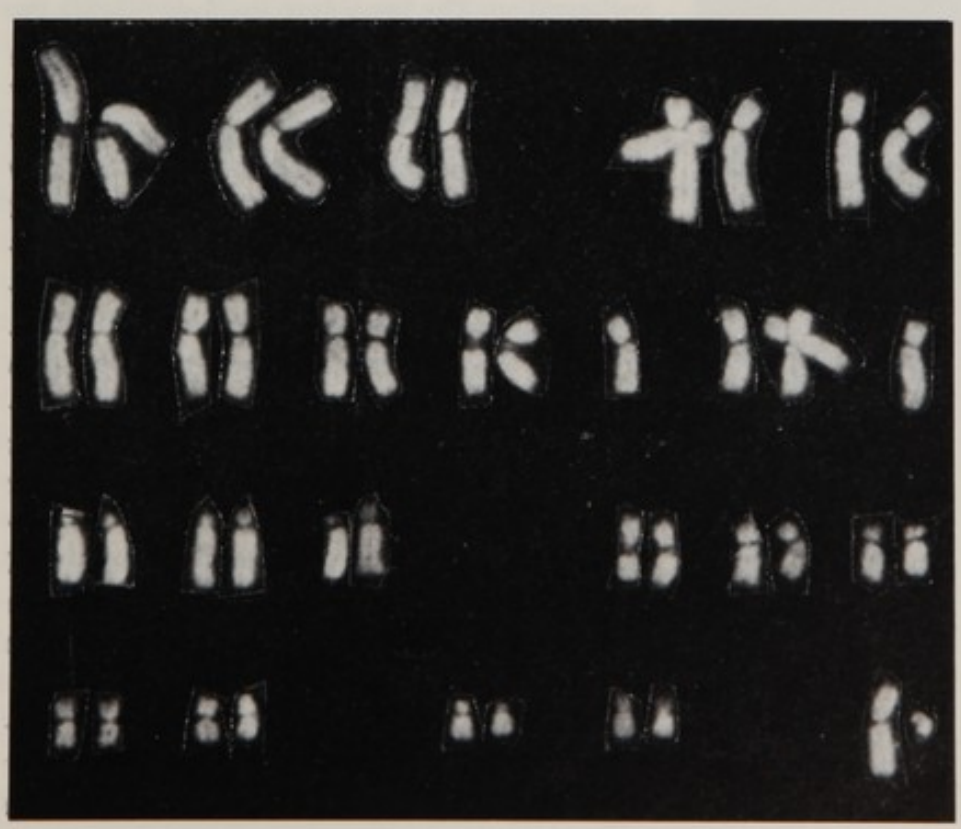
(fig. 3). As with anti-A, the most consistent feature was a non-fluorescent region at the centromere of each chromosome, including the secondary constriction of chromosomes 1, 9 and 16, and at the distal end of the Y. Anti-T and anti-C produced similarly banded chromosomes, and it seems reasonable to conclude that the lack of antibody binding in certain areas is the result of their lack of single-stranded DNA. The non-staining regions contain repetitious DNA which is capable of rapid reassociation and is therefore difficult to maintain in single-stranded form in fixed chromosomes.

Another problem remains to be explained. Although the banding patterns of anti-A were more distinctive than those produced by other antisera, the areas of brighter fluorescence along formamide-denatured chromosomes appeared to be the same no matter what the base specificity of the antibody. This could be due to the greater ease of denaturing regions of DNA that are AT-rich, because these have fewer hydrogen bonds stabilizing the double helix. In partially denatured chromosomes one might thus expect binding of anti-G and anti-C as well as of anti-A and anti-T antisera in AT-rich regions.

Fig. 3. Immunofluorescent male karyotype showing differential binding of anti-guanosine to formamide-denatured human chromosomes. The patterns are less distinctive than those produced by anti-adenosine.

Reaction with Photo-oxidized Chromosomes

We have tried to resolve this problem by using a different method for generating single-stranded regions of chromosomal DNA, a method that



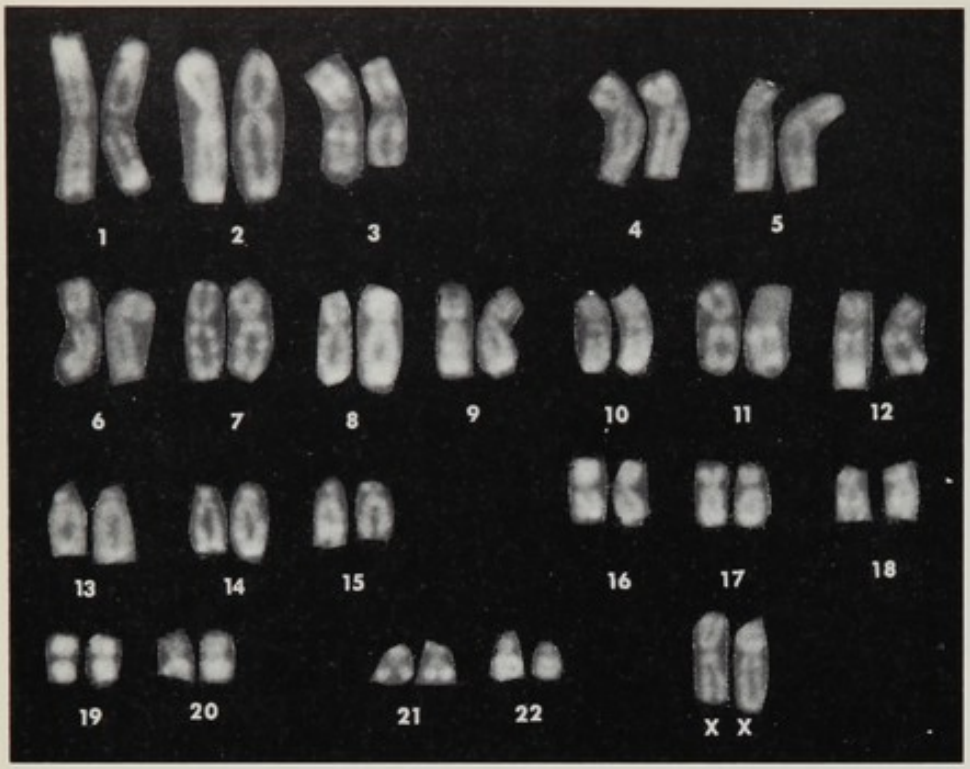
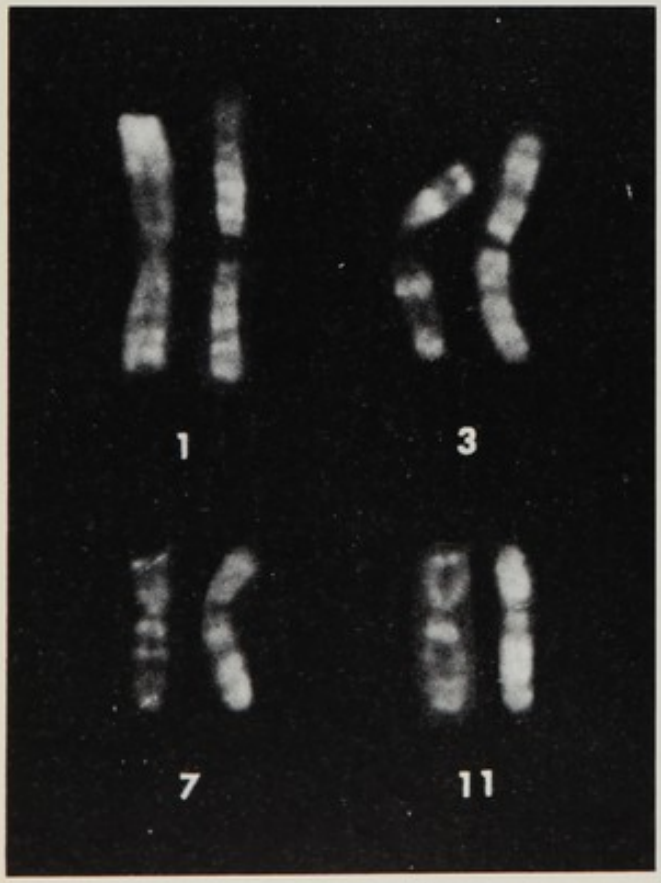


Fig. 4. Immunofluorescent karyotype showing the characteristic "reverse" banding patterns resulting from anti-cytidine binding to photo-oxidized chromosomes.

Fig. 5. Comparison of the immunofluorescent banding patterns produced by anti-cytidine binding to photo-oxidized chromosomes (*left*) with those produced by anti-adenosine binding to formamide-denatured chromosomes (*right*).



would permit a more direct investigation of GC-rich regions. Simon & Van Vunakis [16] showed that photo-oxidation of DNA selectively destroys guanine bases, with no destruction of adenine, cytidine or thymine. This should expose cytidine residues which were formerly hydrogen bonded to guanine. Photo-oxidized DNA in solution does bind anti-cytidine selectively [9]. We have now shown that photo-oxidized DNA in fixed metaphase chromosomes also selectively binds anti-cytidine. The procedure involves placing the slides in a methylene blue solution saturated with oxygen and exposing them to bright light for 15–18 h at 25°C. Indirect immunofluorescence is then carried out as before. Slides similarly prepared but treated with anti-thymine do not show chromosome banding. The banding patterns with anti-C, on the other hand, are characteristic, and consistent between homologues (fig. 4). The brightly fluorescent bands correspond to the weakly or non-fluorescent bands of chromosomes showing anti-A immunofluorescence after formamide denaturation (fig. 5). The underlying banding pattern of each chromosome is virtually identical by the two methods, but the pattern of light and dark is reversed. It seems likely therefore that the chromosome regions which bind the most anti-C after selective destruction of guanine by photo-oxidation are GC-rich. The anti-C banding patterns are quite similar to the reverse, or R-banding, patterns by Dutrillaux & Lejeune

[5], and, like them, are the reverse of the Q- and G-banding patterns.

Our results suggest that the banding patterns of chromosomes reflect localization of AT-rich and GC-rich DNA in surprisingly large chromosome regions, with quinacrine and quinacrine mustard fluorescence being brightest in AT-rich regions. Further support for this idea comes from our observation that after the photo-oxidation procedure, if one stains with quinacrine mustard instead of fluorescent antibody, one sees the normal quinacrine banding patterns. This would not be expected if bright quinacrine fluorescence occurred in GC-rich regions, since so many of the guanine moieties are destroyed by photo-oxidation.

Studies with Less Specific Anti-nuclear Antibodies

Other attempts to apply immunofluorescent techniques to chromosomes have met with less success, in part because the chromosome preparations in earlier years were not as good, but also because the antibodies used were less specific. Several investigators used antinuclear sera from patients with lupus erythematosus [1, 10, 13, 14, 18]. In none of these studies was there evidence of chromosome banding comparable to what I have described. Razavi [14] did obtain remarkably uneven fluorescence of metaphase chromosomes using lupus sera specific for single-stranded DNA, but homologous chromatids showed quite different intensities of fluorescence, suggesting the operation of some non-biological factor in his system. Mace et al. [11] have recently reinvestigated this problem, and observed even fluorescence except in C-banding regions. The non-fluorescent areas usually corresponded to the centromeric region of telocentric and biarmed chromosomes of mouse origin, but a few were located elsewhere, although still in regions which were C-banded. The lack of antibody uptake in these regions is analogous to what we observed in the centromeric region of the human chromosomes, and presumably reflects the limited dissociation or rapid reassociation of DNA in the regions. Desai and his associates [3] have shown that antibodies to pooled histone fractions can be prepared and that they react with chromosomes. Whether there is differential uptake of anti-histone along human chromosomes is not clear.

In conclusion, it is abundantly clear that immunofluorescent techniques, using highly specific antibodies to nucleoprotein components, have a great deal to offer in the analysis of the chemical structure and function of chromosomes.

The work reported here was supported in part by grants from the National Institute of General Medical Sciences (GM 18153), the National Cancer Institute (CA 12504), the National Institute of Allergy and Infectious Diseases (AI 06860) and the National Foundation – March of Dimes. O. J. M. is a Career Scientist of the Health Research Council of the City of New York. R. R. S. is a pre-doctoral trainee (HD-00349).

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Discussion

Modest: Is it generally true that the anti-adenosine fluorescent antibody does not visualize *any* of the polymorphic fluorescent variants (including the Y)? Your slides so indicate.

Miller: I think your suggestion is quite correct. We have not identified any of the polymorphic quinacrine fluorescent variants by immunofluorescence, but on the other hand we have not really looked for them very hard. Certainly no complete study has been done.

Rigler: Part of the difference between Q-bands and anti-adenosine-antibody bands you pointed out in the X-chromosome might possibly be due to the fact that your technique requires single-strandedness. A simple explanation—which of course has to be tested—would be that this particular Q-band could be—for yet unknown reasons—thermally more stable than the other Q-bands.

Menezes: Did you always use the direct immunofluorescent staining procedure, and what is the type of globulin you get as anti-nucleoside antibody? I would also like to know your opinion on double staining of the chromosomes with specific antibodies, against two different nucleosides, conjugated, e.g., one with fluorescein-isothiocyanate and the other with rhodamine. This type of staining may give more detailed fluorescence patterns and may also give further information on the specificity of fluorescent antibody binding in chromosomes.

Miller: We used an indirect immunofluorescent technique, using anti-nucleoside antibodies formed in rabbits and fluorescein-tagged anti-rabbit globulin prepared in sheep. The double staining technique you propose might be informative, although no more so than successive staining of the same metaphase cells with different antibodies. This could be done using black-and-white film as we do now, whereas the procedure you propose would require color film, and we have not yet assured ourselves this would be as satisfactory.

Chromosome Banding Techniques

Surveys of techniques for the study of pattern constancy and for rapid karyotype analysis

Procedures for the Study of the Reproducibility of Normal Mammalian Banding Patterns and for Analysis of Aberrant Chromosome Patterns

T. Caspersson

Institute for Medical Cell Research and Genetics, Medical Nobel Institute,
Karolinska Institutet, S-104 01 Stockholm 60, Sweden

The basic prerequisites for the use of any banding technique in biology and medicine is that it can be shown that the patterns differ for all the various chromosome types and the main regions within the chromosomes, and furthermore that they are constant and reproducible.

It should be pointed out that the only method for which at present a solid statistically evaluated basis for such claims exists is the fluorescence method and that most other methods rely on and are dependent upon comparisons with the fluorescence patterns. Because of the importance of this question it seems proper at this Symposium to present briefly the ways and tools used for the analysis of the fluorescence patterns which are described by Zech (pp. 28–31). The emphasis will be on the human karyotype.

The basic reason for this situation is obviously to be found in the fact that fluorescence banding was the first technique giving many intrachromosomal details and thus quite a large material of the type indicated was needed in order to convince ourselves and others that the technique could be of practical use. Furthermore, the cytological technique works in a very reliable and reproducible way and the fluorescence patterns can be measured and recorded objectively rather easily, involving much simpler instrumentation than would be needed for all other banding techniques described as yet. For many of the latter methods objective recording of patterns is of no practical value because of irregularities in the staining and, especially, shrinkage of chromosome parts due to various factors active during the staining procedures.

In my introductory remarks to this meeting I described how the demonstration that banding pattern analysis — at first DNA-patterns and then fluorescence patterns — could provide a means for the identification of chromosomes and chromosome regions was a direct result of quantitative cytochemical work involving measurements on metaphase chromosomes.

In the continued work it proved necessary to modify or develop suitable instrumentation, based on cytochemical techniques and instruments earlier constructed in our laboratory.

To summarize: The following 4 fields were those where special biophysical tools were needed and developed.

(1) Objective recording of fluorescence patterns with very high optical resolution.

(2) Development of such rapid instrumentation for pattern recording that solid evidence of reproducibility and/or variability could be obtained. This calls for pattern measurements in the 10 000 range at least, in order that statistical treatment of the material would be meaningful.

(3) Identification of chromosome regions, in order to make a detailed analysis of chromosome aberrations feasible.

(4) Procedures for rapid identification of aberrations. Exploitation of the new possibilities which the banding techniques afford for studies on such important problems as, for instance, induction of chromosome aberrations by external mutagens and carcinogens, chemicals or radiations, demands analysis of such large materials of metaphases that specially fast techniques are necessary.

Literature reference [1] contains references to the several publications which cover the work on these fields until the end of 1971. Reference [2] presents the basic instrumentation for quantitative cytochemistry.

Fig. 1 shows a fluorometer developed for high resolution chromosome analysis and fig. 2 gives the principle for the instrument. Important prob-

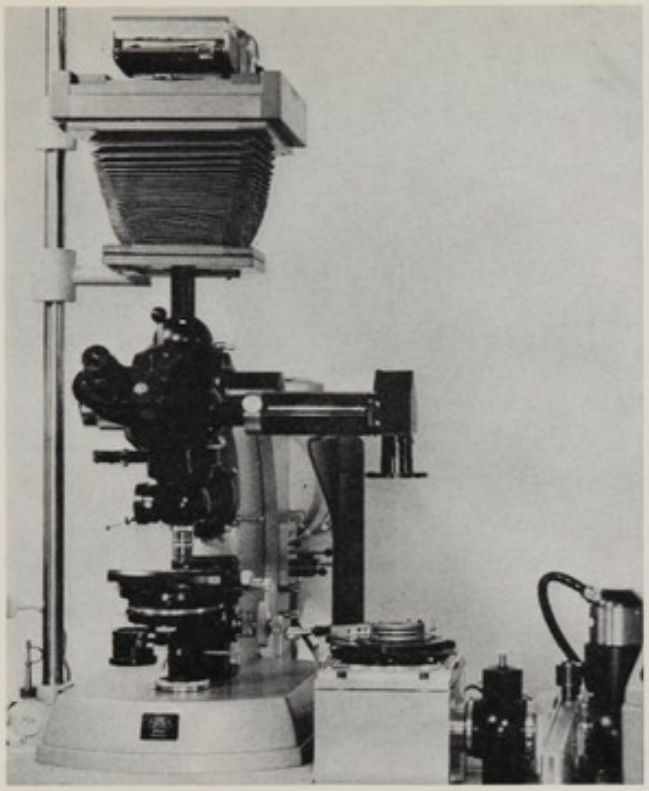


Fig. 1. The high resolution ultramicrofluorometer for measurements directly in the specimen.

lems in fluorometry, especially in work at high resolution, are the avoidance of overexposure of the object, and the selection and exact identification of the region to be measured. All settings before the measurement under UV-illumination can thus be made with the aid of conventional phase contrast optics. For the precise identification of the small area to be measured a special device is employed, whose principles are illustrated in fig. 2. The fluorescence picture is projected onto a movable stage, on which adjustable diaphragms of different shapes and sizes can be placed. The light passing through the diaphragm proceeds via the field lens to the photomultiplier. A special device makes it possible for the diaphragm to be illuminated from behind with red light, a picture of the diaphragm being fed back to the eye via a system of prisms. Thus the eye sees the complete fluorescence picture at the same time as it also sees the diaphragm aperture in the form of a red area. Scanning measurements can be made by moving the stage. An "Interferenzverlauffilter" is applied in front of the photocell for closer spectral analysis.

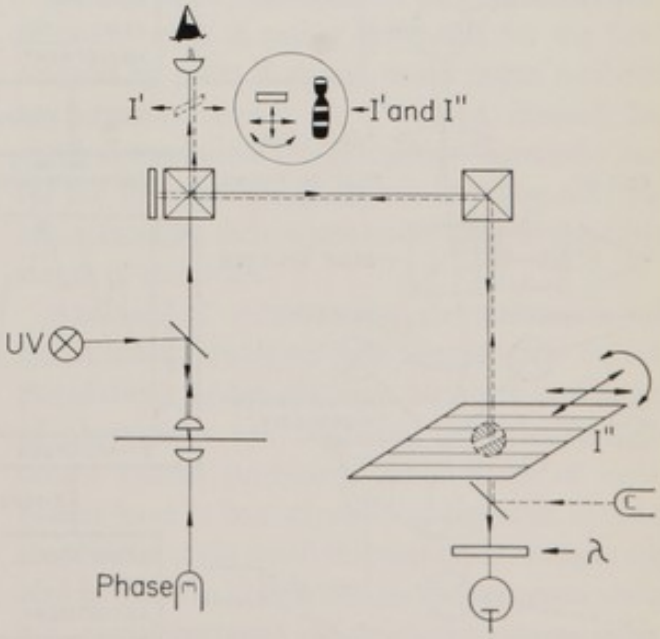
In routine work the pattern measurements are made by using as diaphragm a very narrow slit, perpendicular to the length axis of the chromosome and moved along the length of the chromosome. The paper by Zech (p. 28) shows such curves.

With the instrument described, measurements can be made with an optical resolution very close to the physical limits. The primary observations of the differences in the patterns in human chromosomes were won in this way. In order to investigate the reproducibility or variability of the patterns, however, a machine working very much faster was needed—point 2 in the list on p. 50.

The crucial characteristics in the patterns used for the identification work are the location of the individual fluorescent bands and their relative intensities within the same chromosome. Absolute intensity measurements would add very little and also the need for precision is not very great in the relative intensity measurements within the individual chromosome. This makes it possible to build quite convenient measuring devices working very much faster than the quite precise but also quite slow instrument shown in fig. 1.

An arrangement working with photographic fluorometry is depicted in fig. 3. A photograph is taken of the metaphase in the fluorescence microscope and either the photographic negative or positive print thereof is evaluated in the densitometer/reflectometer, in which the photograph is automatically moved through a measuring system and the patterns recorded on an X–Y-recorder. Fig. 4 shows a diagram of the instrument.

Fig. 2. Diagram of the ultramicrofluorometer. For details see text.



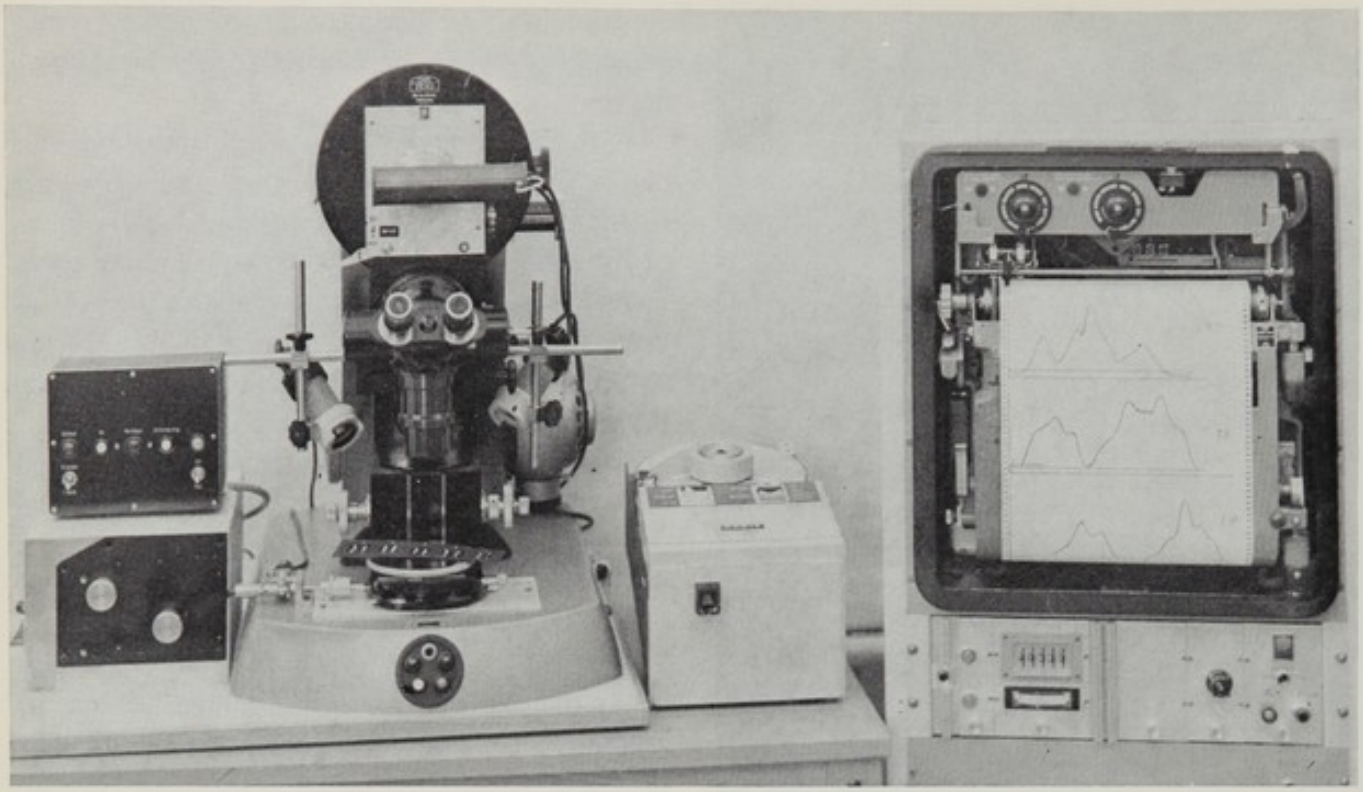


Fig. 3. Combined densitometer-reflectometer for recording of fluorescence patterns from fluorescence microphotographs.

The first description of the 24 fluorescence patterns of the human karyotype was based on a material of 5 000 measured curves. Details about the stability of the patterns and about the short regions which show variability from person to person are given in Zech's paper (p. 28). The observational material has been expanded to include a total of approx. 30 000 measured fluorescence patterns. Apart from negligible details of pattern, the results have entirely corroborated the patterns described above.

In the statistical analysis of the reproducibility of patterns, computerized techniques have been extensively used in several ways. Incorporation of a tape punch in the reflectometer, as indicated in figs 3 and 4, has greatly facilitated the computerized analysis. When the fluorescence pattern is recorded it is simultaneously digitized and punched onto the tape which can then be directly evaluated at the computerized analysis.

In their paper in this volume Møller & Nilsson

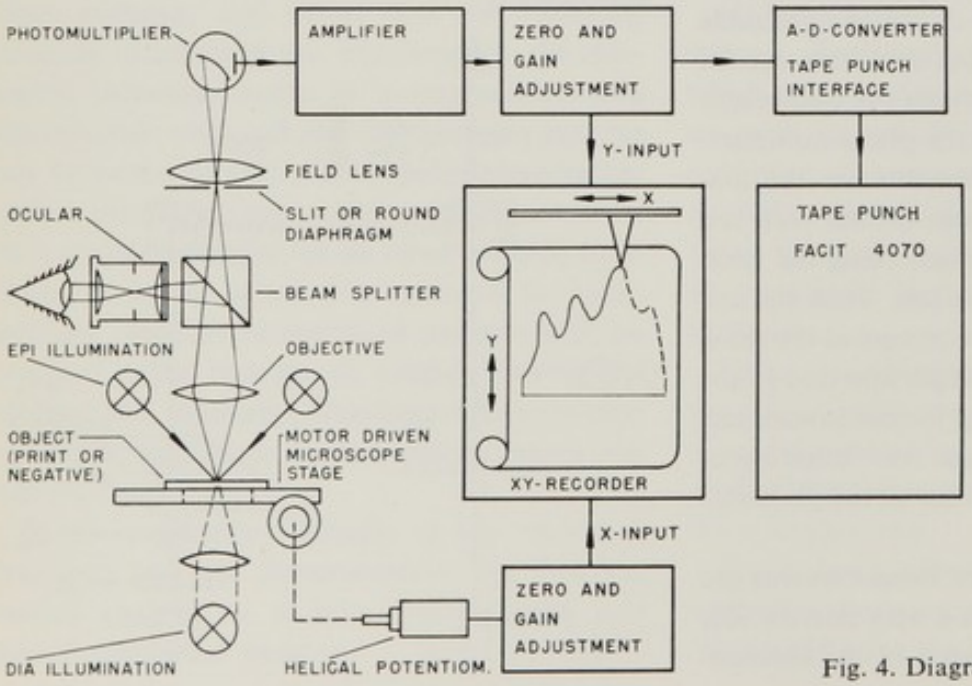


Fig. 4. Diagram of the instrument in fig. 3.

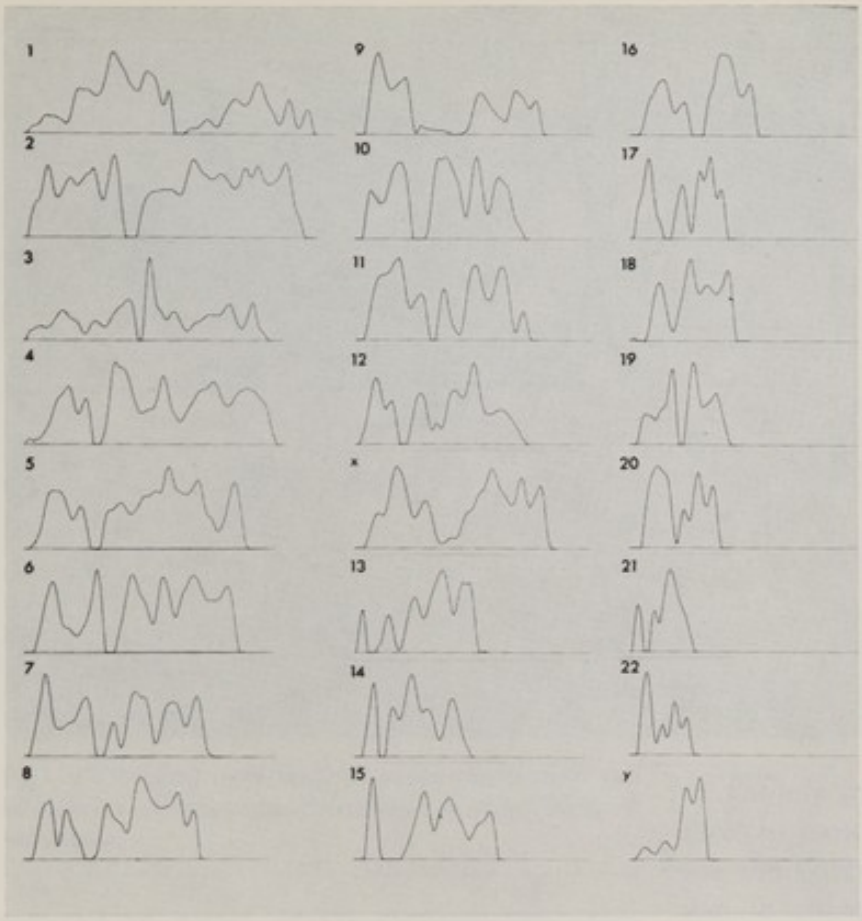


Fig. 5. The human karyotype. Contrast-enhanced patterns.

(p. 56) have described statistical methods used [3] and also the use made of the patterns for computerized recognition of chromosomes.

Point 3 on p. 50 refers to the very important question of defining suitable reference points within the chromosomes for identification of breakage point, etc., in various chromosome aberrations [3, 4]. This calls for identification of the main and most easily recognized fluorescent band in the chromosomes, a task well suited to computerized analysis. Møller & Nilsson describe in their paper (p. 56) the mathematical methods used for contrast enhancement and band localisation and give an example of how a curve appears in different degrees of contrast enhancement. Fig. 5 shows the whole of the human karyotype in very strong contrast enhancement, and fig. 6 the standard, non-enhanced, patterns of the human karyotype with the places for the conspicuous bands marked with vertical lines.

The fact that many constant reference points can be defined in banding patterns makes it possible to analyse chromosome details with greater precision than was earlier possible. As is evident from the presentations on pp. 164–256 such analyses and also general chromosome recognition work can be carried out without measuring devices—these serve the purpose of giving a solid foundation for the banding pattern analysis. However, in the analysis of complex chromosome aberrations, experience has shown that pattern measurement can be of considerable help in the identification of the individual chromosome parts involved.

The visual or photographic chromosome identification work is rather slow, and for the field covered by point 4 above, much faster methods are urgently needed. A TV-based method for rapid chromosome identification—especially useful for fluorescence work but of value also for other banding techniques—was used in the early stages of the work.

Analyses of chromosome aberrations caused by different mutagens and carcinogens in cell populations where different numbers and types of aberrations can be found in different cells create special problems. Large series of metaphases have to be analysed. Furthermore, in order to observe quite small chromosomal changes and also in order to obtain detailed information about e.g. translocations, it is necessary to compare

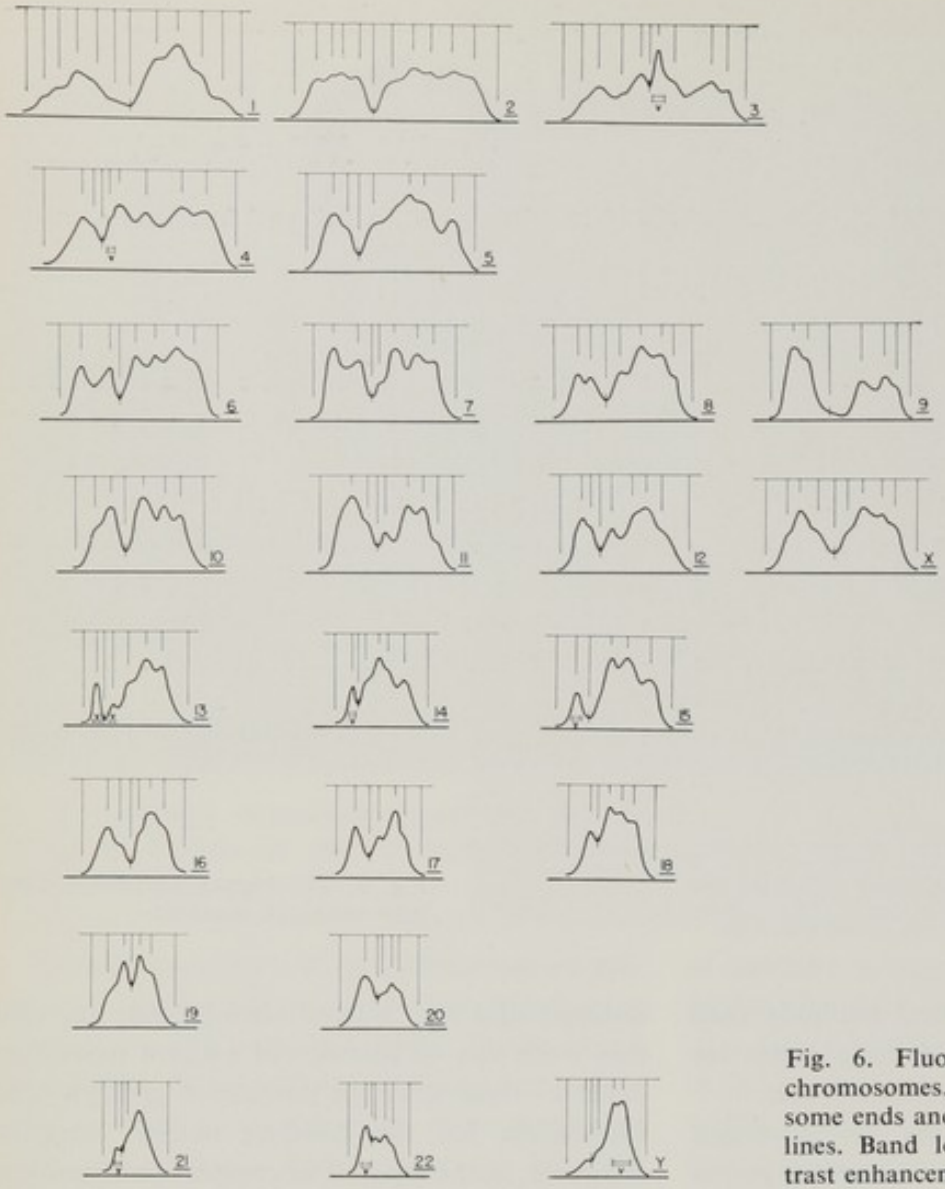


Fig. 6. Fluorescence patterns of the human chromosomes. Prominent bands (and chromosome ends and centromere) marked by vertical lines. Band locations determined by aid of contrast enhancement through digital filtering.

the detailed patterns in different chromosomes.

Fig. 7 shows a TV-based instrument for such analyses [4, 5] and fig. 8 is a diagram of the arrangement. The instrument can be used for detailed studies on banding patterns produced by different techniques, but is of especial advantage for work on fluorescence preparations. There are reasons to assume that the fluorescence techniques are the best of the presently available procedures for detailed analysis of chromosome aberrations, because of the good reproducibility in the preparations and the fact that the staining appears to have little influence on the shape of the chromosomes.

The principle is the following.

The TV-cameras focus either via the microscopes or, as is usually more convenient, on photographic negatives or positives. In the fluorescence picture, various chromosomes show very different overall levels of fluorescence, which means that some fine pattern details are lying in highly fluorescent parts of the metaphase and some in the faintly fluorescent chromosome regions. An electronic arrangement for selective contrast gain is included in the instrument. By turning two dials one can easily bring out on the monitor any fine pattern details, whether they are lying in intensely or weakly fluorescent regions. The pictures from the two cameras can be brought together on the same divided monitor screen, which makes very detailed comparisons between chromosomes possible.

Primarily, this dual TV-equipment was developed for analysis of aberrations induced by external mutagens [5] and it has greatly facilitated and speeded up that work so that with a reasonable amount of manual work such a large observational material can be obtained that it can be analysed statistically. This would hardly be possible with earlier techniques. For instance, a

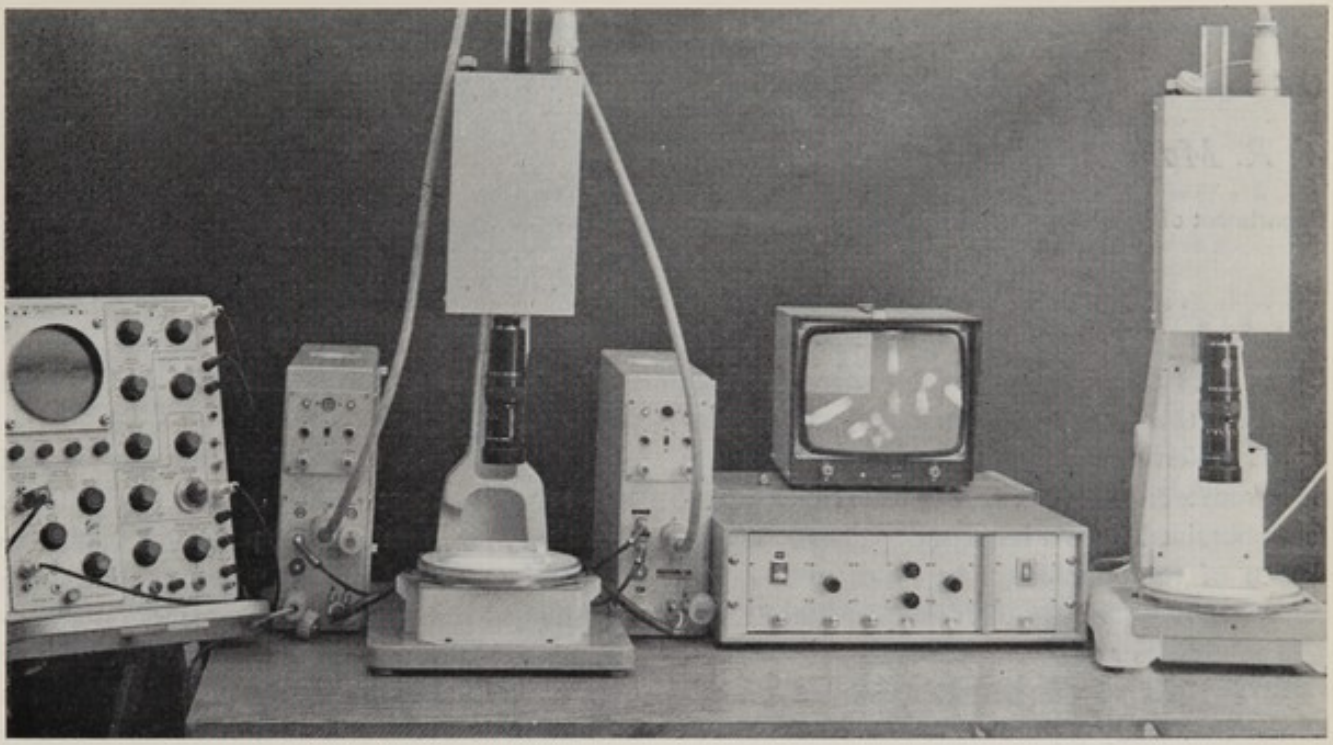


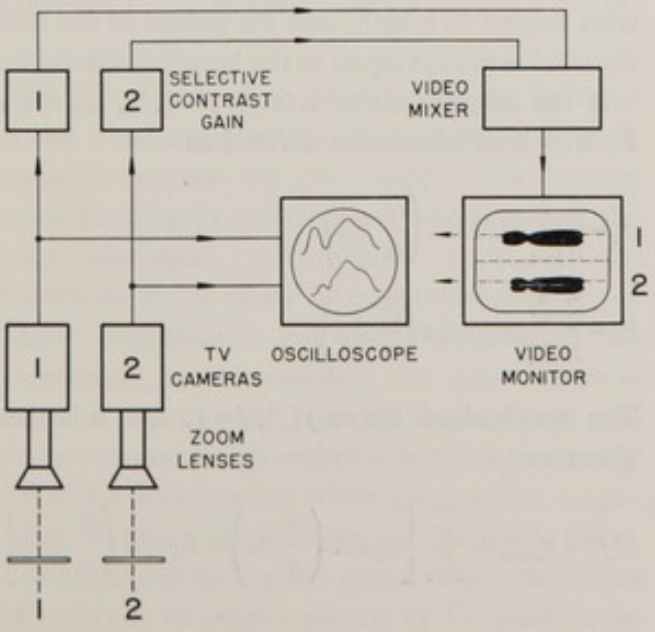
Fig. 7. TV-based intrument for rapid observation and analysis of chromosome aberrations in large series of metaphases.

material of about 20 000 chromosomes from X-irradiated cells has been analysed and it was convincingly shown that the distribution of breaks was non-random [6].

Furthermore, the instrument is a great help in the comparison between the details of the banding patterns produced by different methods. This is important for the further work on the chemical organisation of the metaphase chromosome. As indicated above it is technically almost or entirely impossible to measure the patterns given by most existing banding techniques, except for the fluorescence procedure. This is mainly due to changes occurring in the chromosome during the pretreatments for different types of staining. In the dual TV-arrangement it has proved possible to make quite precise comparisons between patterns even in somewhat distorted chromosomes.

The work described has been financed in its entirety by the Swedish Cancer Society, the Swedish Natural Science Research Council, the Damon Runyon Fund and funds from Karolinska Institutet.

Fig. 8. General arrangement of the instrument in fig. 7.



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Computerized Statistical Analysis of Banding Patterns

A. R. Møller and H. Nilsson

Department of Physiology II, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

It has been shown that the fluorescence pattern of quinacrine mustard stained human chromosomes makes it possible to identify all 24 types of chromosomes [1, 2]. These fluorescence patterns obtained from a large material show a high degree of reproducibility. In order to test this reproducibility and the significance of each pattern in a more rigid statistical sense, a computerized analysis of the patterns was introduced. In the first study [3] the eight chromosomes belonging to the C-group (6–12 and X) were analysed. The individual chromosomes in this group are of approximately the same length and centromere index and they could therefore not be identified before the quinacrine mustard fluorescence method was introduced. The fluorescence along the chromosomes was recorded using reflection photometry in photographic prints of fluorescence microphotographs of metaphase plates. The intensity scale was chosen separately for each chromosome curve so that the maximum amplitudes of the curves were of approximately equal size. The amplitudes were then digitized manually at 10 points/μm and the results punched on cards. These cards were later fed into a general-purpose computer for processing. The second study [4] comprised about 1 000 chromosomes belonging to all the 24 chromosomes types. The chromosomes were described in the same way as in the first study using the fluorescence along the chromosomes. Those data were, however, obtained in a semiautomatic way in order to reduce the manual work. This was accomplished by connecting an analog-digital converter and a paper tape punch to the reflectometer. The patterns were measured at 25 points/μm.

Statistical Methods

Any pattern recognition problem can usually be divided into two parts:
(1) Extraction of significant features.
(2) Statistical classification based on the extracted features.

In the first step, the original data are reduced to a set of numbers, which describe the pattern. By using these numbers, the pattern is then categorized into one of several pattern groups.

In the present study, Fourier analysis was chosen as the method of feature extraction. Fourier analysis, also called harmonic analysis, describes a function as a sum of a number of harmonically related sinusoidal waves. The approximation of such a sum of sinewaves to a given curve represents a best fit with regard to the least mean square errors. How well a curve can be described by such a series of sinewaves depends on how many sinusoids are included in the analysis. This is illustrated in fig. 1 which shows the original curves from four chromosomes (staircase curves) and the synthesized curves based on 1, 2, 3, 4, 6 and 8 harmonically related sinusoids (solid lines). It is seen that the resemblance between the original and the synthetical curve increases with the number of sinewaves included in the synthesized curves. A reasonably good fit is obtained when eight sinewaves are used.

In Fourier analysis all curves are normalized with respect to length, and the period of the first sinusoid is always equal to the length of the curve.

If the original curve is $x(j)$, $j=1, 2, \ldots, n$ the Fourier coefficients are defined as

$$a_k = \frac{2}{n}\sum_{j=1}^{n} x(j)\sin\left(\frac{jk}{n}2\pi\right)$$

$$b_k = \frac{2}{n}\sum_{j=1}^{n} x(j)\cos\left(\frac{jk}{n}2\pi\right) \qquad (1)$$

The synthesized curve $y(j)$, $j=1, 2, \ldots n$ is then given as

$$y(j) = x_{\text{aver}} + \sum_{k=1}^{N}\left[a_k\sin\left(\frac{jk}{n}2\pi\right) + b_k\cos\left(\frac{jk}{n}2\pi\right)\right] \qquad (2)$$

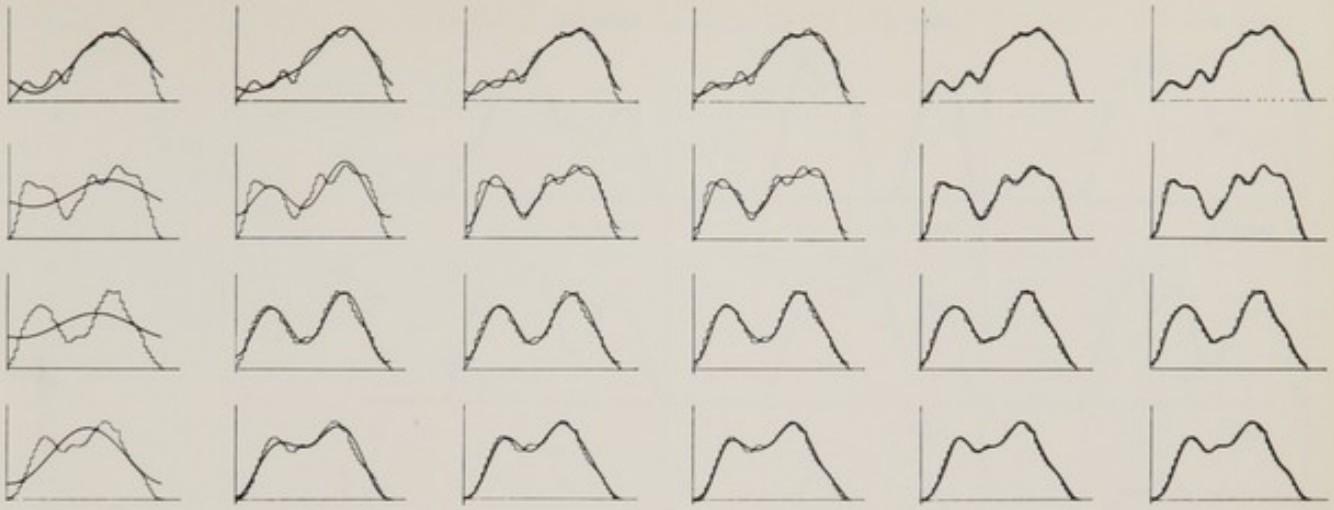


Fig. 1. Photometrically determined fluorescence patterns (stair-case curves) for chromosomes 6, 7, 11 and 12 in order from above. Smooth curves show computer-synthesized functions using, in order from the left, 1, 2, 3, 4, 6 and 8 harmonically related waves.

where

$$x_{\text{aver}} = \frac{1}{n}\sum_{j=1}^{n} x(j)$$

Each harmonic wave is described by two numbers, a_k and b_k, the amplitudes of a sinewave and a cosine wave. As an equivalent description of kth harmonic one can use phase and amplitude of a single wave where

$$A_k^2 = a_k^2 + b_k^2$$
$$\varphi_k = \arctan(b_k/a_k) \qquad (3)$$

In that case the synthesized curve becomes

$$y(j) = x_{\text{aver}} + \sum_{k=1}^{N} A_k \cos\left(\frac{jk}{n} 2\pi + \varphi\right) \qquad (4)$$

In the classification of chromosome patterns of the present study this latter representation is superior because the two components become more statistically independent which makes the later statistical classification more simple. Furthermore the phase information for waves of higher frequency is less useful because of the uncertainty in determining the exact starting point of the curves. Using the amplitude and phase representation makes it possible to discard the phase information while keeping the amplitudes of waves above a certain harmonic order.

Without discarding any phase values, the Fourier analysis of a curve results in 17 numbers describing each curve (8 amplitudes, 8 phases and the length). For statistical classification of the patterns, a method similar to discriminant analysis has been used. First, the mean values and the standard deviations of each of the 17 parameters were computed for each of the 24 groups.

Assuming that the parameters are normally distributed, one can define a probability density function for each parameter x.

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-(x-\mu)^2/2\sigma^2\right] \qquad (5)$$

where μ is the mean and the standard deviation of the parameter. The whole set of parameters for a group then has a multidimensional normal distribution. Assuming that the parameters are independent, this distribution is the product of all the separate distributions (5).

$$f_j(x_1, x_2, \ldots x_m) = \prod_{i=1}^{m} f_{ij}(x_{ij})$$

where j denotes group number and i parameter number. For an unknown chromosome with a set of parameters y_j, we compute the value of the distribution function for all groups and assign the chromosome to the group which gives the highest value.

There is a difficulty in using distributions of phase angles since phase values have an ambiguity of 360°, e.g. an angle of 30° can equally well be 390°. When calculating the mean and standard deviation, the 360° interval is therefore centered for each distribution in a way that gives the smallest standard deviation.

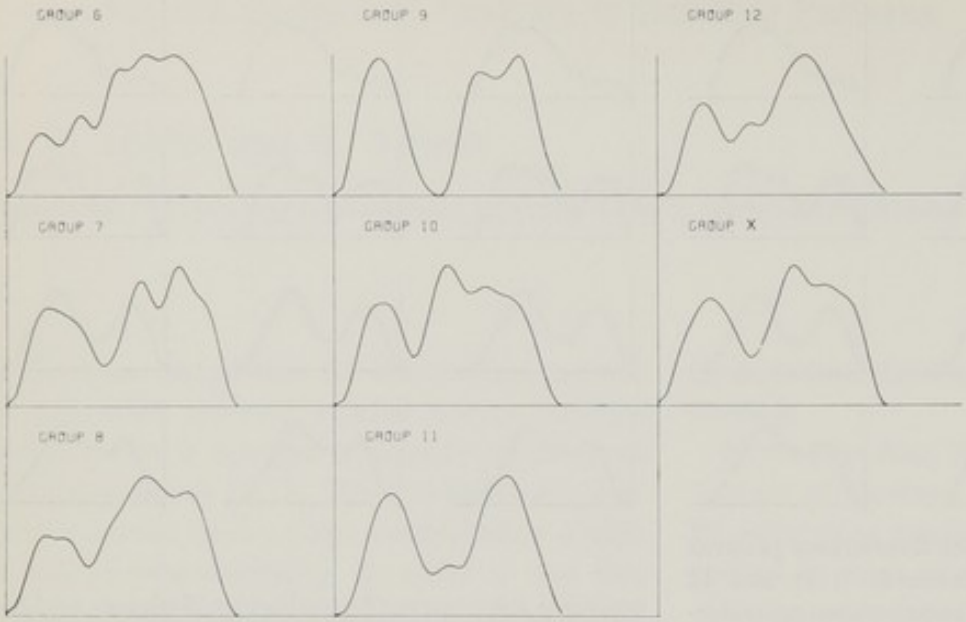


Fig. 2. Type patterns for all the C-group chromosomes, synthesized on the basis of the experimental material.

Results of Classification

In the first study, the 87 chromosomes of the C-group were classified by the method described on pp. 56, 57.

The average of the parameters were used to synthesize average patterns for the groups. These are shown in fig. 2, and are not so fine in detail as the original curves, since the averaging of the parameters smears out small peaks, which are not in exactly the same position in all chromosomes.

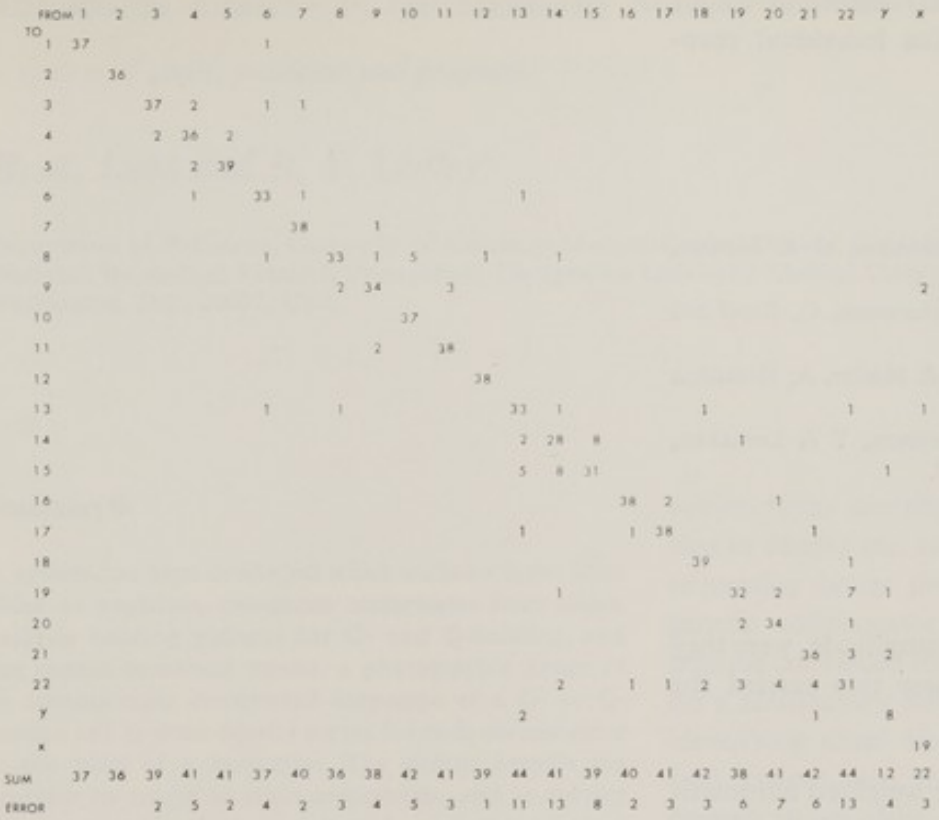
The phase values above the 4th harmonic were discarded. All 87 chromosomes were correctly classified. The same set of chromosome patterns was used to establish the mean values and standard deviations of the parameters (learning test).

Each of the 87 chromosome patterns (test set) were then compared one after another with these mean values and standard deviations. However, this learning set never included the pattern of the chromosome being tested. For every chromosome tested thus, all the other 86 served as the learning set. In the study of the whole karyotype, 913 chromosomes were used. Fourteen Fourier coefficients (amplitude and phase angles) and values of length were used in the classifier described. With this method 803 (88%) chromosomes were classified correctly. Also in this case, the learning set used in classifying each chromosome consisted of the other 912 chromosomes.

Fig. 3 shows the results of this experiment expressed in a confusion matrix. The main reason for misclassification is probably the fact that the chromosomes show different contraction in different regions of the same chromosome. The uncertainty in defining the starting points of chromosome pattern may be the reason for the large error percentage for the short chromosomes. The starting and end points are not determined manually as often is the case, but are defined as the point where the derivative of the curve is above a certain threshold. A third cause of error can be possible erroneous manual classification in the learning set. This last source of errors in addition introduces errors in the average parameter values, and thus may make more than the chromosomes involved misclassified.

Contrast Enhancement and Band Localisation

A description of the position of bands along the chromosome is of great value. In order to do this automatically by computer, one must define a peak mathematically. For sampled curves we have found that a good definition is the following: A peak is a point which has a greater value than the points on both sides of it. This definition used on the curves gives too few peaks on the chromosomes. There are many "shoulders" on the curves which in the fluorescence pattern of the chromosomes appear as distinct bands but which are not peaks by this definition. There is therefore a need to filter the curves in a way that enhances the



FROM / TO	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Y	X
1	37					1																		
2		36																						
3			37	2		1	1																	
4			2	36	2																			
5				2	39																			
6				1		33	1						1											
7							38		1															
8						1		33	1	5		1		1										
9								2	34		3													2
10										37														
11									2		38													
12												38												
13						1		1					33	1				1				1		1
14													2	28	8				1					
15													5	8	31								1	
16																38	2			1				
17													1			1	38				1			
18																		39				1		
19														1					32	2		7	1	
20																			2	34		1		
21																					36	3	2	
22														2		1	1	2	3	4	4	31		
Y													2								1		8	
X																								19
SUM	37	36	39	41	41	37	40	36	38	42	41	39	44	41	39	40	41	42	38	41	42	44	12	22
ERROR			2	5	2	4	2	3	4	5	3	1	11	13	8	2	3	3	6	7	6	13	4	3

Fig. 3. Confusion matrix of 913 chromosomes, sorted by the described algorithm.

contrasts. We used a digital filter with a triangular weighting function shown in fig. 4. Its effect is similar to the lateral inhibition in the eye. The effect of the filter on some of the fluorescence curves of human chromosomes can be seen in fig. 5 for three different degrees of contrast enhacement.

Conclusion

The conclusion of the present study is that the fluorescence pattern of QM-stained human metaphase chromosome are so well defined and reproducible that it is possible to identify all 24 human chromosome types in a strict statistical sense. Probably it would be possible to reduce the error percentage by producing more evenly contracted plates with chromosomes that are more precisely defined in respect to starting points with the use of a more sophisticated algorithm for parameter extraction in which the location of the

Fig. 4. Weighting function (impulse response) of the digital filter used for contrast enhancement.

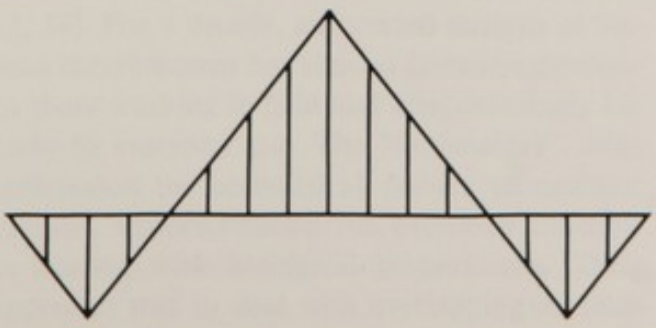
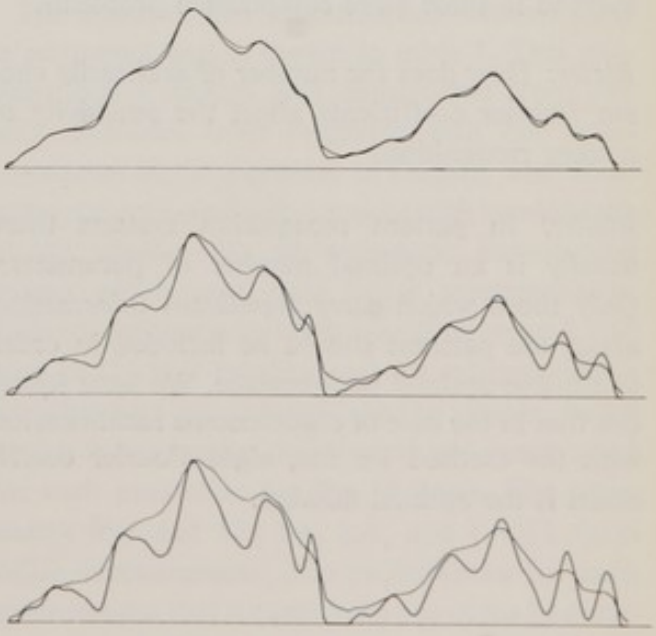


Fig. 5. Fluorescence pattern of chromosome 1 (——) with three different degrees of contrast enhancement (——).



peaks are allowed to vary in compensation for varying contraction along the individual chromosomes.

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Discussion

Bahr: On phase angle versus amplitude, were they weighted? Which one of these two carried the greater weight?

Møller: The parameters were weighted automatically in relation to their standard deviations. One can of course change these weights if one suspects that, e.g., amplitudes carry more information, but this was not done.

Miller: (1) In cultured cell lines, there are usually many structurally rearranged chromosomes. To what extent can your automatic method identify the origin of these chromosomes?

(2) How well do you think your automatic method would stack up against visual analysis and identification of chromosomes in these cell lines?

Møller: It is difficult to apply this automatic method to these more complicated problems.

Rigler: How does the number of arbitrarily chosen Fourier coefficients affect the sensitivity of pattern recognition?

Møller: In pattern recognition systems there usually is an optimal number of parameters. Only those which carry significant information about the patterns should be included in order to achieve optimal performance. We have found out that in the case of chromosome identification with the method we use, eight Fourier coefficients is the optimal number.

Automated Analysis of Differentially Stained Human Chromosomes

A review of goals, problems and progress

H. A. Lubs and R. S. Ledley[1]

Department of Pediatrics, University of Colorado Medical Center, Denver, Colo. 80220, and [1]National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C. 20007, USA

Summary

A system has been developed which includes input from slides or negatives, recognizes metaphases from slides, analyses banding patterns for G- and Q-banding, and can output numerical results, a photographic image of an automatically constructed karyotype of a G- or Q-banded cell or draw density curves for each chromosome in the form of a karyotype. The system permits the operator to accept or reject metaphases, and to correct the automated analysis. Less than 2 min cell is required for certain types of output. Correct placement of chromosomes within the classical group was done more than 98 % of the time using linear measurements, and a Fourier analysis of density curves from 9 stained cells within these groups was 94 % correct. On the average, 3 chromosomes/cell were incorrectly identified.

Prior to the introduction of the banding technics, many important problems in the development of an automated analysis of metaphase chromosomes had either been solved or substantial progress had been made towards their solution. Hardware had been developed for or adapted to scanning and display of results [1, 6, 7, 9, 10, 13, 15]. Systems which were capable of recognizing many metaphases and scanning directly from slides were in their early phases of development [11, 14, 15]. Recognition of chromosomes and analysis of their boundaries and key landmarks were reasonably efficient in several systems [7, 9, 11, 14]. For a decade, automated analysis of human chromosomes has seemed tantalizingly close to those working in field and disappointingly far away to everyone else. The "automators", who understood the potential of pattern recognition systems, underestimated the problems inherent in dealing with biological preparations. Their approach was to deal with overlapping chromosomes, fuzzy morphology and artefactual variation in density etc. as best they could, rather than requesting better preparations from their cytogenetic collaborators. Cytogeneticists generally became convinced that the computer would never do a satisfactory job of karyotyping since many identifying clues were not used in the computerized system and the results were not reliable enough to use after 7 years of work. Perhaps the most important outcome of these frustrations was a bow to reality and acceptance of the idea that only a highly interactive system was likely to produce adequate results on a production line basis. Many major laboratories have now moved in this direction [3, 8, 11, 15]. Although the introduction of operator interaction devices was a necessary step, it was not totally curative and in fact an even more fundamental problem lay beneath the surface. With the similarity in size of many chromosomes, neither the karyotypist nor the computer analysis was really justified in doing more than grouping the chromosomes.

The extent of this limitation only became clear in retrospect and is shown in table 1. This misclassification matrix was constructed from 151 cells measured from negatives with our semi-automated X, Y digitizers in Denver, and compares the cytogeneticist's results with banded cells to the results with an automatic karyotyping system which used only linear measurements. Each chromosome was first identified on an 8×10 print of G- or Q-banding and the cytogeneticist's identification was entered into the system. Based on this data a normal distribution was constructed for each parameter for the 24 types. The parameters included TL, SA, LA, and LA/TL (arm ratio) measurements. The probabilities for each chromosome that it came from one of the 24 distri-

Table 1. *Assignment (%) based on 2 probabilities (TL & AR)*

Assignment by cytogeneticist	1	2	3	4	5	6	7	X	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Y
1	99	.	.																					
2	.	99	.																					
3	2	.	97																					
4				64	35		.		.															
5				30	69	.	.		.															
6			.		1	84	11	3	.															
7			.			12	50	29	5	1	.	2												
X					.	7	28	48	2	3	.	11												
8					.	.	3	1	61	4	17	5	8											
9					2	.	6	7	29	4	28	20	3											
10							.	1	20	3	52	8	15											
11							.	7	2	5	6	79	.											
12					.				7		14	.	77	1		.								
13											1		.	59	29	10								
14										.	.		.	33	30	36		.						
15														10	21	66	.	2	.					
16												1					87	8	1	2				
17																1	7	73	18	.	.			
18																1	2	19	74		.			3
19																	3			60	36		.	
20																	2		.	33	64	.	.	.
21																	.					54	41	4
22																	.		.		1	25	61	12
Y																1	1		3		1	9	27	95

N, 151 cells preidentified by G- or Q-banding. X, Y digitizer data. Normalized by regression correction. Not limited to 2 chromosomes/number. Dot indicates < 1 %.

butions of the 4 parameters were then computed and varying combinations of the 4 parameters were multiplied. The maximum product over all 24 distributions gave the most likely identification. Use of the maximum likelihood method has previously been reported [12]. Results were very similar with all combinations, but slightly better when TL and arm ratio were used, hence the results are presented only for the latter parameters. A limitation of 2 chromosomes/type was not imposed in order to permit subsequent utilization of the system for non-modal cells. (Presumably the results could be improved somewhat with an additional subroutine for cells with 46 chromosomes which would include this restriction.) The cytogeneticist's designation, based on the banding pattern, is shown to the left, and the probability typing along the top. With few exceptions, the probability typing is poor. For a few chromosomes, such as 9, they are extremely poor; 9 is even more variable than expected. The addition of other morphological characters, such as secondary constrictions would likely have improved the results, but the identification of more than a few chromosomes was, in fact, a lost cause. What could be done then, and can be done now on the basis of these simple measurements, is to place chromosomes correctly in the classical groups a very high proportion of the time: better than 98% (table 2). This was the same data as shown in table 1. The starting point of our analysis of banded chromosomes, therefore, is placement in the classical group by the TL and arm ratio measurements using the maximum probability approach. The task of comparing curves is much simpler and more effective because the number of comparisons within a group is much smaller. The success of this first step, however, is based upon 10 years work by the many laboratories [1, 3, 8, 9, 10, 14, 15, 16] which have

Table 2. *Assignment (%) based on probabilities (TL & AR) (same data as shown in table 1)*

Assignment by cytogeneticist	A	B	C&X	D	E	F	G&Y
A	99	—	[a]	—	—	—	—
B	—	99	1	—	—	—	—
C&X	[a]	[a]	99	[a]	—	—	—
D	—	—	1	98	1	—	—
E	—	—	[a]	1	96	1	1
F	—	—	—	—	3	96	1
G&Y	—	—	—	[a]	1	1	98

N, 151 cells. X,Y digitizer data. Normalized by regression correction.
[a] less than 1 %.

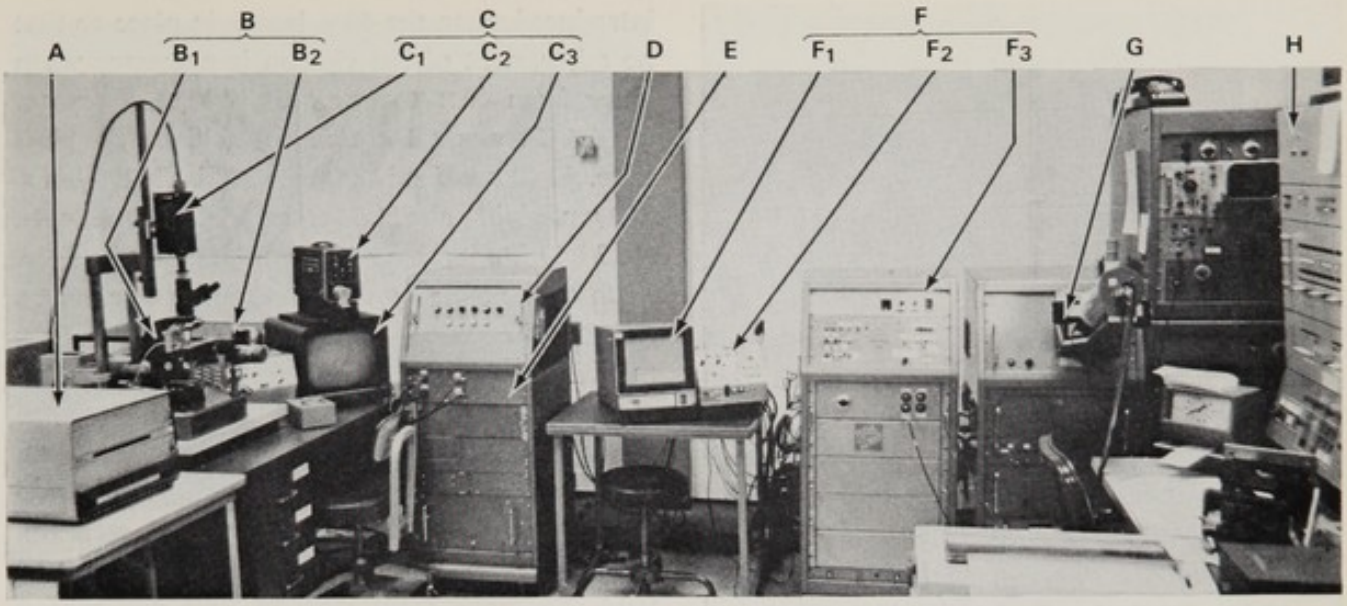


Fig. 1. Current equipment for automated chromosome analysis at National Biomedical Research Foundation. A, Hard Copy Picture Printer; B, SPIDAC (Specimen Input to Digital Automatic Computer); B1, Leitz microscope; B2, motorized stage; C, VIDIAC (Vidicon Input for Automatic Computer): C1, Vidicon scanner C2 control; C3, TV monitor; D, Silicon Video Memory System; E, Automatic stage control unit; F, MACDAC (Man Machine Communication with Digital Automatic Computer): F1, display; F2, control console; F3, control unit; G, FIDAC (Film Input to Digital Automatic Computer); H, IBM 360/44 computer.

contributed to the development of recognition and analysis systems for classically stained chromosomes.

Description of Present System at NBR

Although FIDAC and FIDACSYS are still critical parts of the overall system, several important components which provide entirely new capabilities have been added, and the whole system improved sufficiently that a complete review should be informative. The present equipment is shown in fig. 1. The system consists of an IBM 360/44 computer, high-resolution flying-spot scanner (FIADC), a mechanical scanner with a very great memory system (VIDIAC), a microscope scanning system (SPIDAC), and an interactive communication system (MACDAC). The associated software systems used in the operation of the above hard ware systems consists of FIDACSYS which supervises input of the image into the computer from the scanning devices and accomplishes overall picture manipulation; MACDACSYS, which implements the interactive capabilities of the systems, permits accepting information from and displaying information on the MACDAC unit; and finally SPIDACSYS, which is used to automatically control the SPIDAC microscope state, to detect good chromosome spreads, and to record the coordinates of the center of each such good spread.

Only the FIDAC and computer were part of the system 4 years ago. The operator-intervention device (MACDAC) was built 3 years ago. The storage tubes, which are critical components of the system have been readily available for less than 2 years. SPIDAC is less than 1 year old. The capabilities of these new components are outlined below.

SPIDAC (Specimen Input to Digital Automatic Computer)

The SPIDAC is an instrument for scanning slides directly through a microscope, digitizing the resulting grey-level pattern, and placing the digitized image directly into the computer's memory. The system can automatically manipulate the microscope slide in both the x, y, and z directions, record the coordinates of objects of interest, automatically focus the image instantaneously, and monitor results, all under computer control. The automatic focussing feature is of critical importance, and has worked quite well. SPIDAC, together with the VIDIAC scanning camera is shown in fig. 2. Eliminating the photographic step has the advantage of saving personnel time and effort; it is, therefore, the major step in reducing costs compared below those of conventional

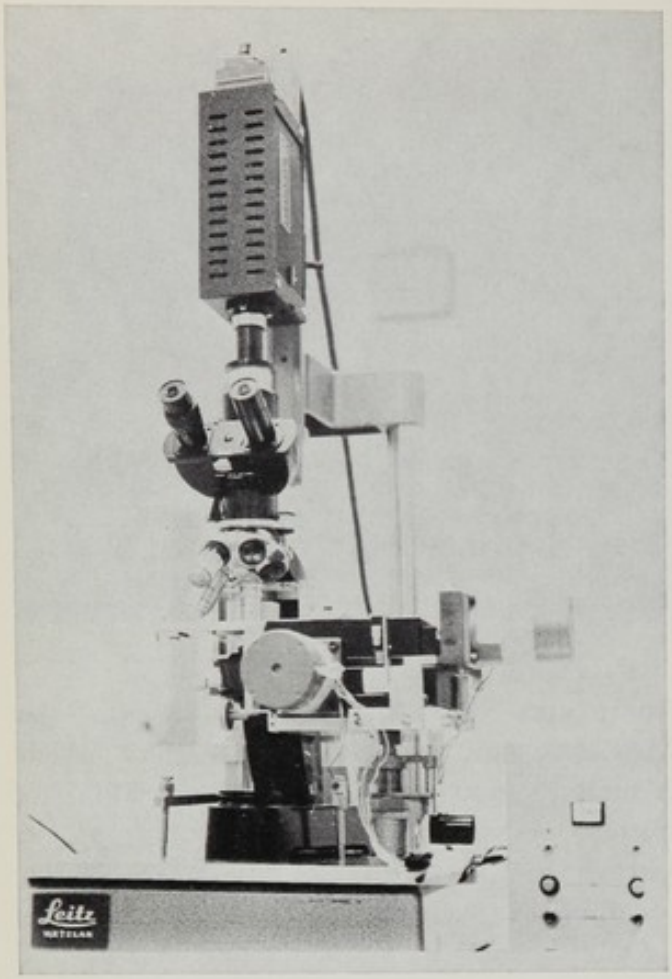


Fig. 2. SPIDAC and VIDIAC scanning camera, showing microscope, motorized stage and a portion of the manual control panel (*rt*).

cytogenetic work. Also, in a number of instances it is possible that such direct digitization may bring more sensitivity to grey-level discrimination and can eliminate certain possible grey-level distortions which can occur when using the film as an intermediate step. Excellent equipment, however, will likely have to be used to improve on the resolution now obtained from negatives.

The SPIDAC system performs its operation in two phases. During the first phase a coarse scan (40× objective) is made to determine where objects of possible interest are located. During the second phase a final and more accurate determination (at 100× objective) is made of whether or not the object located in the first stage is of sufficient interest to be automatically analysed; if so, then its image is scanned by the VIDIAC, digitized, and read into the computer. The pattern-recognition programs in the computer are utilized to compute the desired quantitative information from the pictorial data. The 40× and 100× objectives are parfocal, and a filter is introduced when the 40× lens is in place so that no adjustment of the vidicon is necessary when going from 40× to 100× objective. At present SPIDAC scans a 1 cm×1 cm field on the glass slide. At 40× objective, the frame size is 200 μm× 260 μm, giving about 40 strips of 50 frames each (or 2 000 frames) over the 1 cm² area of the field. Each of the *x* and *y* stepping motors is run at a rate of 650 steps/sec, with 200 steps/revolution, moving the stage 500 μm/revolution. This amounts to 2.50 μm/step, meaning that we can locate any point in the field to the nearest 1.25 μm. The rate of movement is (650×500)/200=1 625 μm/sec, or 10 000/1 625=6 (approx.) sec/strip, or finally 6×40=240 sec for the 40 strips of the field. To move from strip to strip will take a total of 6 sec across the field, so the total scan time limitation due to the motors themselves is 246 sec or 4 min and 6 sec. In addition, each field is examined (in the first phase as described above) for a chromosome spread, and this takes 1/30 sec/frame, or 2 000×(1/30)=66.6 sec or 1 min and 6.6 sec. Thus the total time that SPIDAC takes to search a 1 cm×1 cm field area for chromosome spreads is 5 min and 12.6 sec. Vibration after each step has not been a problem.

VIDIAC

The VIDIAC device was designed either for scanning through a microscope or for scanning large pictures or X-rays. It consists of a high-resolution vidicon system that scans at standard TV rates (i.e. 1/30 sec/frame), coupled to a Silicon Video Memory System. The standard vidicon scanning rate is too fast for most analog-to-digital conversion systems and for data transmission to a digital computer. Hence the Silicon Video Memory System acts as an interface between the vidicon and the computer. VIDIAC is shown in fig. 2. The capabilities of the VIDIAC are as follows: The image scanned by the vidicon can be displayed on a TV monitor for viewing and for optimizing such parameters as focus, intensity, contrast, etc. The image scanned by the vidicon can be written onto the Silicon Video Memory System. The electronic image as stored in this silicon memory can in turn be internally scanned and displayed on either the TV monitor or on the storage tube of the MACDAC. A zoom control on the silicon memory and a bias control on the console of the MACDAC can be used to

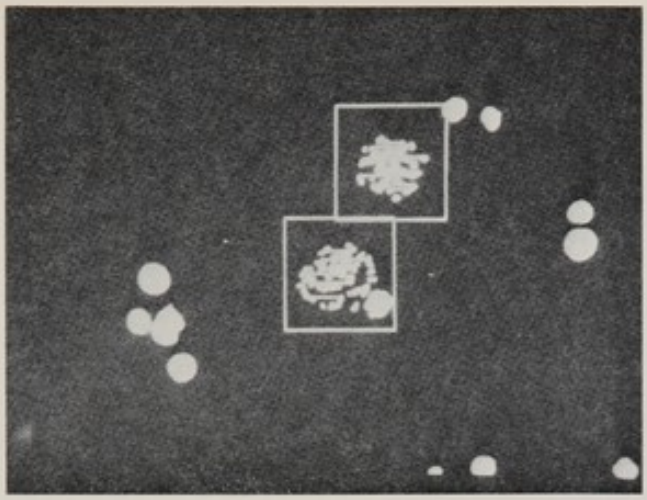


Fig. 3. Illustration of method used to recognize chromosome spreads during a search of the glass microscope slide. The technique employed uses, in effect, a triplet of conceptual cursors in the computer's memory. When a properly spaced 010 triplet occurs, an object of small width is identified and a "cover" is initiated (see horizontal lines on the chromosome spread). If as the scan proceeds, another small object is identified within a prescribed distance from the first, then the cover is continued; otherwise the cover is terminated. A collection of covers, properly placed, is said to be a chromosome spread, and its location is that of the geometric center of the collection of segments making up these covers. The figure shows a square around two chromosome spreads that were so detected, where the center of the square is at the center of the chromosome spread as detected by the computer. The pictures were taken from the MACDAC display scope face.

display any portion of the image. This zoom feature is under computer control. The detection of metaphases with VIDIAC and SPIDAC is described in fig. 3. Metaphases can either be displayed on the VIDICON monitor or MACDAC for operator acceptance or rejection.

DRIDAC (Drum Input to Digital Automatic Computer)

DRIDAC is a high-speed, high-resolution, on-line drum scanner which can scan photographs (X-ray, transparency, or print) of any size up to 16 inches by 12 inches of useful area. The resolution may be varied to as fine as 0.004 inches. This allows it to sample 4 000 × 3 000 spots, for a total of 12 million spots, from a large picture. Each spot can be digitized as required into 1 bit (black and white) or up to 6 bits (64 grey levels/spot). Although not currently in use for cytogenetic analysis, it is part of the system and offers the additional option of rapid input from photographs.

MACDAC (Man Communication with Digital Automatic Computer)

The MACDAC (fig. 4) is an inexpensive instrument that enables rapid man-machine interaction to aid in the automatic computer analysis of pictures of biomedical importance. MACDAC was built at NBR prior to the introduction of banding technics to permit routine automated chromosome analysis. It has proven equally valuable for the analysis of banded chromosomes. The instrument is operated on-line with the computer and in real time. In many biomedical pattern-recognition applications, such as the automatic analysis of human chromosomes, MACDAC can enable a computer operator to edit the pictures prior to their computer analysis, or to monitor the stages of a computer analysis for possible manual intervention during the analysis. The scanned picture is displayed on the MACDAC cathode-ray tube face, and the picture is manually editing by means of a "joystick" cursor. The editing consists in identifying artifacts in the photomicrograph that are not chromosomes but are similar in size, and also in "separating" any touching chromosomes in the picture, or fusing 20 objects that are incorrectly separated. The results of the editing process are put into the computer's core memory by the MACDAC. Then the editing results are taken into account by the computer: A digitized picture from FIDAC or SPIDAC can be either displayed on the MACDAC cathode-ray tube or transmitted directly into the core memory of the computer. The video picture (i.e., not digitized) can also be displayed on the MACDAC CRT In addition, the computer can "write" or draw on the display tube by means of the joystick and cursor. The display tube is a storage cathode-ray tube. Metaphases found by SPIDAC then can be observed directly and immediately rejected or accepted and analysed, as can cells entered from 35 mm film. Moreover, the automated karyotype including density curves can be displayed and edited; improperly paired chromosomes can be corrected by placing the cursor over the chromosomes to be changed and entering appropriate instructions for the control panel.

The MACDAC control panel contains two joysticks. The main joystick controls the position of a cursor, or bright spot, on the display tube. The location or path of the cursor may be stored on the CRT face or not, as desired. The x and y

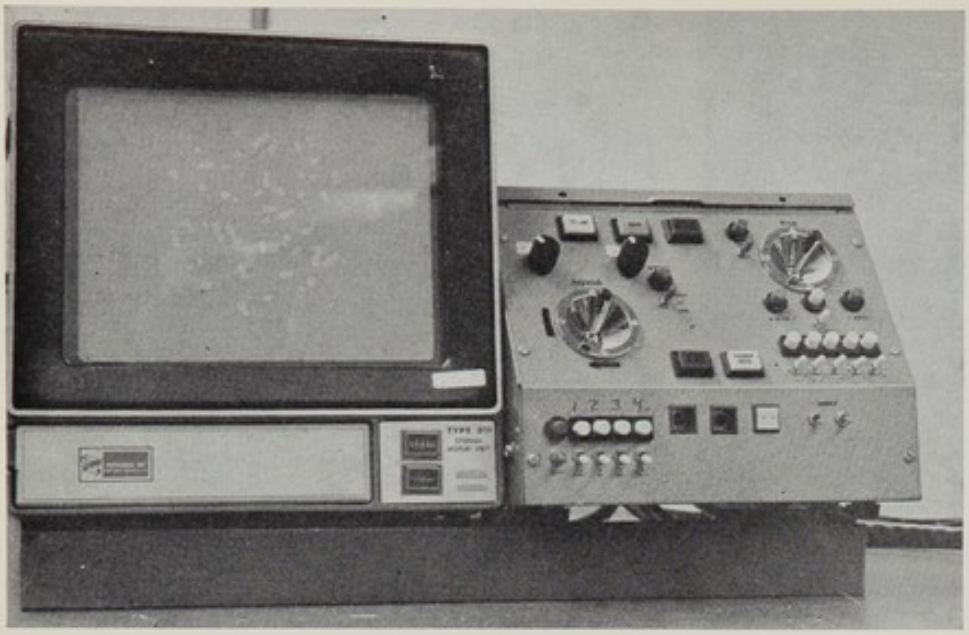


Fig. 4. Diagram of MACDAC console. The left is the display tube; the right the control panel. The lower left (main) joystick controls the cursor; the upper right (bias) joystick controls the zoom feature. The line of five switches below the bias joystick controls the four-bit code read in with the coordinates when the "read spot" button is pushed. Nonstandard codes can be set by means of the five switches below the main joystick.

coordinates of the cursor (10 bits each) can be transmitted to the computer, under the computer-interrupt mode, by pushing the "Read Spot" button. A four-bit code can be associated with each pair of coordinates by setting one of the code switches. In this way the computer program is "told" what to do with the coordinates, by means of the associated code.

The control panel also includes a "zoom" feature that will display an enlargement of any section of the picture for more detailed study. To actuate this feature, the cursor is placed in the center of the area to be enlarged, the bias-joystick and zoom-feature toggle switches are turned on, the bias joystick lever is moved so as to bring the cursor to the center of the display-tube face, and the display button is pushed. Then the enlarged section of the picture is displayed, whether it originates from the FIDAC or from the computer.

Typical operation of the system would proceed as follows, entirely under the control of the computer and the machine operator at the control panel: When the operator pushes the button labeled "FIDAC" (or "SPIDAC"), a scan of the frame is made by the FIDAC (SPIDAC), and the digitized picture is stored on the display tube. The operator now uses the main joystick to move the cursor to, say, a part of the picture that he recognizes as a piece of "dirt". He then selects the proper code switch for the four-bit code for an unwanted particle and pushes the "Read Spot" button. The MACDAC logic interrupts the computer (in its current analysis) to read into the core memory a 24-bit word, which contains the 4 bits of notational information denoting "dirt" and the 10-bit x and 10-bit y coordinates of the spot at which the cursor is located. The computer takes a maximum of only 30 μsec to receive this information, since the analog-to-digital conversion of the coordinates of the cursor is accomplished by the MACDAC. The operator continues to point the cursor at various trouble spots and send the coordinates and the proper codes to the computer. After each spot is read into the computer, the operator can make a check or cross or other mark on that spot for his own reference, by storing a short path of the cursor on the display tube (but not in the computer). When the operator has finished sending the editing data to the computer, he pushes the "End" button, which turns control of the display tube and the FIDAC (SPIDAC) over to the computer.

This process enables a sequence of chromosome spreads on successive film frames to be analysed with no time loss due to the editing procedure. While one film frame is being analysed by the computer, the next frame is being edited, in an overlapping sequence. The ongoing computer

analysis is interrupted only 30 μsec at a time as the computer receives editing data for the next film frame.

After the computer has received the "End" code and finished the previous analysis, it can do any of the following (depending on the computer program): It may write the results of the previous chromosome analysis on the display scope, or it may write some symbol on each of the edited spots to verify their reception, or it may read the picture from the FIDAC and initiate the analysis of that picture, utilizing the editing data.

The MACDACSYS interactive programming language as used for chromosome analysis has the following options:

(1) Control FIDAC and SPIDAC: (*a*) scan frame; (*b*) end operation, i.e., initiate computation; (*c*) accept results; (*d*) end of job; (*e*) end of codes.

(2) Enter edit codes (subprogram CLENUP); (*a*) separate chromosomes; (*b*) connect two objects; (*c*) erase an object; (*d*) partial fence for eliminating artifacts; (*e*) complete fence for eliminating arifacts; (*f*) erase last code put in.

(3) If other than 46 objects found: (*a*) outline plot appears on scope; (*b*) coded dots in upper left corner of display tell number of objects over 40; (*c*) push reset button; (*d*) either rescan or enter code for accepting result as is.

The MACDAC is inexpensive for two reasons: (1) By using a CRT for the display tube, the display does not have to be refreshed. Hence no core or drum storage need be associated with the display, and no flicker problems arise, as with other display methods. (2) By having the operator use a joystick cursor to communicate with the display tube, the programming aspects become greatly simplified over the light-pen method. Using the joystick, the coordinates of the cursor are given directly by the joystick displacement; on the other hand, the light-pen method requires the computer to scan on the display tube for the pen location and hence requires a more complex program. In addition, the joystick and storage-display combination gives the operator greater flexibility, since he can make notations on the display tube or can display a picture completely independently of the computer. The result is economy in both programming and computer-time utilization.

FIDAC (Film Input to Digital Automatic Computer), Mod II

The FIDAC is a high-speed high-resolution flying-spot scanner developed and built at the National Biomedical Research Foundation. It has a maximum capability of scanning a raster of $2\,000 \times 2\,000$, or 4 000 000 sample points, in 4 sec (i.e. at the rate of 1 μsec/sample spot), with up to 16 grey levels/point. The number of sampled spots in the raster is adjustable, as is the number of grey levels. For instance, for the analysis of chromosomes by classical (nonbanding) methods, a nominal 700×500, or 350 000 sample-spot, raster is most efficacious.

The FIDAC is a raster type of flying-spot scanner and has two modes of operation, as follows: (1) A complete picture mode. In this mode the raster's size is nominally 700×500; that is, the picture is sampled at 350 000 spots, and each point can be sampled as black-and-white binary. i.e. one bit/spot, or up to 16 grey levels, i.e. 4 bits/spot. This raster size is used for the examination of biomedical photomicrographs which were obtained from an optical microscope, since this sampling rate exceeds the resolution of the optical microscope. (2) Control word mode. In this mode of operation a resolution of 2 048 spots across each of 1 365 rows (3×2 aspect ratio) with a maximum of 16 intensity (grey) levels can be obtained. This is accomplished by allowing the computer program to select all or any portion of the picture to be scanned, with a corresponding sampling density such that the memory space allocated to the pictures would not be overrun.

The specifications of FIDAC are as follows: The system is completely synchronous and is geared to an easily adjustable master clock which can be clocked at any frequency up to 10 MHz. Thus the clock speed can be increased or decreased, depending upon the characteristics of the computer to be used. The sampling rate used is 1 μsec/spot, and the 4-bit, 16-grey-level analog-to-digital conversion takes place completely within this time period. Thus one frame can be scanned in about 0.35 sec for the complete picture mode (700×500 raster), and 3 sec for a $2\,048 \times 1\,365$ raster. The circuitry matching FIDAC to the computer consists of several replaceable cards. These FIDAC-computer interface cards are the only circuitry that would have to be changed in shifting from one computer system to another.

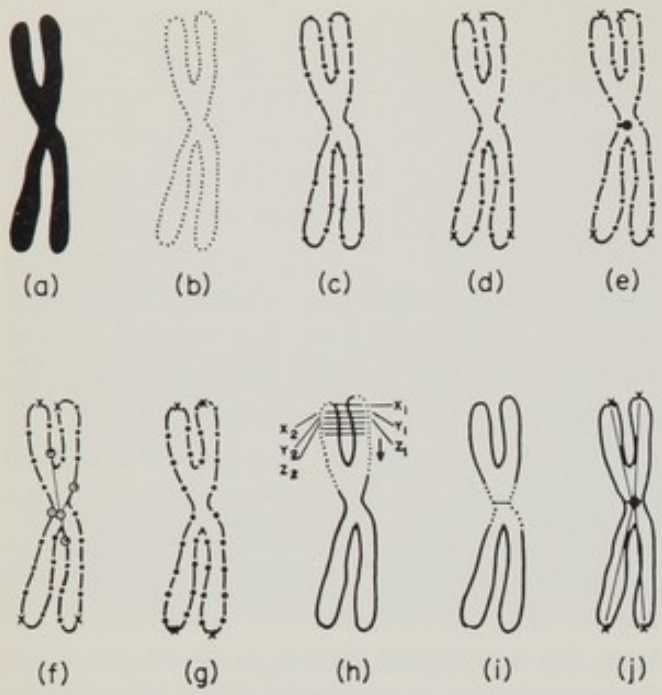


Fig. 5. FIDAC analysis of chromosomes showing (*a*) a chromosome silhouette; (*b*) boundary points; (*c*) segment center points; (*d*) arm ends; (*e*) average of arm ends; (*f*) four quadrants; (*g*) adjusted arm ends; (*h*) beginning search for centromere location; (*i*) adjusted centromere location; (*j*) measurement distances.

Dynamic focusing is incorporated in FIDAC, to produce an almost constant spot size from the center to the edges of the scan. The CRT spot size is 1.5 mils at one foot-Lambert light output. At the 1.5 mil spot size and at 2 000 spots per x sweep of the 3-inch-wide raster, there is no overlap of spots. The rise time of the photomultiplier that reads the intensity of the light transmitted through the film is 2.3 nsec, the video-amplifier bandpass is better than 10 MHz, and the phosphor persistence is 100 nsec. Thus one sample spot/μsec clearly presents nonoverlapping spots.

A 35 mm still-frame film transport is used which can take up to 250 frames. The film frames can be advanced automatically under computer control.

Analysis of Metaphases

The associated software systems used in the operation of the hardware systems described in the previous section include FIDACSYS, SPIDACSYS and MACDACSYS. These have recently been described in detail elsewhere [8] and will not be discussed here. The general working of these programs, however, can be surmised from the descriptions of the hardware in the previous section. The analysis of banded chromosomes begins with the same analysis developed for classically stained metaphases. These have also been recently reviewed [8] and will only be summarized here (fig. 5). First, the boundary of the chromosome is determined as above. In the illustrative drawing, every 5th boundary point is then used as the center of a boundary segment. (Actually, the spacing of the centers of the boundary segments is a variable which depends on the size of the object to be analysed.) The curvatures of the boundary segments are determined, and these data, together with the relative positions of the edge segments around the periphery of the chromosome, are used in a syntactical analysis to identify the provisional ends of the chromosome arms. The provisional arm ends are then used to calculate the center of gravity of the chromosome. Following this calculation, the centers of the boundary segments situated midway between each pair of ends of the chromosome arms are identified, permitting the division of the chromosome into 4 quadrants. Each provisional arm end is moved to the point of maximum distance from the center of gravity within the limits of its quadrant. The proper arm pairs are then identified by pairing the ends that are closest together.

Next, starting at the ends of an arm pair, the narrowest portion of the chromosome is determined by a self-aligning routine which measures widths down the entire length of the chromosome. To do this, three adjacent width measurements are tested at any one time sequentially along the length in overlapping fashion. If X, Y, Z are any series of three width measurements, then $X > Y < Z$ would define a constriction. The narrowest constriction is defined as the centromere.

An alternate method, which has been used with the banding analysis first finds the axes of symmetry of the chromosome. Next, a best-fit parabola is found for the chromosome as a whole. Then, in a parabolic coordinate system with the symmetry parabola as one axis, the ends of the arms are found as the extremes of a set of intersections of a certain family of curves with the boundary of the chromosome. The centromere is found as the narrowest region of the chromosome, where the boundary of the chromosome is nearest to the parabolic symmetry axis. Special cases arise for the acrocentric D and G groups

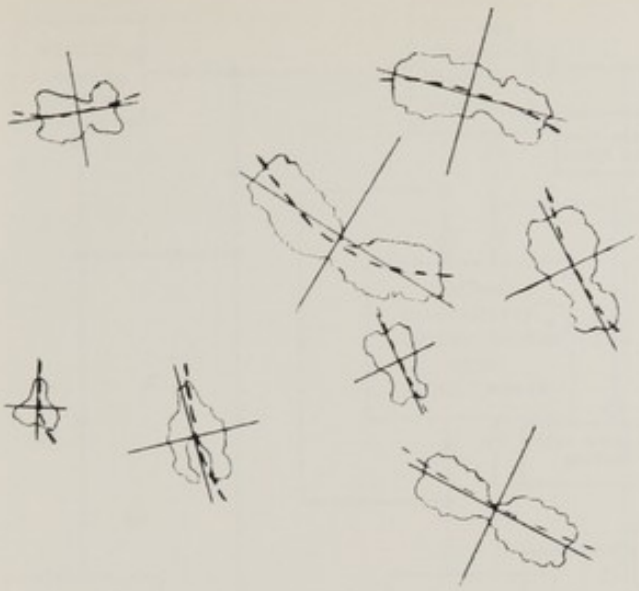


Fig. 6. Parabolic axes of chromosomes. The dashed line shows the best parabolic axis for each chromosome.

of chromosomes. Particular programs identify chromosomes of these types, and for the determination of the arm ends and the centromere special programs are called that depend on local curvature concavities for the centromere location.

This technic is used for the larger chromosomes, and the technic described above (fig. 5) is used for the smaller chromosomes. The first phase in the analysis of chromosome banding is to find the grey level profile along the axis of the chromosome by integrating grey levels on successive lines perpendicular to the axis. Since the chromosomes can be bent, a curvilinear coordinate system is used. The finding of the rectilinear principle axis and the parabolic approximation for the center line of bent chromosome are described in detail in appendix I. A stage in this approach is shown in fig. 6.

The principles involved in finding the density curves of banded chromosomes are simple: The principal axes of the chromosome are found, and modified to parabolic axes. The longer axis is then divided into convenient segments of equal length. The chromosome is examined spot by spot, and the grey-level value of each spot is added to a tally for the longer-axis segment corresponding to the longer-axis coordinate of the spot, as a tally is kept of the number of spots contributing to each segment. When the examination of the chromosome is completed, the grey-level tally for each longer-axis segment is divided by the corresponding spot-count tally to find the average grey-level for that segment.

The spot-by-spot examination of the chromosome requires some programming complexity. The examination proceeds by lines from the top of the chromosome, which is simple until one or the other side of the chromosome starts to rise, to form a hump, or "crest". The coordinates of the line from which the crest rises are stored (in a push-down list), and examination of the chromosome is suspended as the crest is ascended. Once the top of the crest has been surmounted, the line-by-line examination continues, until the stored line at the base of the crest is again encountered. Then the full width of the chromosome is again examined. The lower boundary of the chromosome may also display bifurcations, called "arches". When such an arch is encountered, examination is again suspended, until the top of the arch is passed. Then it is recommenced in descending the arch, but with any material encountered within the arch subtracted from the two tallies; this is done because the material had already been added in erroneously in the descent of the outside arms of the arch. As was the case with the crest, the line from which the arch rose is stored in the push-down list until the arch has been descended. Profile curves are produced by averaging grey-level values of sample points taken in strips perpendicular to the long axis of the chromosome where this long axis is a best-fit parabola as discussed above. The curves are based on the average of the squares of the grey values along the chromosome length to enhance their appearance to the human eye for convenience in detailed study. In addition, the curves represent the results of smoothing the raw (squared) data to make the identification of such critical points of the curve as maximum, minimum, and inflection points more reliable.

The overall flow of the processing of data from the metaphases is given in fig. 7; the interrelationships of the components in the automated analysis system can be understood from the diagram shown in fig. 8. Data can be input from slides for G-banded cells, from negatives or photographs for any type of banded cells. The analysis of classically stained chromosomes, including the MACDAC interaction, can be completed in less than a minute beginning from a negative, provided numerical output is desired.

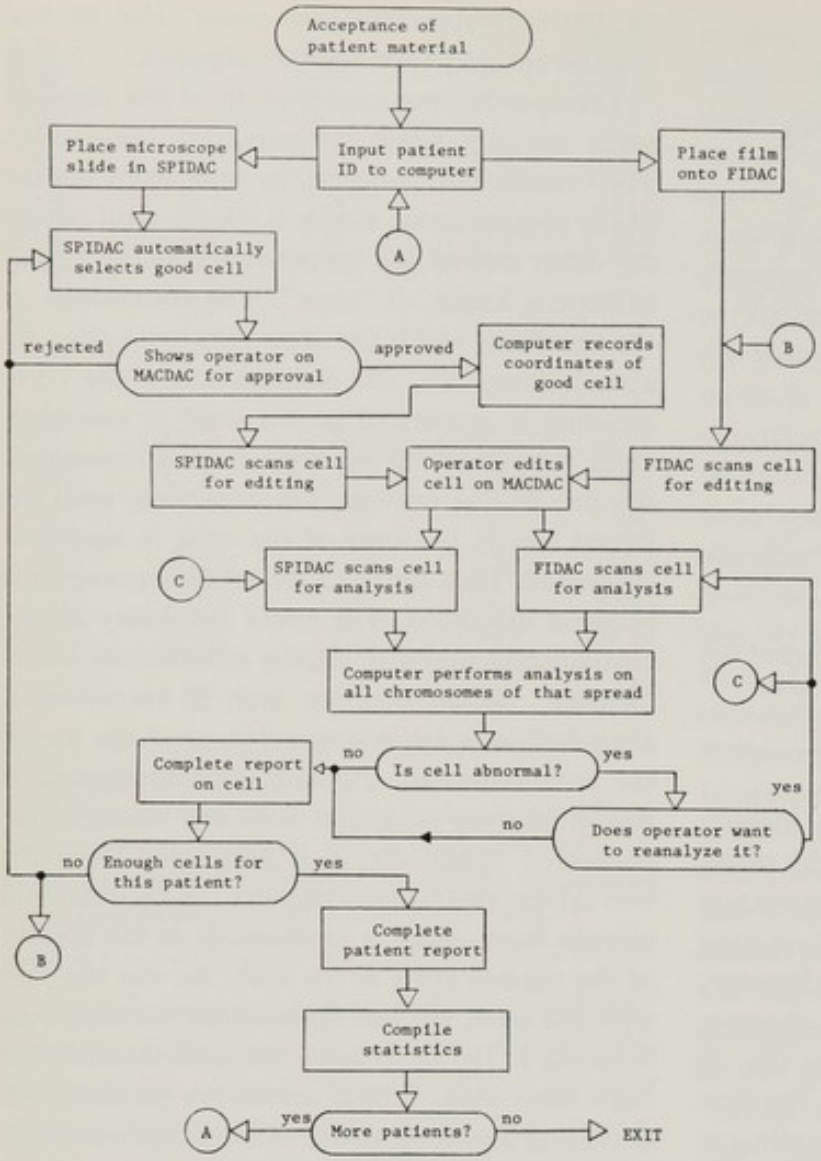


Fig. 7. Flow chart for processing metaphases.

The addition of the banding analysis has not greatly changed the time necessary for the analysis of one cell. Many types of output are possible (see next section) and these influence to a significant degree the total time necessary to analyse a cell. If, for example, data must be transferred to tape for Calcomp plots of the density curves and outlines of each chromosome, the time/cell approaches 2 min. No time study has yet been carried out when input is directly from a slide, since the program is being rewritten in machine language, but in view of the nature of the system in which different cells are being analysed simultaneously 2 min/cell is a reasonable expectation.

Results

Our most active current work is in developing a usable method of identifying each human chromosome. Several approaches are being evaluated. These include the use of Fourier analysis [2], a statistical analysis of differences from the standard curves for each of the 24 pairs, the use of the discriminate function and Bayesian approaches and, lastly, the application of an empiric approach. The latter involves using the most critical features of the curves such as one or two principal maxima or minima. In general these mimic the cytogeneticist's description of the major features. As part of this approach the variation in these points is being evaluated.

To date, the most promising results have been obtained using 9 pairs of Fourier coefficients. These results are shown in table 3. Eleven cells prepared and stained by the Giemsa 9 technic were analysed. Chromosomes were first placed in the classical groups using the cytogeneticist's identification and the results are, therefore, a within-group comparison. (From the data on table 2, nearly as good results would be obtained

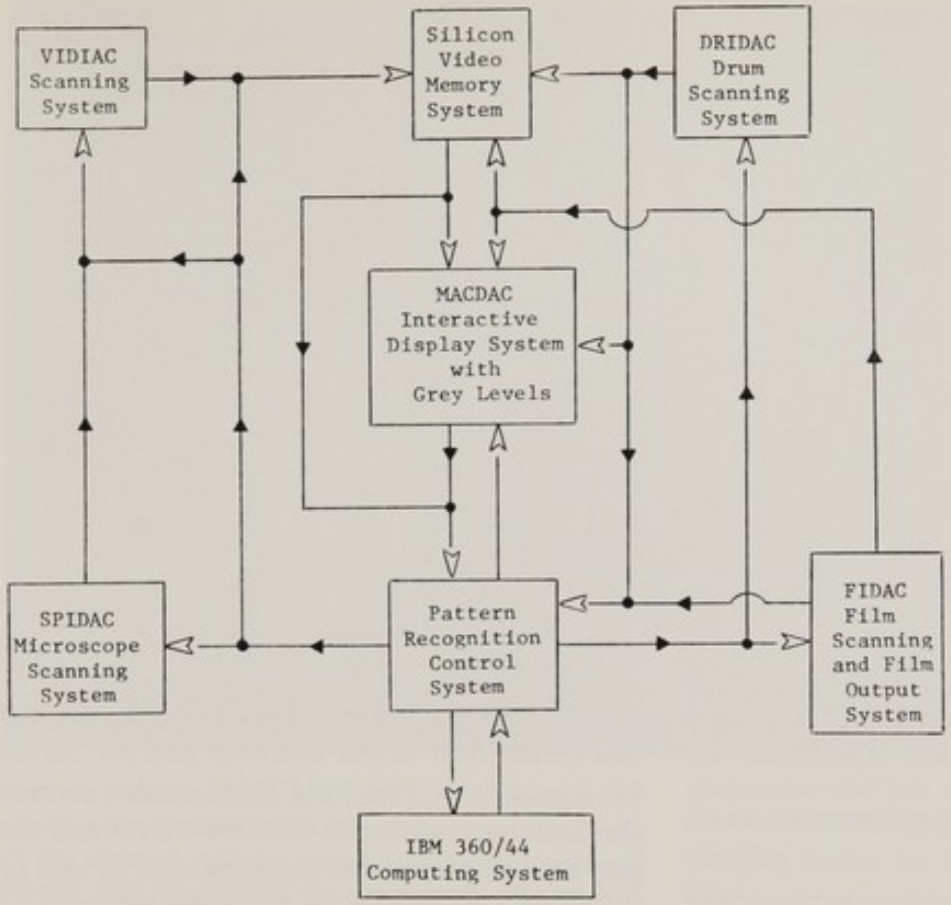


Fig. 8. Interrelationship of components of the automated analyses system.

Table 3. *Assignment by Fourier analysis* (*data given as number of chromosomes*)

Assignment by cytogeneticist	1	2	3	4	5	6	7	X	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Y
1	22																							
2		21	1																					
3			22																					
4				21																				
5					22																			
6						20					1		1											
7							17		1	3	1													
X						1		14	1				1											
8								1	20		1													
9							2		1	18			1											
10						1					20													
11												21												
12													20											
13														22										
14															18	4								
15															1	21								
16																	21		1					
17																		22						
18																			22					
19																				20	2			
20																				2	19			
21																						20		2
22																						3	18	1
Y																						1		4

N, 11 cells.
Data-FIDAC system.

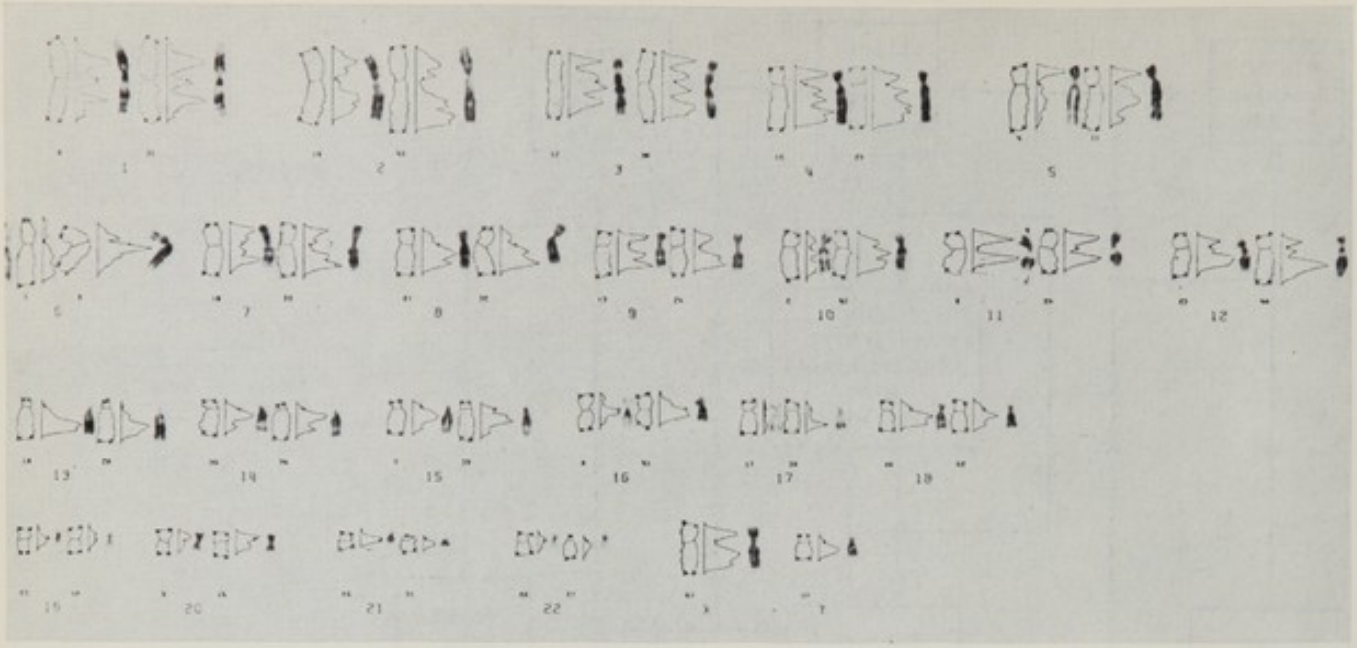


Fig. 9. Grey level profiles for an entire cell where the classical analysis of each chromosome has been plotted by the computer as well. Next to the grey level profile curves a photograph of the actual chromosome was pasted onto the Calcomp plot so that easy comparison can be made between the original chromosome and the grey level profile.

using the maximum probability approach in the first step.) The cytogeneticist's identification (left) was compared to the results of the Fourier analysis (top); 92% of the time these were identical. In only 32 of 506 chromosomes did the Fourier analysis lead to a different identification and it may have been more accurate in some instances. Of more importance is the fact that there were only an average of 3 mistakes/cell. This is an acceptable level for operator decision and correction. The number of cells studied has been kept to minimum number to permit thorough analysis of the results, but these results justify a trial in a larger series in which the norms and test-cells would differ. This will be undertaken shortly. The results using a second set of cells, i.e. not those from which the coefficients were generated will not likely be as good as those presented here. Because of stretching, overlaps, variation in clarity of banding etc, an operator decision for a small number of chromosomes is likely to remain necessary in the near future.

A number of types of output are possible with this system. We have found the most useful to be a Calcomp plot which includes both an outline of the chromosome and its derived density curves (fig. 9). In the latter figure a photograph of the chromosome from which the data were generated was placed next to the Calcomp plot for curves for comparison. The MACDAC system permits the operator to examine both the curves and the grey level display of the chromosomes, as well as the original metaphase, and with this information incorrectly identified chromosomes can be correctly placed in the karyotype. A better grey level display currently available will greatly improve this aspect of analysis. It is also possible to obtain a direct output of this same information in the form of a photograph of the banded karyotype generated through FIDAC (fig. 10). All of the above work has been carried out using cells stained by the Giemsa 9 technic. Adaptation of this system, however, to Q-banding essentially involved only the reversal of a grey levels and the same cell shown in fig. 9 was also stained with quinacrine and analyzed. A comparison of G and Q curve data from this cell is shown elsewhere in this symposium (p. 315–322, fig. 1) and in fig. 11 the G-bands are compared with published results of Q-banding. The similarity between those curves is quite striking, with the exception of certain *h* regions. The degree of variation between homologs and between cells for one chromosome [9] is shown in fig. 12.

Other types of output include the classical measurement data for lineal and areal measurements after correction for differential contraction [5], a statistical analysis of between homolog differences in length, and display of the results in a variety of ways at the choice of the operator.

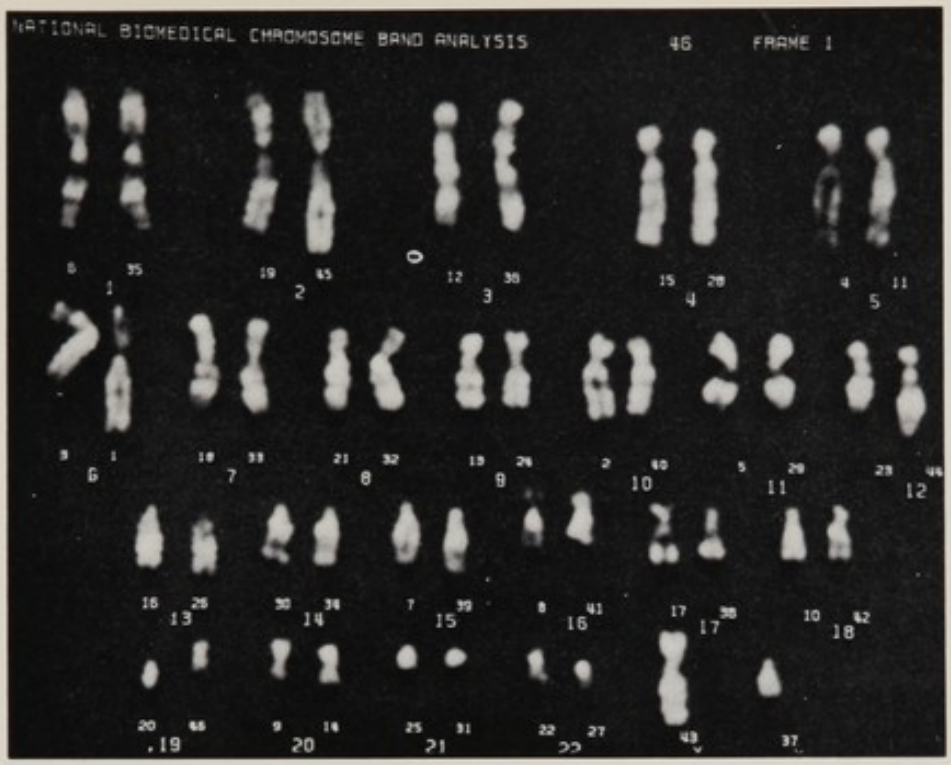
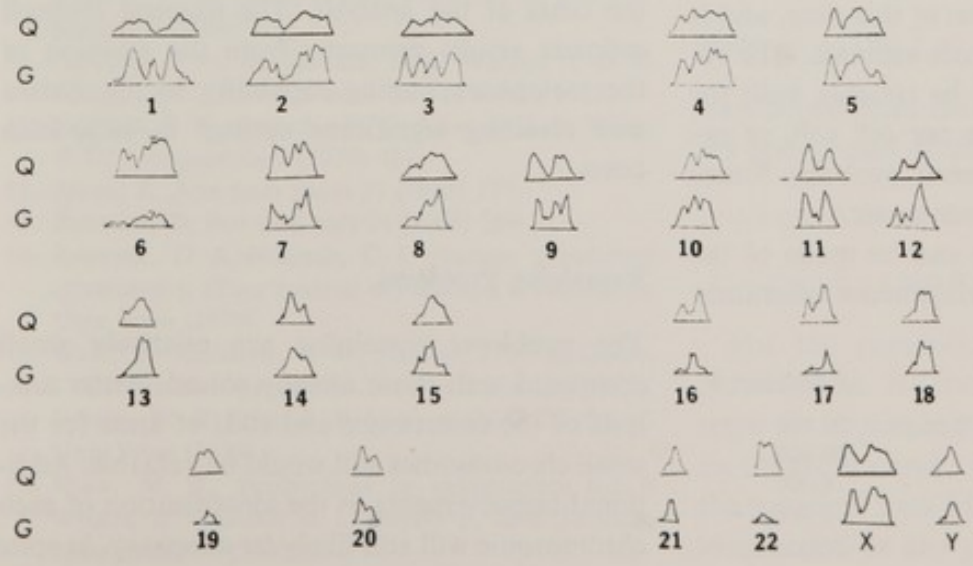


Fig. 10. Picture actually generated by the computer by exposing film in the FIDAC. The picture shows the result of the classical analysis carried out on chromosomes stained with the banding Giemsa 9 stain. The original chromosome spread was scanned, and each chromosome was rotated vertically and placed into the proper position as determined by the computer's classical-chromosome-analysis program. Some of the low grey-level values of the chromosome bands do not come out well in the photographic reproductions shown. (The distortions appearing in the photograph are caused by the photographic enlarger). Same cell as in fig. 7, shown here in negative form.

Fig. 11. Comparison of Q and G density curves. G curves from same cell as in fig. 7, and Q curves from published data of Caspersson.



These include histograms, plots, and displays of banding curves for a particular chromosome (fig. 12).

Review of Goals

Our primary goal can be described simply: We wish to produce an interactive automated system which will work as well as the cytogeneticist using conventional microscopic and photographic technics, but which will be significantly faster and cheaper. We expect the system to be better than the cytogeneticist only in so far as it will provide a quantitative and statistical evaluation of results and ready storage of results.

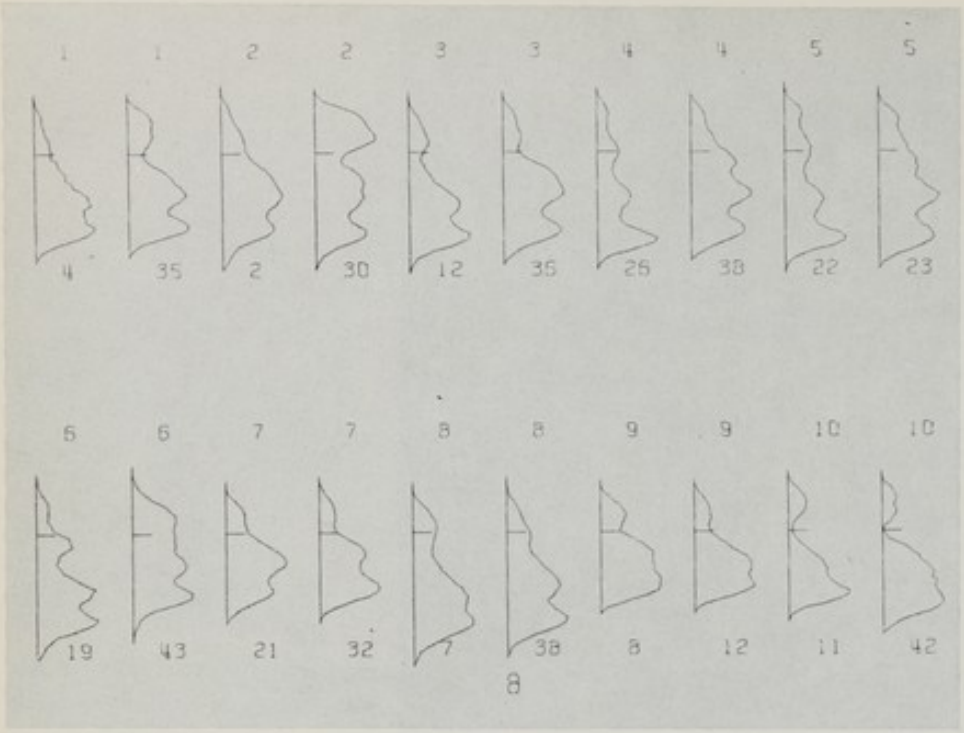


Fig. 12. Calcomp plot of both 8 homologs for 10 cells. The cell number is shown at the top of the curves and the numbers below indicate the order of recognition within the cell. All curves are normalized for height and length by group within a cell, not between cells. Chromosome 8 is shown because an average degree of between homolog and between cell variation occurred. For many chromosomes there is an even greater degree of variation. Centromere location determined by the automated analysis.

More precisely, after the system described above, which requires a large computer, has been proven effective for routine use, a simpler system will be built with a smaller computer (in the range of PDP8 or 12) in which much of the software will be replaced by hardware designed to carry out the same functions. It is our aim to make the total cost of the unit less than $100 000, including the computer. How small the computer can be is the major unknown at this time, and is the major variable in the cost estimate. The remainder of the system can be roughly built for less than $80 000. The time per cell will, of necessity, be longer than at present, probably 5 min/cell because of the smaller computer.

An interesting projection can be made of the capabilities and costs of a cytogenetics laboratory equipped with this device:

Expenses, $	Cost/year, $
Total cost 100 000 divided over 5 years	20 000
Two technicians, each at 10 000	20 000
Media	6 000
Miscellaneous	4 000
	50 000

Costs/cell: Number cells/year at 5 min/cell = 12 h × 8 h/day × 260 days = 25 000 cells (1 shift), Or a cost/cell of $2.

Capabilities at $2/cell over a year: 2 cells at $4/person for 12 500 patients; 10 cells at $20/person for 2 500 patients; 20 cells at $40/person for 1 250 patients.

Even if the cost/cell was $3–4 it would still be lower than the present cost and would include an improvement in capabilities of the laboratory. Three such units would permit the screening of 2 cells from each pregnancy in Colorado (about 40 000 births/year), and the prevention of at least 200 children with major cytogenetic abnormalities. A larger computer, if justified by sufficient volume, would permit a reduction in time/cell. The addition of the banding data has added little to the costs of the analysis. The lowered cost/cell estimate results primarily from the addition of the metaphase finding capability to the system and resulting significant savings in technician costs.

Remaining Problems

The problems remaining are relatively small compared with those already solved. Better analysis of the centromere and ends of arms for the small chromosomes still would be valuable. Additional improvements in the identification of each chromosome will still likely be necessary, in spite

of present promising results. Technics must be worked out to handle banding at difficult stages of compaction. The recognition of polymorphisms and statistical comparison of the curves of homologues over a series of cells is necessary for population studies. The most important problem in the very near future, however, is likely to be the limitation placed on the system by technical variables in the preparations presented for the automated system. This aspect also is a good candidate for automation but work is still in its preliminary stages. There is no inherent reason why we cannot learn to make better preparations more efficiently.

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Appendix I

Method of finding the rectilinear principal axes and the parabolic approximation for centerline of a bent chromosome.

From mechanics, the angle θ of the principal axes of a closed curve C is such that

$$\tan 2\theta = \frac{2\int_C x' y'\, ds}{\int_C x'^2\, ds - \int_C y'^2\, ds}$$

where s is the arc length and x' and y' are coordinates based on any axes with origin at the centroid x_0, y_0; that is, $x' = x - x_0$ and $y' = y - y_0$. Hence

$$\tan 2\theta = \frac{2\int_C (x - x_0)(y - y_0)\, ds}{\int_C (x - x_0)^2\, ds - \int_C (y - y_0)^2\, ds}$$

$$= \frac{2\int_C xy\, ds - 2x_0 \int_C y\, ds - 2y_0 \int_C x\, ds + 2x_0 y_0 s}{\int_C x^2\, ds - 2x_0 \int_C x\, ds + x_0^2 s - \int_C y^2\, ds + 2y_0 \int_C y\, ds - y_0^2 s}$$

But $x_0 = \frac{1}{s}\int_C x\, ds$ and $y_0 = \frac{1}{s}\int_C y\, ds$. Hence

$$\tan 2\theta = \frac{2\int_C xy\, ds - 2sx_0 y_0 - 2sx_0 y_0 + 2sx_0 y_0}{\int_C x^2\, ds - 2sx_0^2 + sx_0^2 - \int_C y^2\, ds + 2sy_0^2 - sy_0^2}$$

$$= \frac{2\int_C xy\, ds - 2sx_0 y_0}{\int_C x^2\, ds - sx_0^2 - \int_C y^2\, ds + sy_0^2}$$

Based on the boundary of a digitized picture, $ds = 1$ and $s =$ the number of points on the boundary. Hence,

$$\tan 2\theta = \frac{2\sum x_i y_i - 2nx_0 y_0}{\sum x_i^2 - nx_0^2 - \sum y_i^2 + ny_0^2}$$

where $x_0 = (1/n)\sum x_i \quad y_0 = (1/n)\sum y_i$.

New coordinates are established for each boundary point based on the principal axes:

$$x' = x\cos\theta + y\sin\theta - x_0\cos\theta - y_0\sin\theta$$

$$y' = x\sin\theta - y\cos\theta - x_0\sin\theta + y_0\cos\theta$$

For the parabolic approximation to a bent chromosome, let the center line of the chromosome be approximated by a parabola $y = px^2 + qx + r$ where x and y are the coordinates based on the principal axes (dropping the primes for convenience). For any point x_i, y_i,

$$\Delta y_i = y_i - y(x_i) = y_i - px_i^2 - px_i - r$$

and

$$(\Delta y_i)^2 = (y_i - px_i^2 - qx_i - r)^2$$

Hence

$$\sum(\Delta y_i)^2 = \sum(y_1 - ps_i^2 - qx_i - r)^2$$

At minimum $\sum(\Delta y_i)^2$, i.e. for best fit, the partials with respect to p, q, and r vanish:

$$\frac{\partial \sum(\Delta y_i)^2}{\partial p} = \sum[(-x_i^2)(y_i - px_i^2 - qx_i - r)] = 0$$

$$\frac{\partial \sum(\Delta y_i)^2}{\partial q} = \sum[(-x_i)(y_i - px_i^2 - qx_i - r)] = 0$$

$$\frac{\partial \sum(\Delta y_i)^2}{\partial r} = \sum[(-1)(y_i - px_i^2 - qx_i - r)] = 0$$

or

$$p\sum x_i^4 + q\sum x_i^3 + r\sum x_i^2 = \sum x_i^2 y_i$$

$$p\sum x_i^3 + q\sum x_i^2 + r\sum x_i = \sum x_i y_i$$

$$p\sum x_i^2 + q\sum x_i + rn = \sum y_i$$

These last three equations constitute a set of linear equations from which p, q, and r are determined by Cramer's rule. New coordinates are established for each boundary point based on the parabola. The new y is the (approximate) perpendicular distance from the point to the curve; and the new x is the (approximate) distance along the curve from the center of the parabola to the intersection of the curve with the normal from the point: y' is approximated as $(y-d)\cos\alpha$, where

$$\tan\alpha = \frac{dy}{dx} = 2px + q \text{ and } d = px^2 + qx + r$$

x' is approximated as the arc length of the parabola (to x) plus $(y-d)\sin\alpha$. Let the parabola $d = px^2 + qx + r$ be represented also by $d - b = p(x-a)^2$; hence

$$a = \frac{q}{2p} \text{ and } br - pa^2.$$

A standard approximation to the arc length of such a parabola is

$$\sqrt{(x-a)^2 + \tfrac{4}{3}(d-b)^2} \simeq (x-a) + \frac{2(d-b)^2}{3(x-a)}$$

$$\simeq (x-a) + 2/3(d-b)\,p(x-a)$$

Hence $x' = x - a + {}^2/_3 p(x-a)\,(d-b) + (y-d)\sin\alpha$

$$y' = (y-d)\cos\alpha$$

where $a = -q/2p$, $b = r - pa^2$, $d = px^2 + qx + r$, and $\alpha = \arctan(2px+q)$.

Discussion

Ford: Under what magnification is the scanning done?

Lubs: Initially, cells are located under a 40× oil immersion lens and these same cells are then relocated automatically with 100× oil immersion. The analysis is done under the latter magnification.

Bahr: Does your system use automatic focusing algorithms or is the focus kept by mechanical means?

Lubs: The principal means of maintaining focus is mechanical although it can be adjusted automatically.

Hamerton: Could you tell us how long the total process from finding a metaphase-spread to complete karyotyping takes including operator–machine interaction?

Lubs: If a negative of a metaphase is used as a starting point, the classical analysis takes from 1 to 2 min depending on the type of output that is desired. For example, Calcomp plots require the longest time, and numerical output the least. The new parts of the program are currently being rewritten in machine language so that it is difficult to know what the final working time will be either from a slide or negative but we do anticipate a time of about 2 min/cell for the analysis of banded material.

Automatic Systems for Chromosome Identification

K. R. Castleman and R. J. Wall

Jet Propulsion Laboratory, California Institute of Technology, Pasadena, Calif. 91103, USA

The recently developed chromosome staining techniques which produce characteristic banding patterns [1–10] provide new promise and new problems for automatic chromosome identification. Automatic systems offer two advantages over manual techniques for chromosome identification. These are (1) high speed which results in lower cost per cell analysed; (2) the ability to extract quantitative measurements and perform analyses which cannot be done otherwise. An automated system for chromosome identification should be viewed as a tool for use in chromosome analysis. While the machine can locate, measure and identify chromosome images, the validation and interpretation of the result is left to the human. The exact nature of the expected machine analysis will vary with the objective of each particular genetic test. The analysis which best serves a mass screening project, for example, where abnormalities are to be detected in a clinically asymptomatic population is somewhat different from that which would best support testing for a specific anomaly. The amount of a priori information which can be used by the machine will also vary with the application. In some cases the chromosomal number and sex are known beforehand, and this information can be used in the analysis to assist identification. In other cases no such assumptions can be made.

We are developing an automatic system to identify chromosomes by type, flag abnormal configurations, and display the karyotype image for validation and interpretation by a human operator [11–14]. The analysis will be flexible enough to make use of whatever a priori information is available.

Automatic Chromosome Identification

The steps involved in automatic chromosome identification are shown in fig. 1. The first step is to position a suitable metaphase spread beneath the microscope objective and bring the image into focus. This can be done by manual or semi-automatic means [13, 14, 20].

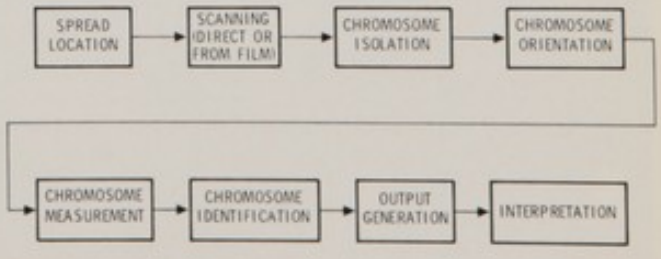
The second step involves conversion of the optical image into numerical form. The image is sampled at equally spaced points in a rectangular scanning pattern. At each point optical density (or fluorescent intensity) is quantified and assigned a grey-level value. The cell image can be digitized by a scanner mounted directly on the microscope or the cell can be photographed and the resulting film scanned by a separate device [11]. Direct scanning requires special purpose equipment but avoids the delay and photometric nonlinearity of the film intermediary.

After the spread image is stored in the computer as a numerical array of grey levels, this digital image is examined by the computer program to detect and isolate the individual chromosome images. The individual chromosomes are detected and separated from the surrounding background on the basis of grey level.

The next step is the orientation of the chromosomes to a consistent frame of reference. Each chromosome image is rotated to bring its principal axis to the horizontal. Our system determines the original angle of the principal axis by enclosing each chromosome in a rectangle of minimum area [11, 13].

After the chromosomes have been oriented,

Fig. 1. Automatic chromosome identification.



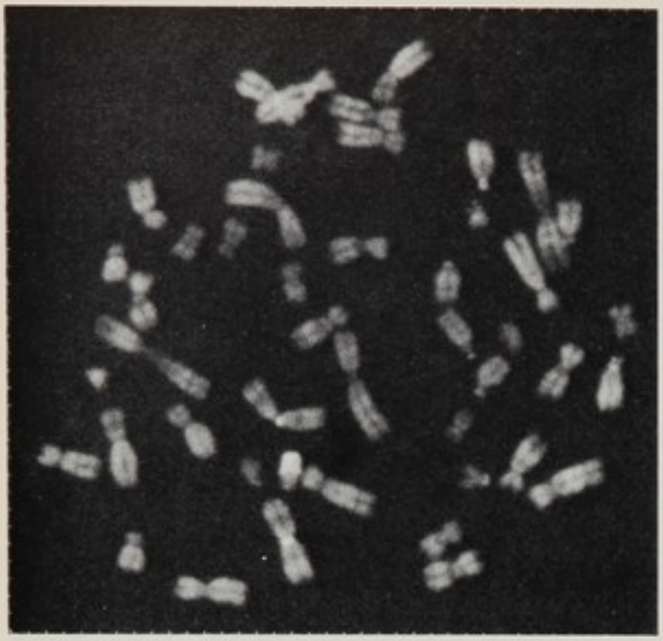


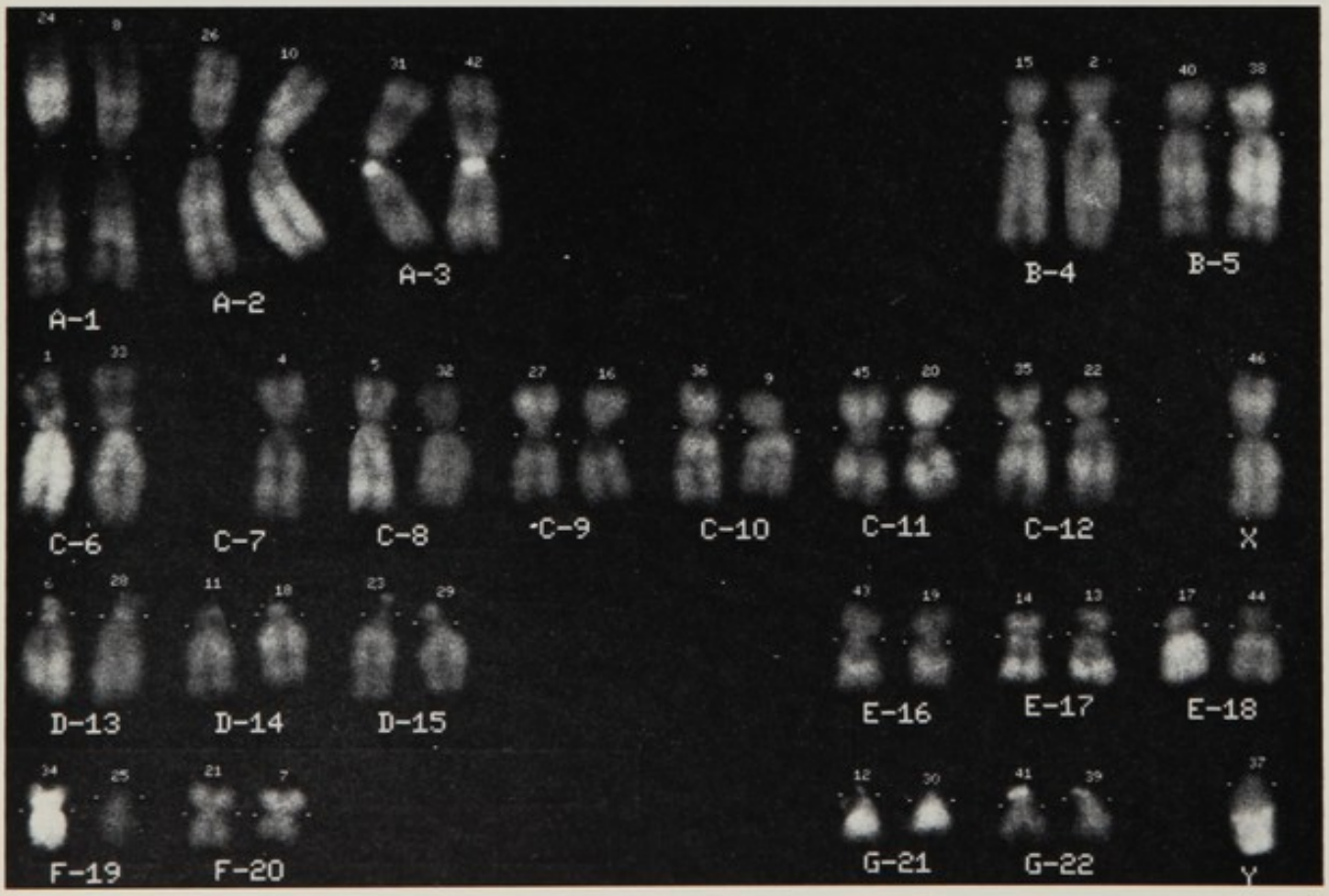
Fig. 2. A QM-stained chromosome spread. This spread image was scanned from a fluorescence photomicrograph negative and reconstructed by a computer display device. Negative by courtesy T. Caspersson, Karolinska Institutet, Stockholm, Sweden.

measurements are extracted. Single quantities such as length and area can be extracted from the chromosome images directly. With banded specimens the chromosome image is reduced to a function which reflects the stain distribution along its length. This is done by computing the average grey level in strips perpendicular to the chromosome axis. The profile waveform, defined as the average optical density at equally spaced points along the chromosome axis, shows the bands as distinct peaks. This function is further analyzed to extract a set of measurements which characterize the banding pattern. These include global measurements such as Fourier coefficients [15, 16] and local measurements such as the location and grey-level amplitude of each band.

The chromosomes are identified on the basis of the measurements extracted in the previous step. The accuracy of the identification is affected by the stability of the chromosome banding pattern, the discrimination afforded by the waveforms, and the reliability of the techniques used to analyse the waveforms. Classifiers are under development in our laboratory and elsewhere using both local and global measurements [11, 12, 15, 16].

The results of the analysis are displayed to the

Fig. 3. Computer-produced karyotype. The chromosome spread in fig. 2 was processed by computer to produce this output. The chromosomes were grouped automatically by arm length measurements and paired by human interaction.



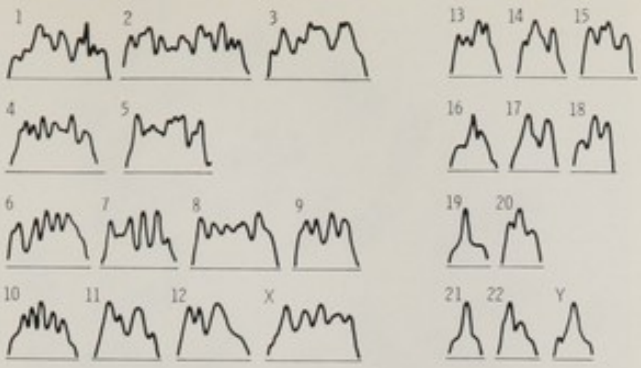


Fig. 4. Trypsin-Giemsa profile waveform ensemble composed of computer-produced profile waveforms from several trypsin-Giemsa spreads.

Fig. 5. *Abscissa:* chromosome type; *ordinate:* grey level.

Relative chromosome contrast. This plot illustrates the contrast variation among chromosomes in (*a*) 10 QM; (*b*) 10 trypsin-Giemsa spreads. The plot shows the difference between the mean interior grey level of each chromosome and the local background grey level. One point is plotted for each chromosome in the test set. The line connects mean values for each type. The grey levels represent equal steps in OD, 256 of which cover the entire contrast range of the specimen.

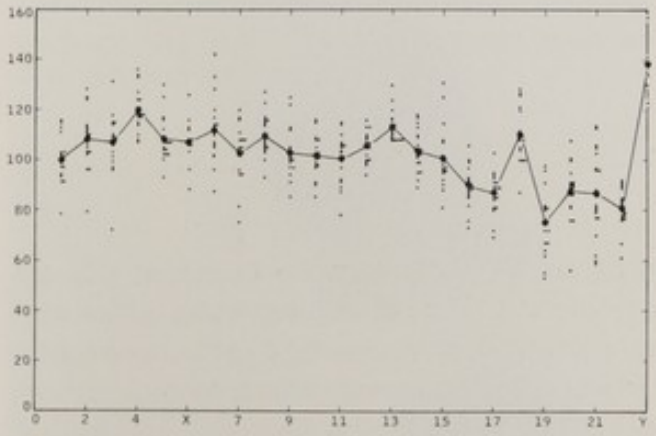


Fig. 5 a

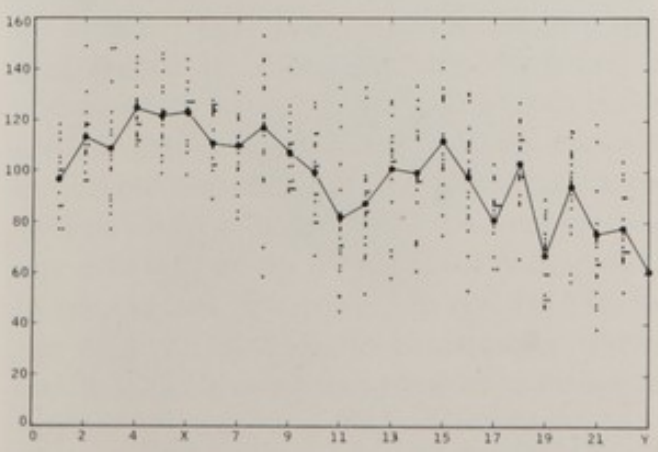


Fig. 5 b

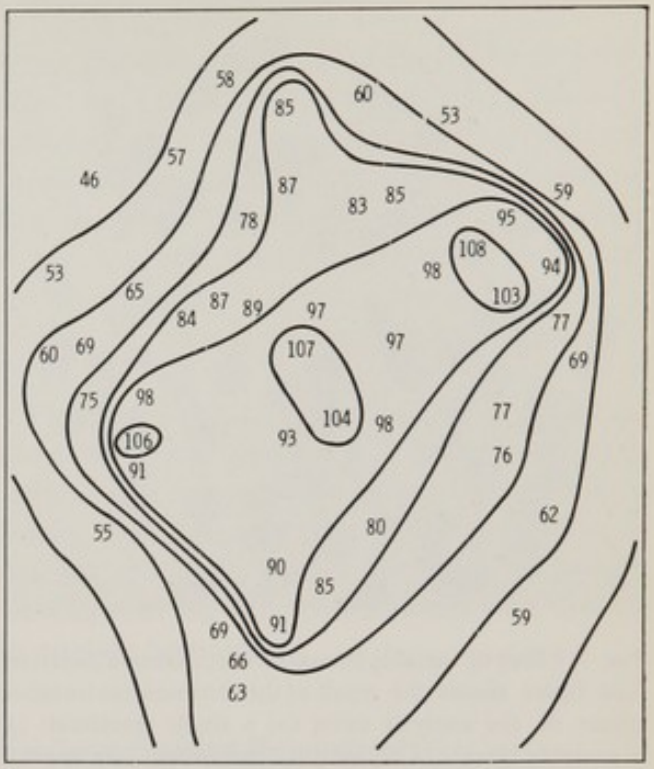


Fig. 6 a

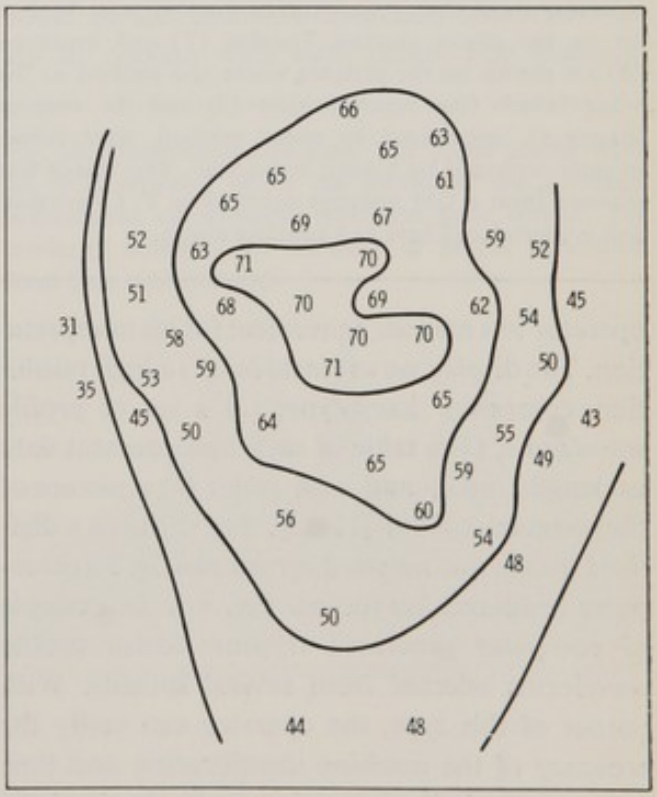
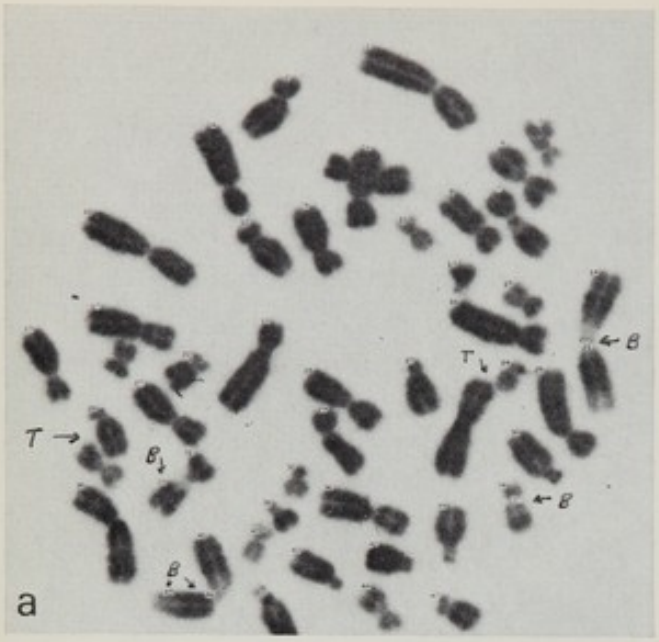


Fig. 6 b

Fig. 6. Background grey-level variation. This plot shows the mean background grey level as a function of position in (*a*) a QM; (*b*) a trypsin-Giemsa spread image. Background levels were determined automatically with contour lines added manually.



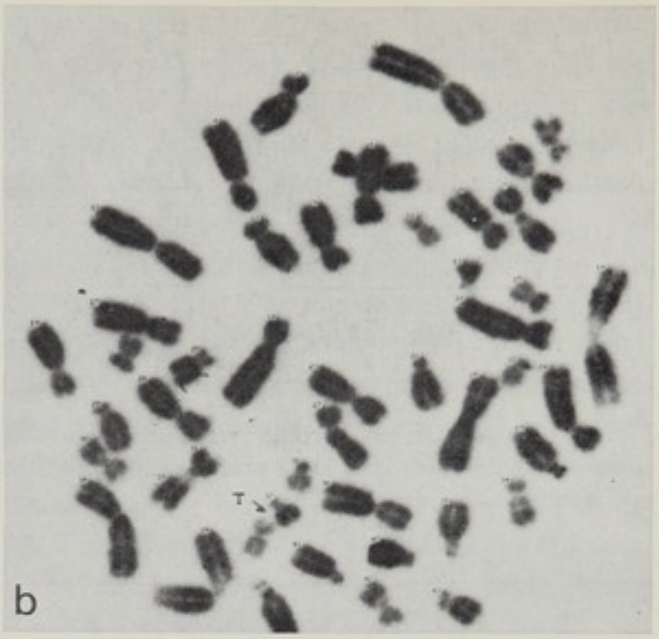


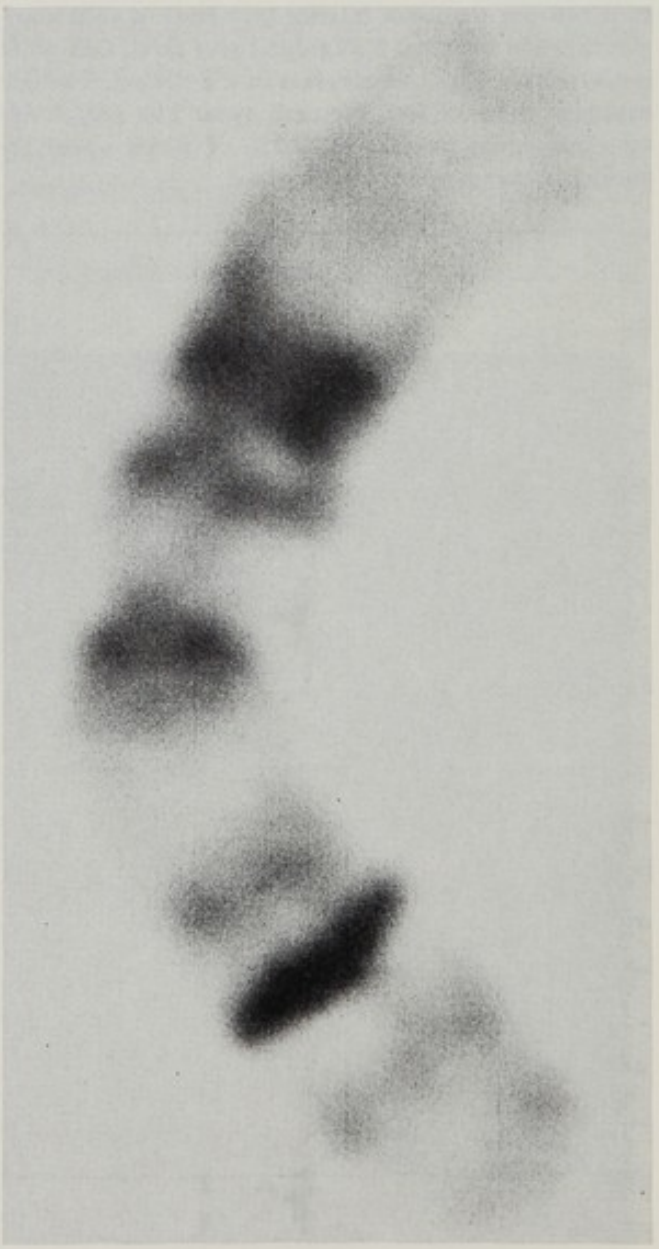
Fig. 7. Effect of variable threshold chromosome isolation. The figure shows the result of the chromosome isolation phase of the analysis using (*a*) a single threshold; (*b*) a variable threshold technique. The system has inserted sequence numbers at the top of each "object" located. Where a chromosome received no number, the analysis failed to separate it from another chromosome. Those receiving more than one sequence number were broken up by the object locator. Touches (*T*) and breakups (*B*) are shown on the pictures where one method or the other failed. One touch (object 17) and the overlap (object 6), unresolved by either method, were subsequently corrected by human interaction. This image was scanned from a QM negative supplied by T. Caspersson and is reproduced here as a negative image.

operator in a manner convenient for his interpretation. The display we use includes (1) a high resolution composite karyotype; (2) a set of profile waveforms; (3) a table of such measurement data as length, area, and arm ratio; (4) a picture of the original spread [11–15]. Fig. 2 shows a digitized quinacrine mustard spread and fig. 3 its computer produced karyotype. Fig. 4 is an example of computer generated trypsin-Giemsa profile waveforms selected from several spreads. With output of this type, the operator can verify the accuracy of the machine identification and then make a genetic interpretation on the basis of the data presented.

Chromosome Isolation

Chromosomes are detected and isolated by a grey-level thresholding procedure [12–14]. In order for this technique to work properly they must be

Fig. 8. A bent trypsin-Giemsa chromosome.



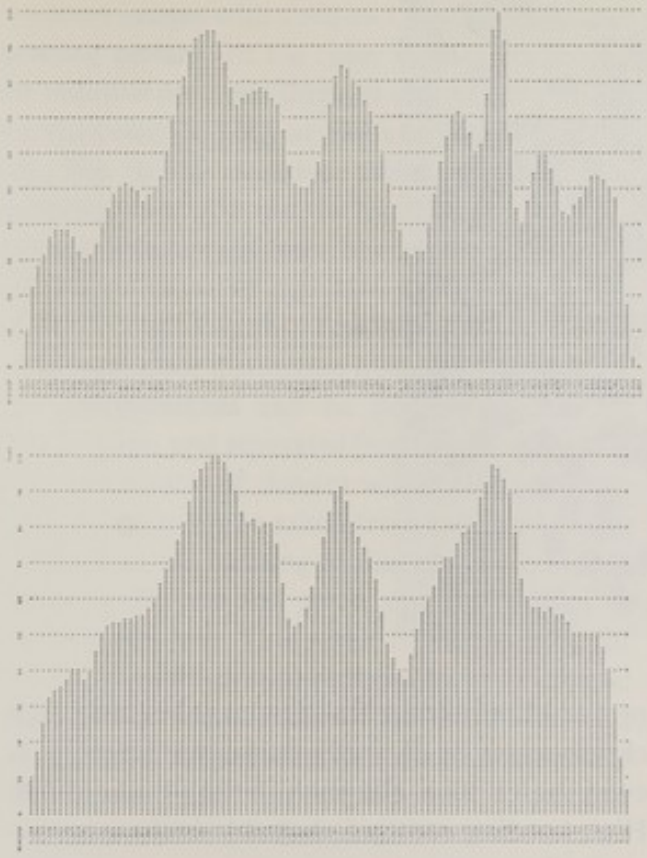


Fig. 9. Profile waveform comparison. These plots show computer-generated profiles for the chromosome in fig. 8. The upper plot accounts for bending while the method used to generate the lower plot assumes the chromosome is straight. The peaks corresponding to the bands are more distinct in the upper plot.

spatially separated within the spread and have reasonably sharp contrast with respect to the background. Fig. 5 illustrates the variation in average contrast among chromosomes in a spread. Background brightness variations also occur due to uneven illumination, glare, cytoplasmic fluorecence, or optical scattering phenomena (fig. 6). To minimize these effects we have designed and programmed an object detection and isolation scheme which starts by determining mean background grey level and chromosome contrast throughout the spread [12]. It then assigns a grey-level threshold to each chromosome commensurate with its contrast and the local background. In practice there is considerable threshold variation from chromosome to chromosome within each spread. This technique provides performance superior to the use of a single threshold throughout the entire image, as illustrated in fig. 7.

Chromosome Measurement

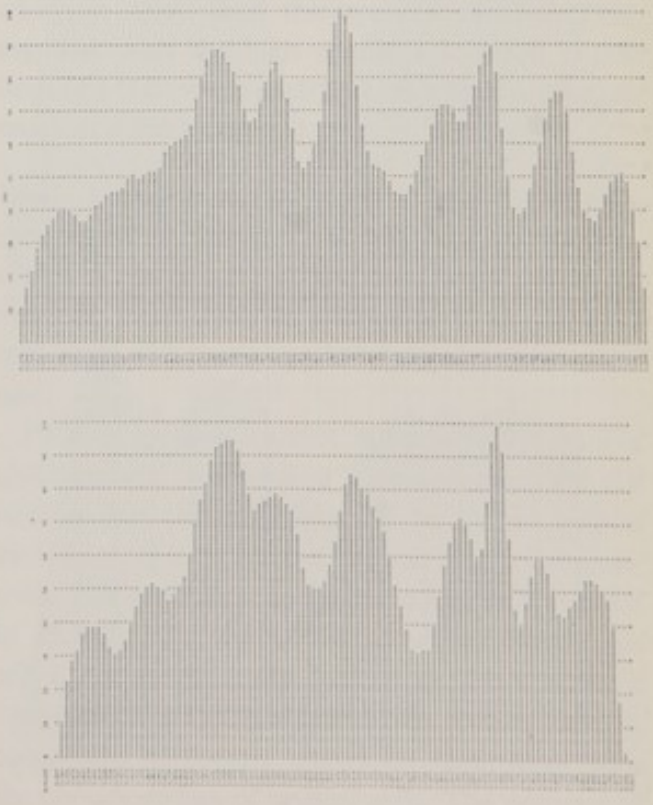
When the chromosome staining profile is generated by simple techniques which assume a straight axis, it can be distorted by chromosome bending. We have developed a waveform generation technique which is insensitive to bending. It uses the medial axis transform to locate the central axis of bent chromosomes and then computes the average grey level normal to this axis [12].

Fig. 8 shows a bent trypsin-Giemsa chromosome. Its profile waveform, as generated by this technique, is compared with the waveform which results from assuming that the chromosome is straight in fig. 9. Fig. 10 compares the profile waveform of the bent chromosome with that of its straight homolog.

Specimen Staining Requirements

Specimen characteristics play an important role in the success or failure of computer analysis. Some of the desirable specimen characteristics are in conflict with each other. For instance,

Fig. 10. Homolog comparison. The upper profile from fig. 9 is repeated below the profile of the (straight) homologous chromosome from the same spread. The similarity indicates the accuracy of profile generation from bent chromosomes.



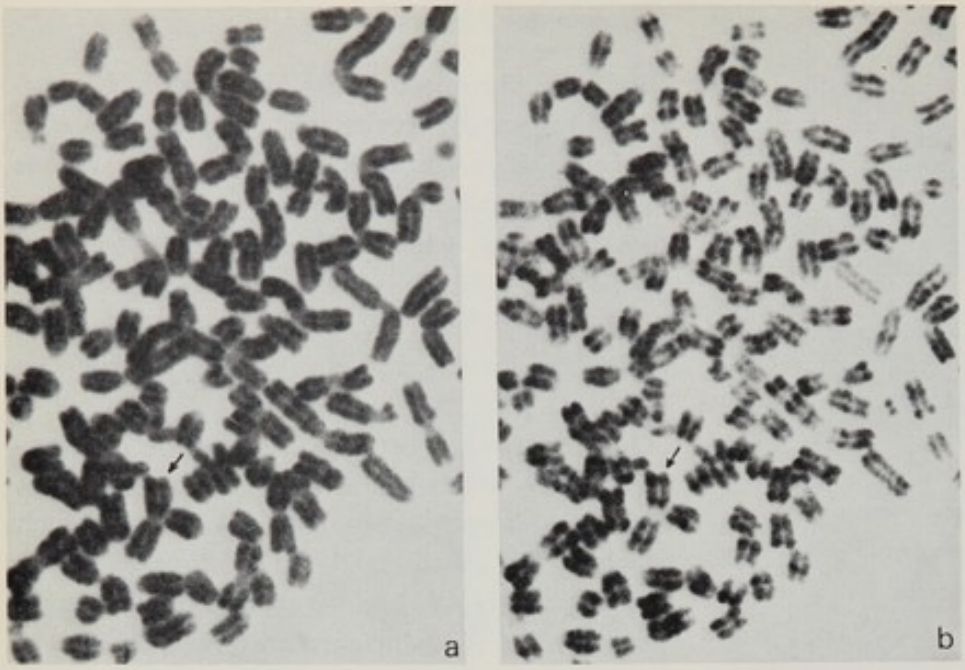
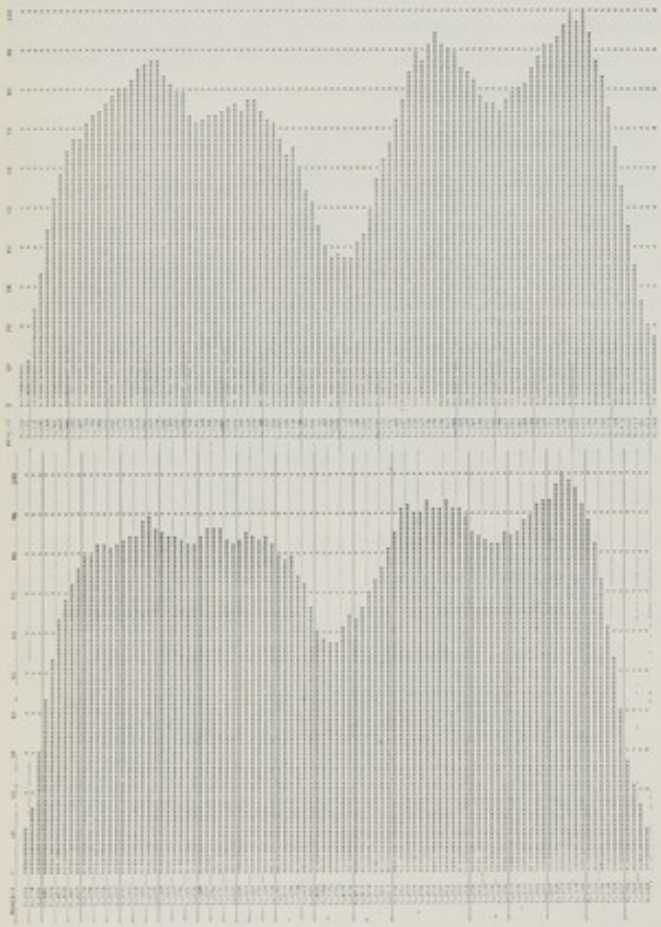


Fig. 11*a*, *b*. Red and green color separation pictures of A + O stained mouse chromosomes.

Fig. 12. Red-and green-derived profiles from one chromosome. The lower plot is a computer-produced profile from the red image of the chromosome marked by the arrow in fig. 11. The upper plot is the profile of the same chromosome in the green image. Note the higher contrast of the banding pattern in the upper plot.



proper chromosome isolation requires that each chromosome be distinctly darker (or brighter) at every point than the surrounding background. High banding contrast, desirable in the measurement phase, may produce specimens with negative bands scarcely distinct from the background. Thus the ideal specimen would have negative bands significantly darker than the background and positive bands significantly darker yet.

The various staining procedures in use at the present time each emphasize specific characteristics of the chromosomes. Some show the traditional banding pattern and some the reverse pattern, while others emphasize characteristics such as heterochromatin, polymorphic regions, and satellites. Each of these characteristics appears useful for identification purposes but no single staining technique appears optimal for them all.

Color Banding and Multispectral Analysis

We have conducted a pilot study to examine the possibility of combining two or more stains of different colors, scanning the chromosome images, and separating the effects of the stains by multispectral analysis. Fig. 11 shows red and green color separation negatives made from a 35 mm color slide of mouse chromosomes stained with acridine orange. The chromosomes were prepared by Dr David Comings who treated the chromosomes with sodium hydroxide and formaldehyde to produce selective denaturation of the DNA [17]. Staining with acridine orange

produces images with a red and green fluorescence banding pattern. In the color separation process the negative obtained through the red filter shows the fluorescence both at 590 and at 530 nm [18, 19]. The green negative shows primarily the fluorescence at 530 nm. In fig. 11 *a* the chromosomes are uniformly dense but contain little banding information. In fig. 11 *b* the chromosomes exhibit a high contrast banding pattern, but in some areas poor distinction from the background. The chromosomes can be isolated in the red (11 *a*) image, and separated from the background. The distribution of density within the boundary thus obtained can be taken from the green (11 *b*) picture and the banding profile obtained. Fig. 12 compares the red and green staining profiles of one chromosome. This result is encouraging for the use of multispectral techniques in automatic chromosome identification. The hardware and analytical techniques exist, but the ultimate utility will depend upon the development of suitable multicolor staining techniques.

Automatic chromosome identification using the banding stains shows great promise for a role in the future of genetics. As preparation techniques and analytical methods are refined such systems are likely to become practical for clinical application.

We are indebted to Howard Frieden and Sayuri Harami for software development support, and to Dr Robert Nathan.

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Discussion

Comings: Was the reason the computer gave such excellent pictures because it had had a large number of shades of grey to work with?

Castleman: Yes. The system uses 256 grey levels which is more than the eye can resolve. This, together with the high spatial resolution (1 000 lines of 1 000 points each), yields a high resolution reconstructed image.

Wall: A major consideration in the utility of a computerized system to identify human chromosomes is the quality of the data with which the machine must deal. In particular it is desirable that each cell have spatially disjoint chromosomes.

Present systems cannot automatically resolve touching and overlapping chromosomes. We are hoping that this problem will be solved by the biologists. If not, we can pursue several alternatives. For example, the system could be designed to identify only those chromosomes from each cell which are spatially disjoint. In this manner a "composite karyotype" could be constructed. A composite karyotype would be assembled from the chromosomes of several cells. The number of

cells involved would depend upon how many the system had to examine in order to find spatially isolated examples of each homologous pair. That is, both homologs would be taken from a single cell.

My second comment relates to the performance of computerized karyotyping systems. It is intimately related to the characteristics of the data supplied. The cytological material from each laboratory and in fact each process can contain subtle idiosyncrasies which can severely impair the performance of an automatic system. Thus the probability of misclassification is only meaningful when the test data is of a quality similar to your own. The only presently feasible means of characterizing data quality for automatic chromosome analysis is by the use of a test set of cells. That is, in order to relate system classification performance to the material produced by a laboratory one should define system misclassification rates by automatically processing his own data.

The Use of Distribution Functions to Describe Integrated Profiles of Human Chromosomes

G. H. Granlund

Linköping University, Department of Electrical Engineering, 581 83 Linköping, Sweden

At MIT we have been working on a method to resolve the curve representing the integrated intensity or density into a number of Gaussian-like distribution functions. A curve can then be described as a number of triplets, each consisting of peak height, width and position of a distribution function. Our underlying assumption is that variations among chromosomes can be adequately described as variations of these distribution function parameters.

The new method can be characterized as an "optimization of heuristic features". The classification procedure assumes that each chromosome to be classified belongs to a certain class of a given set of classes. A curve-fitting procedure is performed where a curve representing an "average" chromosome of the reference class is matched to the actual chromosome by varying the distribution function parameters. When the error between the "optimized mean" and the actual curve is at its minimum, a set of parameters is available that usually gives a good description of the curve to be classified. This procedure is then repeated for the remaining classes. The derived sets of parameters can then be used as input for a classifier.

This procedure differs from a conventional least-square curve fitting procedure in that constraints are imposed on how parameters are allowed to vary during the optimization. This is necessary, first, in order to avoid the nearest local minimum, which usually is not the desired one. Secondly, the constraints can be used to provide information about global relationships so as to ensure a convergence towards a "reasonable and global" minimum or no convergence at all. From this procedure, together with the classification, three products are possible:

(1) The procedure converges and the derived set of parameters is found to be similar to the parameters of the reference set of the class with the given constraints.

(2) The procedure converges, but the set of derived parameters is found to differ from the parameters of the reference set of the class with the given constraints.

(3) The procedure does not converge because the constraints of the reference class are inconsistent with the actual curve.

The restrictions on variations in the parameters, given in the following sequence of iterations, have in all observed cases given a result consistent with the conceptual model.

(1) In the first sequence of iterations only the position of the peak is allowed to vary.

(2) In the second sequence of iterations the position of the peak and its height are allowed to vary.

(3) In the third sequence of iterations all variables are allowed to vary.

The number of distribution functions used to represent a chromosome integrated density profile varies from three to seven. Longer chromosomes require a higher number of distribution functions to provide a good description. Fig. 1 shows the number of functions that have been chosen to represent the various chromosomes. Each curve represents a reconstruction of the averages of the parameters within each class, with the lower curve being the sum of the individual distributions. It is far from clear that given representation is the optimal one for all cases of stains and preparations. However, the current representation has been chosen after an investigation of the material available, keeping the number of peaks as small as possible while including all

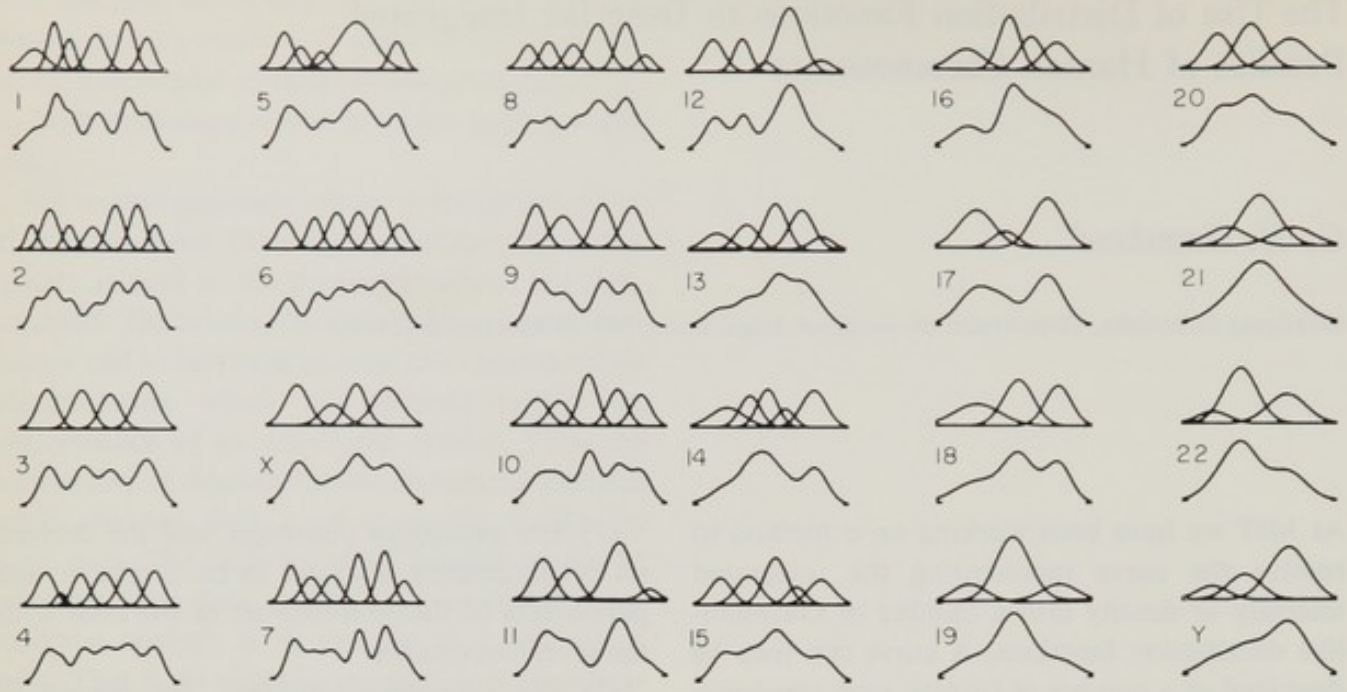

Fig. 1. Integrated density profile averages in terms of distributions.

consistent peaks. The number of peaks displayed here is thus smaller than, for example, what is indicated as possible in literature.

In the distribution representation the position of the centromere can be referenced with respect to the position of a particular peak, normally chosen to be the nearest one in the reference pattern. This results in a decreased sensitivity to local dilations.

It is important to note that this model does not try to give a complete description of a specific chromosome in the way that, for example, Fourier descriptors do. It rather gives a description in relation to a certain heuristic model for this class, with what cannot be described according to this model considered as noise.

There is a difficulty in comparing different numbers of parameters which here is solved by rescaling the errors to a magnitude corresponding to a case of four peaks. This leads, in essence, to a comparison of the error per feature.

In our classification experiments, 240 chromosomes of classes 1–22, X and Y have been classified. Using a distribution function representation, an accuracy of 96% was obtained. The same material was also used for classification using Fourier descriptors with an accuracy of 93%. A comparison using a curve matching procedure gave an accuracy of 90%.

Now, karyotyping is only one procedure that can be performed on chromosome data and it probably is not even the most important one. Karyotyping is a procedure that can be done very well manually with low estimated error rates by a technician. It certainly would be attractive if the tedious and time-consuming karyotyping task could be performed by a computer system.

What seems to be the most useful function of a computer system in this field is to perform statistical analyses on chromosomes. Since the discovery of the quinacrine mustard stain a number of variations among chromosomes have been discovered. Some of these variations have been found to be linked to different clinical syndromes. The detection of such variations is thus an important problem from the scientific, as well as the clinical point of view. In many cases the deviation can be readily observed in a karyotype. In other cases, the observer might suspect an aberration at a specific location on a chromosome, yet be unable to verify the fact. Detection of these smaller deviations is more difficult to do. This is essentially a signal detection problem where noise has to be filtered from a signal. One way to implement a filtering is to perform an averaging over the set of chromosomes.

A computer can use information about a chromosome, derived from 10 to 30 cells from the patient to establish a "filtered prototype" for that chromosome to compare with a reference. Such correlation of information from different

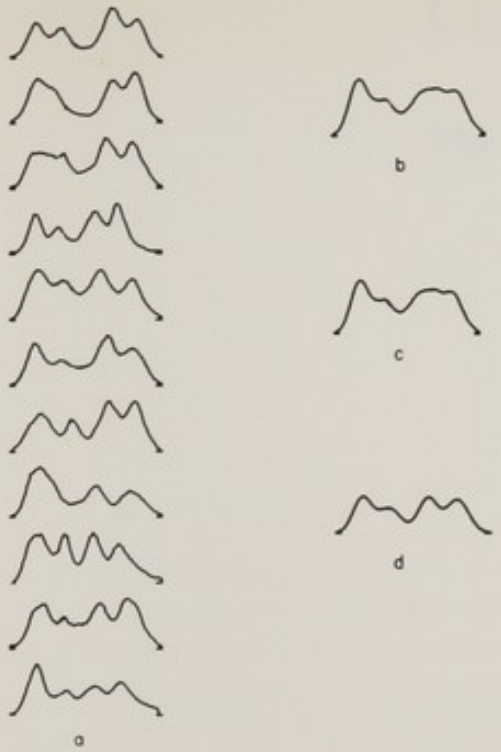

Fig. 2. Computed averages for chromosome No. 9 using different types of descriptors. (*a*) Curve samples; (*b*) average using curve; (*c*) average using Fourier; (*d*) average using distributions.

karyotypes is almost impossible with manual procedures.

The choice of parameters over which to perform the averaging is crucial. If the parameters are not in some way related to the physical mechanism causing the variation, the averaging operation will only give rise to smearing of the information. Fig. 2 gives a simple illustration of this fact. The information from 11 chromosomes number nine is combined by an averaging of different types of parameters. It is observed that the averaging over the curve samples (*b*) and the Fourier parameters (*c*) gives a reconstructed function that has lost some of the characteristic features of chromosome number nine. In the case of distribution parameters (*d*) the reconstructed average clearly displays the two separate peaks to the right.

One application of statistical chromosome analysis has already begun. It concerns a case of suspected aberration on a chromosome No. 9. It was here possible to establish with an extremely high statistical significance that one of the homologs was different from the other homolog; from the chromosomes of the parents and different from the chromosomes of a reference set taken from a number of normal persons. The interpretation and use of such findings is still another difficult problem which, however, is outside the scope of this discussion.

This technique has also been used to "recognize" the chromosomes of different individuals using a number of cells to supply the information for each individual.

Chromosome Banding Techniques

The human karyotype, nomenclature

Chromosome Band Nomenclature—The Paris Conference, 1971

J. L. Hamerton

Department of Paediatrics, Division of Human Genetics, University of Manitoba,
The Children's Hospital of Winnipeg, Winnipeg, Manitoba, Canada

The Conference

The intention of the Paris Conference, held on September 6–11, 1971 at the time of the Fourth International Congress of Human Genetics was to agree on an international classification of the human somatic chromosome complement based on the banding techniques which had been or were in the process of being developed. Historically the meeting was the fourth in a series of International Meetings on human chromosome nomenclature, the first of which was held in Denver in 1960 [1]. The purpose of each of these meetings, the number of participants and the results are summarized in table 1. This shows an expansion of 350% in the number of participants and laboratories during the past 12 years. The necessity of this fourth meeting became apparent at the Colorado Springs Meeting on perspectives in human cytogenetics held in 1970 when Caspersson [2] elegantly demonstrated the fluorescent banding of the human karyotype and showed the relative ease with which each chromosome pair could be identified. It was immediately clear to some of us that these observations were perhaps as significant as those made 15 years earlier, also in Sweden, by Tjio & Levan [3] when they so beautifully demonstrated that the human chromosome number was 46 and not 48. This observation led directly to the publication of the human karyotype by a number of laboratories and so directly to the Denver classification. Caspersson's results demonstrated a vast increase in the precision with which the human complement could be characterized and necessitated the revision of the Chicago Nomenclature [4] and the establishment of an internationally acceptable system for chromosome banding and band nomenclature.

The terms of reference for this meeting were; (1) to agree on an internationally acceptable system of nomenclature for each human chromosome on the basis of the available techniques and to make it sufficiently flexible so that further characterization with future technical advances could be related to this description; (2) to consider amendments to the Chicago Nomenclature; (3) to discuss other areas relating to the human chromosomes and to explore areas in which agreement existed.

The Report

The Report of this Conference (Paris Conference, 1972), obtainable from Dr D. Bergsma, National Foundation, March of Dimes, 1275 Mamaroneck Avenue, White Plains, N.Y. 10605, USA, incorporates the report of the Standing Committee which was appointed at the conclusion of the Meeting and which met in Edinburgh in January, 1972 (see below). The results of these meetings can be summarized as follows:

(*a*) Amendments to the Chicago Nomenclature. A few minor amendments were recommended for the shorthand nomenclature adopted at Chicago in 1966. These related to (1) the position of the "+" and "−" signs; (2) description of the length of the secondary constrictions; (3) the use of parentheses. Other topics discussed apart from banding included autoradiography, measurement, human meiosis and terminology.

(*b*) Methods of chromosome banding using fluorescence [6], Giemsa [7–15] and the denaturation–reassociation [16] techniques were discussed in detail and operational definitions were assigned to each technique as follows: quinacrine mustard and quinacrine—Q-bands or Q-staining techniques; Giemsa and staining techniques using

Table 1. *Chromosome nomenclature conferences*

Year	Place	No. of participants	No. of laboratories	Result
1960	Denver	14	12	Classification of the Human Karyotype
1963	London	26	21	New Methods of Identification of Individual chromosomes
1966	Chicago	37	34	Shorthand Nomenclature
1971	Paris	50	42	Classification of the Human Karyotype in Relation to Banding

Giemsa and related stains after appropriate pretreatments—G-bands or G-staining; methods utilizing Giemsa after heating to 87°C and giving reverse banding [17]; R-bands or R-staining and finally C-bands or C-staining for constitutive heterochromatin, demonstrated by the denaturation–reassociation techniques [16]. The agreement on chromosome numbering was based on Q-banding and the other staining methods were compared to the Q-bands.

Agreement was reached on the definition of a band, as "a part of the chromosome which is clearly distinguishable from its adjacent segments by appearing lighter or darker in the "Q", "G" or "R" techniques". Bands that are dark by one method may appear lighter by another. All areas of the chromosome were specified as belonging to a band and therefore by definition there were no inter-bands.

The karyotype and descriptions originally published by Caspersson et al. [6] were accepted as the basis for describing the human chromosomes and as the standard for identification. The description of the human karyotype was confined to visually recognizable patterns and data obtained by photometric scanning was used only to confirm the previously observed visual impressions. The previously used definitions, comprising length, centromere index, autoradiography and secondary constrictions were retained for chromosomes 1 to 5, 9, 13 to 18, and the Y chromosome, and in each case banding was related to the previous definition. For the remainder the description of each chromosome given by Caspersson [6] was accepted. The principle, accepted by the Denver Conference [1], that the chromosomes should be numbered in descending order of length was confirmed and retained. One exception to that rule was accepted. The number 21 was retained for the chromosome associated with Down's syndrome which, as a result of the measurement of banded chromosomes, is now known to be the shortest of the G autosomes. It was felt that alteration of the designation to No. 22 would create an unacceptable amount of confusion and that this could not therefore be justified. The major diagnostic features for each chromosome were agreed and are tabulated in the report on the basis of Q-bands. The G and R techniques were then compared to this definition. The major variation found between the results obtained with the different techniques related to the centromere, and the heterochromatic regions 1qh, 9qh and 16qh. Apart from these differences there was general agreement between the three banding techniques. The C-band for each chromosome pair was defined by its length and position along the chromosomes.

It was found impossible to agree on a detailed nomenclature for chromosome banding and this problem was referred to a Standing Committee. This consisted of B. Dutrillaux, H. J. Evans, C. E. Ford, J. Lindsten, F. Ruddle and myself, which, with the exception of Dr Ruddle, who was unable to attend, met in Edinburgh in January, 1972. The report of this Committee has now been incorporated in the final report of the Conference [5].

Chromosome Nomenclature

After extensive discussion, it was agreed that the nomenclature finally adopted should:

(*a*) Utilize consistent cytological landmarks which could be used for the division of the chromosome into regions.

(*b*) Allow precise designation of break-points in any chromosome rearrangement.

(*c*) Provide a means of designating normal and rearranged chromosomes according to their banding patterns, with the Q-, G- or R-techniques.

(*d*) Not involve chromosome measurement as this was impractical for routine use.

(*e*) Be designed for the microscopist and hence,

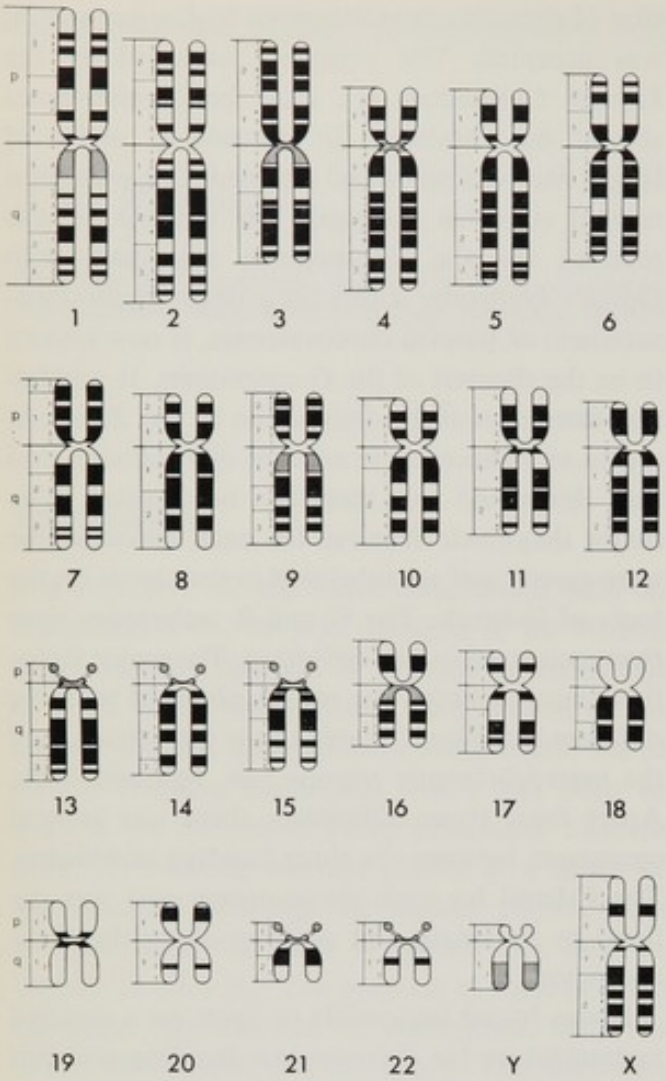

Fig. 1. Diagrammatic representation of chromosome bands as observed with the Q-, G-, and R-staining methods; the centromere is representative of the Q-staining method only. (Note: While the relative sizes of the bands are approximately correct, these are not based on measurement and no attempt has been made to take into account intensity of staining). □, negative or pale staining Q- and G-bands; positive R-bands; ■, positive Q-and G-bands; ▨, variable bands.

not utilize arbitrary divisions of the chromosomes into equal sized or fixed numbers of units.

(*f*) Facilitate communication between laboratories.

(*g*) Be as simple as possible to describe economically the majority of chromosome aberrations and not be over complicated by the excessive use of additional symbols.

(*h*) Be flexible, open ended and capable of expansion and development.

The chromosome banding diagram on which the band nomenclature was based is shown in fig. 1. It was based on the patterns observed in different cells stained with either the Q-, G- or R-techniques and the cells used were not stained sequentially with two or more of these techniques. The relative sizes of the bands shown in the map were felt to be approximately correct but were not based on measurement. In the diagram no attempt was made to indicate intensity of staining or fluorescence.

Each chromosome is considered to consist of a continuous series of bands. The chromosomes were divided into regions which are delimited by specific chromosome landmarks. A landmark is defined "as a consistent and distinct morphological feature that is an important diagnostic aid in identifying a chromosome". Landmarks include the ends of the chromosome arms, the centromeres and certain bands. A chromosome region is defined as any area of a chromosome lying between two adjacent landmarks. In the construction of the diagram each landmark or band is defined by its mid-point and not by its margins and because of variability the diagram of the centromere relates only to its Q-banding pattern.

The system adopted for the designation of arms, regions and bands, retains the designations "p" and "q" for the short and long arms of each chromosome respectively and the regions and bands are numbered consecutively from the centromere outwards. Thus, the region adjacent to the centromere is number "1" in each arm, the next more distal region "2" and so on. A band used as a landmark is considered as belonging entirely to the region distal to the landmark and is accorded the band no. of "1" in that region. The band bisected by the centromere is considered as two bands each labelled band 1 in region 1 of the appropriate chromosome arm. Thus, the full enumeration of a particular band requires four items, the chromosome number, the arm symbol, the region number and the band number in that region. These items are given in order without spacing or punctuation, e.g. 1p33 indicates chromosome no. 1, short arm, region 3, band 3.

If due to improvement in techniques, a band serving as a landmark subsequently requires subdivision, all sub-bands derived from it retain the original region and band number of that landmark, even if such subdivision causes one or more of the sub-bands to lie in an adjacent region (fig. 2). In enumerating sub-bands a decimal point is used and the sub-bands are numbered sequentially from the centromere outwards. Thus,

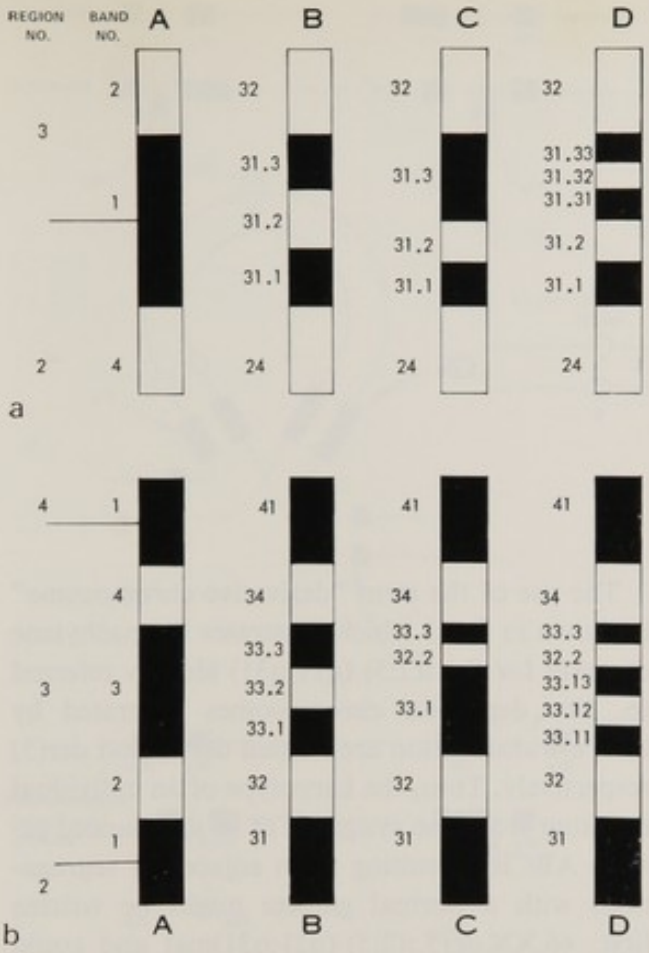

Fig. 2. (*a*) Example illustrating the convention for numbering the subdivisions of a landmark bridging two regions: (A) the original landmark, band 31; (B) subdivision of band 31 into three equal bands 31.1, 31.2, 31.3; (C) alternatively the subdivision of band 31 into three unequal bands. (D) Further subdivision of band 31.3 into three equal bands 31.31, 31.32, and 31.33. (*b*) Example illustrating the convention for numbering the subdivisions of a band within a region (A) the original band 33; (B) three equal bands 33.1, 33.2 and 33.3; (C) alternatively the subdivision of band 33 into three unequal bands; (D) further subdivision of band 33.1 into three equal bands 33.11, 33.12 and 33.13.

if 1p33 were to be divided into three equal or unequal sub-bands, these would be numbered 1p33.1, 1p33.2 and 1p33.3, sub-band 33.1 being proximal and 33.3 distal to the centromere (fig. 2). If one of these sub-bands is further divided, additional digits, but no further punctuation are used, e.g. 1p33.1 could be divided to 1p33.11, 1p33.12, etc. (fig. 2).

Designating Chromosome Structural Rearrangements

The report gives two systems for the designation of chromosome structural rearrangements, the first, a short system in which the nature of the rearrangement and the break-point or points are identified by the bands (or regions) in which they occur. The band composition of the abnormal chromosomes is not given directly in this system, but can be inferred from the information provided. The second is a detailed system which besides identifying the type of rearrangement defines each abnormal chromosome present in terms of its band composition. These two systems are presented in detail in the report and it will suffice here to present one example from each system.

A reciprocal translocation between chromosomes 2 and 5 may be written 46,XY,t(2;5)(q21;q31) implying that breakage and reunion had occurred in bands 2q21 and 5q31 and that the segments distal to these breakpoints had been exchanged. The convention adopted in Chicago is retained here in a modified form; the chromosome number is specified first, then the sex chromosomes, followed by a designation for the rearrangement with the chromosome or chromosomes involved being placed in parentheses and separated by a semi-colon. The break-points are then given and included in the second set of parentheses in the same order as the chromosomes and also separated by a semicolon. The same translocation using the detailed system would be written:

46,XY,t(2;5) (2pter → 2q21::5q31 → 5qter;5pter → 5q31::2q21 → 2qter).

Such a designation includes several new symbols, for instance, ter is terminal or end of chromosome, → is from – to, :: is break and join. In this system the first three terms are identical to those used in the short system and the full designation of the two rearranged chromosomes separated by a semi-colon is given in the second set of parentheses. In general, the rearranged chromosome carrying the lowest numbered centromere is written first and the one with the highest number second. There are, however, certain exceptions to this rule and these are outlined in the report. The bands, in which breakage and rejoining occurs (2q21 and 5q31) are specified in both rearranged chromosomes. One other difference from the short system is the specification of each band in full, thus 2q21 and not q21; without this the composition of the rearranged chromosome

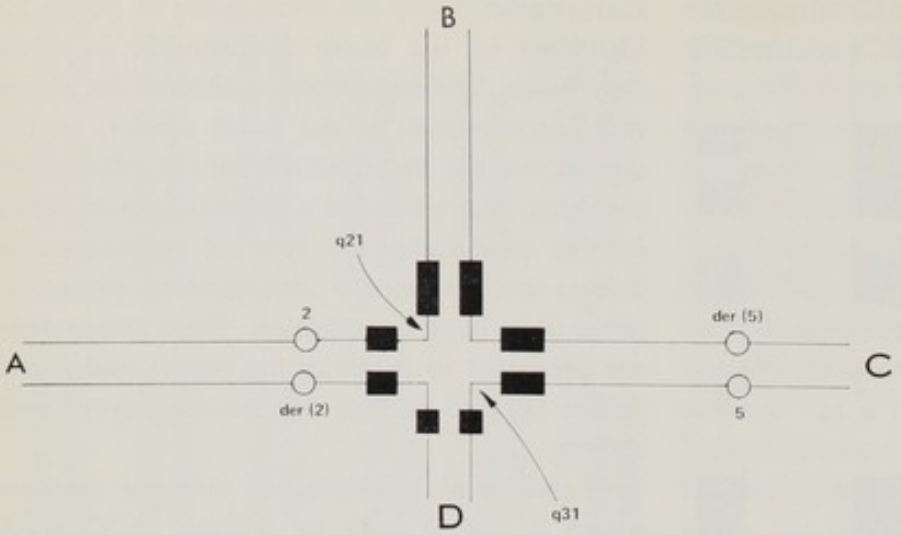

Fig. 3. Pachytene diagram of a t(2;5) (q21;q31) heterozygote used to specify the derivative chromosomes and disjunctional possibilities from such a translocation. The letters *A*, *B*, *C* and *D* designate segments extending from the chromosome ends (telomeres) to breakpoints. Bands delimiting break points only approximately to size are shown.

would become ambiguous. Numerous other examples are given in the report together with the full definitions of all new terms used.

Two new concepts introduced into the report should be mentioned here. These are the derivative ("der") and recombinant ("rec") chromosome. A "derivative chromosome" is defined "as one of the structurally rearranged chromosomes generated by a single rearrangement involving two or more chromosomes". This is necessary because (1) designation by the short system symbolizes the rearrangement as such and not the chromosome generated by the rearrangement; (2) in order to designate unbalanced karyotypes among the offspring of structural heterozygotes, which may include any one or any combination of derivative chromosomes.

A "recombinant chromosome" is defined as "a structurally rearranged chromosome with a new segmental composition resulting from meiotic crossing-over between a displaced segment and its normally located counterpart in certain types of structural heterozygotes".

Thus, derivative chromosomes are the products of original rearrangements and segregate at meiosis without further change. Recombinant chromosomes arise de novo during gametogenesis in appropriate structural heterozygotes as predictable consequences of crossing-over in a displaced segment.

The use of the term "derivative chromosome" is shown in fig. 3 which illustrates the pachytene diagram for the t(2;5) (q21;q31) already referred to. The derivative chromosomes generated by such a translocation are written der(2) and der(5) respectively. Thus, the karyotype of an individual resulting from the syngamy of an unbalanced gamete ABCB, (resulting from adjacent 1 segregation) with a normal gamete might be written first: 46,XX,der5,t(2;5) (q21;q31)mat and could subsequently be abbreviated to 46,XX, der(5)mat.

Recombinant chromosomes will usually result from crossing-over in an inversion or insertion heterozygote. Fig. 4 illustrates a pericentric inversion of chromosome no. 2 [46,XX,inv(2)(p21q31)]. Crossing-over has resulted in a duplication of 2p in one recombinant chromosome and of 2q in the other. These karyotypes are recorded as: 46,XX, red(2),dup p,inv(2) (p21q31) and 46,XX,rec(2), dup q,inv(2) (p21q31) respectively, specifying in the first instance a duplication from 2pter to 2p21 and a deletion from 2qter to 2q31 and in the second, a duplication of 2qter to 2q31 and a deletion from 2pter to 2p21.

Concluding Remarks

The report adopted by this Conference logically follows that published after Denver and Chicago in that it provides a standard system for the classification of the human chromosomes in relation to the various banding techniques which have recently been developed in a number of laboratories. The system adopted is more complex, because banded chromosomes are more complex in appearance than chromosomes stained in a

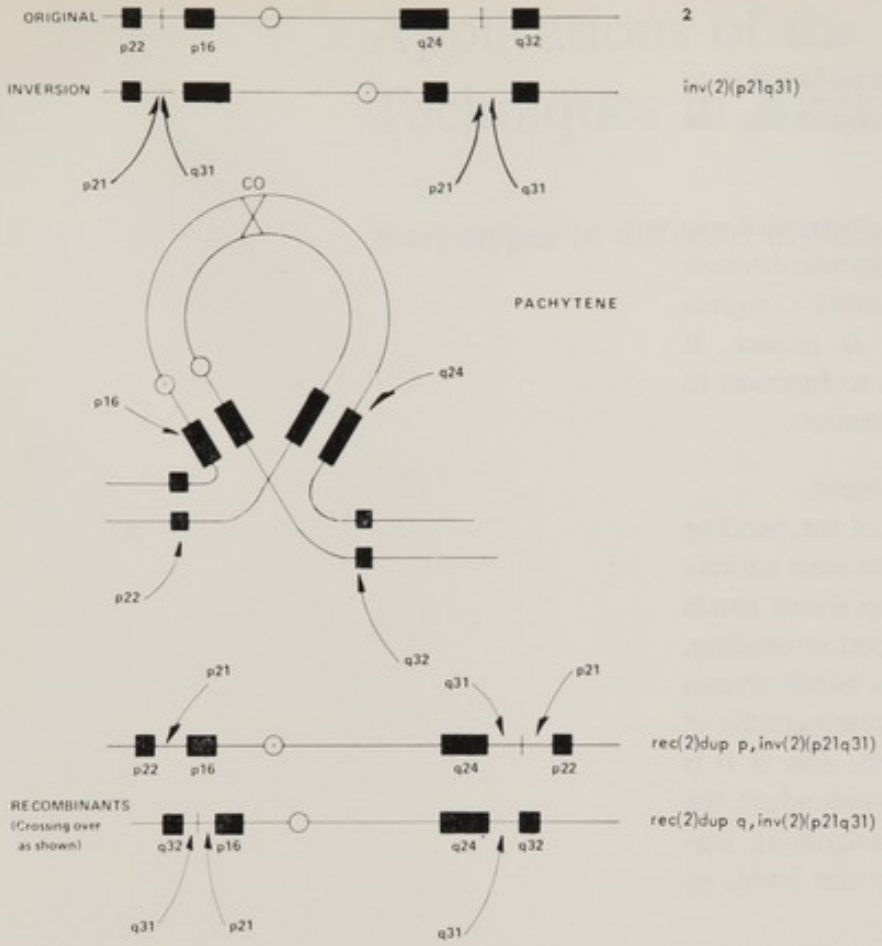

Fig. 4. Designation of recombinant chromosomes in a pericentric inversion of chromosome No. 2 with breakpoints in 2p21 and 2q31.

conventional manner. It is to be hoped, however, that it will find general acceptance among human cytogeneticists and that it will provide a similar degree of uniformity in description and communication to that provided by the Denver system of classification for conventionally stained chromosomes, which was established 12 years ago and which forms the basis for the systems described here.

The development of banding techniques has provided a new impetus for human cytogenetics. These methods, if fully utilized, can provide analytical resolution never before realized; new nosological entities will become evident. If the full benefit of these new techniques is to be realized, communication between laboratories is essential. It is to be hoped that the results of this meeting will provide a lasting basis for such a dialogue.

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Discussion

Lubs: Will the committee continue to function to permit introduction of new information into the system?

Hamerton: It is my belief that the committee was appointed to serve until the next Standardization Conference, or the next International Congress of Human Genetics, whichever is sooner. It presumably will therefore be able to function to allow incorporation of new information.

Ford: I have some comments to make.
(1) I have encountered criticisms of the banding map prepared at Edinburgh. These were entirely of the type "The Edinburgh map shows bands that we don't see". As a non-expert in banding, I was entirely satisfied that the bands shown on the map were present on the photographs of karyotypes we were working with. But if it is thought that parts of the map are redundant, the report provides a method for designating segments that consist of more than one band, as shown in the map.

(2) I think all will appreciate that an abbreviation ("rec") for "recombinant chromosome" is necessary, but some may not yet have been convinced of the need for the abbreviation ("der") proposed for "derivative chromosome". This need arises from the fact that the short system for designating rearrangement defines the rearrangement as a whole and not the individual rearranged chromosomes. Progeny of structural heterozygotes may carry any one or any combination of these individual rearranged chromosomes and to define the karyotypes they must be specified.

Hamerton: I agree with Dr Ford's comments and am grateful to him for clarifying further the need for the abbreviations "rec" and "der".

Applications of the Banding Techniques in Biology and Medicine

Karyotypes in different organisms

Branched Chromosomes

An alternative to the hypothesis of selective endoreduplication

C. E. Ford and K. Madan[1]

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, and
Medical Research Council's Population Genetics Unit, Old Road, Headington, Oxford, UK

Summary

Movement of a chromatid fragment with the intact sister chromatid in anaphase and persistence of the association into and through interphase can explain the origin of "branched chromosomes" and is preferred to the hypothesis of selective endoreduplication. If the latter be true, the sister of a cell containing a branched chromosome should include two normal No. 2 chromosomes. According to the alternative hypothesis, the sister cell should contain a deleted No. 2 chromosome.

Cultures were set up from the blood of a patient known to have a No. 2 chromosome with a fragile site close to the centromere in the long arm. Preparations made on the fourth and fifth day were scanned for pairs of adjacent cells in mitosis but no informative pairs were found. Single metaphases were observed containing one or more acentric chromosomes of three different orders of length. Trypsin-banded preparations demonstrated that the shortest group of acentrics were equivalent to the whole of the long arm of No. 2 lying distal to the fragile site and that a second group consisted of iso-acentric chromosomes, equivalent to two of the shortest acrocentrics joined together proximally. Still longer acentric chromosomes were identified in conventional preparations but not seen in banded preparations. Their origin and constitution is unknown. A single iso-acentric chromosome was observed to be folded back on itself so that presumptively homologous bands in each half of the chromosome were closely opposed. The observations as a whole are thought to favour the alternative interpretation but a fresh search for informative critical sister cell pairs, or newly originated tetraploid cells (which would be equivalent), is desirable.

The term "endoréduplication-selective" was introduced by Lejeune, Berger & Rethoré [12] and first applied to an otherwise normal chromosome spread that included an extra exceptional chromosome consisting of four parallel chromatids, as in a typical endoreduplicated cell. The four chromatids making up this structure were longer and narrower than the chromatids of the remaining chromosomes, which were normally and uniformly contracted. To the best of our knowledge this observation remains unique. The abnormal structure was located peripherally in the spread and we suggest that it could have been a "stranger", that is to say a chromosome that had broken away from a damaged cell during processing and come to rest adjacent to another spread [6].

Lejeune and his colleagues also applied the term to configurations of a different kind, five of which were figured. These could be described as chromosomes that had been duplicated over part of their length so as to appear forked. Lesions ranging from gaps to apparently clear breaks were present in one to four of the strands at the point of forking. The exceptional structures were not additional to the modal complement and appeared to be normally contracted. The pairs of chromatids diverged strongly from one another at the point of forking, thereby contrasting sharply with the parallel alignment of the chromatids in a normally endoreduplicated cell. Two of the five involved a C group chromosome and three a D group chromosome. All were from different patients. The abnormal structures could be described as triradials, but we prefer to retain this term for the breakage and reunion contexts in which it has hitherto exlusively been used and will refer to them by the neutral term "branched chromosomes".

Subsequently, Lejeune et al. [13] reported four more branched chromosomes in a total of 640 cells from a woman aged 31. In all of them the point of branching was close to the centromere in the long arm of a No. 2 chromosome. Breaks or gaps were observed at this point in one of the

[1] Now at Department of Genetics, University of Liverpool, Liverpool 3, UK.

(otherwise normal) No. 2 chromosomes of nearly half the cells examined. A branched chromosome was also seen in a cell from one of her two daughters. Other abnormalities noted in preparations from mother or daughter (or both) included cells with a deleted No. 2 chromosome and cells with one, two or three additional acentric chromosomes. The latter were of standard length equivalent to the segment of 2q from the point of breakage to the end of the arm. None of these specific types of abnormality was observed in preparations from the second daughter.

Further examples of chromosomes with similar properties have been reported in single individuals and in families [1–5, 7–11, 14–16, 18]. The chromosomes concerned have included further examples involving the same site in a No. 2 chromosome, one or more C group chromosomes, No. 16 and No. 18. The positions at which the lesions are observed have been called "fragile sites". In several pedigrees the evidence shows that the fragile site is a fixed property of the chromosome concerned that is transmitted to progeny in a normal Mendelian manner.

The explanation for the branching phenomenon put forward by Lejeune and his colleagues is implicit in the concept of "endoréduplication selective", namely that a specific segment of a specific chromosome underwent *two* phases of replication during the S phase while the remainder of that chromosome and all the others had replicated once only.

An Alternative Hypothesis

We consider that the assumption of selective endoreduplication, which invokes a new principle in cytogenetics, is unnecessary and that the following alternative interpretation is to be preferred. We suppose a chromatid break occurs at the fragile site, that the intact chromatid proceeds to one pole in anaphase together with the acentric fragment, and that the acentric fragment may remain attached to the intact daughter chromosome at the fragile site through the following interphase. A single replication event in each chromatid would then be sufficient to account for the branching phenomenon. This hypothesis has also been advanced by Ferguson-Smith [5] and considered, but not favoured, by Fraccaro et al. [8].

Should the acentric fragment separate from the intact daughter chromosome during interphase, it should appear as an independent acentric chromosome at the next metaphase, not necessarily related in position to the No. 2 chromosome that had been its sister in the previous cycle. That acentric chromatid and chromosome fragments could sometimes be incorporated passively into a daughter nucleus was established by the classical studies of chromosome breakage. The one minor new assumption required by the alternative hypothesis is that the broken end of a chromatid fragment may sometimes remain attached to its intact sister through anaphase into the next interphase. The two assumptions, passive inclusion of acentrics and facultative retention of interchromatid attachment at the fragile site, account for the branching phenomenon and for the other types of abnormality reported by Lejeune et al. [13]. They suggest that still other abnormal cell types might also be found.

In some of the branched chromosomes figured, two of the four chromatids are joined at the point of branching to produce a double-length acentric chromatid. Passive inclusion of such a structure into a daughter nucleus at telophase should then lead to the appearance of an extra long *isoacentric* fragment at the next metaphase. Isoacentrics would also be generated directly by an isochromatid break at the fragile site followed by sister union. Should non-disjunction of the daughter centromeres occur, four other types of abnormality might be expected at the next metaphase: (1) a cell with 47 bodies, the deleted No. 2 chromosome (2q –) being represented twice; (2) a cell with one normal No. 2 chromosome replaced by an 'iso-dicentric' chromosome, produced by duplication of a 2q – chromosome about the break point; (3) a branched chromosome in which the short arm of the No. 2, the centromere and the short proximal segment of the long arm are duplicated; (4) a doubly branched chromosome produced by duplication of the segments on both sides of the fragile site. The last type would be equivalent to a quadriradial configuration produced by chromatid interchange at identical loci in homologous chromosomes.

Selective endoreduplication can explain the observation of branched chromosomes but it does not account for cells containing the deleted No. 2 chromosome or extra acentric chromo-

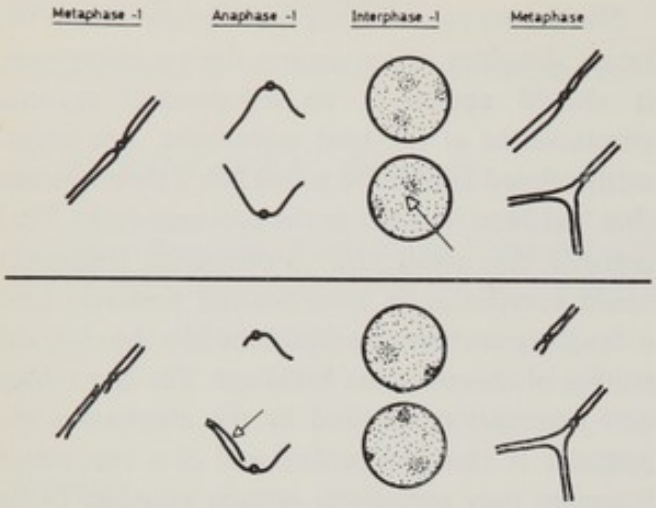


Fig. 1. Diagram to demonstrate a method for distinguishing between the two hypotheses. (*Top*) selective endoreduplication hypothesis: the postulated critical event occurs in the immediately preceding interphase and the corresponding chromosome in the sister cell is normal. (*Bottom*) the alternative hypothesis: the postulated critical event occurs in the preceding anaphase and the corresponding chromosome in the sister cell is deleted.

somes. The additional assumption of passive inclusion of acentric fragments in daughter nuclei is necessary. A more serious objection is the striking contrast, already mentioned, between the characteristically parallel chromatids in typical endoreduplicated chromosomes and the strongly divergent forking of the pairs of chromatids in the branched chromosomes. Neither objection, however, is decisive.

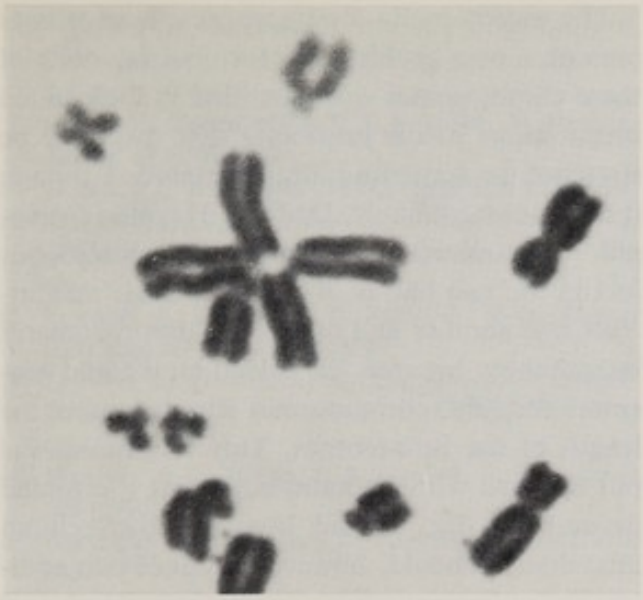
The two hypotheses might be discriminated by an appropriately designed ^{3}H-thymidine labeling experiment [8] but we have not been able to devise an autoradiographic test that would be decisive. They may be distinguished, however, by considering pairs of sister cells that enter mitosis together. According to the selective endoreduplication hypothesis the critical event takes place in the interphase immediately preceding the metaphase in which the branched chromosome is observed. The sister nucleus should have received two normal No. 2 chromosomes at the previous anaphase and therefore have a normal karyotype. According to the alternative hypothesis, the critical event occurs at the previous anaphase, when an acentric chromatid fragment proceeds to the one pole in the company of an intact daughter chromosome, leaving the complementary centric fragment to move to the other. A branched chromosome in one of two sister mitoses should then be accompanied by a deleted No. 2 chromosome in the other (fig. 1).

The requirement for a critical test, therefore, is to find sister cells that have entered mitosis together. We had noted that pairs of adjoining cells in mitosis were not uncommon in preparations made from blood cultures, particularly if harvested late on the third or on the fourth day, and had supposed they were sister cells. There is no assurance that adjacent mitoses are indeed sister cells, but the point can be tested by culturing mixed blood from male and female donors and determining whether mixed pairs occur.

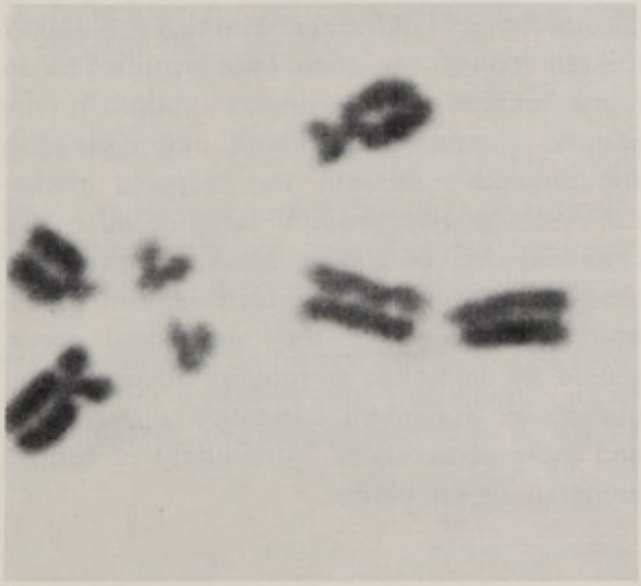
Observations

Through the courtesy of Dr B. Noel and Mlle B. Quack of Chambery, France, we were provided with a specimen of peripheral blood from a female patient who had a No. 2 chromosome with a fragile site close to the centromere in the long arm in a position apparently identical to the one in the chromosome studied by Lejeune et al. [12]. Mlle Quack (unpublished) had already established that mitoses with various abnormalities, including branched chromosomes, were frequent in preparations made from cultures of this patient's blood. She had noted many cells with a lesion at the fragile site; others with a deleted No. 2 chromosome (2q −); still others containing one or more acentric chromosomes corresponding in length to the segment of 2q from the fragile site to the end of the arm, or approximately twice this length; and, finally, long chromosomes with a beaded (moniliform) appearance somewhat resembling banded chromosomes and contrasting sharply with the appearance of the remainder of the chromosomes in the spread.

Seven cultures were set up. Two of them were seeded with whole blood and five with leucocytes, following sedimentation of the red cells by plasmagel. One of the whole blood cultures was seeded additionally with three drops of whole blood from a normal male donor. They were harvested after 72, 96 or 120 h incubation, the last two of which were in the presence of approx. 5×10^{-6} M Colcemid. Preparations were made by a standard air-drying method and stained with toluidine blue or orcein, or by Leishman's stain following brief tryptic digestion [17].



a



b

Fig. 2. Groups of (*a*) four; (*b*) two associated acentric chromosomes of the first order. Toluidine blue, × 3 500.

Five hundred single cells from four of the cultures have been studied and the observations made by Mlle Quack have been confirmed. We have noted the following additional features: (1) when two or more of the shorter acentric chromosomes (corresponding to the segment of 2q distal to the fragile site) are present together in one cell they are often closely associated at one end (fig. 2); (2) the longer iso-acentric chromosomes are not so associated; (3) one cell with an isochromatid break at the fragile site contained a classic U-shaped acentric fragment, indicative of distal sister union; (4) some cells contained an exceptionally long acentric chromosome with chromatids that were uniform in thickness but

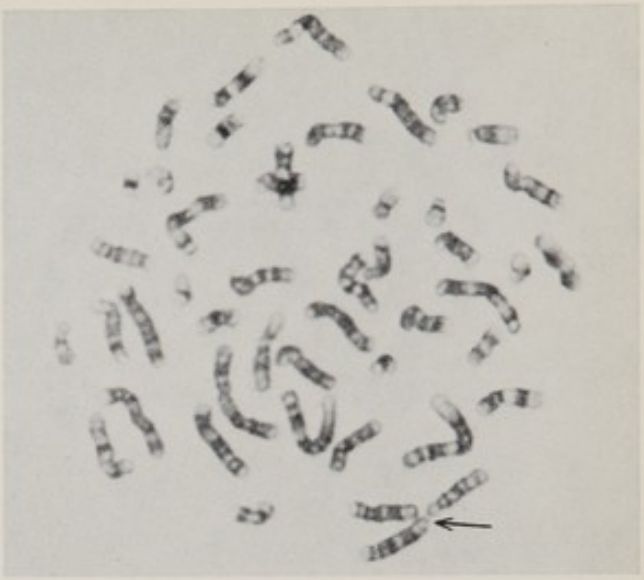
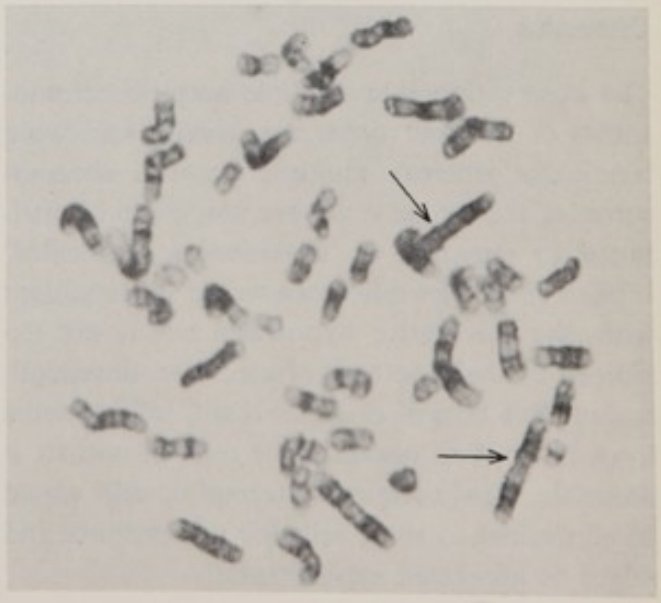


Fig. 3. Spread including an acentric chromosome of the first order associated with a No. 2 chromosome. Trypsin, Leishman, × 1 600.

invariably somewhat narrower than those of the remaining chromosomes in the cell; (5) two cells contained a dicentric chromosome that presumptively involved, as one component, the short arm and centromere of the fragile No. 2; (6) chromatid gaps, breaks and one dicentric were seen among the other chromosomes though the total frequency was considered probably to have been within the normal range; (7) many interphase nuclei had associated micronuclei.

Trypsin-banded preparations confirmed that the acentric chromosomes of the first order were

Fig. 4. Spread including two acentric chromosomes of the second order (iso-acentrics). Note bilateral symmetry of banding. Trypsin, Leishman, × 1 600.



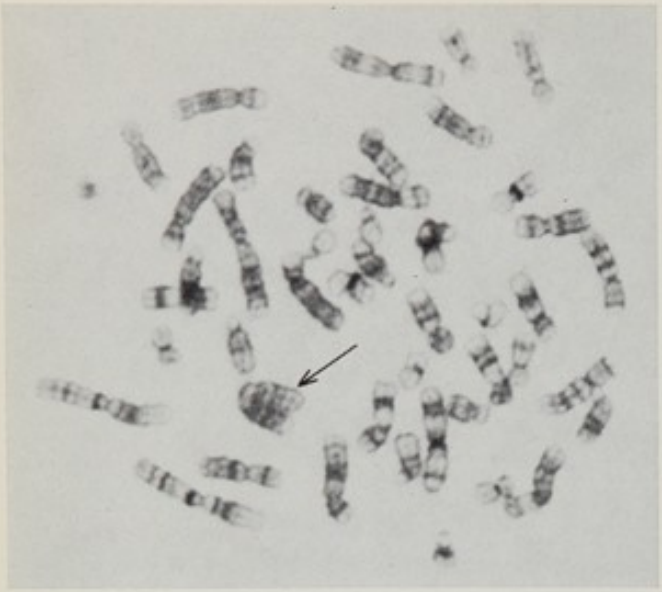


Fig. 5. Spread including a single acentric chromosome of the second order (iso-acentric) with lateral association of presumptively homologous sites. Trypsin, Leishman, ×1 600.

equivalent to the segment of the long arm of No. 2 lying distal to the fragile site (fig. 3), and that the double-length acentric chromosomes were indeed iso-acentrics derived by symmetrical doubling of the first order acentrics about the point of breakage (fig. 4). In one cell an iso-acentric was observed to be folded back on itself in such a way that presumptively homologous bands were associated (fig. 5).

Unfortunately there were few pairs of associated cells in mitosis. Ten pairs were analysed but none was informative. The test with mixed blood was also uninformative: a Y chromosome was not identified in any one of a sample of 150 cells analysed.

Discussion

The observation that multiple acentric chromosomes of the first order are usually associated terminally whereas multiple acentric chromosomes of the second order are not, could be attributed to retention of 'stickiness' in 'unhealed' broken ends. The difference would be consistent with the alternative hypothesis but is not explained by the hypothesis of selective endoreduplication. But critical evidence is still lacking and a fresh attempt to obtain sister cells in mitosis is desirable. Newly originated tetraploid cells would be equivalent to sister cells for this purpose and might be generated experimentally.

The exceptionally long acentric chromosomes present a new problem. Unfortunately, none of these chromosomes was identified in the banded preparations so the possibility that they may be stretched iso-acentrics is not yet excluded, though it does not seem likely. Dr D. L. Hayman (personal communication) suggests that homologous loci in the two halves of an iso-acentric may attract one another and points out that chromatid intrachanges between the folded arms could generate acentric chromosomes up to twice the length of the iso-acentrics. This suggestion was put forward without knowledge that the folded iso-acentric (fig. 5) had been found. Such an intrachange should, however, produce two acentric chromosomes whose total length equals two iso-acentrics. One cell with two long acentric chromosomes of different sizes which may satisfy this requirement has indeed been identified but in four other cells there is a single exceptionally long acentric chromosome without any sign of a complementary element. The proposed mechanism should also produce large acentric ring chromosomes, depending upon the particular chromatids involved in the rearrangement. Moreover, intrachanges, if they do indeed occur, should be detectable as primary aberrations at metaphase. Hayman's suggestion is intriguing and more observations, particularly of banded preparations, are needed.

We are grateful to Mr G. Breckon for setting up the blood cultures for photography and for some of the preparations, to Mrs M. Seabright for some trypsin-banded preparations and to Mr B. Purvis for drawing fig. 1.

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Discussion

Hsu: I think your explanation is quite plausible but I object to the term "branched chromosomes" It is an additional piece, not a branch.

Ford: I adopted the expression "branched chromosome" as a neutral term, unrelated to either interpretation, and simply descriptive of what I saw.

Autosomal Heterochromatin in Some Carnivores

K. Fredga and N. Mandahl

Institute of Genetics, University of Lund, 223 62 Lund, Sweden

Summary

Carnivores in general possess small amounts of heterochromatin. Some species, however, were discovered with relatively large blocks of autosomal heterochromatin and three *Mustela putorius* ($2n = 40$), *Mustela rixosa* ($2n = 42$) and *Herpestes edwardsi* ($2n = 35/36$), having 3, 7 and 2 chromosome pairs with heterochromatic blocks, respectively were studied in detail. Autoradiographic studies with ^{3}H-thymidine show that these regions synthesize DNA late. The effect of various recently developed staining techniques was tested and it was found that the heterochromatic regions in the two *Mustela* species showed similar properties: They were often differentially stained with orcein, showed dark fluorescence when stained with quinacrine mustard, were faintly stained and without banding structure with various G-band techniques, and were heavily stained with the C-band technique only after prolonged denaturation with NaOH. Experiments with acridine orange indicated presence of fast reassociating DNA in the differential regions. The staining properties of the two late replicating regions in *Herpestes* were different: They did not show dark fluorescence, and after G-band treatment did not stain differentially from the rest of the karyotype. No heavily stained regions could be demonstrated in *Herpestes*, neither in the late replicating regions, nor in the centromeric areas, although various modifications of the C-band technique were tested.

A comparison is made with the staining reactions of heterochromatic regions in other mammals, including man. The role of constitutive heterochromatin in creating new karyotypes, and its significance for the speciation process are discussed.

For many years one of us (K. F.) has studied the chromosomes of various carnivores, mainly belonging to the families *Mustelidae* [1, 2] and *Viverridae* [3]. The new banding techniques offer improved possibilities for chromosome identification and are of outstanding value for studies concerned with karyotype evolution and cytotaxonomy. In the present communication, however, we will not describe the banding patterns in detail, but only report on the staining properties of certain large, heterochromatic regions in the metaphase chromosomes of the ferret (*Mustela putorius furo* L), the polecat (*Mustela putorius putorius* L), the weasel (*Mustela nivalis* L), the pygmy weasel (*Mustela rixosa* Bangs) and the Indian mongoose (*Herpestes edwardsi* Geoffroy). The heterochromatic blocks of these species were discovered with the "old-fashioned" acetic orcein squash technique and/or with autoradiography.

The present findings indicate that there exists in the Carnivora constitutive heterochromatin with different properties. These results are discussed in relation to similar findings in other mammals.

Material and Methods

The chromosomes were studied in adult specimens of 8 ferrets (4 ♂♂ and 4 ♀♀), 6 polecats (all ♂♂), 7 weasels (3 ♂♂ and 4 ♀♀), 3 pygmy weasels (2 ♂♂ and 1 ♀) and 4 Indian mongooses (3 ♂♂ and 1 ♀).

Of the ferrets, 5 were obtained by the courtesy of Dr J. Hammond, School of Agriculture, University of Cambridge, and 3 by Mr N. Borgström, Sövdeborg, southern Sweden, who also supplied 4 of the polecats. The 2 remaining polecats were also trapped in southern Sweden, at Trolleholm, and were obtained by the courtesy of Dr I. Hansson, Institute of Zoology, University of Lund, who in addition supplied us with 6 of the weasels. One weasel was obtained from Mr A. Rünow, Institute of Genetics, University of Lund. All weasels were trapped in the province of Skåne. Of the pygmy weasels, two were from the province of Lappland and one from Jämtland, and were kindly put at our disposal by Dr I. Hansson (1 animal) and by the late Dr U. Bergström, Swedish Museum of Natural History, Stockholm (2 animals). To all these persons we are very much obliged.

The taxonomic identity and nomenclature of Scandinavian weasels have been most confusing, and the chromosome studies were primarily initiated with the hope of casting some light on this problem, but no difference in the karyotypes of the two types could be detected [2].

We have here followed the opinion of Siivonen [4],

namely that there are two species of weasels in Sweden, *Mustela nivalis* and *Mustela rixosa*, and that the pygmy weasel of Scandinavia belongs to the species *M. rixosa*, that is the same as the pygmy weasel of N. Asia and N. America.

Acetic orcein squash preparations were made from all of the above-mentioned specimens. Successful autoradiographic studies were performed on 1 male polecat, 2 male pygmy weasels and 3 mongooses, 1 female and 2 males. Some of the new staining techniques mentioned below were applied to preparations from 1 male ferret (wild-type coloured), 2 male pygmy weasels and 1 male and 1 female Indian mongoose. In some animals direct squash preparations were made from bone marrow of the femur after 3 h colchicine treatment in vivo (0.04 % colchicine, 0.5 ml/100 g body wt) hypotonic treatment of the cells for 30 min in 1 % sodium citrate at 37°C, fixation in 60 % acetic acid : 1 N HCl (9:1) and staining with orcein.

In the majority of animals fibroblast cultures were established from skin, lung, heart or testis [3], and chromosome preparations were made by the acetic orcein squash technique or by the conventional air drying procedure (hypotonic treatment for 15–20 min by addition of 2 parts of distilled water to the cell suspension, fixation in methanol/acetic acid (3:1), no heating or flaming).

Autoradiography

The DNA synthesis of the chromosomes at the end of the S period was studied. ^{3}H-Thymidine was added to the cultures (1 μCi/ml medium) 4–6 h prior to fixation. Colcemid treatment, trypsinization, hypotonic treatment, fixation and staining were according to our routine squash method [3]. The autoradiographic stripping film technique of Schmid [5] was used with minor modifications [6].

Fluorescence

(1) The Q-band technique [7]. The preparations were stained without any pretreatment in a solution of quinacrine mustard (50 μg/ml in McIlvaine's buffer, pH 7) for 5–10 min, washed for 25–30 sec in tap water and mounted in distilled water with Krönig cement. Some of the preparations were afterwards stained with Giemsa.

(2) The AO technique [8]. The chromosomes were denaturated for 5 min at 100°C in 0.1 × SSC and reassociated for 30 sec at 65°C in 2 × SSC, followed by addition of formaldehyde (at a final concentration of 4 %) to prevent further reassociation. The preparations were stained for 5 min in 0.125 mg/ml acridine orange solution (pH 6.0).

The preparations were viewed with a Leitz Orthoplan microscope fitted with a Xenon light source and using exciter filter BG 12 and barrier filter K 510. The photographs were taken on 24 × 36 mm Scientia 50 B 65 film which was developed with Refinex (7 min, 20°C).

Giemsa

(1) The G-band technique of Schnedl [9] was slightly modified: The preparations were treated in 0.007 N NaOH at room temperature for 90 sec, rinsed in several changes of 70 % ethanol, transferred to 95 % and absolute ethanol and dried. The slides were incubated for 24 h in Sörensen's buffer (pH 6.8) at 59°C rinsed in newly prepared buffer, 70 % and 95 % ethanol, air dried and stained in buffered Giemsa solution (1:50, pH 7.0) for 5 min.

(2) The G-band technique of Wang & Fedoroff [10] was applied as follows: The preparations were treated in a 0.025 % trypsin–versene solution at pH 7 for 2–5 min at room temperature, rinsed in a balanced salt solution and stained in Giemsa as above.

(3) The C-band technique of Arrighi & Hsu [11] was slightly modified: The preparations were treated in 0.2 N HCl at room temperature for 30 min, rinsed in distilled water, kept in 0.07 N NaOH at room temperature for 5 min and rinsed in 70 % and 95 % ethanol. The slides were incubated in 2 × SSC at 65°C for 18–20 h, rinsed in 2 × SSC, 70 % and 95 % ethanol, dried and stained in buffered Giemsa solution (1:10, pH 7.0) for 15–20 min.

In many experiments with C- and G-band techniques preparations of human and *Microtus agrestis* chromosomes were included for comparison. In spite of identical treatment differences were found among the materials; thus with the ordinary C band technique stained regions were induced in the human and *Microtus* chromosomes but not in the *Mustela* and *Herpestes* chromosomes.

Observations

The polecat and the ferret

The karyotypes of the wild polecat (*M. p. putorius*) and the domestic ferret (*M. p. furo*) are identical [2, 12]. The chromosome number is $2n = 40$. According to Frykman [12] the karyotype consists of 5 m, 5 sm, 4 st and 5 t autosome pairs (nomenclature based on centromere location, Levan et al. [13]). Both sex chromosomes are m, the X is of intermediate size and of original type [14], the Y is the smallest chromosome of the complement. The chromosomes in figs 1–4 are arranged into the four classes *m*, *sm*, *st* and *t*, and the autosomes are numbered within each class according to decreasing length. The pair t4 has a secondary constriction in the proximal part of the long arm, and the size of the constriction varies often between the homologues in a cell and also between cells of the same animal. This secondary constriction may be confused with the centromere in Q- and G-stained preparations. As pointed out by Fredga [1] a striking feature of the karyotype is that three pairs of autosomes have one arm positively heteropycnotic at metaphase when stained with orcein (fig. 1). In two pairs (sm2 and st3) it is the short arms and in one pair (sm4) it is the long arm that is heteropyc-

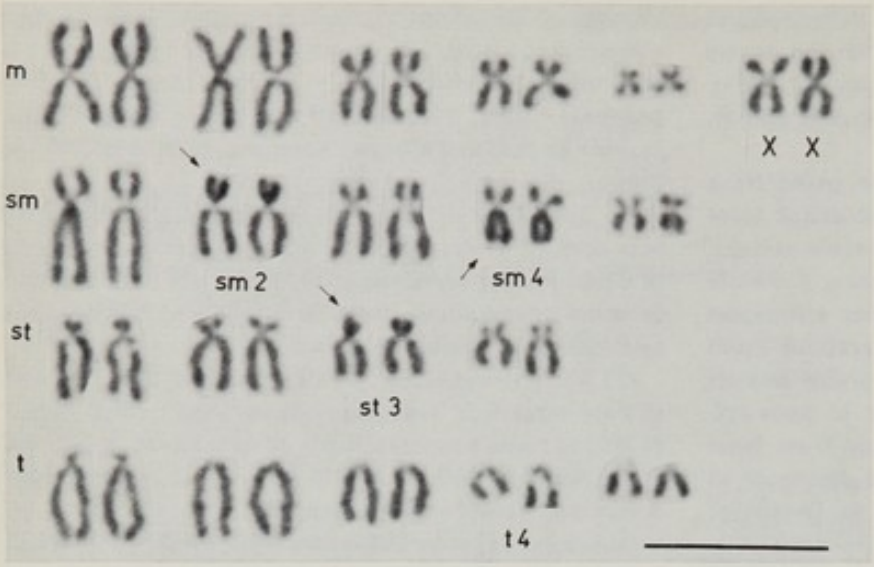


Fig. 1. Karyotype of *Mustela putorius furo*, female, $2n=40$, stained with orcein. Note positively heteropycnotic arms of chromosomes sm2, sm4 and st3 (*arrows*). Direct squash preparation from bone marrow after colchicine injection in vivo. Scale, 10 μm.

notic. These regions, which are present in both *M. p. p.* and *M. p. f.*, are most conspicuous in direct preparations from bone marrow, but are also seen in preparations from fibroblast cultures of different origin. They constitute together 6.5% of the female haploid set (A+X).

The late labelling pattern of the chromosomes was studied in passage 1 of a lung fibroblast culture, ^{3}H-thymidine being added to the medium 6 h prior to fixation. The heteropycnotic regions were regularly heavily labelled (fig. 2), and this was particularly conspicuous in the two largest blocks, viz. the long arm of sm4 and the short arm of sm2. However, the three heteropycnotic regions were not outstandingly late replicating,

Fig. 2. Duplicate karyotypes of *Mustela putorius putorius*, male, $2n=40$. The chromosomes were photographed before (*upper rows*) and after (*lower rows*) application of the autoradiographic film (cells exposed to ^{3}H-thymidine at the end of DNA synthesis). Note heavily labelled arms of sm2, sm4 and st3 (*arrows*). Autoradiography, acetic orcein squash. Scale, 10 μm.

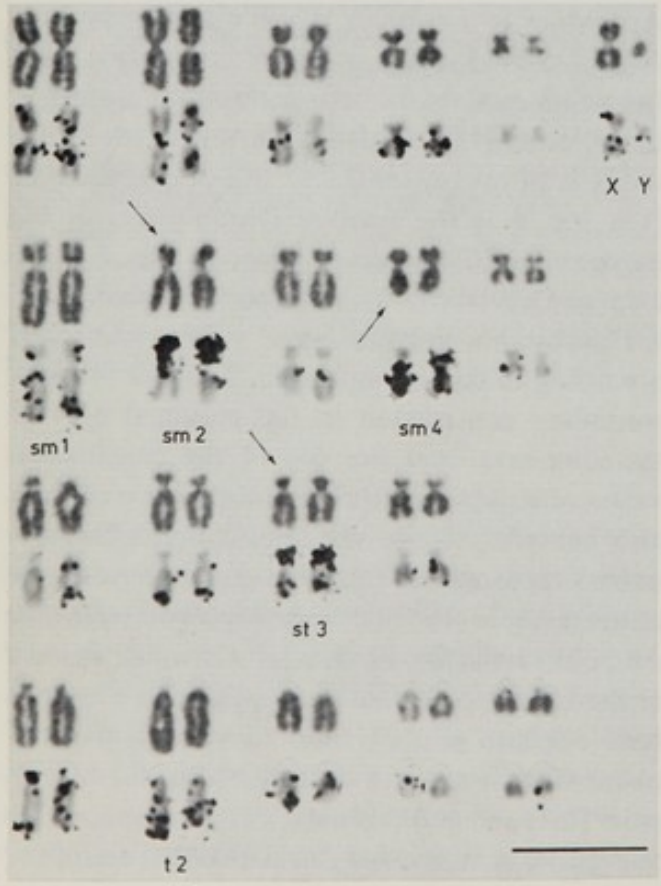
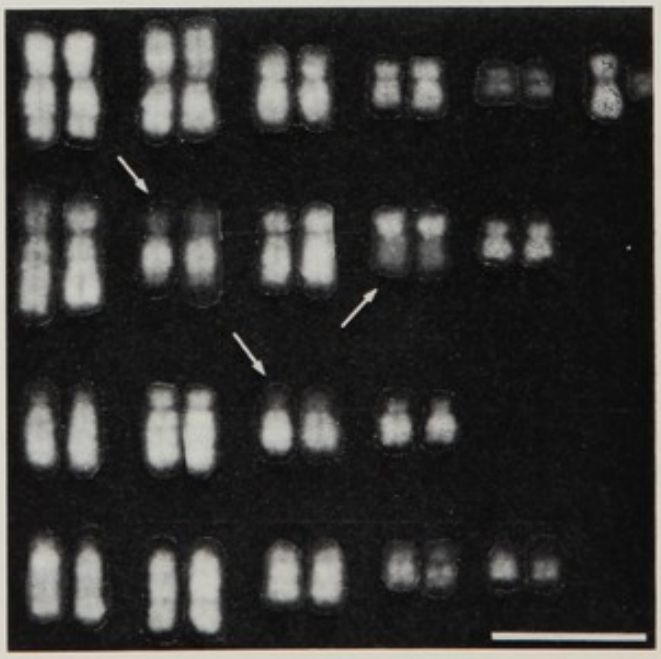


Fig. 3. Karyotype of *Mustela putorius furo*, male, stained with quinacrine mustard. Note dark fluorescence in the arms of sm2, sm4 and st3 (*arrows*). Scale, 10 μm.



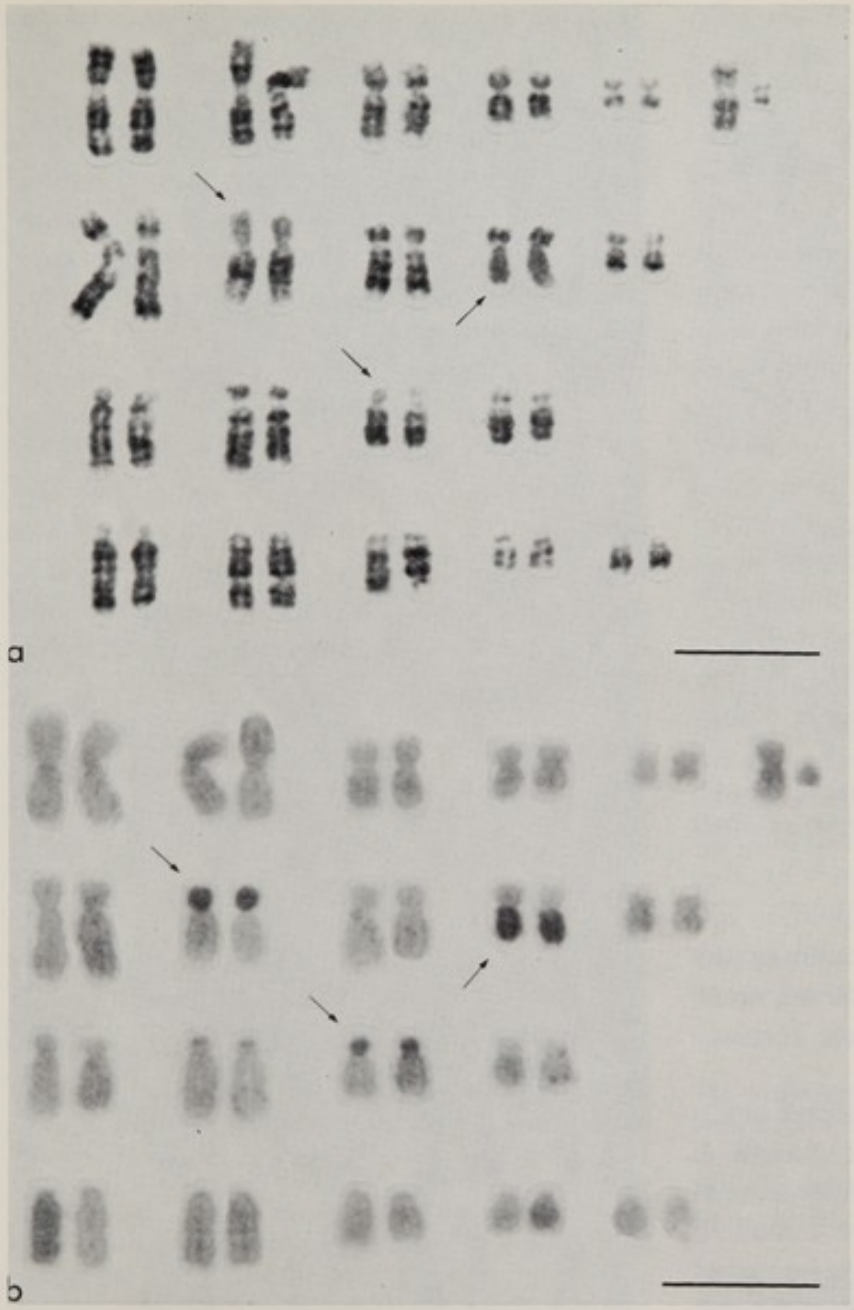


Fig. 4. Karyotypes of *Mustela putorius furo*, male, stained with Giemsa. (*a*) G-bands produced by the trypsin technique. Note no banding structure in the arms of sm2, sm4 and st3 (*arrows*). (*b*) Heavily stained regions (*arrows*) produced by a modified C-band technique. Note absence of centromeric heterochromatin. Scale, 10 μm.

silver grains always being present to a lesser extent in other chromosomes of the complement, e.g. in the distal part of the long arm of sm1, in the t2 and in the Y.

When preparations of the ferret were stained with quinacrine mustard, the condensed regions showed dark fluorescence (nomenclature according to Fredga [15]) (fig. 3). If the preparations were stained with acridine orange as described above, it was possible to obtain differential fluorescence of fast and slow reassociating DNA. The heteropycnotic regions showed bright yellow fluorescence (in contrast to the rest of the chromosome complement) thus indicating presence of fast reassociating DNA in the heterochromatic chromosome arms of sm2, sm4 and st3. If just the denaturation was carried out, all chromosomes showed a uniform orange-red fluorescence (indicating single-stranded DNA); if the denaturation was followed by 3 min instead of 30 sec of reassociation, all chromosomes showed a uniform yellow-green fluorescence (indicating double-stranded DNA).

The trypsin technique for producing G-bands revealed another characteristic feature of these

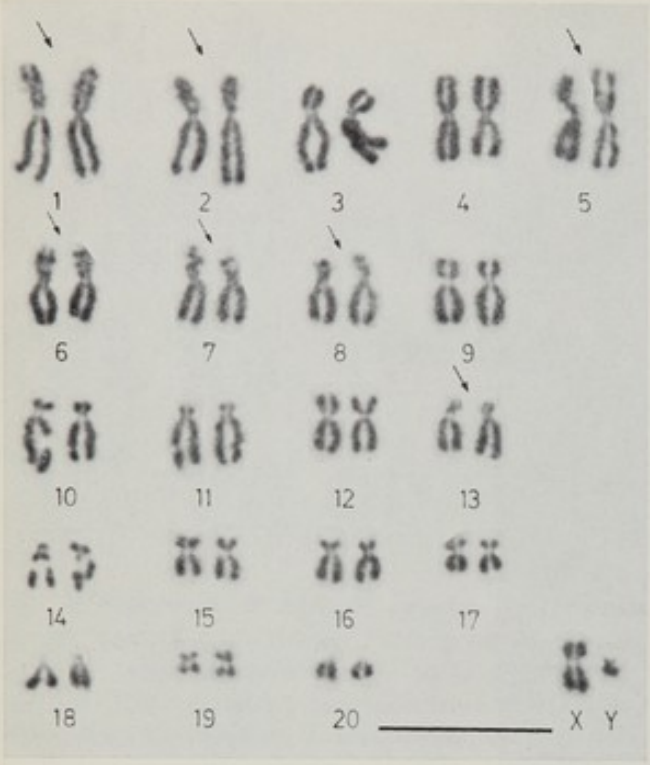


Fig. 5. Karyotype of *Mustela nivalis*, male, $2n=42$, stained with orcein. Note fuzzy appearance of distal part of 7 arms (*arrows*). Scale, 10 μm.

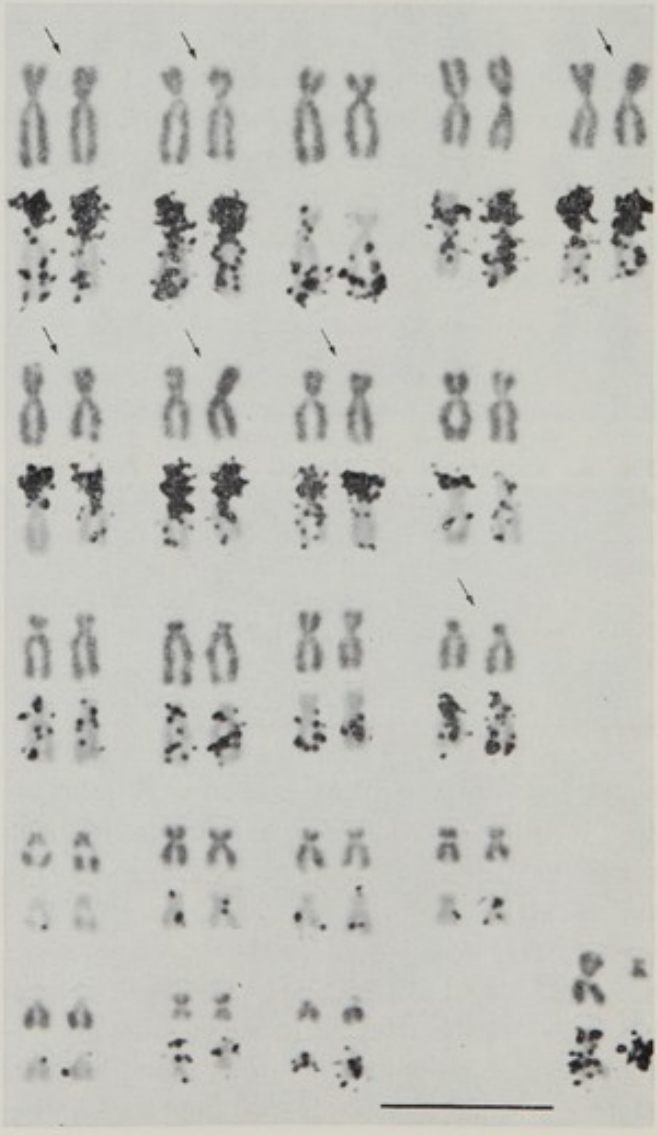
regions: they were stained uniformly without any banding structure and were usually stained weakly in comparison with the rest of the chromosomes (fig. 4*a*).

It was necessary to make one important modification of the C-band technique of Arrighi & Hsu [11] to obtain any heavily stained regions at all in this material: the denaturation treatment with 0.07 N NaOH had to be for 5 min instead of only 2 min or less as recommended. With 2–4 min treatment the heterochromatic segments became weakly stained as described above for the G-band technique, or no differentiation whatsoever was apparent in the chromosomes. After 5 min of NaOH treatment and incubation in 2 × SSC at 65°C for 18 h, the 3 heteropycnotic arms were heavily stained in contrast to the rest of the chromosome complement (fig. 4*b*). It must be pointed out that no centromeric heterochromatin could be demonstrated (apart from the proximal, centromeric parts of the 3 heteropycnotic arms).

The weasel and the pygmy weasel

The weasel (*Mustela nivalis*) and the pygmy weasel (*Mustela rixosa*) have both $2n=42$. Their karyotypes, studied with the acetic orcein squash technique, were identical. Although often no obvious difference in staining properties was seen in the karyotype, sometimes the major, distal part of 7 chromosome arms was poorly stained and fuzzy (arrows in fig. 5), or at other times darkly stained and positively heteropycnotic. These 7 regions comprise approx. 11% of (A+X). When the chromosomes are numbered consecutively from the longest to the shortest, the differential segments are on the short arms of nos. 1, 2, 5, 6, 7, 8 and 13. The autosomes 2–3, 4–5 and 6–9 which are usually difficult or impossible to distinguish from one another with conventional staining techniques, are identifiable with the Q- and G-band techniques. The other autosomes may be identified by their relative sizes and centromeric indices. No. 14 has a prominent secondary constriction in the proximal half of the

Fig. 6. Duplicate karyotypes of *Mustela rixosa*, male, $2n=42$. Note heavy labelling in the 7 arms (*arrows*) indicating late DNA synthesis. Autoradiography, acetic orcein squash. Scale, 10 μm.



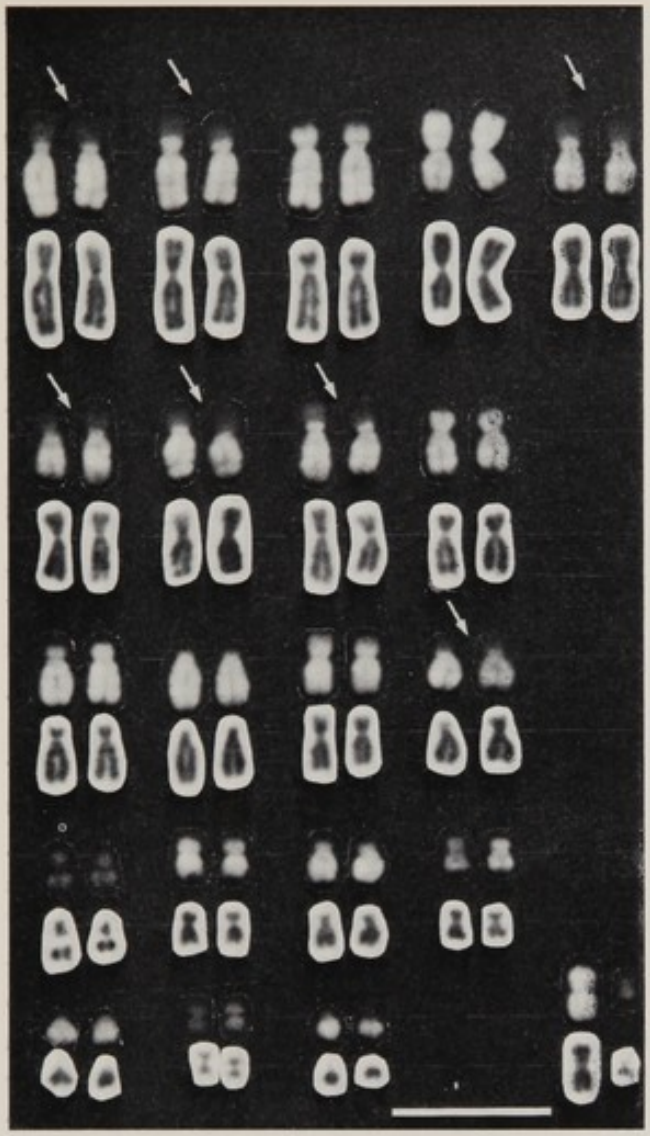


Fig. 7. Duplicate karyotypes of *Mustela rixosa*, male, stained with quinacrine mustard (*upper rows*) and Giemsa (*lower rows*). Note dark fluorescence in the distal part of the 7 arms (*arrows*). Scale, 10 μm.

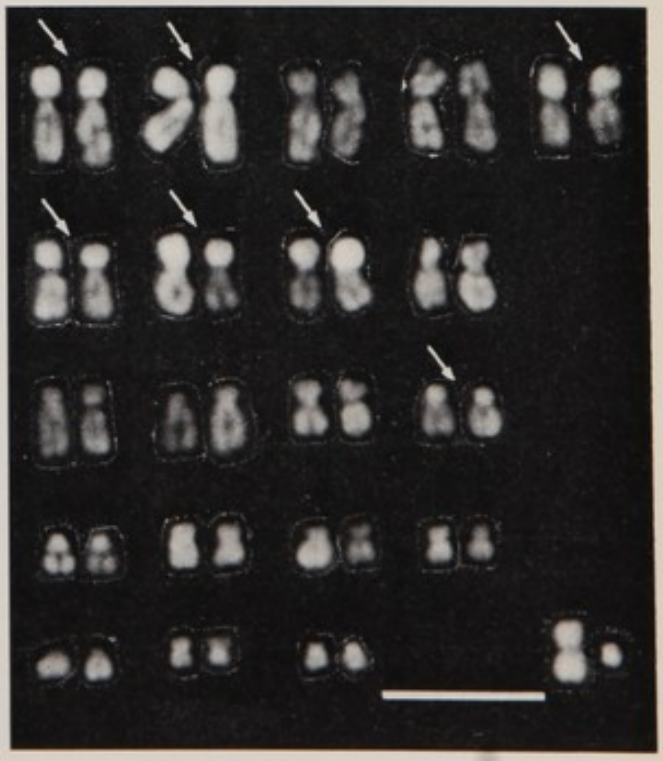
long arm. The X chromosome, which is of original type [14], may be confused with No. 12, but is slightly smaller and its centromere has a more median position. The Y chromosome, which also is an m chromosome, is the smallest element of the karyotype.

The late labelling pattern of the chromosomes was studied in passages 2 and 3 of lung fibroblast cultures; ^{3}H-thymidine was added to the medium 5 and 6 h prior to fixation. The 7 differential regions were heavily labelled (fig. 6), although not outstandingly so. Some other regions, e.g. the distal part of the long arm of No. 3, the proximal and median parts of both arms of No. 4, and the Y chromosome were also late replicating.

After staining of the preparations with quinacrine mustard, the 7 regions showed dark fluorescence as indicated by arrows in the upper rows of fig. 7. The lower rows in the figure show the corresponding chromosomes after staining with Giemsa. It should be noted that it is not the entire short arms, but only the distal—and major—parts which show dark fluorescence. The 7 regions are in contrast to other late replicating autosomal regions, e.g. in chromosomes 3 and 4, which exhibit bright fluorescence. The fluorescence of the X chromosome is bright, that of the Y dark to dim, and this is in agreement with the fluorescence of the sex chromosomes of the ferret. In preparations stained with acridine orange after denaturation followed by 30 sec reassociation, the 7 regions showed bright fluorescence indicating presence of fast reassociating DNA (fig. 8).

The modified G-band technique of Schnedl [9] gave a drastic effect. The 7 regions were now weakly stained and showed a characteristic balloon-like appearance (fig. 9*a*). The borderline between the differential regions and the proximal parts of the short arms were very clear also with this technique. According to Schnedl [16] the differential centromeric regions of cattle chromosomes, processed according to this technique,

Fig. 8. Karyotype of *Mustela rixosa*, male, stained with acridine orange after denaturation followed by 30 sec reassociation. The 7 regions show bright fluorescence (*arrows*), indicating presence of fast reassociating DNA. Scale, 10 μm.



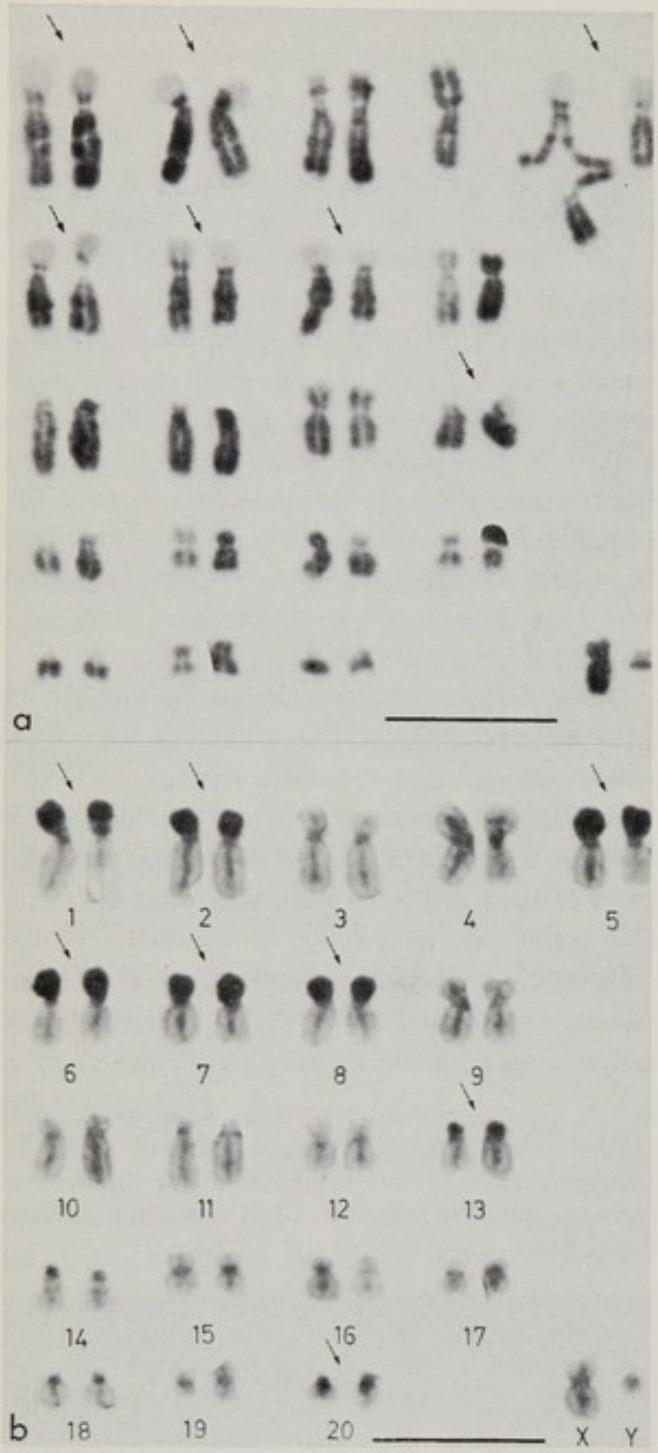


Fig. 9. Karyotypes of *Mustela rixosa*, male, stained with Giemsa. (*a*) A modified G-band technique of Schnedl [9]. The regions are weakly stained and show a balloon-like appearance (*arrows*). (*b*) Heavily stained regions (*arrows*) produced by a modified C-band technique. Note heavy staining in No. 20 in addition to the 7 regions. Scale, 10 μm.

show striking crater-like structures in interference contrast. We did not see any such structures, however, in the pygmy weasel chromosomes when studying them in a Leitz Orthoplan microscope equipped with a Nomarsky interference contrast device.

A 5-min treatment with NaOH was needed to induce heavily stained regions, primarily corresponding to the 7 heteropycnotic regions (fig. 9*b*). In addition, the centromeric regions of many chromosomes (e.g. Nos. 4, 14, 18, 20 and the Y) were more darkly stained than the rest of the chromosomes, but with the exception of No. 20 they were less dark than the 7 differential blocks. The centromeric region of No. 20 very likely belongs to the same category of heterochromatin as the differential blocks, but due to its small size its characteristic properties did not show up clearly in all the techniques tested.

The Indian mongoose

Some of the differential staining techniques were also applied to chromosome preparations of *Herpestes edwardsi*. The karyotype of this species is remarkable because two pairs of chromosomes, designated A6 and C1 in fig. 10, are larger than the corresponding chromosomes in all other species of mongooses [3]. This is due to addition of chromatin to the short arms of both pairs, in chromosome A6 to such an extent that the originally shorter arm becomes the longer (turned upwards in the karyotypes). The amount of additional chromatin varies among individuals, two different chromosome types being recognized in each pair [3]. These areas together constitute 3.6–5.0% of (A+X). In conventional orcein-stained preparations, these regions usually do not show any differential properties. Sometimes a secondary constriction is visible at the border-line between the "standard" C1 chromosome and the new material, and sometimes the additional chromatin has a fuzzy appearance. Autoradiographic studies [17] revealed that the additional regions were clearly late replicating, thus in agreement with the heteropycnotic regions of the mustelides just described. The staining properties of these regions were different, however, in the mongoose.

With the same quinacrine mustard staining as in the ferret and the pygmy weasel, the late replicating regions of the mongoose did not show dark fluorescence. It is true, that the late replicating part of A6 showed fairly weak fluorescence ("dim"), but the late replicating, distal part of the short arm of C1 showed relatively strong fluorescence ("light") (fig. 11). The difference in size of the short arms between the two C1 chromosomes in fig. 11 should be noted, the arrow indicating the borderline between the

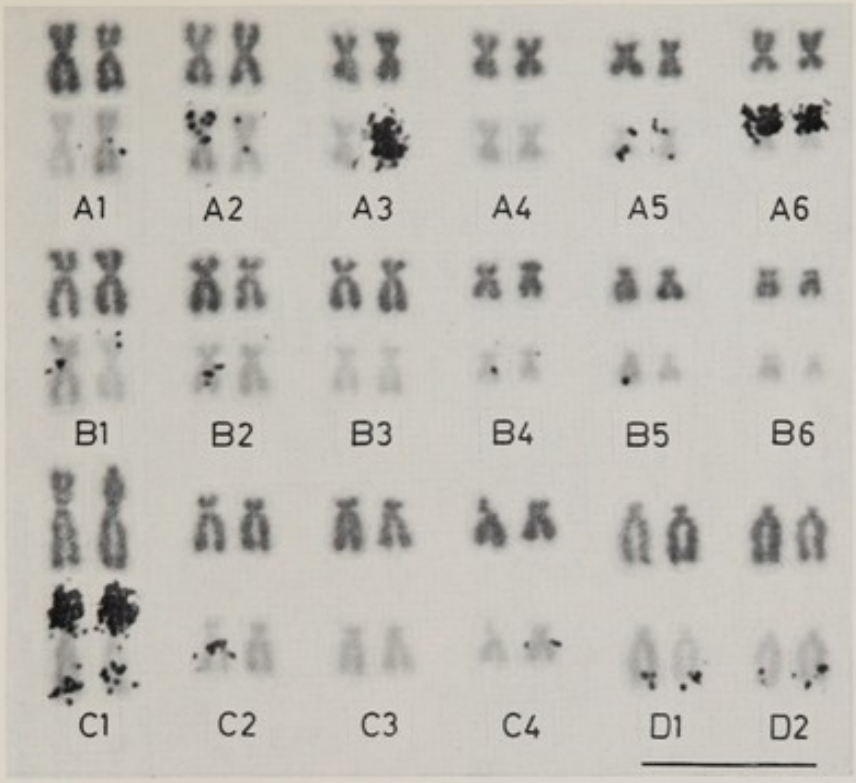


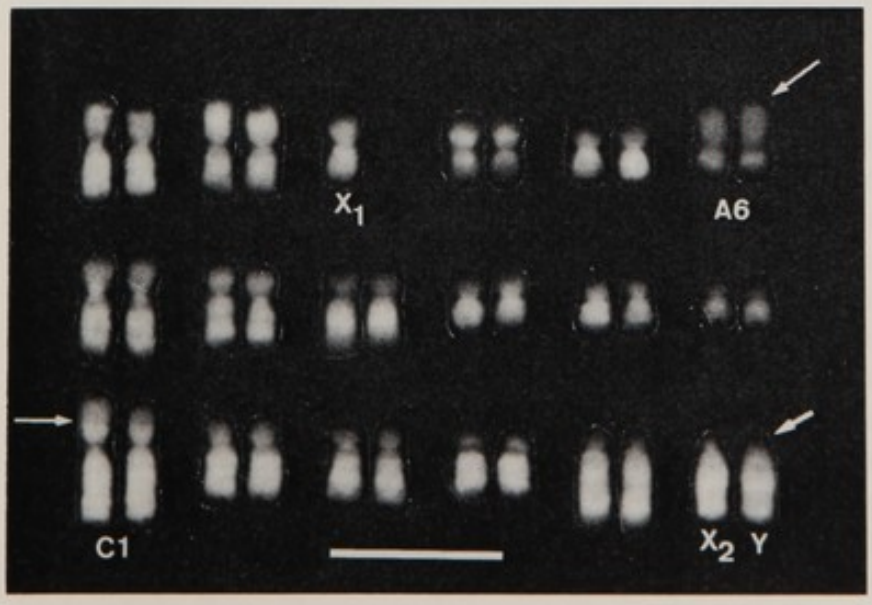
Fig. 10. Duplicate karyotypes of *Herpestes edwardsi*, female, $2n = 36$. Note heavy labelling in two autosome pairs (A6 and C1) and the late replicating X (designated A3). Autoradiography, acetic orcein squash. Scale, 10 μm.

"standard" C1 chromosome and additional chromosome material. The karyotype is from a male ($2n = 35$); the very short arm of the Y (neo-Y) is devoid of fluorescent chromatin (arrow in fig. 11).

Fig. 11. Karyotype of *Herpestes edwardsi*, male, $2n = 35$, stained with quinacrine mustard. Note that distal part of C1 does not show dark fluorescence (see text on p. 110). Scale, 10 μm.

G-bands were demonstrated with the trypsin technique, but the results in regard to the heterochromatic regions were not as clearcut in this species as in the previous ones. A relatively weak trypsin treatment produced clear bands in both regions (fig. 12*a*), whereas a stronger trypsin treatment gave poor or no banding at all (fig. 12*b*)

The C-band technique was also tried in the mongoose preparations, but in spite of many modifications, including various lengths of NaOH treatment, no differentiation whatsoever was obtained. Intensely stained regions were seen neither in the A6 and C1 chromosomes, nor in the centromeric regions of any chromosomes.



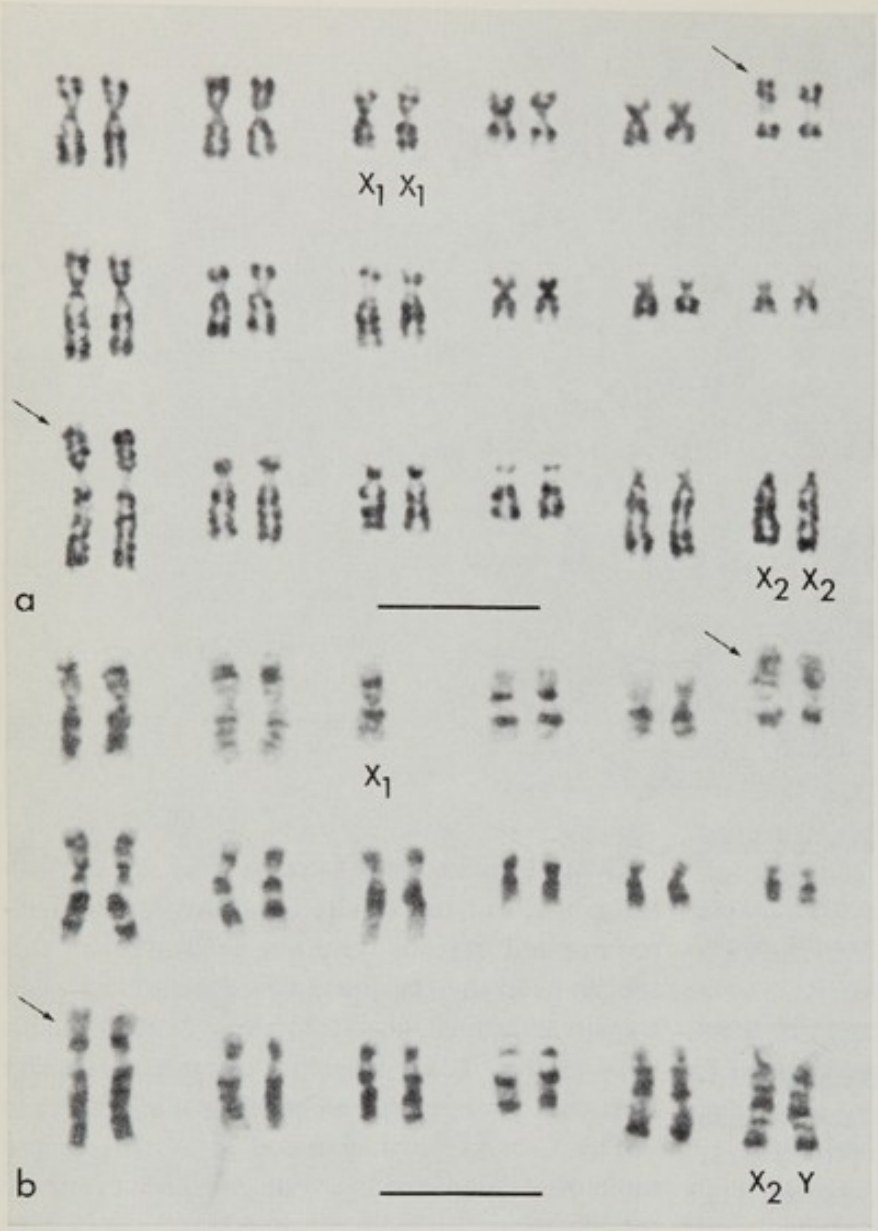


Fig. 12. Karyotypes of *Herpestes edwardsi* stained with Giemsa. (*a*) Female, weak trypsin treatment; (*b*) male, strong trypsin treatment. Distinct banding in heterochromatic regions in (*a*) but not in (*b*) (*arrows*). Scale, 10 μm.

Discussion

The old concept of heterochromatin [18] is now usually divided into two main types, facultative and constitutive, which both have been intensively studied in recent years (see e.g. [19]). Characteristic features of constitutive heterochromatin include differential staining, late DNA replication and presence of repeated nucleotide sequences. By means of new and relatively simple staining techniques it is possible to divide constitutive heterochromatin into different subtypes.

The amount and distribution of constitutive heterochromatin varies even between closely related species. E.g. *Microtus agrestis* possesses about 7 times as much constitutive heterochromatin as *Microtus pennsylvanicus* [20]. In different species of hedgehogs, the amount of constitutive heterochromatin is largely the same, but the distribution in the karyotypes varies: Large blocks of distally located heterochromatin are found in two autosomal pairs in *Erinaceus* (*Aethechinus*) *algirus*, in three pairs in *E. europeus* and *roumanicus*, whereas in *Hemiechinus auritus* and *megalotis* [21, 22] the heterochromatin is distributed in several pairs. Usually, mammals seem to have no large blocks of heterochromatin, but have it distributed in the majority of autosomes and located in the centromeric areas [23], which is the case in e.g. mouse and man.

Practically nothing is found in the literature about the amount of constitutive heterochromatin in carnivores, its distribution in the karyotypes and

Table 1. *Staining reactions of constitutive heterochromatin in somatic metaphase chromosomes of some mammals*

Order	Species	2*n*	Region (haploid set)	Orcein	C	G	Q	DNA-replication	Polymorphism
Insectivora	*Erinaceus europeus*	48	3 autos. reg.	0	?	−[29]	?	Late [22]	Yes [29]
	roumanicus	48	3 autos. reg.	0	?	?	?	Late	
	algirus	48	2 autos. reg.	(−)	+*[21]	−[21]	−[21]	Late [21]	
Primates	*Homo sapiens*	46	cen reg.	(−)	+	(+−)	−	(Late)	
			1q12; 16q11	(−)	+	+	−	Late	Yes [35]
			9q12	(−)	+ [11]	− [9, 30, 31]	− [7, 32]	Late [33, 34]	Yes [35]
			3q11; Yq12	(+, Y)	+	+	++	Late	Yes [36, 37]
			sD and sG	(−)	+	−	++	(Late)	Yes [35]
Rodentia	*Mesocricetus auratus*	44	11 autos. reg., ~1/2X, Y	0 (+)	+[23]	?	?	Late [24, 20]	(Yes) [38]
	newtoni	38	8 autos. reg., 1/3X, Y	0	+[25]	+[25]	+[25]	Late [25]	
	Microtus agrestis	50	5/7X, Y	0	+[39, 40, 41]	+[40, 41]	+[42, 43, 44]	Late [20, 45]	Yes [46, 47]
	Mus musculus	40	cen reg. (not Y)	(−)	+[48, 19]	+[49]	−[49]	(Late) [23, 34]	
	Cavia porcellus	64	18 autos. reg.	0 (+)	+[27]	?	?	Late [20]	Yes [27, 50]
Carnivora	*Mustela rixosa*	42	7 autos. reg.	(+−)	+*	−	−	Late [51]	
	putorius	40	3 autos. reg.	+	+* [51]	− [51]	− [51]	Late [51]	
	Herpestes edwardsi	35/36	2 autos. reg.	0	−	(+)	+(−)	Late [17]	Yes [3]
Artiodactyla	*Bos taurus*	60	cen reg. autos., Yp	0	+[19]	−[16]	−[16]	Late [16]	

+, positive (dark) staining or positive (light) fluorescence (Q); −, negative (pale) staining or negative (faint) fluorescence; 0, no differential staining; ( ), doubtful, variable or controversial expression; ?, no information available, +*, positive staining only after prolonged denaturation. Polymorphism only indicated when demonstrated. Nomenclature of human chromosomes is according to Chicago Conference [52], and band numbering according to Paris Conference [32].

Table 2. *Survey of staining reactions in table 1*

No.	Staining C	G	Q	Material	Location
1	+*	−	−	*Erinaceus, Mustela*	autos. reg.
2	+	−	−	*Homo*	9q12
				Bos	autos. cen reg., Yp
3	+	+	−	*Homo*	1q12, 16q11
				Mus	cen reg., except Y
4	+	+	+	*Mesocricetus, Microtus*	autos. reg., Y, part X
5	+	+	++	*Homo*	3q11, Yp12
6	+	−	++	*Homo*	sD, sG
7	−	(±)	+(−)	*Herpestes*	autos. reg.

its staining properties. Indications of large blocks of heterochromatin in carnivores are rare, and in our experience the species reported on here are exceptions. *Mustela rixosa* has 7, *M. putorius* 3 and *Herpestes edwardsi* 2 relatively large blocks of heterochromatin, mainly located in the short arms. The heterochromatic segments of the ferret and the pygmy weasel have many properties in common: (1) they are often out of phase in contraction in comparison with the rest of the karyotype, which results in differential staining with orcein; (2) they synthesize DNA late; (3) they show dark fluorescence when stained with quinacrine mustard; (4) they are faintly stained with Giemsa after mild denaturation and reassociation or after treatment with trypsin; (5) they are heavily stained after prolonged denaturation and reassociation; (6) they are composed of repetitive, fast-reassociating DNA as indicated by the acridine orange experiments.

The late-replicating autosomal segments of the Indian mongoose differ in some respects from those of the mustelides. The two regions in the mongoose did not fluoresce particularly faintly when stained with quinacrine mustard, as a matter of fact the hot region in the C1 chromosome fluoresced more strongly than many other parts of the karyotype. With the G-band technique the two regions did not differ clearly from other parts of the karyotype, and so far we have not been able to demonstrate any heavily stained regions at all with the C-band technique, even though various modifications were tested on preparations made on different occasions and from two different animals. It thus seems reasonable to conclude that the late replicating autosomal regions of the Indian mongoose belong to another category of heterochromatin than those of the mustelides. It is possible that the additional heterochromatin of the Indian mongoose is unique in its composition (cf table 1).

Large blocks of autosomal heterochromatin have been reported in a few mammals belonging to different orders: 3 hedgehog species, *Erinaceus algirus*, *E. europeus* and *E. roumanicus* [21, 22], the bat *Carollia perspicillata* [23], the Syrian and Roumanian hamsters, *Mesocricetus auratus* and *M. newtoni* [20, 24, 25], *Peromyscus maniculatus* [23, 26] and the guinea pig, *Cavia porcellus* [20, 27]. In addition, constitutive heterochromatin associated with any or both sex chromosomes has been reported in a number of mammals with sex chromosomes of the composite type (see [28]), the ones most studied being the field vole, *Microtus agrestis* and the Chinese hamster, *Cricetulus griseus*. The chromosomes of some of the above-mentioned species have been studied with the new banding techniques, and these are included in table 1 together with some other intensively studied species, namely man, mouse and cattle. As is clear from the table, differences occur in the staining reactions of the regions considered, although all are late replicating (with the possible exceptions of the human satellites and of some centromeric areas in man and mouse). It is possible to distinguish different tentative groups of constitutive heterochromatin, and their characteristics are summarized in table 2 (for explanations see the comments to table 1). Typical of the first group is that prolonged denaturation and reassociation is necessary for obtaining positive reaction with the C-band technique. The two regions of *Herpestes edwardsi* (group 7) might perhaps be divided into separate groups due to differences in fluorescence. Complete information is not available for the guinea pig, but we guess that it will be included in group 4 together with the other rodents.

It is apparent from the present survey that constitutive heterochromatin in mammals includes a number of groups, characterized by different combinations of reactions to the C-, G- and Q-band techniques. The further analysis will show whether the differences in staining capacity are based on well-defined differences in molecular organization and composition.

Chromosomal polymorphism in mammals seems to be more common than earlier assumed, polymorphism of many different kinds having been reported in recent years. The most common type is rearrangements of the centric fusion/fission type, but also various kinds of translocations, pericentric inversion, duplications and deficiencies have been identified before the era of chromosome banding. However, the new banding techniques greatly facilitate the interpretation of these structural rearrangements, and recently also insertions were demonstrated in man [53]. Heterochromatic segments are often involved in structural rearrangements and this seems natural when some of the properties of constitutive heterochromatin are taken into account such as its susceptibility to chromosome breaking agents and tendency to bring chromosomes together, thus facilitating exchange of chromosome material. Polymorphism due to variation in size of heterochromatic regions may also be explained by unequal crossing-over in the tandemly repetitive sequences [35]. Gain or loss of heterochromatin without phenotypic effects implies genetic inertness.

The type of polymorphism recorded in the last column of table 1 includes mainly loss of heterochromatic material, and also pericentric inversions. Since the human cases are comparatively well known, only those other than human will be briefly commented on below. Gropp et al. [22] studied 15 specimens of *Erinaceus europeus* from West Germany and found similar karyotypes in all of them without any indications of polymorphism. We have recently studied the chromosomes of one pregnant female from the area of Malmö in southern Sweden and her 7 fetuses (4 ♂♂ and 3 ♀♀). All eight specimens were heterozygous in two chromosome pairs due to variations in the amount of heterochromatin [29]. The only case of polymorphism reported in *Mesocricetus auratus* involves the X chromosome [38]. One phenotypically normal female had only one normal X chromosome and, likely, one deleted X. It was not established clearly which arm of the X was partially deleted, whether the somewhat larger one, composed of constitutive heterochromatin, or the smaller one, composed of facultative heterochromatin.

In *Microtus agrestis* heteromorphism has been observed in both sex chromosomes. Two out of 100 phenotypically normal males captured in the wild had approx. 1/2 and 1/3 of the long arm of the X deleted [46]. Male field voles in southern Sweden have Y chromosomes deviating morphologically from the normal Y. Due to a pericentric inversion, the "Lund Y" chromosome has a longer short arm and a shorter long arm than the standard Y [47].

In *Cavia porcellus* polymorphism due to size variations of the short arm of chromosome 1 has been described by many authors [20, 50, 54, 55]. Recently Bianchi & Ayres [27] reported smaller size of a heteromorphic X chromosome, probably due to lack of heterochromatin in its short arm, and also one small autosome pair with heteromorphism due to differences in amount and localization of heterochromatin.

The size variations in the heterochromatic areas of *Herpestes edwardsi* have been mentioned above.

The role of constitutive heterochromatin in mammals has been discussed by many authors (e.g. [19, 20, 22]) most of which agree as to its evolutionary significance. Schmid [20] sums up the situation as follows: "... groups of plants and animals possessing relatively much heterochromatin are—under otherwise identical conditions—expected to be more successful in speciation, thereby exhibiting a great array of different karyotypes. The occurrence of a greater number of viable chromosomal rearrangements favors reproductive isolation which is an important step in the formation of new species."

Production of new sources of constitutive heterochromatin (repetitive DNA) by saltatory replication [56] may constitute a new impulse for speciation in an old species which has entered a blind alley in evolution. Is this what has happened in the species of the old genera *Erinaceus*, *Mustela* and *Herpestes* discussed above? It is worthy of mention that many of the species listed in table 1 offer taxonomic problems of a species/subspecies type (*Erinaceus europeus–roumanicus*, *Mesocricetus auratus–newtoni*, *Microtus agrestis – pennsylvanicus*, *Mus musculus – poschiavinus*, *Cavia porcellus – cobaya*, *Mustela putorius – furo*, *Mustela nivalis* (*vulgaris*) *– rixosa* (*minuta*)).

Only one example will be commented on: Ellerman & Morrison-Scott [57] are of the opin-

ion that there is only one valid species of the genus *Mesocricetus*, whereas Russian authors [58], supported by karyological evidence, distinguish four valid species: *M. auratus*, $2n=44$, *M. brandti*, $2n=42$, *M. raddei*, $2n=42$–44 and *M. newtoni*, $2n=38$. All species have different N. Fa. ("nombre fondamental autosomique") values, 74, 78, 72 and 70 respectively, gain or loss of entire heterochromatic arms being the most likely explanation of this variation. Centric fusion/fission mechanisms are responsible for the differences in chromosome number.

There is no doubt that constitutive heterochromatin plays an important role in the speciation process and that we witness this evolution taking place in certain mammalian species of different orders.

We wish to express our sincere thanks to those friends and colleagues (p. 104) who kindly provided us with the material for this study. We are also indebted to Professor Albert Levan for critical reading of the manuscript. The financial support of the Swedish Cancer Society and the John and Augusta Persson Foundation is gratefully acknowledged.

Note added in proof

By a modification of the "BSG" technique (Sumner, A T, Exptl cell res 75 (1972) 304) we eventually succeeded in obtaining dark staining in the heterochromatic parts of the short arms of chromosomes A 6 and C 1 in *Herpestes edwardsi*.

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Discussion

Comings: In relation to the reversed C-banding Dr Fredga described, this may not be all that atypical. We find that simple Giemsa staining of mouse chromosomes shows that C-band regions actually stain more lightly than the arms while this is reversed after C-banding.

Hsu: Since you used a prolonged (5 min) NaOH treatment to reveal these particular heterochromatic arms, you may have overtreated the centromeric heterochromatin of the rest of the chromosomes. Have you tried a more standard time of NaOH treatment and examined the centromeric regions?

Fredga: Shorter treatment gave no differentiation whatsoever, and, particularly in the ferret, no heavily stained centromeric heterochromatin could be demonstrated.

Gropp: The regions displaying heteromorphism in your specimens of *Erinaceus europaeus* seem to me to correspond exactly to the regions of autosomal heterochromatin. What struck me particularly is the similarity of the results of staining of the heterochromatic segments in the carnivore species and in the hedgehogs, as far as the observations of our laboratory are concerned. Do you agree with this statement?

Fredga: Yes, particularly the heterochromatic segments in the mustelides show striking similarities to those in the hedgehogs.

Comings: One possibility is that you had a set of identical triplets and one of identical quadruplets, in which case the chance that all seven would carry the translocation is more reasonable.

Fredga: The hypothesis of identical triplets and quadruplets is attractive. In the nine-banded armadillo (*Dasypus novemcinctus*), the four young are known to be monozygotic quadruplets, but whether sets of monozygotic young occur also in the hedgehog is unknown to me; however, I find it unlikely: Unlike the armadillo, the hedgehog has a bicornuate uterus, and in the present case the sequence of the fetuses was: left uterine horn: M, M, M, right: F, M, F, F. Since the statistical likelihood is negligible (1 : 16,384) that all 7 fetuses were heterozygous for 2 chromosome pairs, some other factor must be assumed as influencing either the chromosome segregation at meiosis, or the amount and distribution of heterochromatin. Due to the size of the difference between the homologues of each of the 2 pairs involved, mere contraction effects can be excluded.

Identification of Metacentric Marker Chromosomes in the Mouse by Use of Banding Techniques

A. Gropp and Lore Zech

Institut für Pathologie der Medizinischen Akademie Lübeck, Lübeck, BDR,
and Karolinska Institutet, S-10401 Stockholm 60, Sweden

Identification of the individual chromosomes of the mouse and the assignment of known linkage groups may be reckoned among the most important achievements in the fields of experimental genetics and cytogenetics, because they compensate the imbalance which exists between the considerable amount of genetic information on mutational events and on linkage groups of genes in the mouse, on the one hand, and the limited suitability of this species for cytological work, on the other.

Beyond these possibilities, the availability of marker chromosomes of known identity and origin seems to be of great importance for many experimental procedures, mainly those in which the use or the introduction of an easily identifiable chromosome with cytological methods is necessary. Therefore, our interest has been directed to the search for metacentric marker chromosomes derived from Robertsonian centric fusion in feral mouse populations and to their identification and isolation [14]. These marker chromosomes of the mouse have been found to be particularly useful, e.g. in the study of the cytogenetic factors involved in the impairment of fertility resulting from abnormal meiotic segregation and in the experimental production and analysis of aneuploid embryos of known identity of the extra chromosome [8, 10, 11].

The purpose of this report is the presentation of a series of metacentric marker chromosomes in the mouse and the description of their staining properties using quinacrine mustard [3] and Giemsa [2, 17] banding techniques. These procedures, combined with the meiotic assay of chromosomal homologies in double heterozygotes, were designed to permit the identification of the individual metacentric marker chromosomes and of the chromosome arms involved in their formation.

Until recently, it had been generally admitted that feral populations as well as the laboratory strains of the mouse have a constant karyotype with 40 acrocentrics. According to our own observations in collaboration with Dr J. Marshall, Bangkok, this is true at least for habitats in Northern and Eastern Europe, on the Iberian peninsula, in India, South-East Asia and Japan. However, an extreme chromosomal variant of the domestic mouse, commonly called tobacco mouse, homozygous for a series of seven fusion metacentrics, was found 3 years ago in the Valle di Poschiavo in Southeastern Switzerland [12]. For these metacentrics the designations T1 to T7Bnr (*Bonn*/*R*hein) have been proposed [13]. The particular phenotype of this small, dark-coloured mouse was first described by Fatio [7] who proposed the species designation *M. poschiavinus*.

Banding patterns and identification of the T1 to T7Bnr metacentrics of the tobacco mouse

The fluorescence pattern of the chromosomes of *M. poschiavinus* as revealed by quinacrine mustard (QM) staining is demonstrated in fig. 1. Careful comparison of the individual chromosome arms of *M. poschiavinus* with the chromosomes of the laboratory mouse [9, 16] shows that there is a complete mutual identity of the bands. The assumption of the homology of the chromosomes of the tobacco and the laboratory mouse seems to be confirmed by the formation of regularly paired trivalents in their F_1-hybrids. It is therefore possible to translate the designations

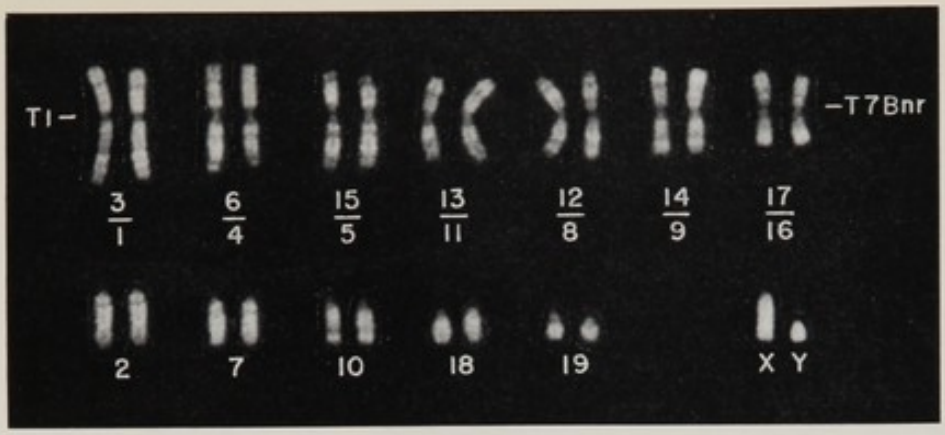


Fig. 1. Karyotype of the tobacco mouse (*M. poschiavinus*). QM-fluorescence banding pattern. Numbering of the chromosome arms according to the recommendation of the Committee for the Standardization of Genetic Nomenclature for Mice [5].

of the mouse chromosomes proposed by the Committee for Standardisation of Genetic Nomenclature for Mice [5] to the tobacco mouse metacentrics. Table 1 records their assignment to the chromosome arms involved in the formation of the T1 to T7Bnr metacentrics of the tobacco mouse together with the corresponding linkage groups which can be coordinated with these chromosomes [4, 5, 16]. Thus, the seven metacentric marker chromosomes of the tobacco mouse series comprise 10 out of the 18 known linkage groups of the mouse.

Banding patterns, identity and homologies of the T8 to T10Bnr metacentrics isolated from other feral mouse populations

Since these observations, other feral mouse populations with metacentric chromosomes have been detected in neighbouring areas outside Valle di Poschiavo, thus providing evidence of the presence of an intensive intraspecific variation of the Robertsonian type in mice from Alpine valleys of Southeastern Switzerland [14]. In a few cases the metacentric chromosomes from other areas were found to be identical with metacentrics of the tobacco mouse series. However, independence prevails among the metacentric chromosomes, if the findings in the different localities of Southeastern Switzerland and of the North Italian lake district are compared [14]. Thus, it was possible to identify and to isolate by breeding procedures three new metacentric chromosomes

Table 1

Designation of metacentric chromosome	Locality	Coordination of chromosome arms[a]	Linkage group[a]
T1Bnr	Valle di Poschiavo	3/1	—/XIII
T2Brn	Valle di Poschiavo	6/4	XI/VIII
T3Bnr	Valle di Poschiavo	15/5	VI/XVII
T4Bnr	Valle di Poschiavo	13/11	XIV/—
T5Bnr	Valle di Poschiavo	12/8	—/XVIII
T6Bnr	Valle di Poschiavo	14/9	III/II
T7Bnr	Valle di Poschiavo	17/16	IX/—
T8Bnr	Val Bregaglia	11/10	—/X
T9Bnr	Albula valley Area of Basel	12/4	—/VIII
T10Bnr	Val Bregaglia	10/1	X/XIII
T1Ald	ref. [15]	15/6	—/XI
T163H	ref. [6]	19/9	XII/II
T1JeM	ref. [1]	17/8	IX/XVIII

[a] Designation of chromosome arms and assignment of L. G. according to the Committee on the Standardization of the Genetic Nomenclature for Mice; Standard karyotype of the mouse [5], and to Cattanach et al. [4].

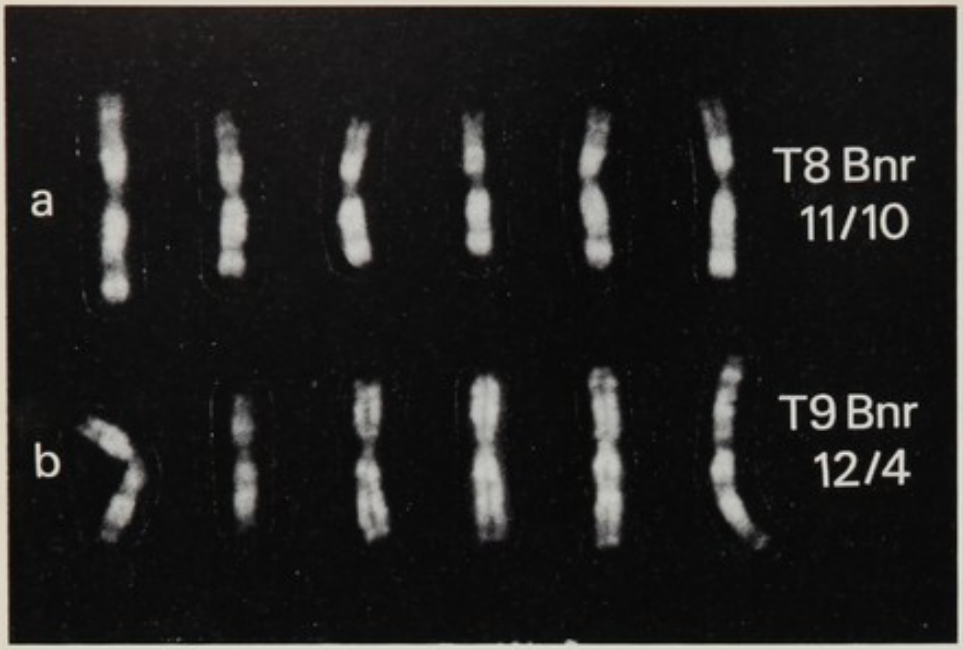


Fig. 2. Metacentrics T8Bnr (*a*) and T9Bnr (*b*) selected from several karyotypes. QM-fluorescence banding.

besides the T1–7Bnr metacentrics of the tobacco mouse. They display, however, mono- or heterobrachial homologies with the individual elements of this series. For these chromosomes, which were present in mice caught in the Val Bregaglia (T8Bnr and T10Bnr) and in mouse populations down the River Rhine from the upper tributary valleys to Basel (T9Bnr), the designations T8, T9 and T10Bnr (table 1) are proposed. Mice carrying the T8Bnr or the T10Bnr metacentric were trapped in places of about 3 to 4 km apart within the same valley.

The banding patterns of the T8 to T10Bnr chromosomes as shown by QM-fluorescence are presented in figs 2*a*, *b*, 3*a*–*c* and 4*b*, *c*, whereas fig. 5*a* and *b* demonstrate the corresponding patterns of the T8 and T10Bnr metacentrics stained with the Giemsa ASG technique [2].

The QM- and G-banding of the arms of the T8–10Bnr chromosomes correspond to that present in 5 of the acrocentrics of mice with the regular $2n=40$ karyotype [2, 9]. This similarity of the longitudinal differentiation lends support

Fig. 3. Metacentrics T8, T9, T10Bnr and T1Ald. QM-fluorescence banding.

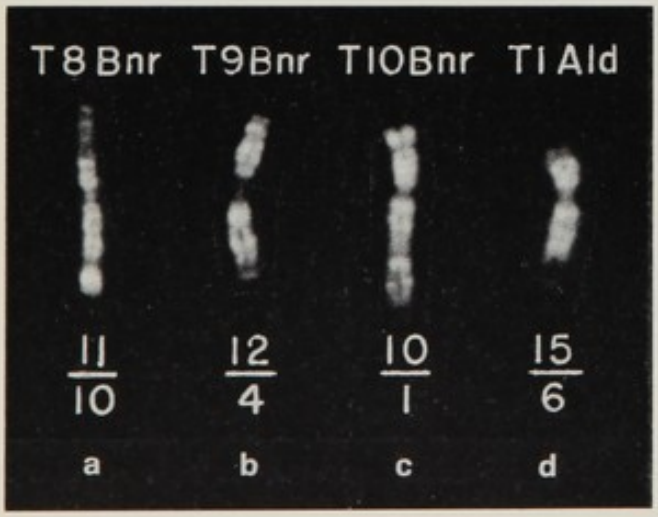
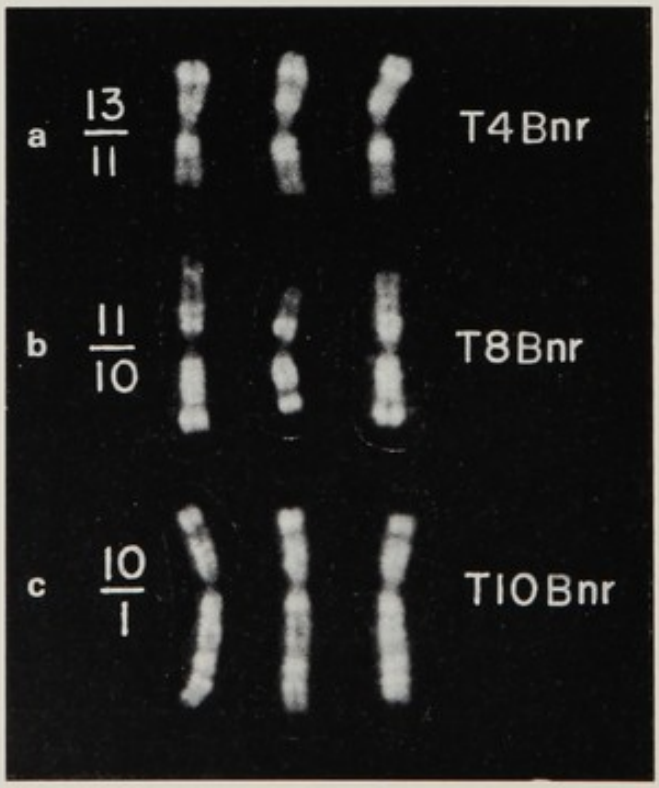


Fig. 4. Metacentrics T4, T8 and T10Bnr. QM-fluorescence banding reveals the monobrachial homologies of T4Bnr and T8Bnr and of T8Bnr and T10Bnr.



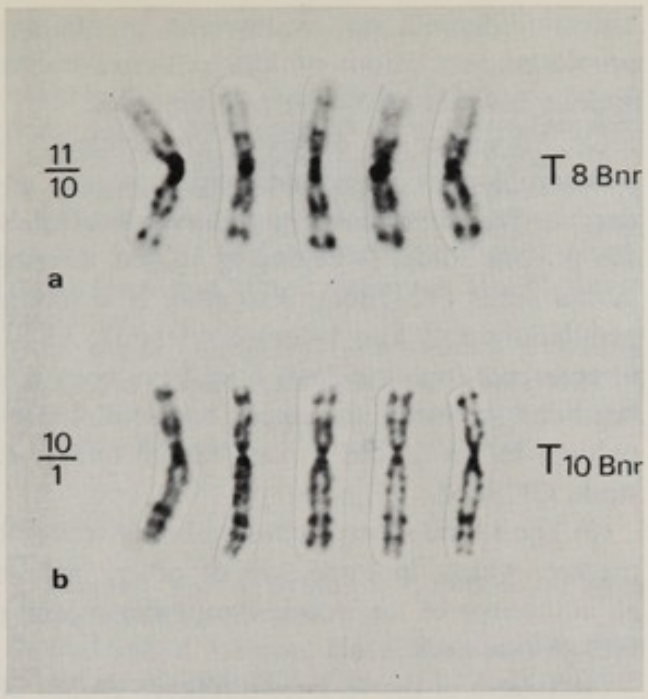


Fig. 5. Giemsa (ASG) banding pattern of chromosomes T8 and T10Bnr.

to the assumption of the identity and homology also of the arms of the T8–10Bnr metacentrics with those of the laboratory mouse acrocentrics. The designation of numbers to these arms according to the recommendation of the Committee for Standardization of Genetic Nomenclature for Mice [5] is therefore feasible (table 1). Two of these metacentrics contain mouse chromosome No. 10 which does not participate in the formation of the metacentrics of the tobacco mouse series at all. This brings to 15, the number of individual mouse chromosomes, and to 11, the number of linkage groups carried and represented by the marker metacentrics which have so far been isolated from feral mouse populations [4, 8, 14].

Cross-checks using the diakinesis analysis, in males or females, doubly heterozygous for all possible combinations between the T1, T4, T5, T7 and T8–10Bnr metacentrics confirmed these assignments. Thus, the double heterozygotes carrying the T8Bnr and T10Bnr show in meiosis I, a large chain tetravalent (fig. 6), because both chromosomes have a monobrachial homology for mouse chromosome No. 10 (figs 3*a*, *c*, 4*b*, *c*).

At present, the introgression of the T8 to T10Bnr as well as of the T4 and T7Bnr metacentrics, in the C57Bl/6J background by consecutive breeding is being performed by Radbruch & Gropp.

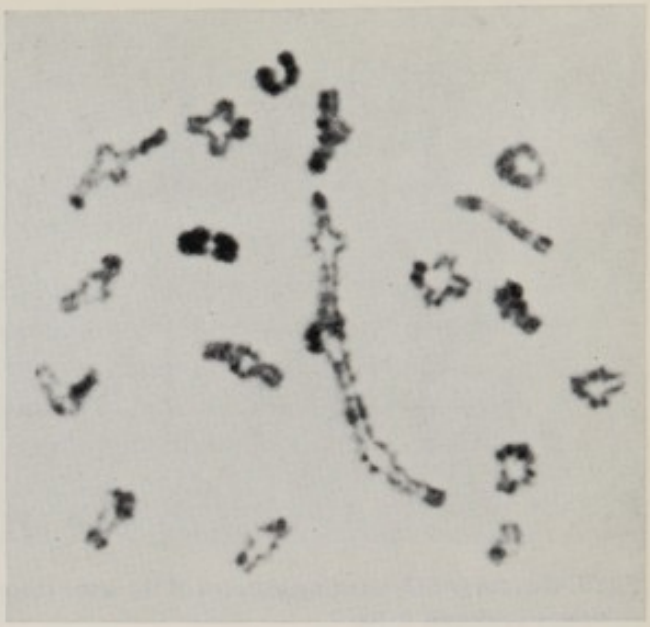
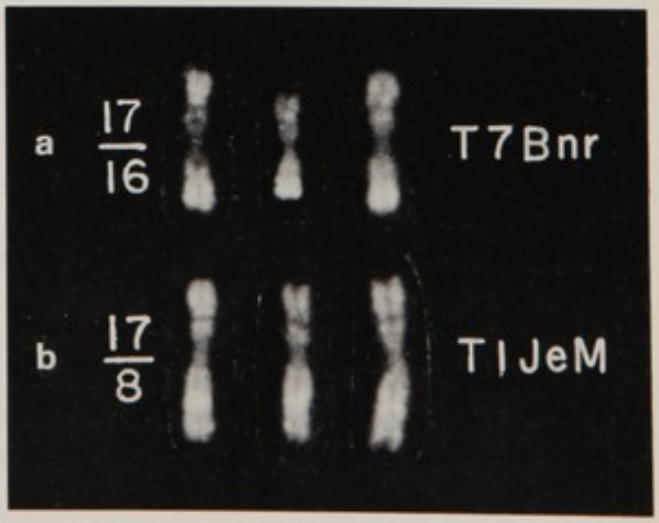


Fig. 6. Late diakinesis of F_1 doubly heterozygous for T8 and T10Bnr, both forming a tetravalent.

Banding patterns and homologies of metacentric chromosomes observed in laboratory strains of the mouse

Some few metacentric chromosomes derived from Robertsonian fusion have been described in laboratory mouse strains, T1Wh by White & Tjio [18], T1Ald by Leonard & Deknudt [15], T163H by Evans et al. [6], and T1JeM by Baranov & Dyban [1]. These metacentrics could be studied by banding techniques, with the exception of T1Wh. Confirming the former results of Miller et al. [16] the T1Ald (fig. 3*d*) and the T163H metacentrics involve mouse chromosomes 15/6 and

Fig. 7. Metacentrics T7Bnr and T1Jem. QM fluorescence banding patterns disclosing monobrachial homology of the arm corresponding to mouse chromosome No. 17 (see fig. 8).



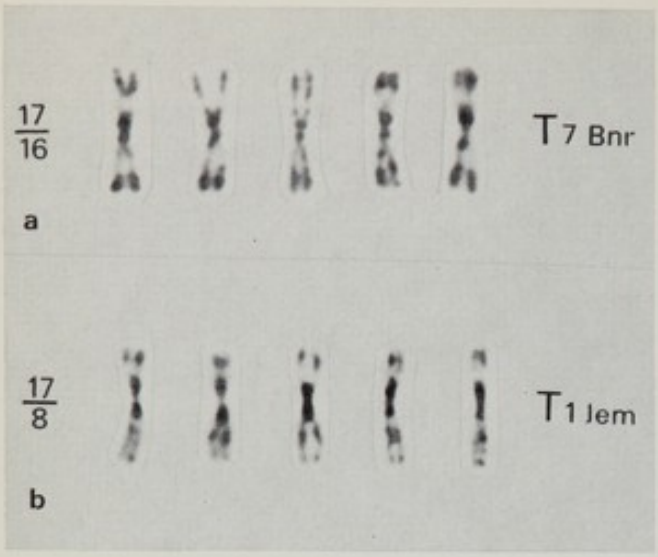


Fig. 8. Giemsa (ASG) banding patterns of the same chromosomes as shown in fig. 7.

19/9, respectively (table 1). The T1Jem metacentric represents a Robertsonian fusion product of mouse chromosomes 17 and 8, as shown by the use of QM-fluorescence (fig. 7*b*) and by Giemsa (ASG) staining (fig. 8*b*).

These coordinations were corroborated by meiotic studies of double and multiple heterozygotes of the T1Ald, T163H and T1JeM metacentrics on the one hand, and the tobacco mouse metacentrics on the other hand. Thus, crosses between mice carrying the T1Jem chromosome and tobacco mice led to the appearance of a pentavalent in the F_1 generation, while in crosses with mice carrying the single chromosomes T5Bnr and T7Bnr of the tobacco mouse series a tetravalent was observed. The monobrachial homology of the T7Bnr and the T1Jem, which is caused by the common possession of mouse chromosome No. 17, is illustrated by figs 7 and 8, and further documented by the tetravalent in the meiotic preparation of fig. 9.

Conclusions

A few points emerge from the present results recorded in table 1:

(1) The numbering of the chromosomes of the laboratory mouse recommended by the 'Committee of Standardization of Genetics of the Mouse' [5] can be assigned to the individual arms of the Robertsonian fusion metacentrics described in this report (T1–10Bnr). This is possible because there is not only a similarity of the longitudinal differentiation as revealed by staining procedures, but also homology as shown by the meiotic analysis of double heterozygotes.

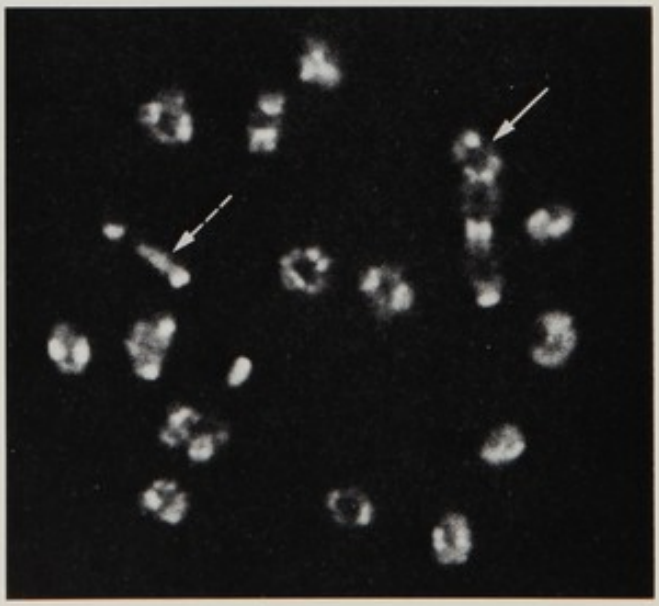
(2) Altogether 13 different, independent, or non-homologous metacentric chromosomes derived by fusion processes have been identified in the present study, 7 belonging to the tobacco mouse series (T1–7Bnr), 3 to other feral mouse populations and 3 to laboratory strains. All 10 metacentrics found in feral mice have been isolated and are being introduced by breeding procedures in an inbred strain background, e.g. strain C57Bl/6J.

(3) The 13 metacentric chromosomes represent markers which, in some way or other, include all autosomes of the mouse except chromosome No. 18 (see table 1).

(4) Twelve of the 18 known linkage groups of the mouse can be associated individually with at least one, mostly with two or several different metacentrics.

(5) Thus, the metacentrics described here are available for genetic studies and for experimental procedures in which the use or the introduction of a metacentric marker chromosome carrying known linkage groups is desirable.

Fig. 9. Late diakinesis of F_1 doubly heterozygous for T7Bnr and T1JeM. Both form a tetravalent due to the common possession of chromosome No. 17. Bright fluorescence of the centromeres after staining with a benzimidazol-derivative (see [14]). Thus, the tetravalent displays four bright blobs (→). Within the XY-bivalent the location of the centromere region of the X chromosome is opposite to the Y chromosome (⇢).



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The authors are grateful to Professor A. P. Dyban, Institute of Experimental Medicine, Acad Med Sci USSR, Leningrad, for supplying us (A. G.) with mice of the T1JeM strain.

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Discussion

Lejeune: Do you believe you will be able to construct such a structural heterozygote that all the autosomes will form a ring at meiosis like *Oenothera?*

Gropp: It may be possible to breed a multiple heterozygote displaying very complex multivalents in diakinesis. But as long as only Robertsonian fusions are involved, only chain multivalents would result.

Chromosomal Evolution of Man and the Primates (*Pan troglodytes, Gorilla gorilla, Pongo pygmaeus*)

J. de Grouchy, Catherine Turleau, Michelle Roubin and Françoise Chavin Colin

Clinique de Génétique Médicale et Unité de Recherche de l'INSERM, Hôpital des Enfants Malades, 75015 Paris, France

One of the obvious applications of the new cytogenetic techniques is the elucidation of the chromosomal evolution of species, and among these man and the hominoid apes are undoubtedly the most tempting candidates for study. Conventional techniques have shown similarities between the karyotypes of these species [2, 3, 4, 5, 6, 9, 10, 11, 16, 19]. The possibilities of precise comparisons were limited, however, due to the absence of any criteria other than the size and centromeric index of chromosomes. When discussing the chromosomal evolution of species, Hamerton [10] nevertheless emphasized the possible role of fusions and pericentric inversions. More recently, Pearson [15], using fluorescence, found evidence of homology in certain chromosomes of man and primates.

Using the R-banding technique, we first compared the karyotypes of man and chimpanzee (*Pan troglodytes*) and demonstrated the major role played in evolution by centric fusions and mostly by pericentric inversions [8, 17]. We have now applied the same study to the gorilla (*Gorilla gorilla*) and the orangutang (*Pongo pygmaeus*). Our observations will be discussed in this presentation.

Material and Methods

Four chimpanzees (2 males and 2 females), 2 gorillas, 2 orangutangs (1 male and 1 female of each species) were studied.

Karyotypic preparations obtained from whole blood cultures were submitted to controlled heating denaturation according to Dutrillaux & Lejeune [7] and stained with Giemsa.

Results

The technique is directly applicable to the hominoid apes. Banding patterns comparable to those observed in man are obtained.

The phylogenic tree shown in fig. 1 and proposed by Valois [18] is generally accepted, although estimates of the temporal separation of man and the apes may still be a matter of debate [13]. Hence, the following two-by-two comparisons were made: man–chimpanzee, man–orangutang, and chimpanzee–gorilla. They are summarized in figs 2, 3 and 4. For each comparison the karyotype of the first-mentioned species was established. Then, for each particular chromosome we have selected the best-matching chromosome from the second species, on the basis of size and banding pattern. This comparison allows a reconstitution within each phylum of the chromosomal rearrangements which have occurred during evolution.

Chromosome No. 1. In each of the hominoid apes there is a submediocentric chromosome which matches the human 1 on the assumption that the subcentromeric constriction present in the latter is not present in the apes. This has been confirmed by using the C-banding technique according to Yunis et al. [20] and Arrighi & Hsu [1] (fig. 5). Chromosome No. 1, in the ancestor common to the four species, must therefore have been similar to that observed today in the apes, and the secondary constriction acquired only in the human phylum.

Chromosome No. 2. None of the three apes possesses a chromosome similar to the human 2. There are, however, two acrocentric chromo-

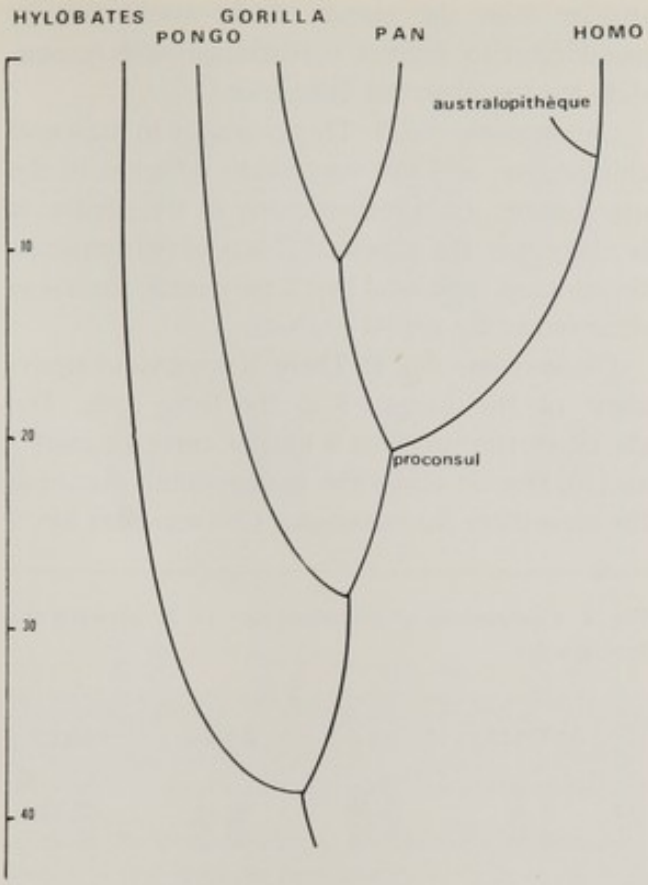


Fig. 1. *Ordinate:* million years.
Phylogenic tree of man and the anthropoid apes.

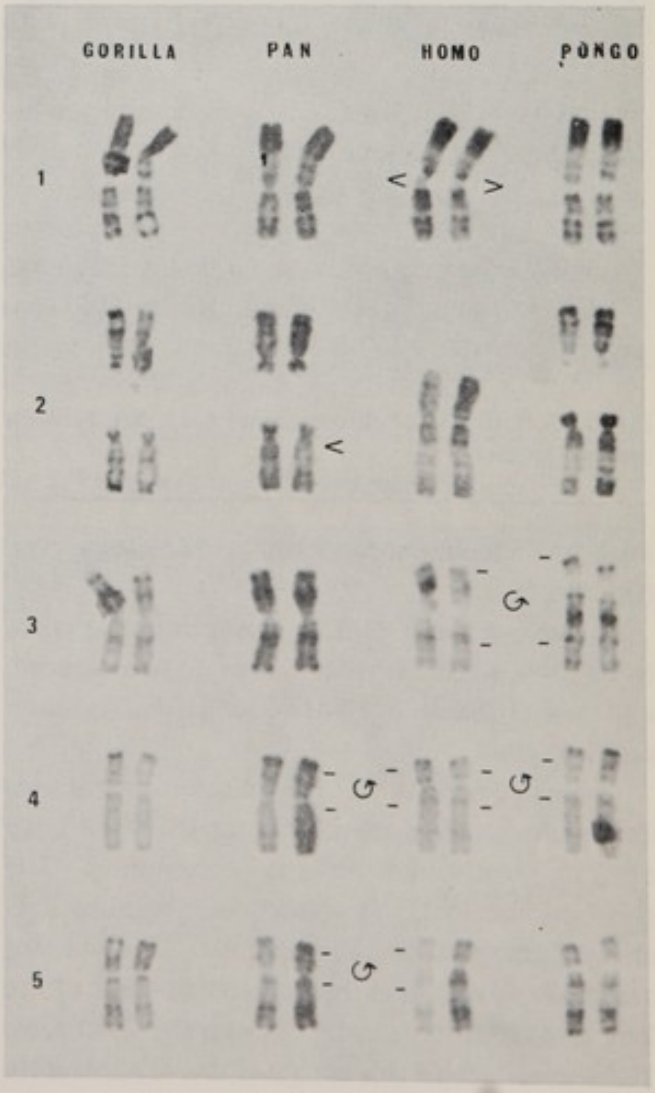
somes, designated [2p] and [2q], which are equivalent by their banding pattern. The homology is good in the orangutang. In the chimpanzee and the gorilla the proximal portion of [2q] is apparently missing. It is therefore likely that the ancestral 2 consisted of two independent acrocentrics similar to those observed in the orangutang. Secondarily, the proximal portion of [2q] was deleted in the chimpanzee–gorilla phylum, and a fusion, of the Robertsonian type, or more complex, occurred in the human phylum (fig. 6).

Chromosome No. 3. Man, chimpanzee and gorilla possess an apparently identical chromosome 3. The orangutang differs by the presence of a chromosome having the same size but subtelocentric. This difference may be explained by a large pericentric inversion within the limits indicated in fig. 2. It is impossible to conclude which of the two types of chromosome was present in the common ancestor. Only the karyotype of the gibbon (*Hylobates*) might help to solve this ambiguity. According to the karyotype of the gibbon, established with conventional methods by Hamerton et al. [10], the common ancestor would have a human type chromosome No. 3 and the pericentric inversion would have occurred in the orangutang phylum.

Chromosome No. 4. Chimpanzee, gorilla and orangutang possess a chromosome equivalent to the human 4 but which is more mediocentric. This difference may be explained by a pericentric inversion having occurred in the human phylum, the ancestral 4 being similar to that observed in the apes (fig. 7).

Chromosome No. 5. Man and orangutang possess the same 5 while gorilla and chimpanzee possess a more mediocentric chromosome similar to the 4 observed in the apes. This may be explained by a pericentric inversion having occurred in the chimpanzee-gorilla phylum, the

Fig. 2. Comparison of chromosomes 1–5 between the four species. Circular arrows indicate pericentric inversions; the < or > signs indicate loss or acquisition of chromosomal material; the question mark indicates undetermined rearrangements. Comparisons are made two-by-two between species: homo–pan, homo–pongo, and pan–gorilla.



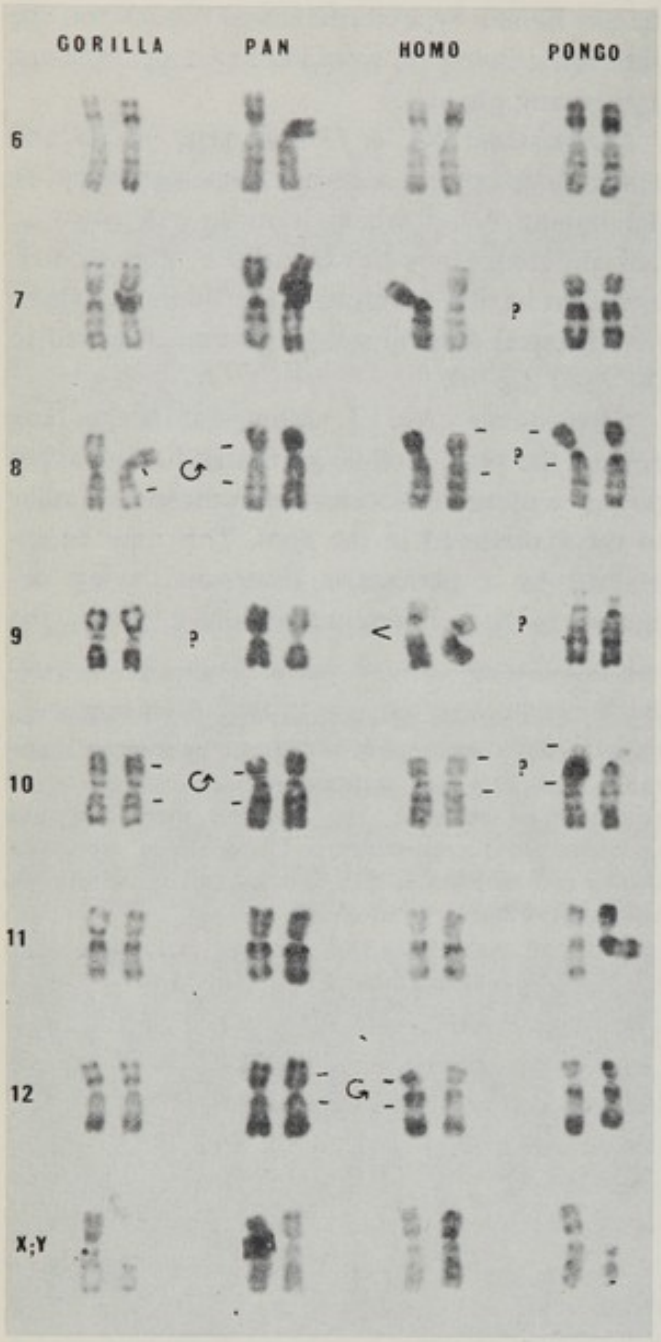


Fig. 3. Comparison of chromosomes 6–12, XY between the four species.

ancestral 5 being of the human–orangutang type (fig. 8).

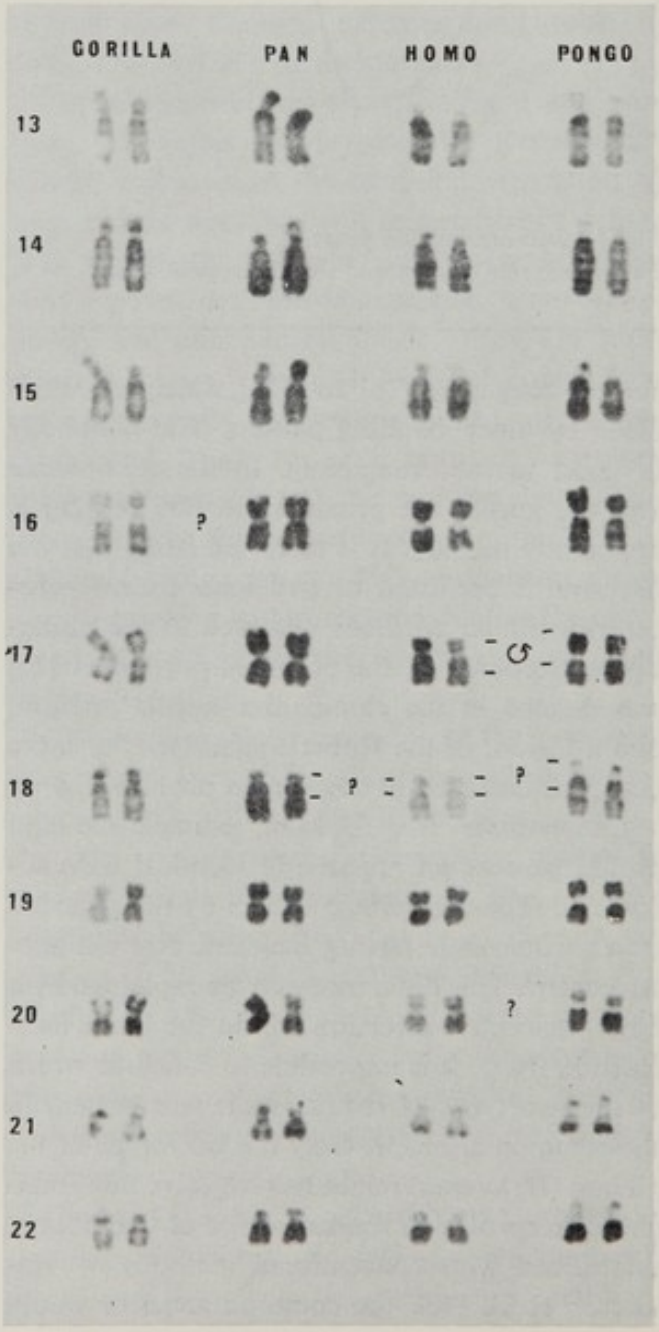
Chromosome No. 6. This chromosome appears to be most stable. It is similar in all four species, and has probably not varied since the common ancestor.

Chromosome No. 7. This chromosome is similar in man, chimpanzee and gorilla. There is no obvious equivalent in the orangutang. The chromosome in fig. 3 was chosen because it is always heavily stained as the human 7. Its banding pattern is similar, mostly for the distal part of the long arm. It differs markedly from the short arm. The overall size of this chromosome is apparently smaller than the human 7. It would not be meaningful to suggest a rearrangement responsible for the observed difference.

Chromosome No. 8. This is similar in man and chimpanzee, and not very much different in the orangutang. It is mediocentric in the gorilla. It is likely that the ancestral 8 was of the human or orangutang type, and that a pericentric inversion occurred in the gorilla phylum.

Chromosome No. 9. There is no obvious equivalent of the human 9 in the three apes. The choice of chromosome 9 and of chromosomes 7 and 10 (fig. 3) offers the best possible matching for these three chromosomes. Chromosome No. 9

Fig. 4. Comparison of chromosomes 13–22 between the four species.



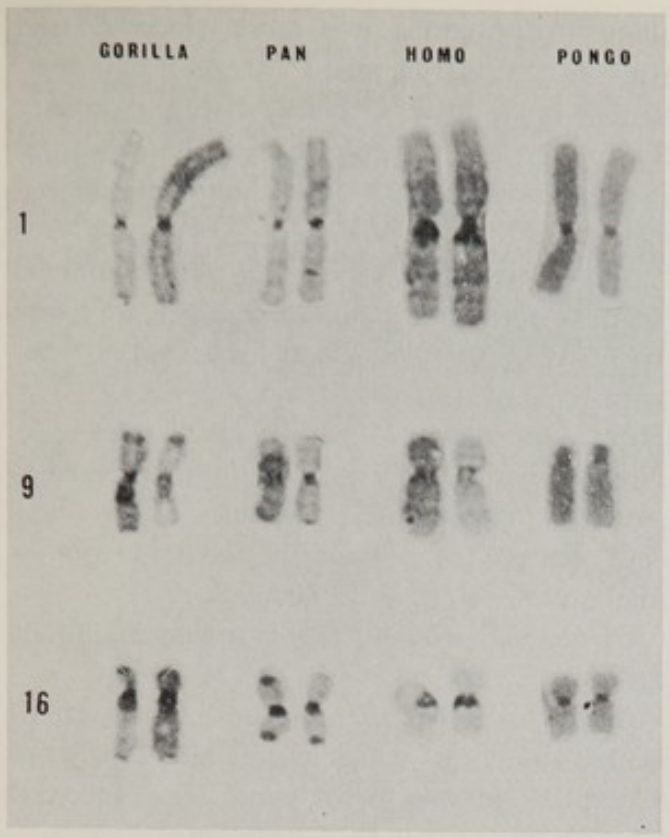
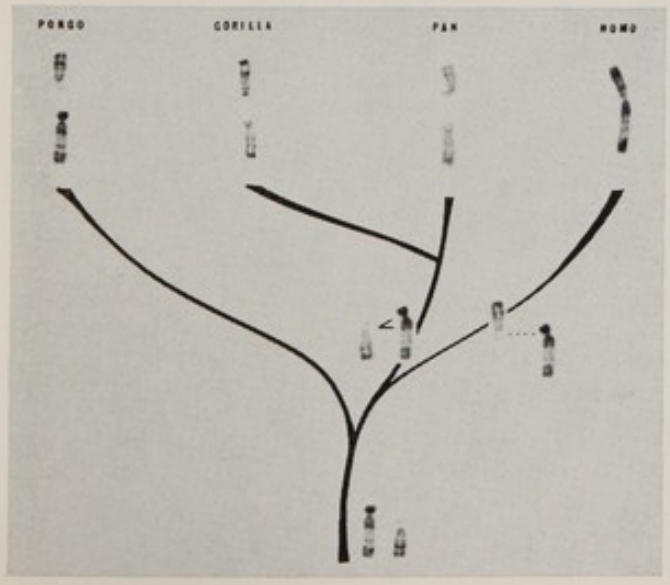


Fig. 5. C-banding technique. Comparison of chromosomes 1, 9 and 16 in the four species.

is different in all four species. It is acrocentric in the orangutang; mediocentric and small in the gorilla, with a secondary constriction (demonstrated by the C-banding technique); also mediocentric in the chimpanzee, but with no constriction and a different banding pattern. The most economical choice for the ancestral chromosome would be a chromosome similar to that of man or orang-utang.

Fig. 6. Phylogenic tree of chromosome No. 2.



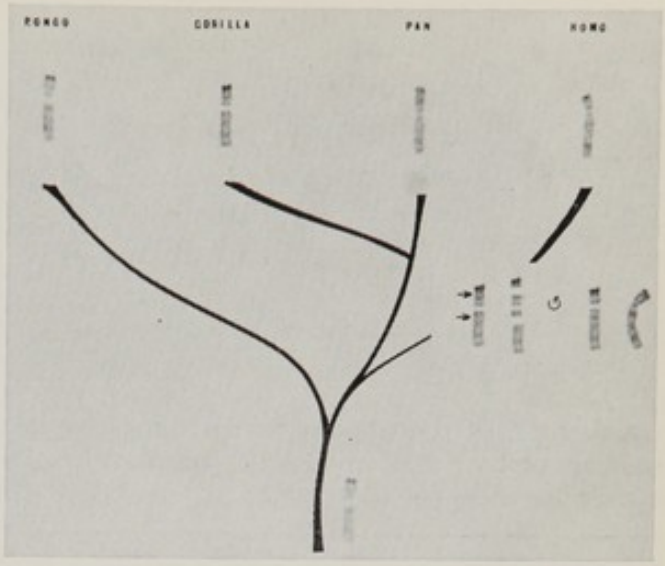


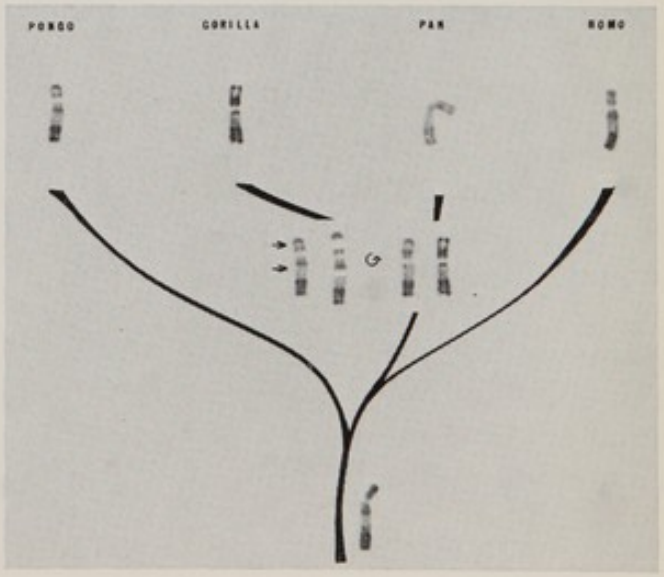
Fig. 7. Phylogenic tree of chromosome No. 4.

Chromosome No. 10. This chromosome is similar in man and chimpanzee. It is mediocentric in the gorilla, and acrocentric in the orangutang. The ancestral chromosome may have been of the human or orangutang type. A pericentric inversion occurred in the gorilla.

Chromosome No. 11. As for the 6, chromosome No. 11 appears to have been remarkably stable during evolution.

Chromosome No. 12. This chromosome is similar in man and orangutang. It is identical in the chimpanzee and the gorilla, but more mediocentric when compared with man and orangutang. Therefore, the ancestral chromosome must have been of the human–orangutang type, and a pericentric inversion (fig. 9) must have occurred in the gorilla–chimpanzee phylum.

Fig. 8. Phylogenic tree of chromosome No. 5.



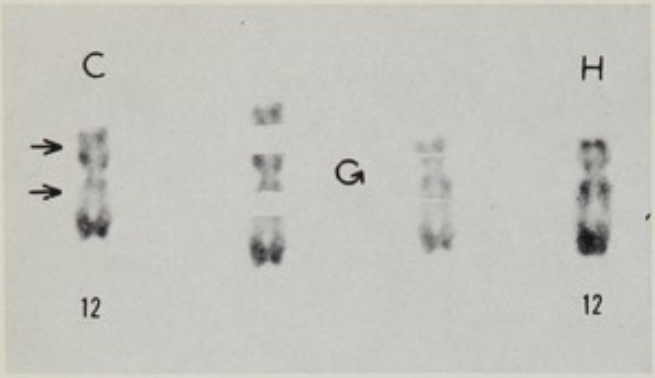


Fig. 9. Artificial pericentric inversion reproducing the rearrangement which occurred for chromosome No. 12 in the chimpanzee–gorilla phylum.

D-Group chromosomes. Within the limits of accuracy of the method, the D-group chromosomes appear to have been stable during evolution.

Chromosome No. 16. This chromosome is more or less mediocentric in all four species. It carries in man a secondary constriction which is not present in the chimpanzee and the orangutang. In the gorilla it is more telocentric and carries a secondary constriction. As for chromosome No. 1, it is probable that a secondary constriction was acquired in man. The ancestral 16 must have been of the chimpanzee–orang-utang type.

Chromosome No. 17. This is mediocentric in the three species of apes. A pericentric inversion must have occurred in man, the ancestral chromosome being of the simian type.

Chromosome No. 18. Similar in the three apes, chromosome No. 18 is slightly more mediocentric in man. A small pericentric inversion may have occurred in the latter. The ancestral chromosome was therefore of the simian type.

Chromosome No. 19. This is similar in all four species.

Chromosome No. 20. Similar in man, chimpanzee and gorilla, chromosome No. 20 is more telocentric in the orangutang. The ancestral chromosome cannot be determined.

Chromosome No. 21. This is similar in all four species.

Chromosome No. 22. This too is similar in all species.

Chromosome X. Once again this chromosome is similar in all species.

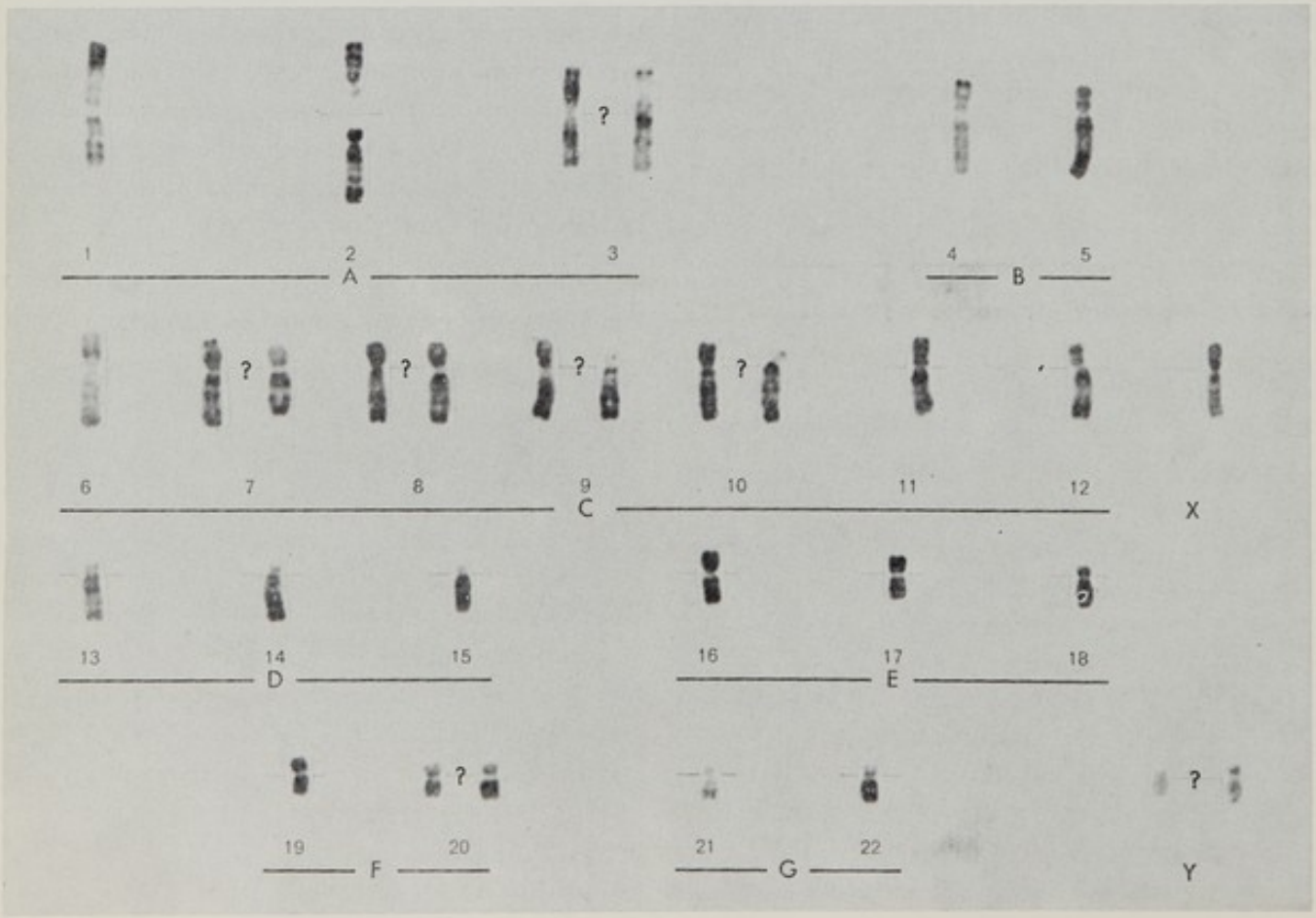


Fig. 10. The karyotype of the common ancestor to man, chimpanzee, gorilla and orangutang. The presence of two chromosomes separated by a question mark indicates an ambiguity which cannot be solved in the present state of knowledge.

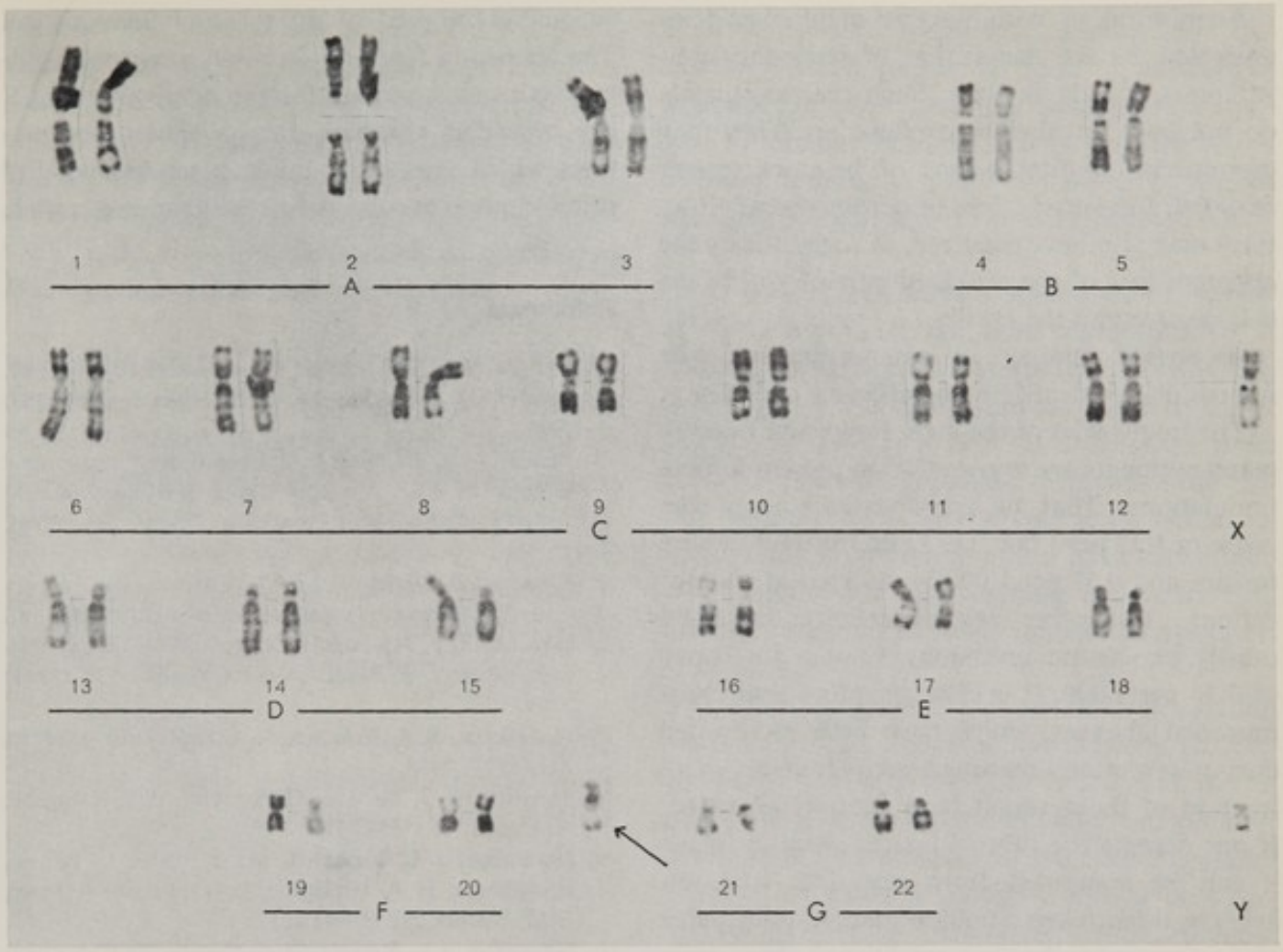


Fig. 11. Karyotype showing a supernumerary marker chromosome (probably a 21) in a male gorilla.

Chromosome Y. Apparently smaller in the chimpanzee than in the three other species, the Y chromosome may have lost the distal part of its long arm in the former.

The common ancestor

Fig. 10 shows the proposed karyotype of the common ancestor of man and the three hominoid apes. When two chromosomes are possible candidates, they are shown with a question mark. These ambiguities may be solved in the future by the study of the gibbon.

It is not our purpose, but rather that of the anthropologists, to identify this common ancestor.

The case of the 49,XY,mar+ gorilla

The karyotype of the male gorilla studied by us showed the presence of a supernumerary marker chromosome. This marker is of the size of a G and carries in good metaphases up to three satellites, the medium one being more conspicuous than the two others. The denaturation banding pattern is compatible with trisomy 21 (fig. 11). It must be stressed, however, that the behaviour and physical appearance of the ape were apparently normal and had never worried the highly competent personnel who were taking care of it. The other chromosomes' banding patterns were identical to those observed in the female gorilla.

This is reminiscent of the observation of Jama, the chimpanzee trisomic for possibly chromosome 21. In this instance, however, the ape was considered mentally and physically retarded [14].

Discussion

Interspecific comparisons of the karyotypes of man and the hominoid apes show that the rearrangements which have occurred during evolution in the different phyla include:

One fusion between acrocentrics, probably of the Robertsonian type. It occurred in the human phylum, thus reducing the chromosome number from 48 in the apes to 46 in man, and producing chromosome No. 2.

At least eight pericentric inversions.

Acquisition of chromosomal material as demonstrated by the acquisition of secondary constrictions, mostly in man. Such rearrangements do not have an obvious explanation. They may correspond to duplications of heterochromatic material. Conversely, loss of chromosomal structures may also have occurred, as suggested by the apparent loss of the proximal part of [2q] in the chimpanzee and the gorilla.

A certain number of rearrangements were impossible to identify with sufficient confidence.

The frequencies of the most important of these rearrangements are known for the present human populations. That of translocations is of the order of 1.57 per 1 000, i.e. 1 per 1 000 for centric fusions and 0.57 per 1 000 for reciprocal translocations. The other rearrangements, including mostly pericentric inversions, have a frequency of 0.36 per 1 000. It is clear therefore that chromosomal changes, which have been established during evolution, represent an extremely small fraction of those which have actually occurred. If one accepts the time estimates given in fig. 1, it can be computed from our data that, on average, it has taken 5 million years for one major rearrangement to become established. This value is, by and large, of the same magnitude as that of genic mutations such as in the case of the hemoglobin genes.

When studying evolution from a more general standpoint, it is impossible to consider gene mutations per se as possible interspecific barriers. These barriers can only consist of chromosomal rearrangements which alone can guarantee the isolation of species. This they may do brutally, as in the case of fusions between homologous acrocentrics [12], or progressively as in the case of the rearrangements we have identified, namely fusions between non-homologous acrocentrics or pericentric inversions.

These rearrangements are known to be harmful in the human populations today. The actual degree of harmfulness is, however, difficult to evaluate and may not be extremely high.

One is therefore faced with the paradoxical situation that evolution proceeds through unfavourable chromosomal changes. This paradox may be explained if one admits that these changes are unfavourable only in the heterozygous state, but not in the homozygous state which is when they isolate an individual and his offspring, thus becoming the seed of an eventual new species. The transition from the heterozygous to the homozygous state may in fact be relatively rapid if one considers the high rate of incestuous matings which must have taken place in ancestral primate populations. A few generations suffice.

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Discussion

Comings: We have heard about the similarity of the chromosomes of animals separated by 30 million years. I would like to compare the chromosomes of man to himself. Ohno (Hereditas 59 (1968) 169) has suggested that during the early

evolution of mammals a polyploid event took place. If this were so one might expect to find pairs of pairs in different animals. In the human and Chinese hamster karyotype such sets of pairs can be observed (Comings, D E, Nature 238 (1972) 455), suggesting that there has been relatively little alteration of some banding patterns over a period of 200–300 million years.

Lejeune: I would like your comment on a fact demonstrated by your remarkable analysis. The chromosomes 13, 18 and 21 (human numbers) are more prone to malsegregation in man (at least in viable trisomies) and 21 and 13 are very often involved in translocations. Curiously they seem quite stable in evolution. Could that be related to their structural make-up or to their genic content (regulation inside the chromosome itself)?

Polani: It should be remembered that a small, young chimpanzee was described, trisomic for a small acrocentric autosome, with signs of mongolism [14]. Anecdotally one should also remember Fredga's water vole (Chromosoma 25 (1968) 75).

Pearson: The specific chromosome was not identified and the chimpanzee is dead, I think, following cardiac catheterization.

Östergren: A chromosome that is labile may still remain unchanged in evolution if the products of changes in this chromosome are of especially low selective value.

Hamerton: I would like to refer to Dr de Grouchy's statement that centric fusion was necessarily harmful and also to refer to his last series of slides showing the origins of an inversion homozygote due to incest. (1) There seems some evidence that some t(DqDq) in man do not have any reproductive disadvantage; (2) that when one takes into consideration the types of small family band in which our ancestors may have lived and the absence of the "Incest Barrier" referred to by Darlington which has developed during human evolution, it seems likely that if a centric fusion arose in such a band (and at a 1 : 1 000 frequency this is not unlikely) then it might, especially through incest, become homozygous and hence fixed. If it had a selective advantage it would then spread through the population.

Hamerton: One example of Robertsonian translocations which seemed to have no deleterious reproductive effect were the rearrangements studied by Charles Ford and myself in populations of *Sorex araneus* in which a number of fusions segregate without any obvious deleterious effect.

Ford: Two of the shrew populations mentioned by Professor Hamerton were segregating for 4 different Robertsonian translocations. (Ford, C E & Hamerton, J L, Symp zool soc Lond vol 26, p. 223 (ed R J Berry & H N Southern). Academic Press (1970)). We supposed there was little meiotic irregularity in heterozygotes, for otherwise the associated infertility would have constituted a strong selective disadvantage, but we have no direct information either on disjunctional patterns or fertility.

There is, however, some relevant information from the mouse. First, the spontaneous Robertsonian translocation found by Evans et al. (Cytogenetics 6 (1967) 105) showed only one per cent disjunctional irregularity in male heterozygotes as inferred from a sample of 600 cells examined at second metaphase. More recently, through the kindness of Professor Gropp, we have been able to study both the progeny karyotypes and disjunctional behaviour of male heterozygotes for single metacentric chromosomes. These have been obtained by backcrossing F_1 male hybrids between tobacco mouse and laboratory mouse to laboratory mouse females and in some instances crossing again to the laboratory mouse. The results are, broadly, that the frequency of trisomic zygotes corresponds to the frequency of hyperploid second metaphase counts and that the incidence of disjunctional irregularity ranges from about 10 to about 40% in the four types of heterozygote we have studied. Different Robertsonian chromosomes may therefore have very different levels of associated infertility and presumptively correlated likelihoods of qualifying for incorporation in the course of karyotype evolution. Although not directly relevant to the present discussion, I would also point out that the differences pose an interesting problem in chromosome mechanics.

The Karyotype and Chromosome Map of the Mouse

O. J. Miller

Departments of Human Genetics and Development, and of Obstetrics and Gynecology,
College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032, USA

Our interest in mouse chromosomes began with the realization that the presence of a variable number of unidentified chromosomes in man–mouse somatic hybrid cells made the identification of human chromosomes less accurate than it could otherwise be and thus hampered the use of such hybrids in assigning genes to human chromosomes. We therefore decided to see if normal mouse chromosomes could be identified by their fluorescent banding patterns, and if so, whether one could extend this technique to the identification of the chromosomes of mouse origin in cell lines and somatic hybrids. This seemed a worthwhile undertaking because the mouse karyotype was notoriously difficult to analyze. The mouse has 20 pairs of telocentric chromosomes of rather similar length and only the smallest of these pairs, pair number 19, could be identified by classical methods [4]. Fig. 1 shows the marked improvement in chromosome identification that is possible using quinacrine mustard. Each chromosome can be identified and homologues paired on the basis of banding patterns, although 9 and 13 are sometimes rather difficult to distinguish [8, 11, 21]. Schnedl [25] and Buckland, Evans & Sumner [5] have reported similar findings using Giemsa banding techniques. So the first part of the question has been answered: it is possible to identify normal mouse chromosomes by their banding patterns.

Before turning to the more difficult problem of the identification of mouse chromosomes in cell lines and interspecific hybrids, we asked whether there were not some immediate applications of the ability to identify every mouse chromosome. The most obvious problem, of course, was the one we were studying in man: chromosome mapping. In January 1971, when we began this study, there was no published report of a gene assignment to a specific autosome of the mouse in spite of the wealth of genetic information available.

Genetic Data

The mouse has been particularly useful for mammalian genetic studies because of its small size, ease of handling, short generation time and ability to survive even as highly inbred strains, of which there are several hundred. Large amounts of genetic data have been obtained by analysis of differences in such traits as coat color, behavior, antigenic make-up and biochemical markers, and mutant genes at nearly 500 gene loci are known. Nearly half of these loci have been shown to be genetically linked to one or more other loci, forming a series of linkage groups (LG), whose numbering corresponds to their order of discovery. For example, 11 loci have been mapped on LG II (fig. 2), the outside or distal markers being curly whiskers (*cw*) and ducky (*du*). With sufficient information, each collection of linked genes will correspond to one chromosome. In the mouse there should be 20 linkage groups, one for each autosome, and one for the X. Nineteen linkage groups have been identified, each containing 2–24 mapped gene loci.

A useful tool for examining mouse linkage groups is provided by translocations. A large number of reciprocal translocations have been produced by radiation. In addition, a few naturally occurring translocations of the Robertsonian type have been observed, with the breakpoint in each chromosome at or near the centromere. At least 40 or 50 stocks of mice, each carrying a specific translocation, have been developed and maintained. References to many of

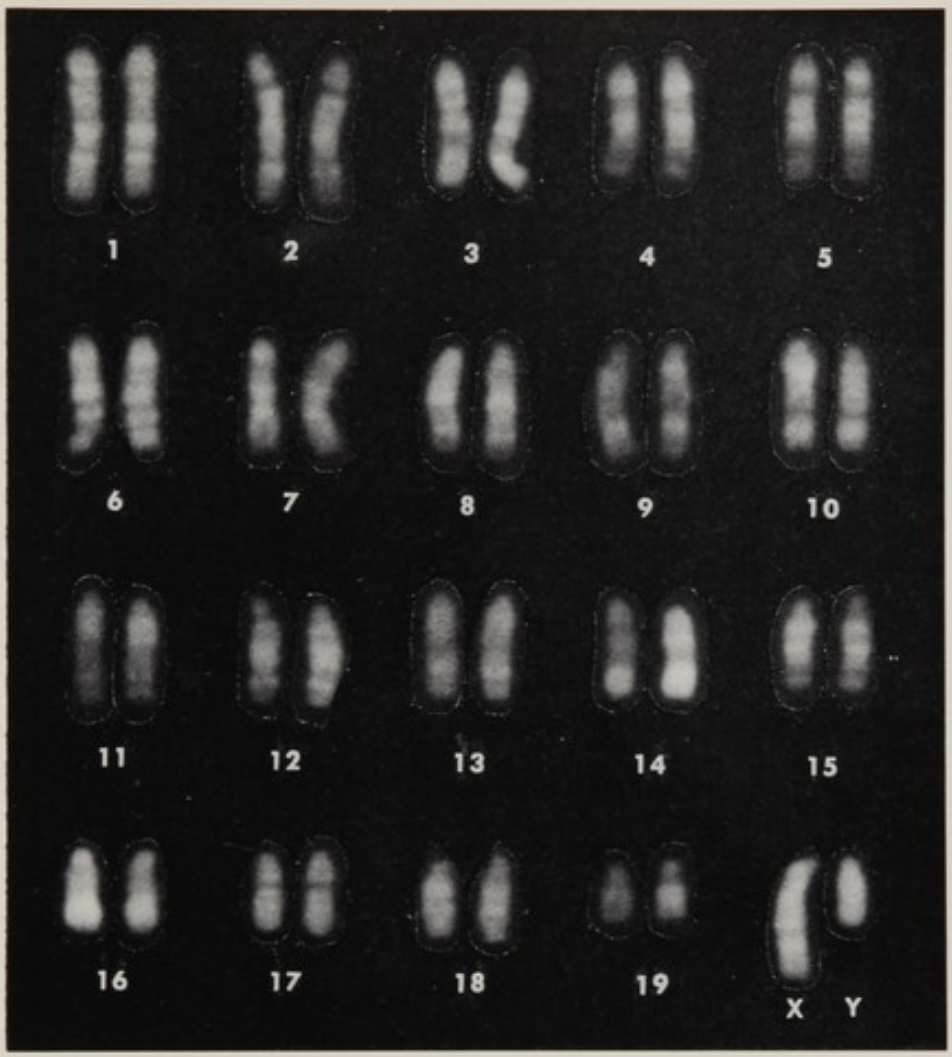


Fig. 1. Quinacrine fluorescent karyotype of a normal mouse cell. Every chromosome pair can be identified. (Committee [7].)

these, including those used in the studies to be described, are given in a recent review [19]. Genetic studies have been carried out to determine the linkage groups involved in many of these translocations and the location of the breakpoint (bp) within each linkage group. The location of the breakpoints of two translocations involving LG II are shown in fig. 2: the *T163H* breakpoint at the centromere, and the *T138Ca* breakpoint between the luxoid (*lu*) and malate dehydrogenase-1 (*Mdh*-1) loci.

Assignment of Linkage Groups to Chromosomes

If it were possible to identify the chromosomes included in the *T163H* and *T138Ca* translocations using one of the new banding techniques, one could assign the common linkage group, LG II, to the common chromosome. Examination of typical karyotypes (figs 3, 4) shows that in each translocation chromosome 9 has been replaced by an abnormal chromosome. Therefore LG II must be located on chromosome 9. In addition we can see from fig. 4 that, provided the two linkage groups which are known to be part of the *T138Ca* translocation, LG II and LG IX, are not on the same chromosome, LG IX must be located on chromosome 17. We tested this by identifying the chromosomes in the *T190Ca* translocation, which also involves LG IX. It too was found to involve chromosome 17, so the assignment of LG IX to chromosome 17 was confirmed. Table 1 summarizes this type of information on five translocations. As it shows, we used the same method to predict that the second linkage group in the *T190Ca* translocation, LG XIII, is on chromosome 1. We confirmed this by showing that the translocation in the *T70H* stock, which also involves LG XIII, involves chromosome 1. But now we run into a problem. The second linkage group carried by *T70H* has not been determined by genetic analysis. The cytological identification of the other chromosome in this

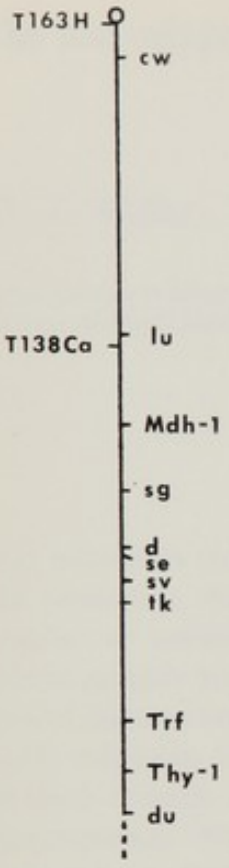


Fig. 2. Linkage group II of the mouse. The symbols and locations of 11 gene loci are listed to the right of the line, while the symbols and locations of the genetic breakpoints of two translocations are to the left of the line. (After C. V. Beechey. Personal communication.)

translocation indicates that either 9 or 13 is involved (our best guess is that it is a 13) but proof is needed.

We have resolved this problem by using a classical genetic method: examination of the meiotic pairing configurations in mice heterozygous for two translocations. If the two translocations in a double heterozygote have one common chromosome, a single translocation figure, a hexavalent, will be present, in addition to 17 bivalents. If the two translocations involve four independent chromosomes, two separate trivalent or quadrivalent translocation figures, plus 16 bivalents should be present. In the case of *T70H*, either it involves chromosome 9 and should form a hexavalent with *T163H*, or it involves chromosome 13 and should form a hexavalent with *T264Ca*. Fig. 5 shows our findings. The *T163H* does not form a hexavalent with either the *T264Ca* (fig. 5*a*) or the *T70H* (fig. 5*b*), but when the *T70H* and the *T264Ca* are both present, a single complex figure is found (fig. 5*c*, *d*) [1, 2]. Several methods for staining the meiotic chromosomes are illustrated. A particularly useful one is the C-banding technique, which allows the positions of the centromeres to be distinguished. The method we used is based on denaturing fixed chromosome preparations with formamide for 8–30 min and then staining with Giemsa; no special period of reassociation is necessary to obtain C-banding in the laboratory mouse [9]. By combining mitotic and meiotic information, the hexavalent can be analysed as shown in fig. 5*e*. Going back to table 1, we can now make use of the information that *T70H* shares a chromosome with *T264Ca* but not with *T163H*. The chromosome in common is number 13, and we can conclude that linkage group XIV is involved in the translocation in the *T70H* stock.

The 14 linkage group assignments we have been able to make to specific chromosomes [1, 13, 18, 20, 21], building on the large amount of pre-existing information about the genetics of the mouse, are shown in table 2. Francke & Nesbitt [12] and Nesbitt & Francke [23, 24] independently confirmed the identification of the chromosomes involved in 4 of the 18 translocations. Table 2 shows the number of translocations involving each chromosome which were analysed with quinacrine fluorescence. With overlapping translocations, and with such a large proportion of all the chromosomes involved in one or more of the translocations, one can feel confident of the validity of these assignments. Further confirmation has come from meiotic studies [6] involving many of the naturally occurring biarmed chromosomes described by Dr Gropp (p. 118). The Committee for Standardized Nomenclature for Mice is con-

Table 1. *Chromosomes and linkage groups involved in five translocations, arranged in an order which illustrates the method of assigning each linkage group to its chromosome*

Chromosome numbers		Translocation	Linkage groups
9	19	*T163H*	II XII
9	17	*T138Ca*	II IX
1	17	*T190Ca*	XIII IX
1	9 or 13	*T70H*	XIII ?
5	13	*T264Ca*	XVII XIV

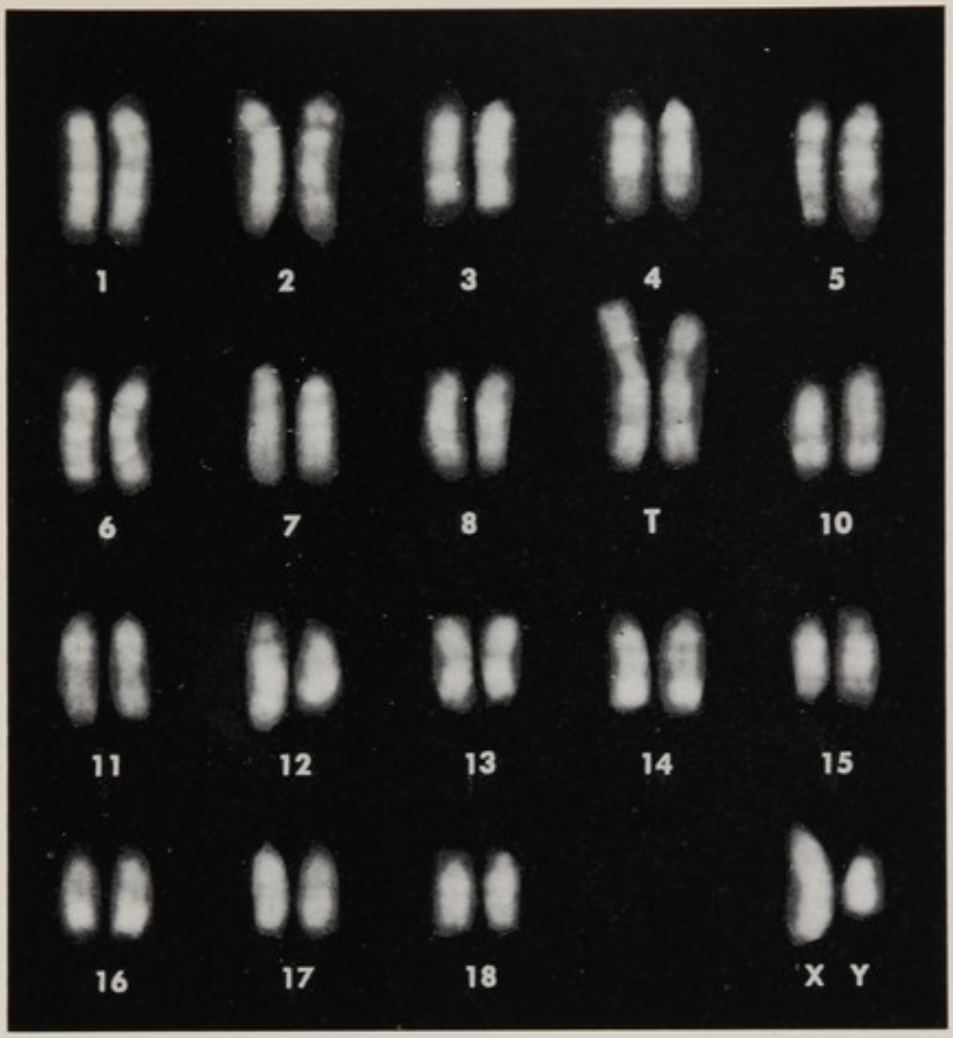


Fig. 3. Quinacrine fluorescent karyotype of a cell from an embryonic mouse homozygous for the *T163H* translocation. (Modified, from Miller et al. [21].)

vinced that the assignments are correct and it has published a standard numbering system [7] and recommended that one no longer refer to linkage groups, but to chromosomes to which the linkage groups have been assigned, where known. They have also recommended that in the designation of each translocation, chromosome numbers should be included rather than linkage group numbers, as before. Table 3 lists the 18 translocations we used in our studies, using the new nomenclature.

I have heard recently that a 15th assignment has been made, that of LG VI to chromosome 15 with the gene locus underwhite (*uw*) at the

Table 2. *A summary of the linkage group assignments in the mouse*

Chromosome	Linkage group	Number of translocations analysed	Chromosome	Linkage group	Number translocations analysed
1	XIII	2	11		
2	V	5	12		
3		1	13	XIV	3
4	VIII	2	14	III	1
5	XVII	1	15		2
6	XI	2	16		1
7	I	3	17	IX	2
8	XVIII	2	18		2
9	II	2	19	XII	2
10	X	2	X	XX	1

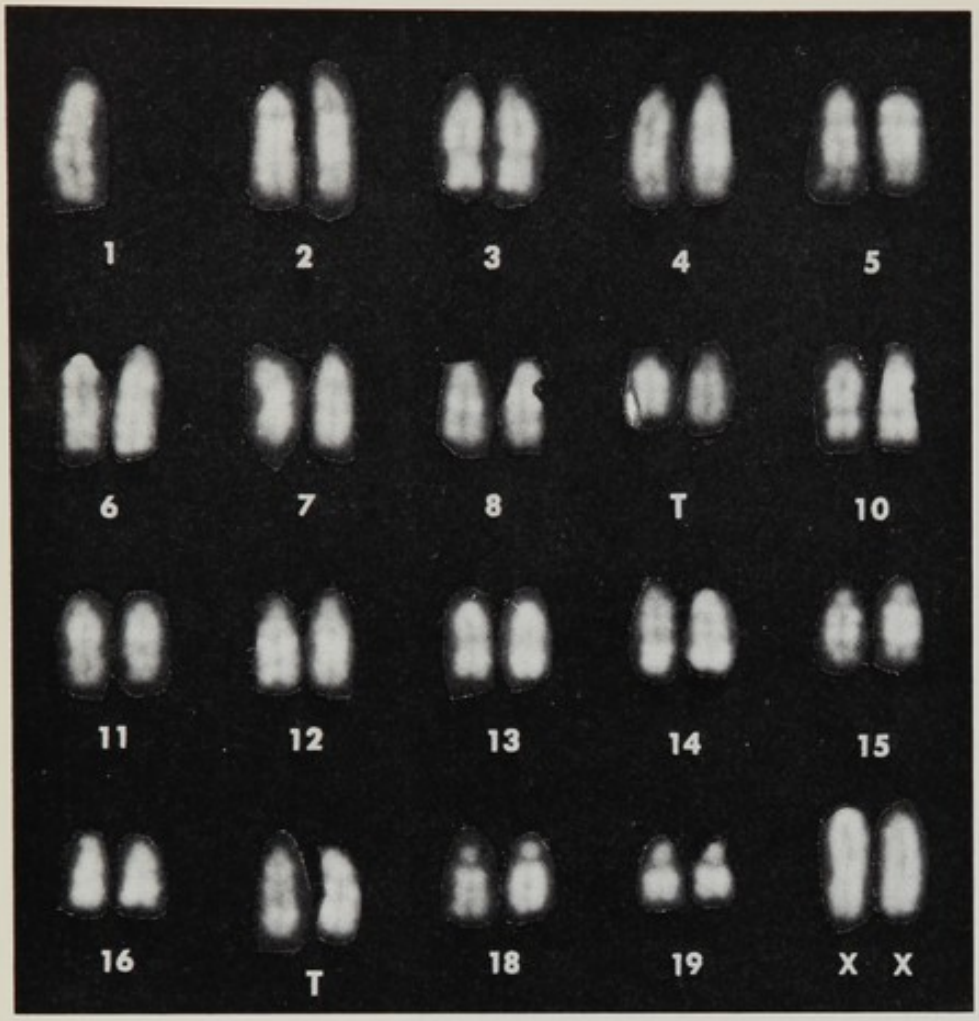


Fig. 4. Quinacrine fluorescent karyotype of a cell from an embryonic mouse homozygous for the *T138Ca* translocation. (Modified, from Miller et al. [22].)

centromeric end (M C Green & E M Eicher. Personal communication). This leaves only four of the known linkage groups unassigned, and at least two of these will probably be assigned within the year, taking advantage of the availability of translocations which carry these linkage groups. Although we did not find any translocations involving either chromosomes 11 or 12, Dr Gropp and his associates did (p. 300) so that the solution of the problem of assigning mouse linkage groups to these chromosomes should be a straightforward one utilizing readily available genetic and chromosomal markers. This means that in the future, new translocations can be quickly checked cytologically to see if they are going to be of interest, and that a much smaller number of test crosses need be carried out in order to confirm the identification of the chromosomes involved and to determine the exact locations of the genetic breakpoint.

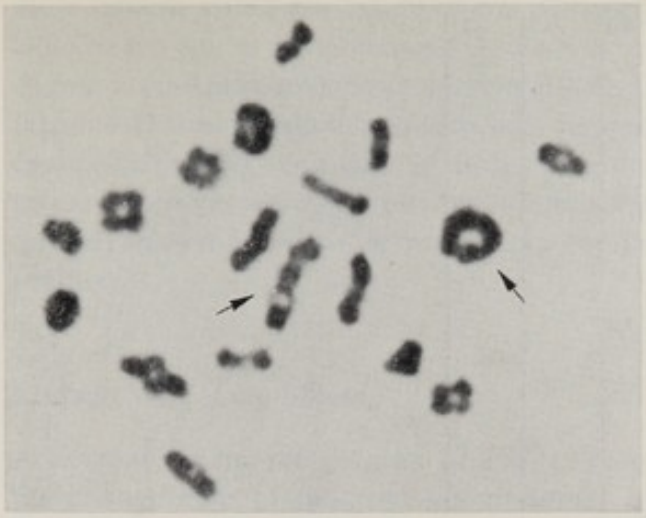
Table 3. *Translocations used in making mouse linkage group assignments*

Chromosome numbers are included in parentheses

T(6;15)1Ald	*T(7;X)Ct*	*T(7;18)50H*
T(14;15)6Ca	*T(2;4)13H*	*T(1;13)70H*
T(2;6)7Ca	*T(8;16)17H*	*T(7;19)145H*
T(9;17)138Ca	*T(10;18)18H*	*T(9;19)163H*
T(1;17)190Ca	*T(2;3)24H*	*T(10;13)199H*
T(5;13)264Ca	*T(2;8)26H*	*T(2;4)Sn*

Localization of Centromeric Ends of Linkage Groups

The fluorescent banding technique will also make it possible to identify the centromeric end of each linkage group after the position of translocation breakpoints has been mapped genetically [18]. Fig. 6 shows two ways of doing this: the one breakpoint method, which is applicable if the breakpoint is near either end of the chromosome, and the two breakpoint method, which correlates the polarity of the breakpoints in the linkage group with that in the chromosome. The latter method requires a bit more work but is much



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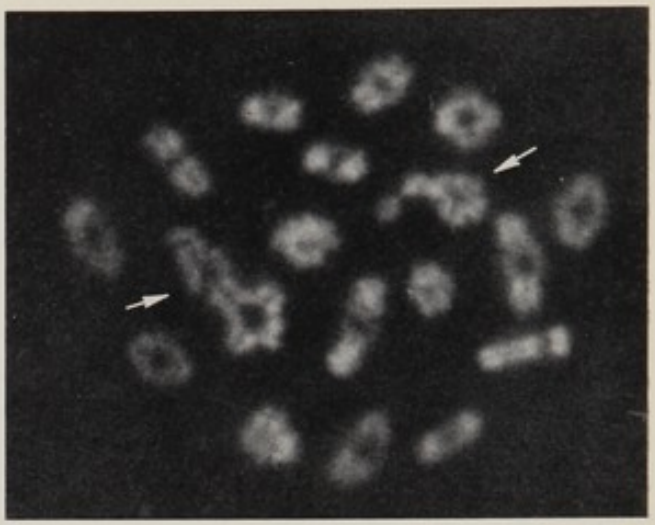

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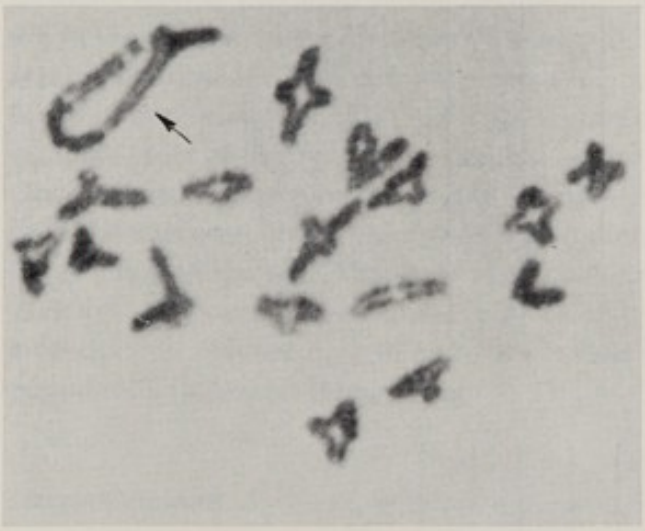

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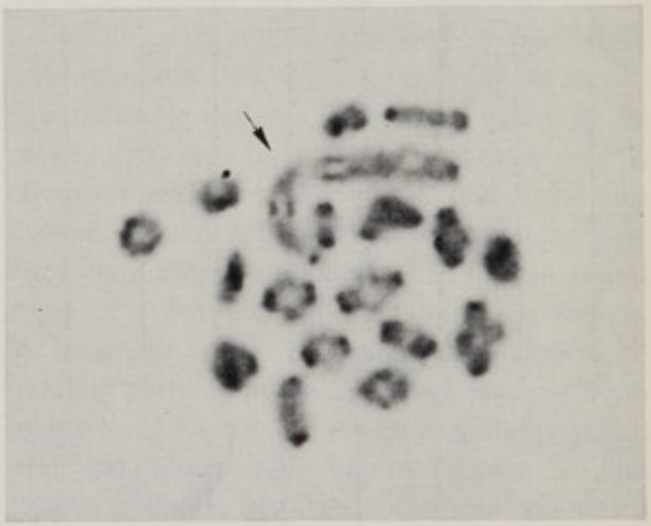

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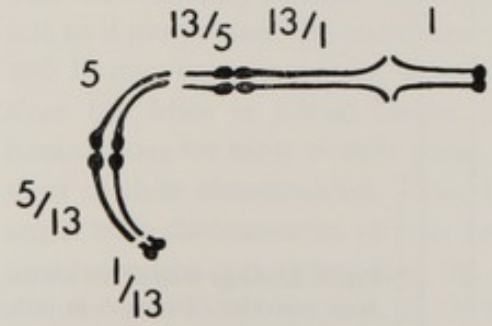

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Fig. 5. Meiotic configurations in mice doubly heterozygous for two translocations: (*a*) from a *T163H* X *T264Ca* male, Giemsa staining, showing separate trivalent and quadrivalent figures plus 16 bivalents; (*b*) from a *T163H* X *T70H* male, quinacrine fluorescence, showing separate trivalent and quadrivalent figures; (*c*) from a *T70H* X *T264Ca* female, toluidine blue staining, showing a single hexavalent ring plus 17 bivalents; (*d*) from a *T70H* X *T264Ca* male, modified C-banding technique, showing a hexavalent chain and 17 bivalents; (*e*) diagrammatic interpretation of the hexavalent shown in (*d*). In the diagram a single number designates an intact chromosome while two numbers, separated by a diagonal line, designate a translocation chromosome whose centromere is derived from the chromosome represented by the number to the left of the diagonal; chromosome 13 is involved in both translocations. (Modified, from Allderdice et al. [1].)

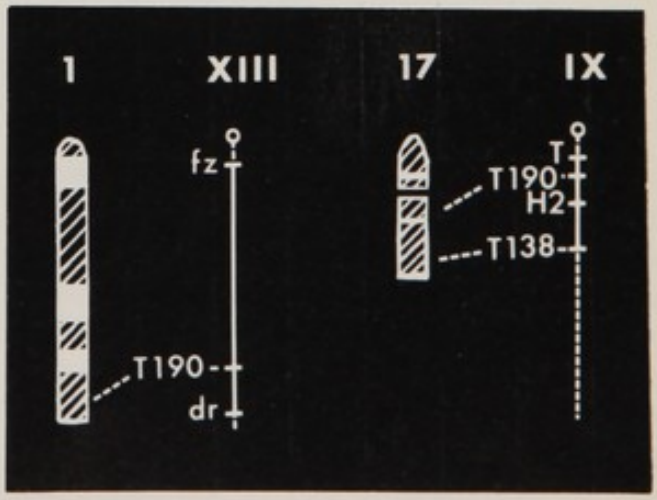


Fig. 6. Diagrams illustrating two methods for determining the centromeric end of a linkage group. On the left, the one breakpoint method; on the right, the two breakpoint method. (Modified, from Miller et al. [18].)

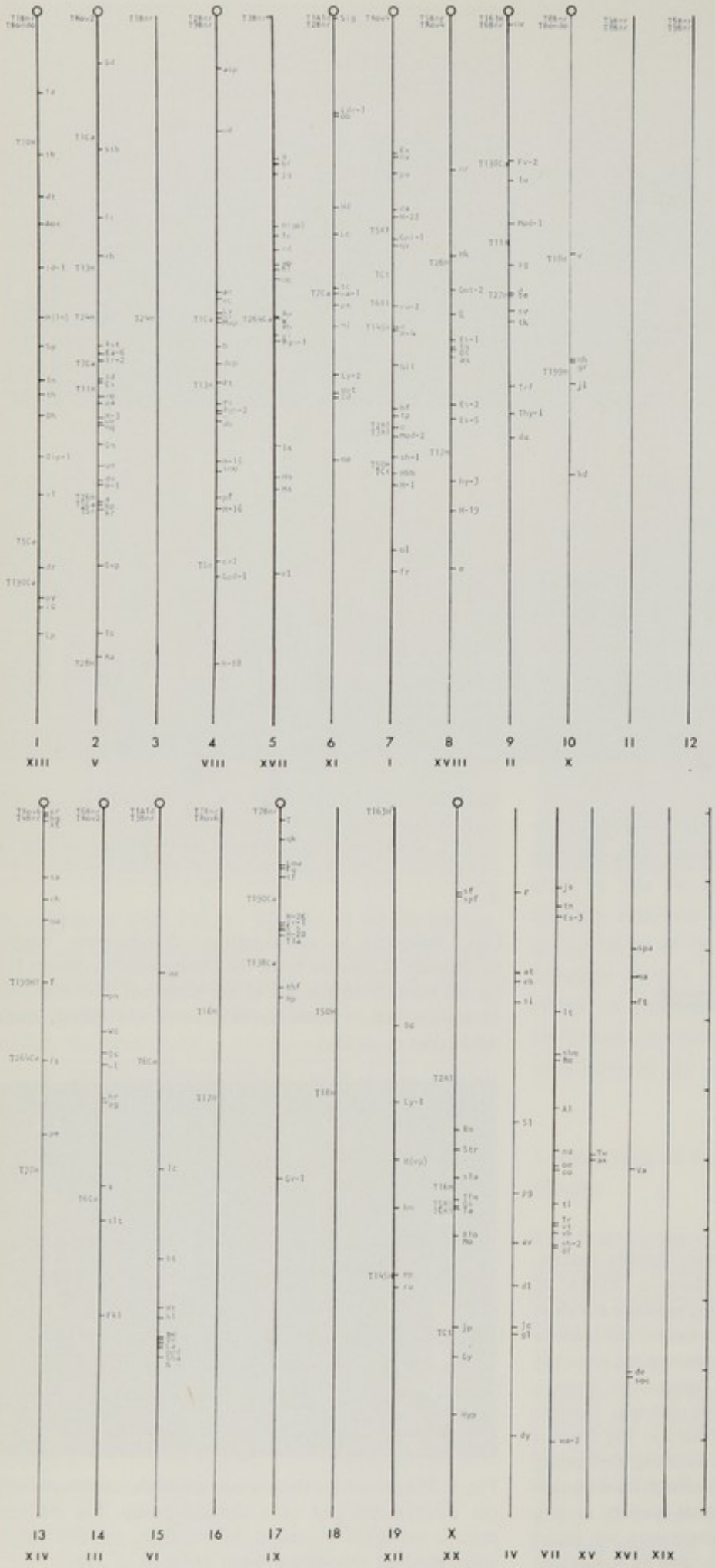


Fig. 7. Linkage map of the mouse, *Mus musculus*, arranged in order of chromosome number, i.e. decreasing length of chromosome. Gene symbols and locations are shown on the right of each chromosome and translocation symbols and their approximate locations on the left. The centromeric end of each linkage group, where known, is indicated by a circle. The polarity of the linkage groups assigned to chromosomes 5 and 19 is uncertain. The five unassigned linkage groups are shown to the right of the 20 chromosomes. (Prepared by D A Miller, using genetic information provided by M C Green & C V Beechey.)

more reliable unless the breakpoint is extremely close to the end of the chromosome—as it is, of course, in the Robertsonian translocations. With 32 reciprocal translocation breakpoints now mapped cytologically [19], and centric fusion translocations known involving all but one chromosome (p. 118) this problem, too, is nearing its complete solution.

Linkage Map of the Mouse

A summary of the present state of knowledge of the linkage map of the mouse is presented in fig. 7. This differs from the maps which were formerly used in that most of the linkage groups are now assigned to specific chromosomes, and arranged in order of chromosome number, i.e. in order of decreasing chromosome length, instead of in the order of discovery of the linkage groups. Gene loci are indicated by symbols to the right of each chromosome, and translocation breakpoints by symbols to the left. The centromeric end of each linkage group, where known, is indicated by a small circle. Mouse linkage maps are updated periodically in Mouse News Letter.

Normal Variants

The quinacrine banding technique has made it possible to identify normal variants in the mouse, just as it has in man: chromosomes 12, 15, 16, 18 and 19 may have prominent secondary constrictions [8]. Mice in inbred strains are not only homozygous for most of their genes, but also for most of their chromosomes. Thus, in an inbred strain both chromosomes 18 may have a prominent secondary constriction (e.g., fig. 4) or neither chromosome 18 has one (e.g., fig. 3). Strains differ in this trait, and if you prepare F1 hybrids between mice of two strains, one with and one without prominent secondary constrictions on a given chromosome pair, the F1 hybrids have one homologue of each type. Since these variants are heritable, one can use them as chromosome markers in genetic studies, much as translocations have been used in the past. Such normal variants should be even more useful as markers than translocations because they are not associated with the reduced fertility seen in translocation heterozygotes.

Evolution

We have recently used the quinacrine fluorescent technique to investigate an evolutionary problem [10]. Dr T. C. Hsu made available to us a wild mouse from Thailand, called *Mus cervicolor*. Markvong and coworkers [15] had shown that this mouse has 20 pairs of telocentric chromosomes, like *musculus*. The quinacrine fluorescent pattern of the chromosomes in *M. cervicolor* shows one striking difference from those of *M. musculus*; there is a spot of bright fluorescence at each centromere, with an adjacent dull zone (fig. 8), while the corresponding region in *musculus* has a much more even fluorescence of moderate intensity. Except for these changes in the C-banding region, the banded karyotype of *cervicolor* resembles that of *musculus*. After the regular C-banding procedure, the stained areas in the centromeric region are of comparable size in *cervicolor* and *musculus*, and if one stains with Giemsa immediately after denaturation, without allowing prolonged reassociation, the C-banding areas in *cervicolor* are still the same as those in *musculus*. The changes in fluorescent Q-banding patterns probably reflect changes in repetitious DNA in the heterochromatin region near the centromere, i.e. the mouse satellite DNA, without detectable changes elsewhere. Sutton & McCallum [26] found limited homology between the satellite DNA of these two species, based on molecular hybridization. They have also shown that the satellite DNA of *cervicolor* is heterogeneous, which fits with the presence of both bright and dull fluorescent bands in the C-banded region.

Analysis of a Histocompatibility Loss Variant

Let me now describe briefly how the assignment of linkage groups to chromosomes in the mouse can be applied to a problem of some interest to immunogeneticists: the cause of the loss of the *H-2* histocompatibility antigen derived from one parent when tumors arising in F1 hybrid mice are successfully transplanted back into mice of the other parental strain which carries a different *H-2* antigen (fig. 9). One explanation of the loss of the $H\text{-}2^a$ antigen is that an entire chromosome is lost, or else a large segment of it is deleted. Dr Penny Allderdice and I have investigated this

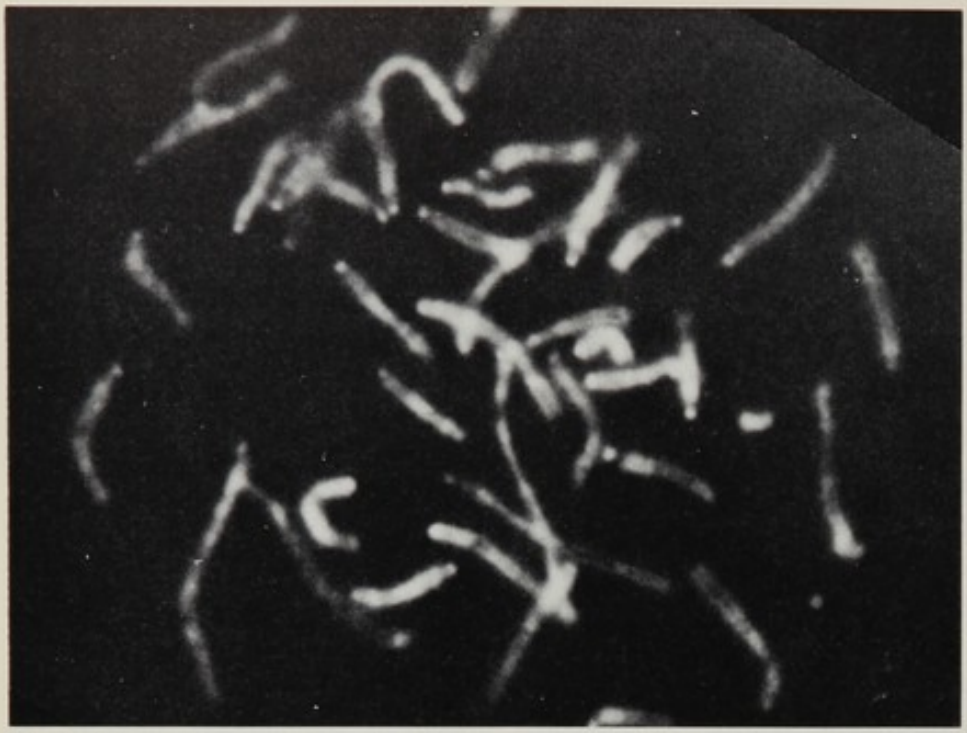


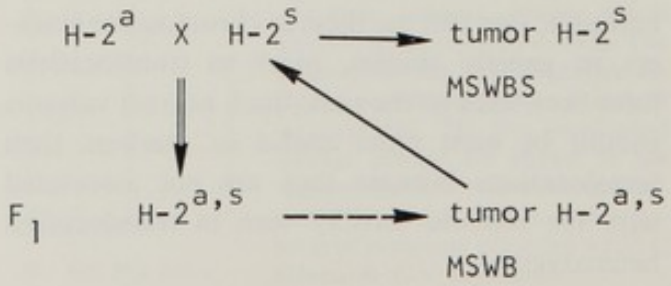
Fig. 8. *Mus cervicolor* prometaphase chromosome spread, by quinacrine fluorescence. Note the bright spot of fluorescence separated by an area of dull fluorescence at the centromeric end of each chromosome except the Y. (Prepared by V. G. Dev.)

problem in collaboration with Drs George Klein and Francis Wiener, using cells of one particular loss variant, called MSWBS, for which this model fits. Professor Levan and his associates [14] have characterized this line by classical methods, and found it to have a fairly sharp modal number of chromosomes with a fairly constant number of biarmed chromosomes. A difference in the distribution of arm lengths in comparison to that seen in the normal mouse karyotype indicated the presence of "cryptostructural" changes in many chromosomes. This is borne out by fluorescent banding studies. Fig. 10 shows the karyotype of one cell from the MSWBS line. Every chromosome can be identified by its distinctive quinacrine fluorescent banding pattern, although the origin of many of the chromosomes or chromosome arms is uncertain. Chromosome 17, which carries the *H-2* locus, is readily recognized, and this cell, like most of the others examined, contains two copies of this chromosome, each involved in what looks like a Robertsonian translocation with a longer chromosome, one a number 1, the other a different but unidentified chromosome of similar length. If both of these translocation chromosomes were also present in the original tumor growing in F1 mice and expressing both *H-2* antigens, we would be able to conclude that the lack of expression of the $H\text{-}2^a$ allele in MSWBS cells is not due to loss of an entire chromosome 17 or to a detectable deletion of part of the chromosome. Even without the information from the MSWB parental tumor, this conclusion seems very likely.

Heteroploid and Interspecific Hybrid Cell Lines

Let me tell you a bit more about this MSWBS tumor line. This particular cell (fig. 10) has 29 chromosomes, only two of which are the same. Other cells in the line have about the same total number of chromosomes, but the karyotype of

Fig. 9. Diagram illustrating the co-dominance of mouse histocompatibility alleles at the *H–2* locus, with both alleles expressed in MSWB tumors arising in F1 hybrids between strains carrying different *H–2* alleles, and the absence of expression of the $H\text{–}2^a$ allele in MSWBS tumors which were derived from the MSWB line by transplantation into mice of the $H\text{–}2^s$ parental strain.



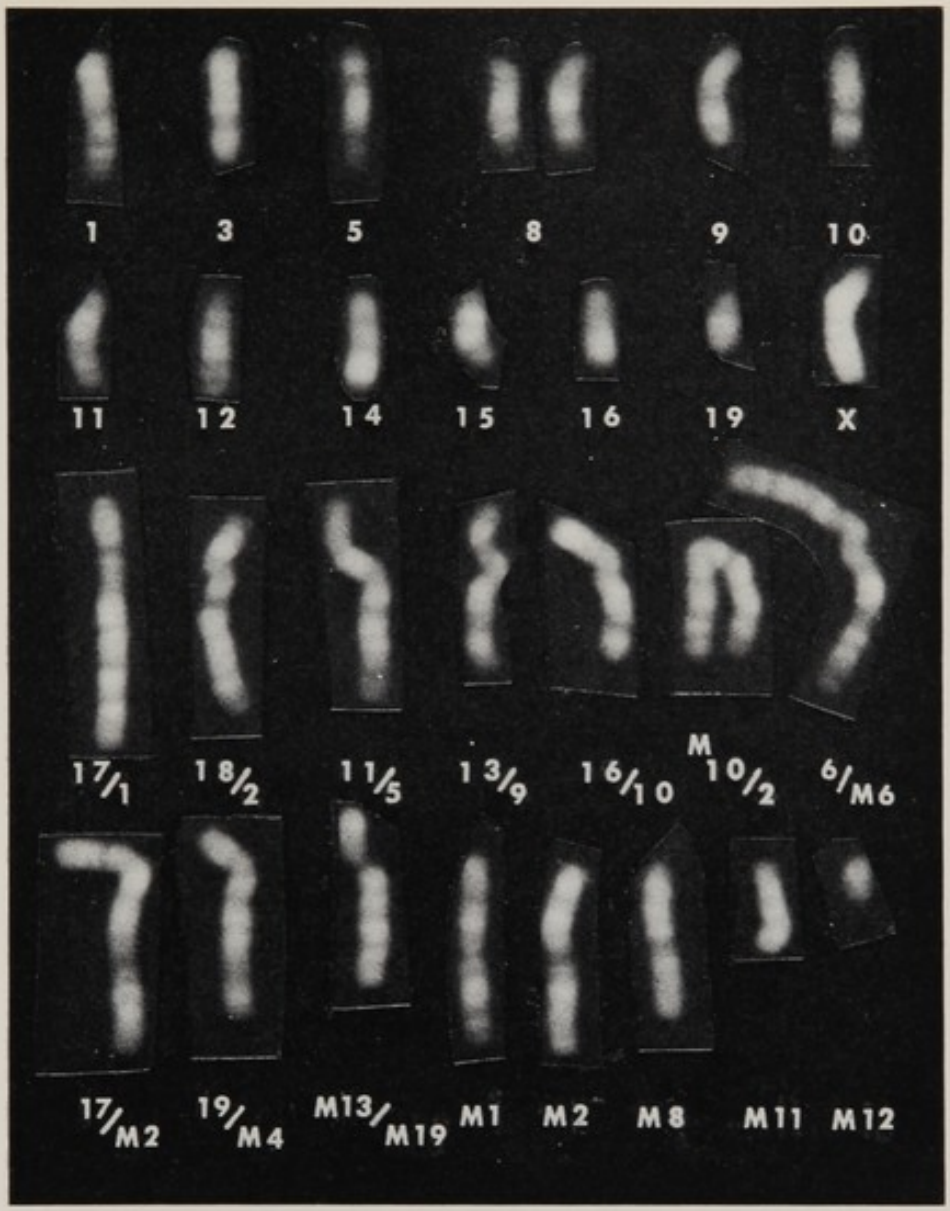


Fig. 10. Quinacrine fluorescent karyotype of a cell from the MSWBS tumor line. (Prepared by P. W. Allderdice.)

each cell is different. My associate, Dr Allderdice, has observed nearly 70 different chromosomes in a sample of only 20 cells from this line, indicating the presence of an enormous amount of unsuspected heterogeneity of the type we have found in established lines from several species: human [20], rat [17] and mouse [2, 3]. This kind of heterogeneity is seen in many of the mouse cell lines used for producing man–mouse hybrids, and that brings me back to the question I posed at the beginning: can one identify, by their banding patterns, the chromosomes of mouse origin in man–mouse hybrid cells? The answer, very briefly, is yes.

A single example will suffice to illustrate what can be done by analysis of quinacrine fluorescent banding patterns. Fig. 11 shows the karyotype of a man–mouse hybrid cell that contains six human chromosomes. All the other chromosomes in this cell have been identified as of probable mouse origin. Some of these exist as normal mouse chromosomes, some as telocentric markers of unknown origin, others as biarmed translocation chromosomes of known origin and the remainder as biarmed marker chromosomes. The mouse origin of these chromosomes has been confirmed by the modified C-banding technique referred to earlier (fig. 12). Human chromosomes show minimal or no C-banding by this modified technique, which makes it useful in checking quickly on the number of human chromosomes in man–mouse hybrids. However, the Q-banding technique provides far more information, which can be used to identify individual human and mouse

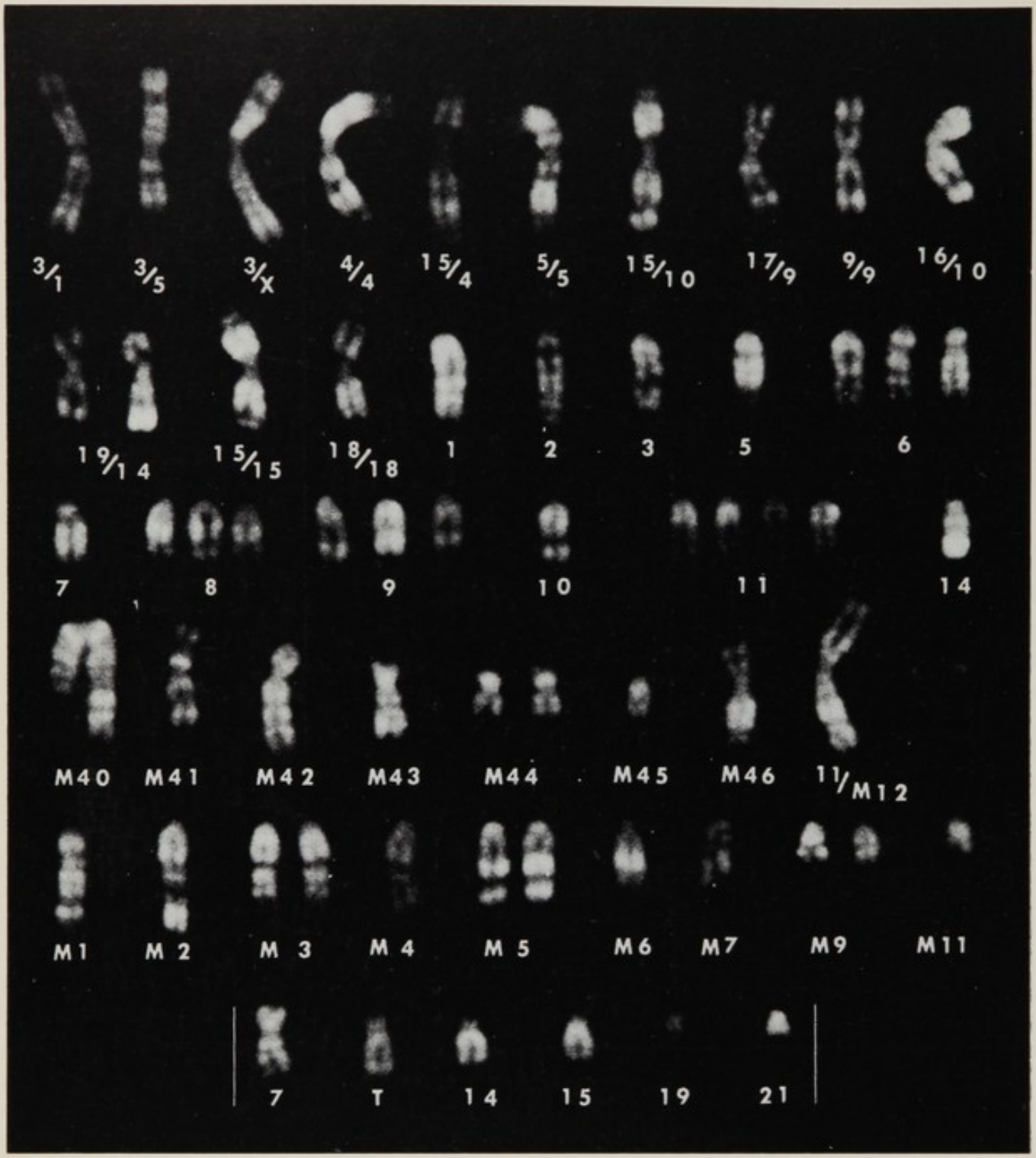


Fig. 11. Quinacrine fluorescent karyotype of a man–mouse hybrid cell from the A9/Daudi line. The six chromosomes of human origin are shown on the bottom line. (Allderdice et al. [3].)

chromosomes and to recognize structural variants. This can be very useful in analyzing man–mouse hybrid cells (e.g., see [3]).

In conclusion, I would like to emphasize the remarkably increased power of resolution of difficult problems in chromosome identification made possible by the advent of the new banding techniques. Many old problems in genetics are now being quickly resolved, and not surprisingly, new problems are coming to light which may engage our attention for quite some time to come.

The work reported here was supported in part by grants from the National Foundation–March of Dimes, the Roche Institute, the National Cancer Institute (CA 12504) and the National Institute of General Medical Sciences (GM 18153). O. J. M. is a Career Scientist of the Health Research Council of the City of New York.

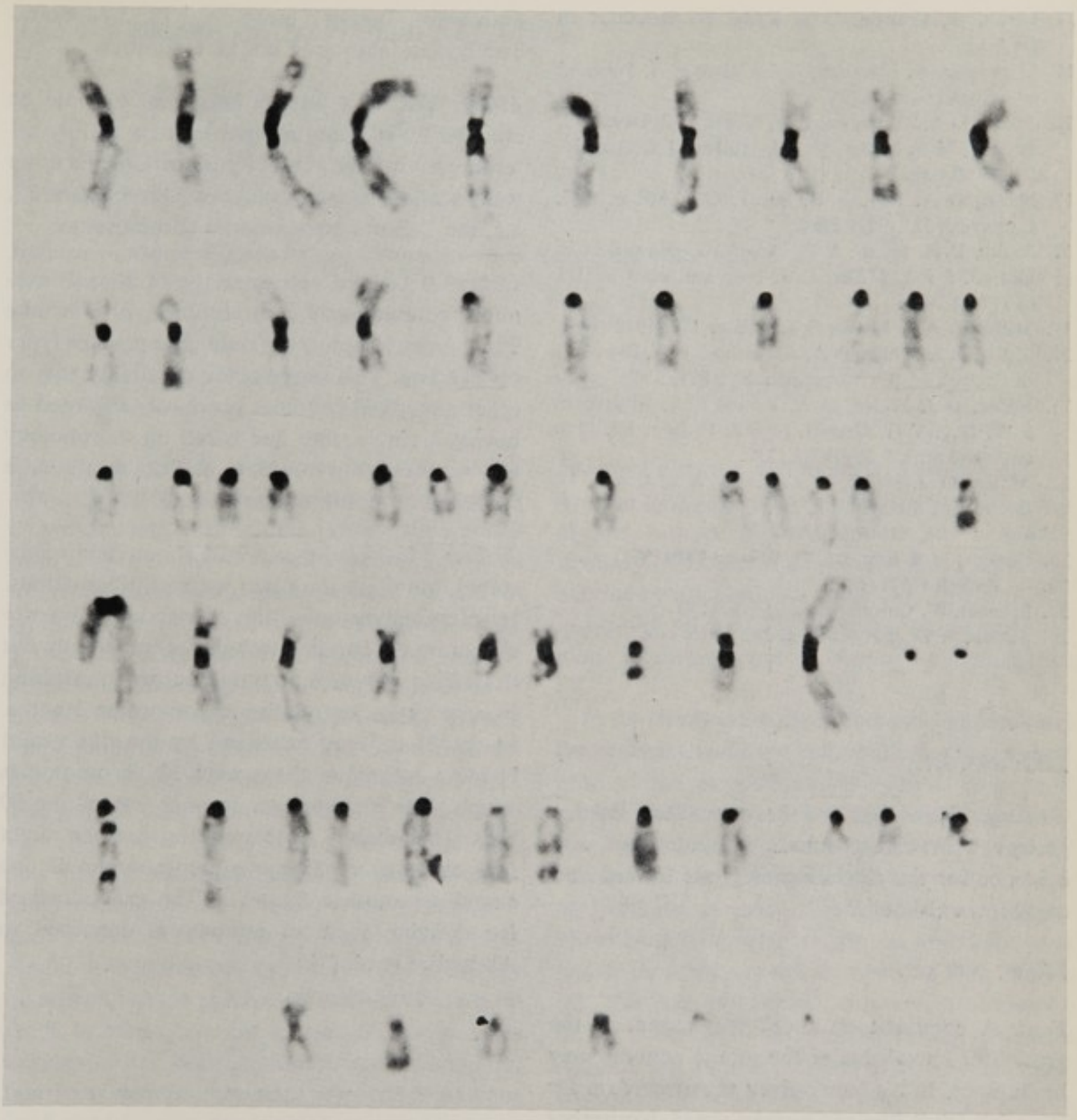


Fig. 12. Modified C-banding of the cell shown in fig. 11. Only the human chromosomes (bottom row) show no C-banding. (Allderdice et al. [3].)

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Discussion

Comings: Now that you have matched linkage groups with chromosomes do you have any evidence for the clustering of genes toward and away from G-bands?

Miller: Not yet.

Ford: A comment on a different aspect of the growth of knowledge of the mouse genome may be in place. In his paper given at Amsterdam Dr Renwick (Bull Eur soc human genet. November issue (1972) 108.) referred to the new linkage map of the mouse prepared by Dr Margaret Green of Bar Harbor (Ann rep Jackson lab (1972)). The total autosomal length covered by the map is 1020 centimorgans. By allowing for the two autosomes with zero map length and for the unmapped proximal and terminal segments in the remainder, she arrived at an estimated total genome length of approx. 13 Morgans. Chiasma counts I have made over the years in spermatocytes and more recently in oocytes give means of approx. 25 and approx. 30, respectively, corresponding to 12.5 and 15 Morgans. Recombination is in general more frequent in females and the map is based on data from both sexes. Taking a mean gives 13.75 Morgans. The concordance may not be fortuitous.

Miller: Yes, it is just an inference. It could be checked by studying meiosis in mice doubly heterozygous for the TTOH translocation, and other translocations shown to involve chromosome 13, e.g. the T4Bnr tobacco mouse chromosome.

Ockey: If I heard you correctly, of 20 cells examined containing 29 chromosomes—give or take 1 or 2—you found 80 different chromosome types on banding. This suggests the possibility that in other aneuploid cell lines previously regarded as having a single stem line based on morphology and number, they would, in reality, have a large variation of multiple stem lines.

Miller: Most of the cells had about 29 chromosomes, but there is a small amount of variations. By classical methods, the tremendous diversity shown by the banding techniques was simply not detectable, although one could see some variation in arm ratios or relative chromosome lengths. In the 20 cells we examined by the fluorescent banding technique there were 38 chromosomes which were each present in only one of the 20 cells. This enables us to estimate that one might find as many as 38 more chromosomes if one examined another 20 cells. (The exact method for deriving such an estimate is described in Allderdice et al. [2].)

The Uniqueness of the Human Karyotype

P. Pearson

Instituut voor Anthropogenetica der Rijksuniversiteit te Leiden, The Netherlands

The introduction of techniques in the late 1950's and early 1960's which permitted the preparation and examination of chromosome preparations from a wide range of animal species quickly led to comparisons being made between the karyotypes of man and his primate cousins [1, 2, 3]. These established that the chromosome number in the chimpanzee, gorilla and orangutan was 48 and not 46 as in man and, on the basis of size and shape parameters, compared individual chromosomes to their supposed human counterparts. The general conclusion was that the chimpanzee karyotype most closely resembled that of man and that the orangutan, although possessing the same chromosome number as the chimpanzee and gorilla, had a very different karyotype. Despite the limitations of analysis based entirely on size and shape parameters attempts have been made to assess how the human 46 chromosome karyotype has been derived from an ancestral line with 48 chromosomes [4] and deductions made which went far beyond reasonable levels permitted by morphological criteria alone.

The discovery that the human Y chromosome had an intensely fluorescent region [5] and that characteristic banding patterns produced either by quinacrine fluorescence [6] or Giemsa staining [7] permitted the unequivocable identification of individual human chromosomes now enables the fine karyotypic features of man and the hominoid apes to be reevaluated on a firmer basis than previously. The present text is concerned with identifying those features of the human karyotype which appear to be unique and those which have apparently remained invariate over a long period of time in the evolution of the hominoid apes.

Intensely Fluorescent Chromatin Distribution

In man the chromatin comprising the distal end of the human Y chromosome has a unique intensity of fluorescence when stained with quinacrine hydrochloride or mustard. This property is variably shared by restricted paracentric regions in chromosomes 3, 4 and the acrocentrics [7].

In the chimpanzee the Y chromosome does not fluoresce intensely but the satellites and/or short arms of the acrocentrics do (fig. 1). In a small sample of chimpanzees of the species *Pan troglodytes* (10 animals studied to date) between 5 and 7 acrocentric chromosomes had intensely fluorescent regions. It seems that the frequency of apparently polymorphic regions is stabilised at a higher frequency in the chimpanzee than in man [8]. The lack of intensely fluorescent chromatin on the Y chromosome is not surprising in view of its small size and the fact that in man, where the Y chromosome is variably larger, all the variation can be accounted for by changes in the length of the intensely fluorescent portion [9]. In *Pan paniscus* which was reported to differ from *Pan troglodytes* by having a larger Y and a pair of small acrocentrics replaced by a pair of small metacentrics [1], the Y does not fluoresce either, but the extra metacentrics appear to be formed by the addition of intensely fluorescent short arms to the pair of chimpanzee acrocentrics, which are homologous in banding criteria to the human chromosome 22.

In the lowland gorilla (*Gorilla gorilla*) the Y chromosome is large and has an intensely fluorescent region similar to that of the human (fig. 2). In addition intensely fluorescent paracentric bands are present on acrocentric chromosomes

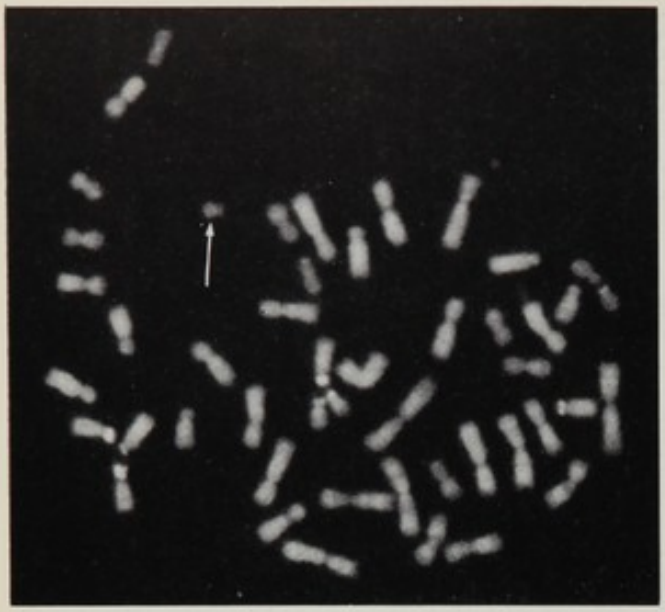


Fig. 1. Fluorescent mitosis of chimpanzee with intense fluorescent short arms on the acrocentrics and a Y without intense fluorescence (*arrowed*).

and also on one pair of submetacentric chromosomes. This latter band can exhibit extreme size differences between homologues (fig. 2).

Despite the presence of a large Y chromosome in the orangutan (*Pongo pygmaeus*) intensely fluorescent chromatin is not found in that species or elsewhere in the primate and mammalian kingdom indicating that it must have had a relatively recent origin, that man, chimpanzee, and gorilla form a natural group and that they have had a recent common ancestor [10].

The nature of the intensely fluorescent chromatin is obscure. Its renaturation kinetics suggest that it consists of repetitive nucleotide sequences and the failure to extract out a light DNA satellite from human chromatin [11] corresponding to it makes the proposal, that it is made up of adenine-thymine-rich sequences, unlikely [12]. Functionally, it seems to be associated with the nucleolus in the interphase nucleus [13] and the suggestion has been made that such heterochromatic chromatin serves the purpose of a genetic buffer by isolating and preventing crossing over between regions known to be ultra-conservative in evolution such as the 5S RNA cistrons and other parts of the chromosome [14].

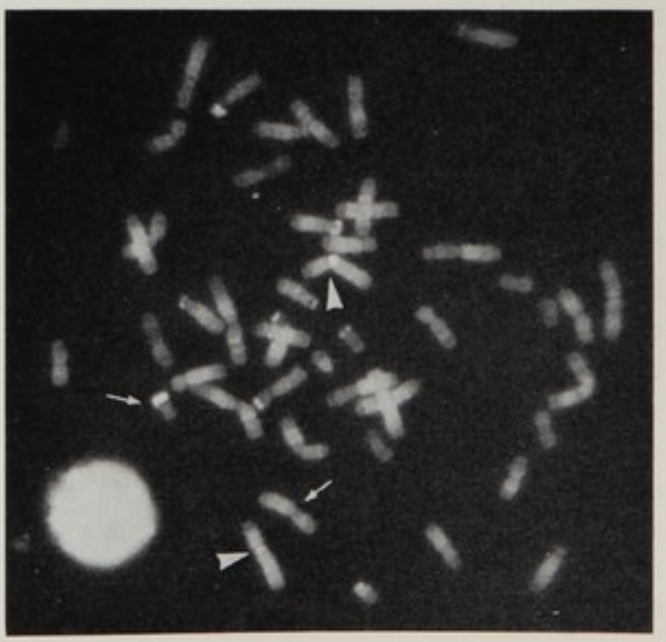
Whilst the purpose of the chromatin is still in the realms of speculation its unique character, both as an indicator of human and hominoid chromatin and direction of evolution, cannot be doubted.

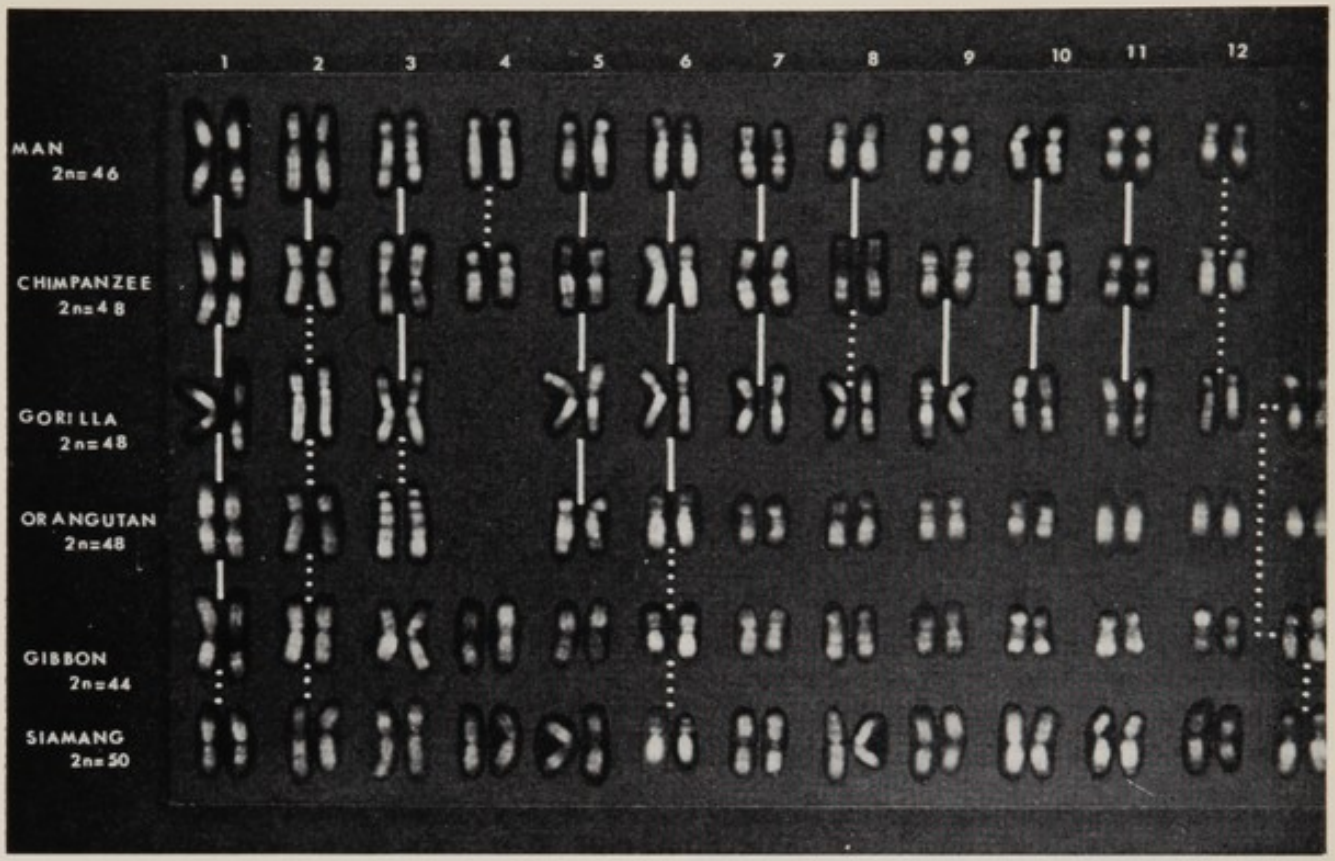
Secondary Constrictions

In fig. 3 is shown a comparative fluorescent karyotype of the hominoid apes. White lines have been drawn connecting up those chromosomes believed to have some evidence of chromosome homology on the basis of banding patterns. The human chromosomes 1, 9 and 16 have an obvious paracentric secondary constriction in their long arms which manifests as an extended quinacrine negative region. For chromosomes 1 and 16 similar regions are lacking in the great apes and for chromosome 9 the identity of the entire chromosome is under some doubt. It can be concluded that the chromatin comprising the No 1 and 16 secondary constrictions has either had a unique development in man or has increased in quantity above that found in comparable positions in the karyotypes of the great apes. This is verified by C-banding techniques which fail to demonstrate comparable blocks of repetitive chromatin in the ape chromosomes to those found in the human 1 and 16's.

The discovery that the secondary constriction region of the human chromosome 9 had a unique differential staining behaviour with alkaline Giemsa [15] prompted a search for comparable chromatin in the karyotypes of the great apes.

Fig. 2. Fluorescent mitosis of gorilla with an intensely fluorescent Y and clear X chromosome (*small arrows*), intensely fluorescent short arms on the acrocentric and a pair of sub median chromosomes with an intensely fluorescent band exhibiting size polymorphism (*large arrows*).





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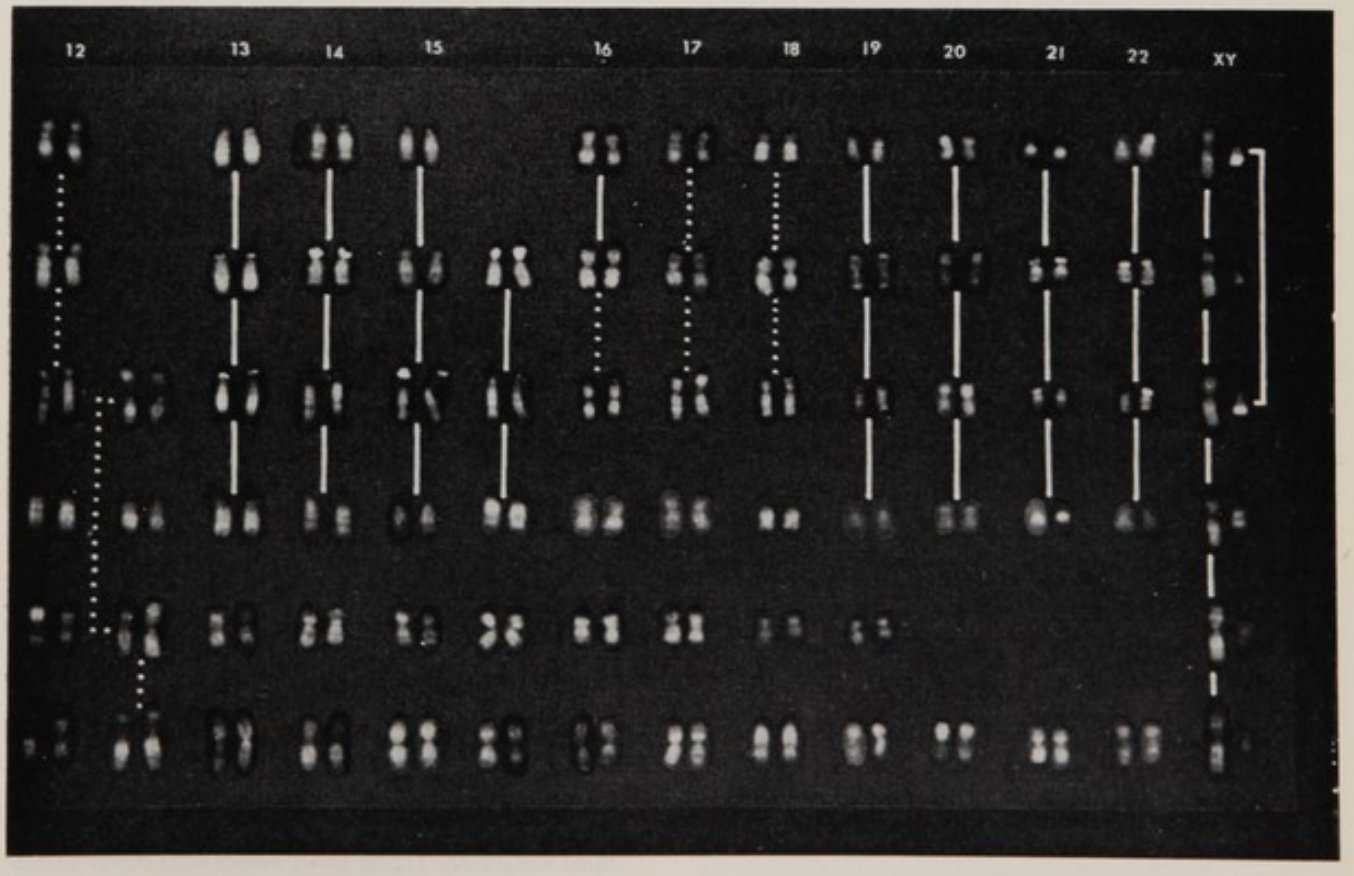


b

Fig. 3. *a*, *b*, Karyotype of the hominoid apes. White lines have been drawn between those chromosomes which show some evidence of banding homology and dotted lines between those where the evidence for homology is tentative.

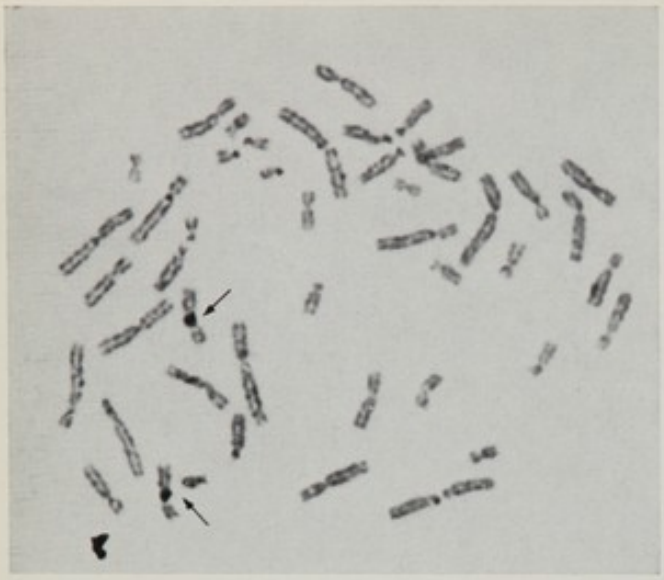
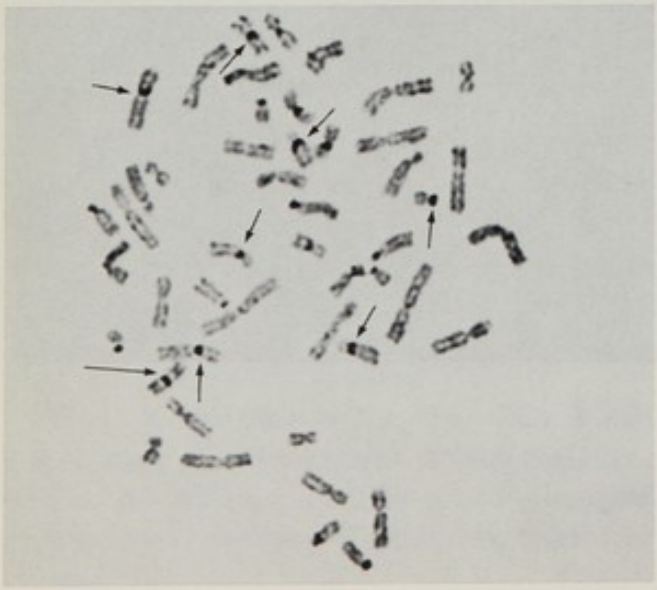


Fig. 4. Human metaphase showing a differential staining of the 9 chromatin (*arrowed*).

Surprisingly, there appears to be much more chromatin of this type in the chimpanzee (fig. 5) than in man but it is more widely distributed around the karyotype. This observation has been verified [16] using the in situ hybridisation technique and demonstrates that the No. 9 chromatin consists of DNA sequences of human satellite 3 [11] which appears to be homologous to chimpanzee satellite A [16].

The pattern of variation in the gorilla is astonishing, since large blocks of chromatin appear to stain up. These correspond in part to the short arms of at least one pair of C-group chromosomes and probably includes the X chromosome (fig. 6).

Fig. 5. Chimpanzee metaphase, showing a wide distribution of the "9" chromatin (*arrowed*).



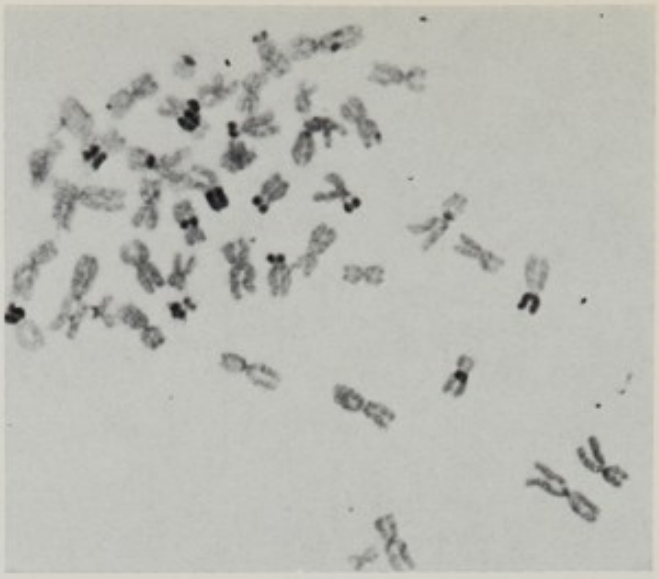


Fig. 6. Gorilla metaphase showing the distribution of the "9" chromatin.

This latter observation made on only one specimen requires to be verified on other animals.

At present then it seems that the secondary constriction chromatin of the human No. 1 and 16 is relatively unique. This has been verified by the inability of human satellite 2 to bind locally to chimpanzee chromosomes with in situ hybridisation (K Jones Personal communication) whilst it does so to chromosomes 1 and 16 in man [17]. On the other hand the chromatin comprising the secondary constriction of the human 9 is distributed widely throughout the karyotypes of both the chimpanzee and gorilla. This observation requires further confirmation in a wider range of specimens of both animals and other members of the hominoidea.

Both Change and Conservatism in the Hominoid Karyotype

Certain facts can be deduced from the overall pattern of variation present in the karyotypes shown in fig. 3.

A, The gibbon and siamang have few autosomal karyotypic features common to other members of the hominoidea and, more surprising, to each other. We may conclude that these genera split off, both from each other and other members of the hominoidea, at a very early stage in the evolution of the taxon.

B, The X chromosome is identical in all members and this fits well with Ohno's theories on the conservation of the X [18].

C, With minor banding variations chromosomes 1, 5, 6, 13, 14, 15, 19, 20, 21, 22 and X are found in man, chimpanzee, gorilla and orang-utan, and chromosomes 3, 7, 8, 10 and 11 in man, chimpanzee and gorilla.

D, For chromosomes 3 and 5 there seems to be a pericentric inversion difference between some species. The fluorescent pattern does not at present permit postulating wide spread pericentric inversion differences between species as suggested by de Grouchy [19] and also in this symposium.

E, Certain human chromosomes appear to have had a more recent origin in that they are present only in man or in both man and the chimpanzee. These include chromosomes 2, 4, 9, 17 and 18.

F, It can be concluded that different chromosomes have evolved at different rates. Some, including the 1,21 and X have remained unchanged for a major portion of the evolutionary time scale of the hominoidea whilst others have apparently evolved rapidly. It seems likely that differential mutation rates would be unable to account for these discrepancies and that the retention of favourable gene linkages on particular chromosomes is the most probable explanation for the conservation of particular chromosomes.

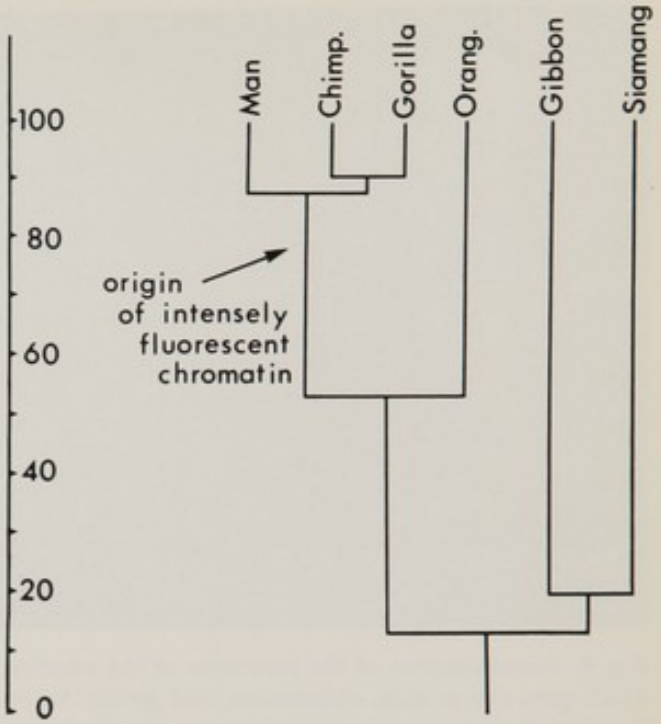


Fig. 7. *Ordinate*: % homology.
A cladogram showing the genetical relationship of the hominoid apes.

Phylogenetical Considerations

It is possible, by calculating the proportion of homologous chromosome bands present in the karyotypes of pairs of species, to construct a comparison matrix for all species combinations. The values which represent the percentage homology between species, will give a crude assessment of the genetical divergence between species. The relationships present in the matrix can be represented in a two dimensional form to give a cladogram of the type shown in fig. 7 by using a simple unweighted cluster analysis technique [20]. From the figure it can be seen that man, chimpanzee and gorilla are closely grouped and that the orangutan has a much lower percentage homology. The gibbon and the siamang appear to have little homology with each other or to other members of the hominoidea. Assuming that the rate of genetical divergence has been constant over time then it is possible to equate the cladogram to a phylogenetical tree. From this it is clear that man, chimpanzee and gorilla had a common ancestor in which intensely fluorescent chromatin arose at some point in the lineage after the branching off of the oran-gutan.

It is interesting that the spatial arrangement shown in the cladogram is very similar to that recently calculated for fibrino-peptide sequence divergence [21] in the hominoidea and follows the general pattern proposed on the basis of other forms of molecular divergence [22]. An absolute time scale cannot be put on the tree owing to lack of basic reference points and attempts to do so have involved making too many assumptions [23].

On the basis that a karyotypic feature present in at least two of the three members in the group formed by man, chimpanzee and gorilla was also present in their common ancestor it is possible to deduce at least part of the karyotype of the ancestor. This is shown in fig. 8 and it seems that the ancestor would have had a karyotype rather similar to that of the chimpanzee but having a fluorescent Y chromosome.

The 48 to 46 Dogma

In the argument given above it is assumed that the common ancestor described had 48 chromo-

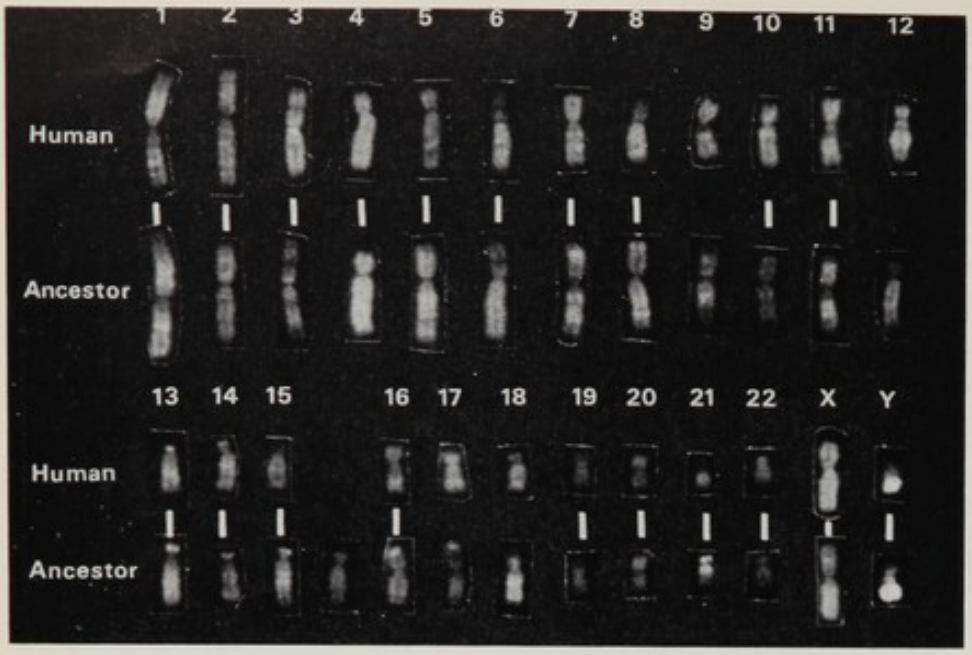


Fig. 8. Reconstruction of the karyotype of the ancestor which gave rise to man, chimpanzee, and gorilla. White lines have been drawn between homologous chromosomes.

somes and therefore ancestral man must have lost two chromosomes. Early proposals (1 and 3) pointed out that this might have occurred by centric fusion of two acrocentric chromosomes. Recently [19] this has been further sponsored by the suggestion that the human two chromosome has originated de novo from centric fusion of two non homologous acrocentrics. Although the idea seems attractive at first sight, there are certain problems involved in that the banding patterns of at least one of the pairs of acrocentrics suggested does not fit the required banding pattern needed to constitute a chromosome two in man.

One of the dangers involved in comparative cytogenetics is of oversimplifying the parameters involved. Thus, in the present context, a reasonable approach is to match up all those chromosomes whose banding patterns show homology between species pairs and then to see to what extent the remainder can be fitted into the pattern. What is not permissible is to make comparisons and create "homologies" where neither the morphology nor banding criteria give firm support for this course of action In the present study several areas of such uncertainty exist, including the precise origin of the 46 chromosome karyotype from an ancestral 48 chromosome line.

New Approaches

The present observations will have to be confirmed in different laboratories using a variety of differential staining techniques and larger samples of animals concerned. In particular, however, the in situ hybridisation technique should be used for localising those DNA sequences complementary to various types of human and primate satellite DNA to establish which bands are homologous. The localisation of the human ribosomal cistrons [24] must be repeated in the hominoid apes and use made of the apparent localisation of the 10 S RNA cistrons to the long arm of chromosome 2 in man [25] to test whether or not the human 2 has been produced from centric fusion of two long acrocentric chromosomes present in the great ape karyotypes.

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Discussion

Gropp: Do you (or Dr K W Jones) think that the human satellite No. 3 (or chimpanzee satellite A) is only more dispersed in the chimpanzee and gorilla—or is there evidence to assume also a higher amount of this satellite in the apes?

Pearson: Both from the Giemsa 11 staining reaction and from the hybridisation data it seems that there is a greater amount of this satellite (approx. 3 times as much) in the chimpanzee karyotype.

Lejeune: Is there an explanation for the apparent stability of 21 and 22—which is surprising regarding the instability of 21 in man?

Pearson: No good explanation is available since other chromosomes are present in the karyotype which have also evolved only slightly and which are never found in trisomic conditions.

Heterochromatin and Chromosome Structure

C. G. Vosa

Botany School, Oxford, UK

Heitz [14] defined heterochromatin as chromosome material which, as distinct from euchromatin, maintains the metaphase condensation at telophase. Following this definition, heterochromatin can be distinguished from euchromatin during interphase when it is observable in the form of heteropycnotic chromocentres. Autoradiographic studies have demonstrated [5] the general rule that heterochromatin replicates its DNA towards the end of the S period [16, 17]. Heterochromatin can also be defined as chromosomes or chromosome segments which, during the various mitotic or meiotic stages, possess a cycle of condensation different from that of euchromatin.

The term allocyclic [6] indicates both the differences in the condensation cycle of the chromosome segments and the differences in the DNA synthetic activities. The term heterochromatin, therefore, covers a number of behavioural situations in the chromosomes with segments having various cycles of condensation and synthesis.

Types of Heterochromatin

Heterochromatin can be subdivided into two main types: facultative and constitutive. Facultative heterochromatin is exemplified by the behaviour of the inactivated, heteropycnotic X chromosome in female mammals and by the heteropycnosis of the single X in male locusts and grasshoppers. Both types of heterochromatin form Feulgen-positive chromocentres which are readily observable in the interphase nuclei. Constitutive heterochromatin includes a multitude of types characterized by their differential reaction when stained with quinacrine or with the Giemsa technique, and by their sensitivity to cold [24, 26]. Cytochemical tests on facultative heterochromatin, such as those provided by quinacrine staining and the various Giemsa techniques, have failed to show any difference from normal euchromatin.

Cold "Starvation"

The cold induced "starvation" effect on heterochromatin was discovered by Darlington & La Cour [6]. It is an extremely useful tool for cytogenetic research and it has been used principally in *Trillium* and in *Fritillaria* by a large number of authors [7, 11, 12, 13, 15]. Its relationship with quinacrine fluorescence has been studied by Caspersson et al. [4] and by Vosa [24]. Cold sensitive chromosome segments may show enhanced or reduced fluorescence according to the species [24]. A third method of differentiation is provided by the Giemsa technique: Vosa & Marchi [26], using a denaturation–reannealing method, found a correlation between the quinacrine bands, the Giemsa reaction and the cold "starved" segments. The Giemsa method, however, like cold, does not discriminate between enhanced or reduced quinacrine fluorescence.

The mechanism of the cold "starvation" effect is still largely unknown. Microdensitometry studies by Woodard et al. [31] have shown that cold treated chromosomes contain the same amount of DNA as untreated ones. Baumann [2] working on *Scilla sibirica* chromosomes, suggested that the cold effect might be due to stopped or delayed specific protein synthesis in the "starved" segments.

Genetic Properties of Heterochromatin

Breeding experiments in a great number of organisms have shown that the role of heterochromatin on heredity is different from that of euchro-

matin. Its influence is somewhat generalized and quantitative rather than specific or qualitative. Mather [18] and Goldsmidt [10] considered that heterochromatin may consist of repetitive series of genes governing a type of quantitative variation. In fact Mather's polygene hypothesis was based on his studies on continuous variation in *Drosophila*. The inheritance of the bristle characters in this organism can be selected for, and modified by, various types of Y chromosomes which are considered completely heterochromatic. The widespread occurrence of repetitive DNA sequences in heterochromatin may well have played an important role in evolution. These sequences could not survive evolutionary pressures unless they were involved in the very survival of the species. Heterochromatin would then be the source of a kind of replicatory polymorphism to be contrasted with the allelic or phenotypically recognizable polymorphism [7].

The New Techniques: Quinacrine Fluorescence and the Giemsa methods

The use of quinacrine as a fluorochrome for the linear differentiation of chromosomes pioneered by Caspersson and his colleagues [3], has been one of the major advances in cytogenetic research. The quinacrine reaction is very specific and repeatable but the exact mechanism of its specificity is still not completely known. Recent studies by Ellison & Barr [9] on *Samoaia leonensis*, a drosophilid fly, using quinacrine and autoradiographic techniques, have shown an exact correlation between enhanced fluorescence and late replicating heterochromatin. This was already noted by Vosa [25] in relation to *Drosophila melanogaster* where the correlation is, however, not so precise.

Furthermore, Ellison & Barr found that the chromosome segments with enhanced quinacrine fluorescence, are characterized by an extremely high, and probably exclusive, adenine-thymine content. This finding has been confirmed by Weisblum & de Haseth [29] and by Pachmann & Rigler [19] who tested the DNA base specificity of quinacrine fluorescence in in vitro experiments. These authors found that while DNA with a high A–T content enhanced fluorescence, DNA with a high G–C ratio had a quenching effect. Fluorescence quenching was, in fact, found to be proportional to the G–C content of the DNA sample tested. Thus quinacrine binds to the repetitive DNA sequences with a specificity towards their base composition, so that fluorescence intensity is determined by the type of nucleotide involved in the binding. The intermediate intensity of the bands found in mammalian chromosomes may be due to differences in the DNA base ratio along their length. In this context it is interesting to note that in 1961 White suggested that euchromatin consists of A–T and G–C nucleotide pairs arranged in a great variety of sequences while heterochromatin consists mostly of only one kind of nucleotide pair.

One alternative to this binding mechanism might be a special affinity of quinacrine for the various nucleoproteins. In the nucleohistones, the amino groups of the basic amino acids are bound to the phosphate group of the DNA [21, 27]. Quinacrine is structurally an amine and may resemble the basic histones and complex directly with the DNA.

Giemsa

A new method demonstrating constitutive heterochromatin has been introduced by Pardue & Gall [20]. Based on their work on in situ DNA hybridization, it involves alkali denaturation and subsequent reannealing, with Giemsa staining. Arrighi & Hsu [1] and Pardue & Gall [20] used the new technique to study human and mouse chromosomes. They showed that the centromeric regions of most of the chromosomes in the human and mouse complement stain heavily with this method. Mouse satellite DNA, which is known to consist of highly repetitive sequences and is located near the centromere, has been demonstrated in this way. This technique is known as Giemsa C (centromeric heterochromatin). Vosa & Marchi [26] have applied the method to plant chromosomes. They found in most cases a correlation between the cold "starved" segments and the Giemsa stained regions.

A modification of the Giemsa C technique has been proposed by Sumner et al. [23] and used on human chromosomes. This technique, known as ASG where the alkali denaturation step has been completely omitted, produces a complex banding pattern on human chromosomes. The location of the bands is, with some exceptions, almost

identical to the quinacrine pattern. The authors have proposed a DNA denaturing role for the alcohol-acetic fixation, with the repetitive DNA more resistant to denaturation than the non-repetitive kind. The repetitive DNA thus reanneals better when the preparations are air-dried. The resulting banding pattern is due to the preferential binding of the dye to the undenatured DNA. Another technique, producing the same banding pattern as the ASG technique, but showing much finer details of chromosome structure, has been devised by Wang & Fedoroff [28]. These authors worked on the idea that the chromosome bands resulting from the Giemsa techniques, especially ASG, might be due to differences in DNA-nuclear protein association rather than denaturation–reannealing processes. Accordingly they used trypsin to remove partially the protein component of the chromosomes and used Giemsa as stain. Other very similar techniques of protein digestion using strong urea solutions have been proposed by Shiraishi & Yosida [22] and by Dutrillaux et al. [8] using various proteolytic substances.

Conclusions

A considerable advance in our understanding of the problem of heterochromatin and of chromosome structure has been made possible by the introduction of the quinacrine fluorescence technique and the various Giemsa methods. The following are some of the most important points:

(*a*) Recent studies on the quinacrine fluorescence base specificity indicate that the intensely fluorescing chromosome segments are composed of repetitive sequences of adenine-thymine rich DNA. Concurrent in vitro experiments have established that DNA with a high G–C ratio has a quenching effect on quinacrine fluorescence. Fluorescence intensity, it seems, is dependent on DNA base ratio.

(*b*) Heterochromatic segments with enhanced or reduced Q-fluorescence replicate their DNA late in the S period. They show the same reaction when stained with the denaturation–reannealing C Giemsa technique. Thus this technique demonstrates the repetitive DNA sequences of the constitutive heterochromatin regardless of their base composition.

(*c*) In the mouse, constitutive heterochromatin, located near the centromere, is A–T rich and is revealed by the C-Giemsa [20], but shows reduced fluorescence when stained with quinacrine. This is in contrast with the evidence noted in (*a*). It is possible that we are dealing with a system where the ruling molecular mechanisms are not those implied by the theory.

(*d*) The expression of facultative heterochromatin is governed by genetic and developmental factors. It is thought to be structurally very similar to euchromatin.

(*e*) In constitutive heterochromatin the nucleotide base ratio is biased towards one or the other nucleotide pair. The differences in base ratio underlie nucleoprotein variations.

In working with chromosomes we are concerned with complex structures composed of deoxyribonucleic acid and basic and acidic proteins. The protein content of the chromosomes must be kept in mind in the case of chromosome banding whether this is produced by quinacrine staining, by any of the Giemsa methods or by the cold treatment. It is known that single nucleotide changes (substitutions or losses or gains) may give rise to changes in the amino acid sequence of a protein. This implies that protein amino acid base sequences are controlled by the DNA nucleotide sequences. It is probable that all these new cytological techniques are dependent on some kind of protein denaturation.

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Discussion

Rigler: You showed certain differences between QM-bands and Giemsa-bands in your chromosomal material. I wonder what the QM-bands would look like if you treat the chromosomes before QM staining in the same way as you do for Giemsa staining.

Vosa: I have not made this particular experiment on *Allium flavum* chromosomes. I have looked at the chromosomes of *Vicia faba* and *Allium pulchellum.* In these two there seems to be no difference in the banding pattern, with or without the Giemsa pretreatment.

The Enhanced and Reduced Quinacrine Fluorescence Bands and their Relationship to the Giemsa Patterns in *Allium flavum*

C. G. Vosa

Botany School, Oxford, UK

Summary

The chromosomes of *Allium flavum* (2n = 16 + 1B) show a pattern of enhanced and reduced fluorescence bands when stained with quinacrine. A Giemsa staining technique (BaOH-Giemsa), with alkali denaturation and reannealing in a strong salt solution, reveals a pattern of deeply stained bands. Both enhanced and reduced fluorescence bands are stained by BaOH-Giemsa in the same way. In addition other Giemsa bands, not in coincidence with either fluorescence band types, are visible in most chromosomes, and some reduced fluorescence bands are not stained by Giemsa. The two methods are thus revealing 5 types of chromosome segments: (1) enhanced fluorescence; (2) reduced fluorescence; (3) segments demonstrated only by Giemsa; (4) reduced fluorescence bands not stained by Giemsa; (5) normal fluorescing chromatin (euchromatin). Most chromosomes are heteromorphic for their band pattern.

The two staining mechanisms are probably dependent on the interaction of DNA with chromosomal proteins.

The total length of the various bands amount to about 30 % of the karyotype. The degree of chromosome heteromorphism suggests a probable adaptive role for this kind of constitutive heterochromatin.

All species of *Allium* of the group "paniculatum" possess heterochromatin ordinarily visible as Feulgen positive chromocentres in interphase nuclei. Quinacrine staining has demonstrated a pattern of enhanced fluorescence bands in the chromosomes of *Allium carinatum* [6] and in other species of the group. In *Allium flavum* (2n = 16) there exists a pattern of enhanced and reduced fluorescence bands. These bands, varying in size and position in the different chromosomes, are not sensitive to cold treatment [5, 6]. The present study is concerned with the relationship between quinacrine fluorescence bands and those obtained with a modified Giemsa-staining technique [7] in the chromosomes of *Allium flavum*.

Materials and Methods

The plants used in this study belong to a clone of *Allium flavum* (2n = 16 + 1B). They were grown potted in a John Innes compost, either in a cold greenhouse or in the open. For cytological preparations actively growing root tips were pretreated in 0.05 % aqueous colchicine for 4–5 h at room temperature. After overnight fixing in 1 : 3 acetic-alcohol the terminal meristematic tips of the roots were squashed in 45 % acetic acid. After separating the coverslips by inverting the slides in a ridged dish of absolute alcohol, the preparations were stained in 0.5 % quinacrine dihydrochloride (Gurr's Atebrine) in absolute alcohol for 5 min at room temperature. For the Giemsa technique, after separating the coverslips, the preparations were air-dried, immersed in a saturated solution of barium hydroxide for 5 min and incubated for 1 h in 2 × SSC at 60°C. Staining was carried out in Giemsa (R66 Gurr) diluted to 0.5 % with buffer at pH 6.8. Optimum staining time was from 20 to 30 min at room temperature. The depth of the staining may vary with the Giemsa stock and it is usually better to understain.

Results and Discussion

All the observations were made on mitotic metaphases. The results of the two techniques, quinacrine fluorescence and BaOH-Giemsa, are indicated in fig. 1 *a*, *b*. The Q-fluorescence bands include enhanced and reduced types (fig. 2). In all cases the reduced fluorescence bands are contiguous with the enhanced fluorescence and may be on one side only or on both sides of the enhanced bands. In the plant studied there are 51 enhanced fluorescence bands. These are comparatively narrow and are present in all chromosomes. There is some heteromorphism; chromosome pair 4 possess one extra band and pair 6 is heteromorphic for a distal band. The reduced fluorescence bands number 62. They are mostly broad and are found in all chromosomes. The BaOH-

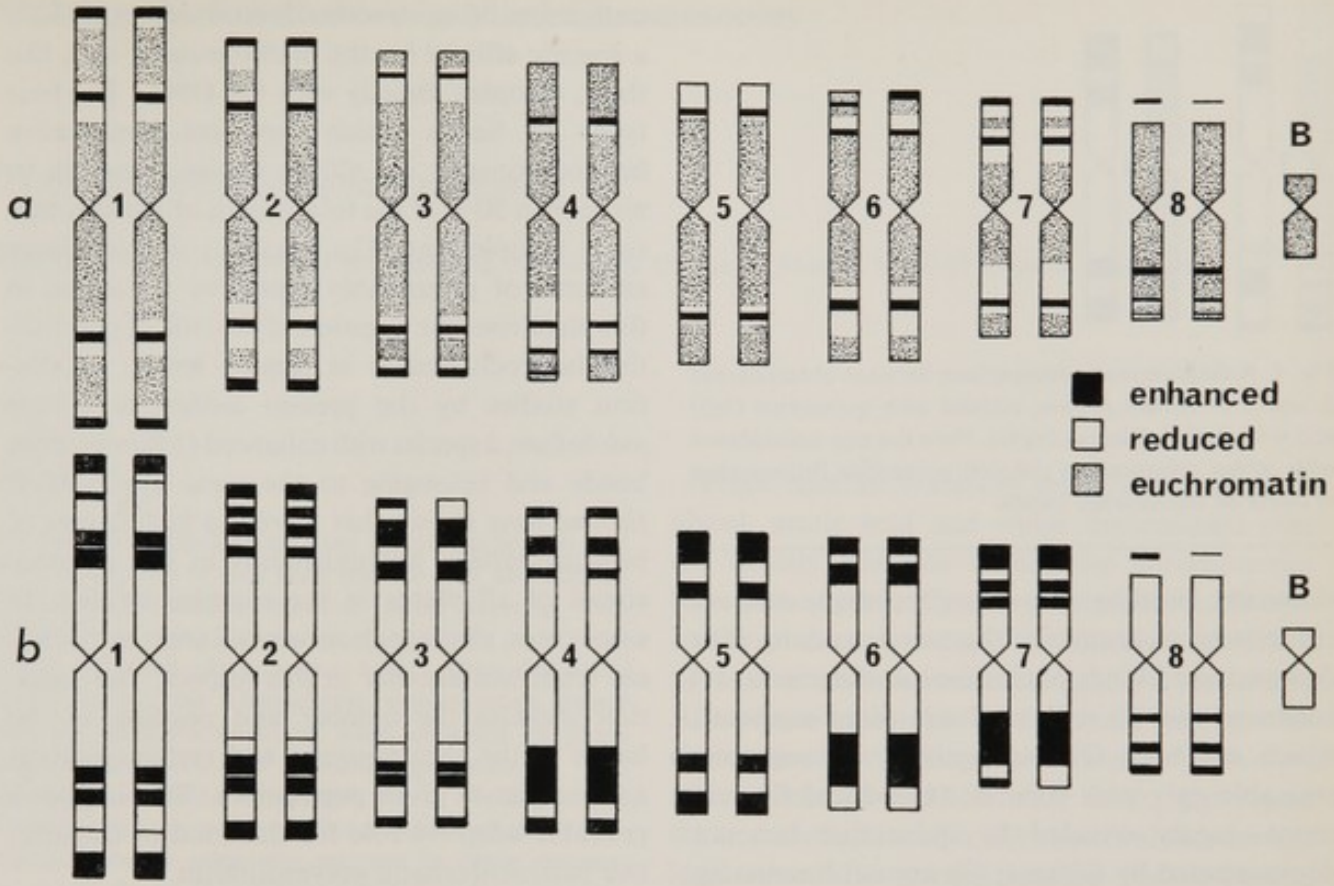


Fig. 1. *a*, Enhanced and reduced quinacrine-fluorescence bands; *b*, BaOH-Giemsa patterns of the chromosomes of *Allium flavum*.

Giemsa technique stains both enhanced and reduced fluorescence bands in the same way forming a pattern of broad and narrow bands (fig. 3).

In addition, on most chromosomes there are Giemsa bands which do not coincide with either Q-fluorescence types. These extra bands are most striking in the case of chromosome 3 (one band in the short arm) and in chromosome 5 (one band in each arm) (fig. 4). Other bands of this type are found on most chromosomes of the complement. On some chromosomes there are reduced fluorescence bands which are not stained with Giemsa. The B chromosome and large portions of all the chromosomes either side of the centromere do not

Fig. 2. Somatic chromosomes of *Allium flavum* stained with quinacrine. Note the pattern of enhanced and reduced fluorescence bands and the B chromosome (*arrow*).

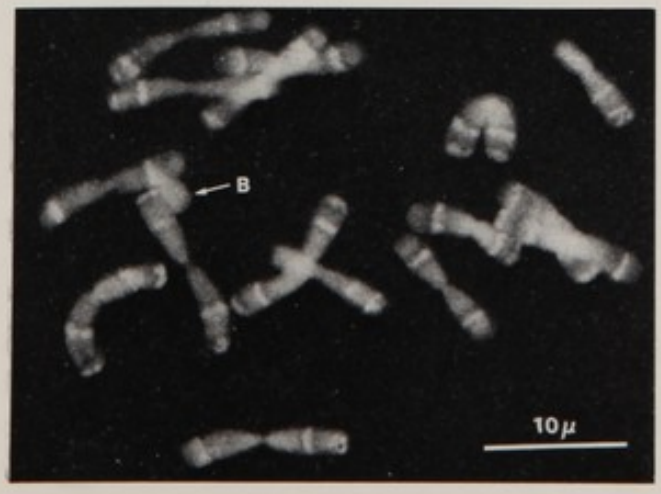
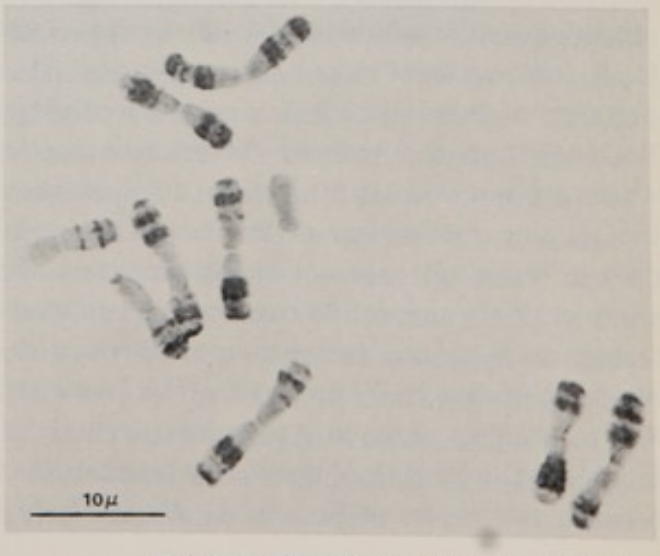


Fig. 3. An incomplete somatic metaphase in *Allium flavum* stained with BaOH-Giemsa. Note the euchromatic B chromosome.



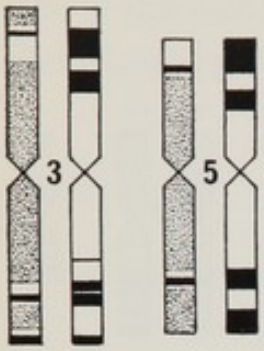


Fig. 4. A diagrammatic comparison between chromosome 3 and 5 of *Allium flavum*, stained with quinacrine (*left*) and with BaOH-Giemsa (*right*). Note the non-coincidence with either enhanced or reduced quinacrine fluorescence of some of the Giemsa bands.

show any banding with either technique and are completely euchromatic. The two procedures thus demonstrate 5 kinds of chromosome segments: (1) enhanced and (2) reduced fluorescence segments, which are both Giemsa positive; (3) segments stainable only with Giemsa; (4) reduced fluorescence bands revealed by quinacrine but not demonstrated by Giemsa; (5) normal fluorescing chromatin (euchromatin). In their studies on the chromosomes of several plant species, Vosa & Marchi [7, 8] already noted, that the BaOH-Giemsa technique does not discriminate between enhanced or reduced Q-fluorescence, staining both in the same way. In *Allium flavum*, where they are present together, the results seem to confirm the non-discriminatory nature of BaOH-Giemsa in respect of the two types of fluorescence bands. This fact would be compatible with the findings on the base specificity of the quinacrine-stain [2, 3, 9]: AT-rich chromosome regions are intensely fluorescent: the presence of GC-rich DNA actually quenches fluorescence. BaOH-Giemsa would then demonstrate the repetitive nature of the DNA of the segments [1, 4], irrespective of their base composition. The presence of bands which are revealed only by BaOH-Giemsa and reduced fluorescence bands which are not stained by it, seem to show that the staining mechanism of the two techniques used in this study may not be dependent exclusively on DNA-nucleotide base sequences. Other factors such as the interaction of DNA with various chromosomal proteins may be involved. The mechanism of quinacrine staining is thought to include the binding of the dye by intercalation between two layers of base pairs. Alternatively, quinacrine being structurally an amine, may show a specific affinity for the nucleoproteins and, like them, complex directly with the DNA. The four types of bands which represent constitutive heterochromatin in *Allium flavum*, amount to more than 30% of the total length of the chromosome complement. The presence of such large amounts of presumably repetitive sequences in this plant rises the question of the role of constitutive heterochromatin in genetic terms. Population studies by the present author in *Allium pulchellum*, a species with enhanced Q-fluorescence bands and belonging to the same group as *A. flavum*, have shown that there is a high degree of banding-pattern polymorphism in the chromosomes of all plants in the samples studied. In some cases, all plants from certain areas are different from one another in this respect. The variation includes the number and position of the bands in the chromosomes and certain patterns are peculiar to given populations. This implies a probable adaptive role for this kind of constitutive heterochromatin polymorphism.

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Staining the Kinetochore in Plant Chromosomes

G. Östergren and Lena Andersson

Department of Genetics and Plant Breeding, Agricultural College of Sweden, S-750 07 Uppsala 7, Sweden

Summary

The cells were fixed in ethylene glycol, acetic acid and water mixed 1:1:1 and later treated with an ordinary chrome-acetic-formalin fixative. After staining with crystal violet, the kinetochores appear dark on a practically unstained chromosome body. After the acetic glycol fixation the kinetochores appear much denser than the chromosome body, when observed by phase contrast microscopy in unstained preparations. This difference in density is probably due to swelling of the chromosome body and extraction of substances therefrom. Crystal violet is selectively retained in dense chromatin, when the stain is differentiated. The dye might also have a selective affinity for substances extracted by the acetic glycol treatment. The higher resistance of the kinetochore to swelling dissolving and extracting influences might be the consequence of a firmer bonding between its constituents, a firmness needed to make the kinetochore resistant to the mitotic forces.

In conventional preparations for chromosome cytology, the kinetic region of a mitotic chromosome usually appears as an unstained or lightly stained constriction. By means of special fixation procedures, the staining properties of the chromosomes can, however, be changed in such a way that the kinetochore appears darkly stained on a very lightly stained chromosome body [1, 2]. In the present study a method introduced by Östergren [2] was investigated in more detail. The cells were fixed from a few hours to a few days in a mixture of ethylene glycol, acetic acid and water in the proportions 1:1:1. Thereafter they were subjected to a second fixation in an ordinary chrome-acetic-formalin fixative. The staining procedure is the usual Newton's crystal-violet-iodine method. This schedule for differential staining of the kinetochore was successfully used on *Hyacinthus orientalis*, *Haemanthus katherinae*, *Scilla sibirica*, *Vicia faba* and *Tradescantia paludosa*.

Phase contrast studies of cells fixed in ethylene glycol, acetic acid and water demonstrate that the density relations within the chromosomes have been profoundly changed by this treatment. In the phase microscope the kinetochores show up as distinct dark bodies on light chromosomes. After fixation in the acetic glycol mixture the chromosomes are large and swollen. However, after combined fixation with acetic glycol and chrome-acetic-formalin, the chromosomes are not larger than after normal fixation. The conclusion is that much substance, presumably histones, has been extracted from the chromosome body, and that the kinetochores are much more resistant to both the swelling and the extraction of substance.

The differential staining of the kinetochores by crystal violet might well be a simple result from the difference in density between the kinetochore and the chromosome body. When cells are stained by crystal violet they are at first strongly overstained by an aqueous solution of the dye. After that, the dye is made less soluble by a treatment with iodine, and finally the slides are differentiated by washing out most of the dye in alcohol and clove oil (or eugenol). During this process of differentiation the crystal violet is more easily washed out from chromatin regions of loose structure than from dense chromatin. This is illustrated by the fact that, in ordinary fixations by means of a chromic fixative, crystal violet stains metaphase and anaphase chromosomes very darkly, while the dye can be nearly completely washed out from the resting nuclei in the same preparations. When the same kind of slides are stained by the Feulgen technique, the resting nuclei are also strongly stained, although the chromosomes at mitosis may be less intensely stained than with crystal violet.

In order to achieve a successful stain of the chromatin by means of crystal violet, it is necessary either to fix the cells in a fixative containing chromic acid or to mordant the cells in chromic acid (or dichromate). This might very well mean that crystal violet in this procedure does not actually stain DNA, but, for instance, histones that have absorbed chromic acid. If this is so, it is easy to understand that the chromosome body is much less stained by crystal violet after our special fixation technique which presumably extracts much histone from the chromosomes. After our special treatment of the chromosomes, they can still be stained by Feulgen.

We have accepted the terminology at present used by electron microscopists, according to which the organelle previously described as the "spindle spherule" is now called the kinetochore. The term kinetochore was earlier used in a wider sense to comprise the whole of the centric constriction. It is clear from our observations that the dense and stainable body that we have studied is located within the primary constriction. Also, it is often pulled out by the chromosomal traction fibre. For these reasons we consider the body in question to be the kinetochore. After application of our special fixation method this body gives a positive Feulgen reaction. Our observations agree with the generally accepted view that each anaphase chromosome has one kinetochore, while the metaphase chromosome has two such bodies, corresponding to the two chromatids.

The mitotic forces (which are capable of breaking chromosome bridges in anaphase) are concentrated on the kinetochore. For this reason, we should expect this body to be especially adapted to resist mechanical forces. The structural reinforcement which produces the mechanical resistance of the kinetochore may imply a firmer mutual bonding of its component constituents. This might very well also decrease the swelling capacity of the kinetochore and the solubility of its constituents, when it is subjected to dissolving or extracting influences. In this manner, our observations on the fixation and staining of the kinetochore may be put in relation to the functional purpose of this body. This kind of firm bonding might conceivably be produced in different ways. To produce this effect the kinetochore might, for instance, contain proteins that differ from those of the chromosome body in general. Another conceivable mechanism to produce mechanical strength might be to let the kinetochore contain repetitive DNA, in which the repeated sequences may be subjected to local separation of the strands of the double helix followed by reassociation with corresponding sequences in other positions, located in other DNA fibres or elsewhere in the same fibre. This would produce an extensive cross-linkage of the DNA, increasing its mechanical resistance in more or less the same way as does vulcanization in rubber. Unfortunately, the small size of the kinetochores might cause considerable difficulties in testing this hypothesis.

This description of our hypothesis might remind of Walker's idea that repetitive DNA in the kinetochore region is located in a proximal heterochromatin which increases the strength of the kinetochore [3]. Actually, however, this is a different hypothesis. By "strength of the centromere" Walker does not imply its resistance to mechanical deformation but its capacity to produce a strong pulling force.

The complete version of the present paper will appear in Hereditas.

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Discussion

Ford: Your hypothesis is intriguing, whether it be true or false. The electronmicrographs, like those demonstrated by Dr Bahr, show some connecting fibres between sister chromatids along the whole length of the chromosome and a greatly increased local concentration of them on each side of the centromere. One wonders about the relative strength of the forces pulling the centromeres apart and holding the chromatids together and the manner in which the interchromatid connections are resolved. Does this process involve physical disruption of the connecting fibres? And if so, is there a related phase of DNA repair?

Östergren: I do not expect the chromatid separation to be due to a disruption of connecting fibres caused by the kinetochore pull. The reason for my opinion is that chromatid separation occurs also, and at the same time, in acentric chromosome fragments. This can be seen from the ciné-films of A. Bajer and J. Molé-Bajer on irradiated endosperm cells of *Haemanthus katherinae*. A normal chromatid separation is also observed in mitoses treated with colchicine which, as a consequence of this, are free from kinetochore forces (ciné-film studies by Molé-Bajer, J, Chromosoma 9 (1958) 322). Further evidence is given by unpaired meiotic chromosomes. In such univalents, the chromatid separation along the chromosome arms at first anaphase occurs at the same time as in paired chromosomes (Geitler, L, Z ind Abst- u Vererblehre 75 (1938) 161–190; Östergren, G, Hereditas 37 (1951) 85). At meiosis, however, the sister chromatids remain mutually associated in the arm regions most proximal to the kinetochore until the onset of the second anaphase, when they suddenly disjoin in these proximal regions (Lima-de-Faria, A, Hereditas 42 (1956) 85). The chromatid separation is obviously an autonomous process of the individual chromatid regions. Due to local processes in the regions in question they separate at specific moments in the cell cycle. In my opinion, one possible mechanism for the sister chromatid pairing might be localized reversible mutual strand exchanges between double helices in a kind of repetitive DNA sequences distributed along the chromosome arms, or even between non-repetitive DNA, as homologous chromatid regions used to be associated. When the chromosomes are prepared for electron microscopy this mechanism could result in pictures of fibre connections between sister chromatids. We may imagine that the chromatid separation could result from an enzyme being suddenly put into action to restore the original strand pairing at these points of association and might call this enzyme *chromatid separase*. When the chromatids fail to separate, as in hybrid grasshoppers (Klingstedt, H, J genetics 3 (1939) 389), some chromosomes are broken by the kinetochore pull in anaphase.

Applications of the Banding Techniques in Biology and Medicine

Somatic cell hybridization

Somatic Cell Hybridization in the Study of Gene-Linkage and Complementation

D. Bootsma,[1] P. Meera Khan,[2] H. van Someren,[3] E. A. de Weerd-Kastelein[1] and A. Westerveld[4]

[1]Department of Cell Biology and Genetics, Medical Faculty, Rotterdam, [2]Department of Human Genetics, [3]Laboratory for Molecular Genetics, Leiden State University, Leiden, and [4]Medical Biological Laboratory TNO, Rijswijk, The Netherlands

When mammalian somatic cells are mixed under appropriate conditions cell fusion can occur resulting in bi- or multinuclear cells. Proliferating hybrid cell lines, containing the genetic information of two or more parental cell lines in one nucleus can be isolated. This cell fusion technique has been profitably applied in the field of cell differentiation and cell genetics.

Certain types of hybrid cells lose their chromosomes during proliferation and such a loss in certain cases was found to be limited to one of the two parental sets. The phenotypic changes resulting from this chromosome loss have given new insight into the mechanisms of regulation of gene expression and facilitated the localization of genes on chromosomes in mammalian cells. A few examples will be discussed in this presentation. The new techniques of chromosome identification have already made important contributions to these studies.

Studies on Regulation of Gene Expression in Hybrid Cells

The work on the regulation of melanin synthesis in mammalian somatic hybrid cells [5, 6] gives a nice demonstration of the application of cell hybridization in the study of gene expression. The melanin production observed in a Syrian hamster melanoma cell line is suppressed in hybrids isolated from fusions between the Syrian hamster cells and non-melanin producing mouse cells. This extinction of a tissue specific ("luxury") function [6] might reflect a mechanism of regulation of gene activity in differentiation, for instance by the production of a diffusible regulator substance coded by the mouse genome. Reexpression of a luxury function in hybrid cells has been observed in several different hybrid systems (e.g. [8, 19]). Karyogram analysis of these hybrids has presented suggestive evidence that reexpression is correlated with the loss of chromosomes in the hybrid cell [8].

Studies on Linkage and Localization of Genes

It has been shown that hybrid cells originating from fusions between human cells and several animal cell lines like mouse, Chinese hamster and Syrian hamster, specifically loose human chromosomes. Most murine and human forms of homologous enzymes are clearly distinguishable by electrophoretic techniques, providing a nice set of genetic markers. Hybrid clones can be obtained having different numbers and combinations of human chromosomes, which can be tested for the retention or loss of human enzyme markers. The simultaneous loss or retention of human enzymes will reveal gene-gene linkage relationships, whereas the identification of the human chromosomes by the new banding techniques provides the basis for gene-chromosome assignments. A few examples taken from the work of our group will be presented in this paper.

The man-Chinese hamster hybrid lines utilized in these studies have been described by Westerveld et al. in 1971 [20]. They were used to prove the X-linkage of the structural locus for human phosphoglycerate kinase (PGK) [9] and to demonstrate the presence of an X-linked component

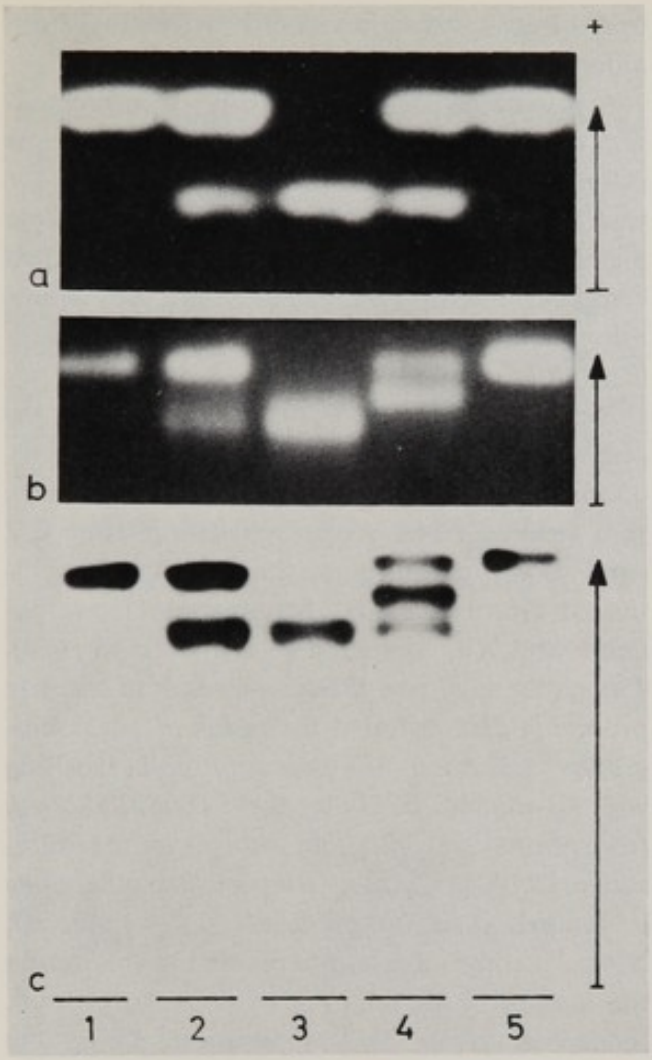


Fig. 1. *a*, Electropherograms of PGK; *b*, α-Gal; *c*, G6 PD showing Chinese hamster (*1*), artifical mixture of Chinese hamster and human (*2*), human (*3*) and man-Chinese hamster somatic cell hybrid (*4*, *5*) patterns. The hybrid in the channel *5* has lost the human loci for PGK and G6PD as well as the additional band for α-Gal. From [7].

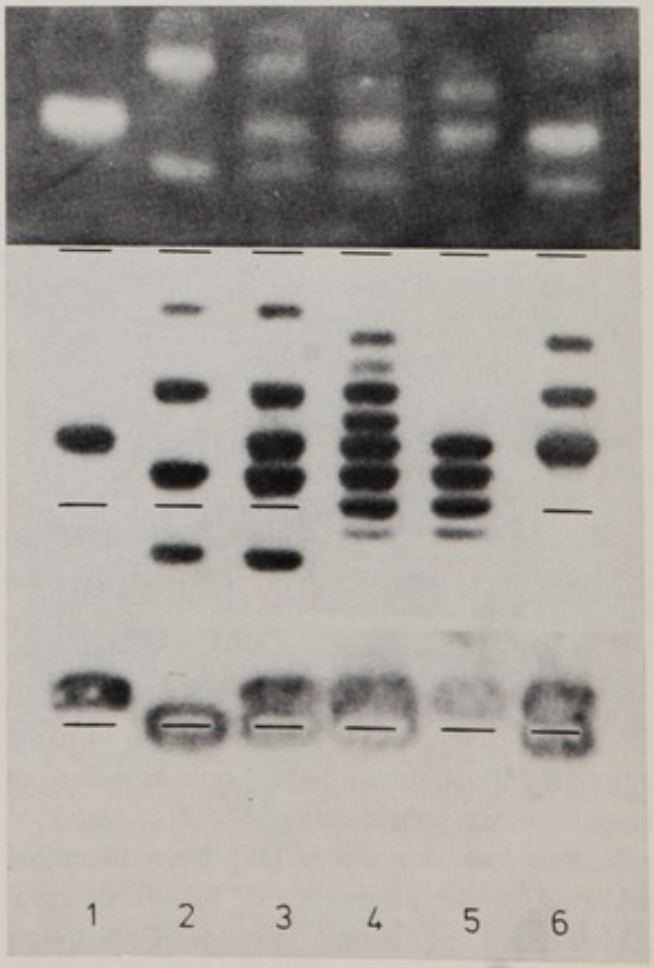
involved in the synthesis of α-galactosidase (α-gal) in man [7]. The latter is illustrated in fig. 1, representing the zymograms of PGK (fig. 1*a*), α-gal (fig. 1*b*) and glucose-6-phosphate dehydrogenase (G6PD, fig. 1*c*) known to be X-linked in man from family studies. The simultaneous retention (fig. 1, channel *4*) in about 125 clones and the simultaneous loss (fig. 1, channel *5*) in about 30 different clones clearly demonstrate the X-linkage of genes involved in the synthesis of these three enzymes.

By using the same systems we have presented evidence for three autosomal linkage groups in man: the linkage between the genes coding for 6-phosphogluconate dehydrogenase (6PGD) and phosphoglucomutase 1 (PGM 1) [21] and two linkage groups both carrying different loci for lactate dehydrogenase (LDH A and LDH B) and glutamic pyruvic transaminase (GPT B and GPT C) [16].

Our studies on the enzyme GPT suggested the existence of at least three independent loci for GPT in man: the locus for the red cell GPT, having a dimeric structure [2] referred to as GPT A and two loci for the white cell and fibroblast GPT: GPT B and GPT C. We got evidence that the fibroblast GPT has a tetrameric structure. Under the conditions leading to a clear distinction between human and Chinese hamster GPT isoenzymes only two of the five bands of the human GPT in fibroblasts appear in the zymogram (fig. 2, channel *2*). Out of 68 hybrid clones 22 have retained the human loci for GPT B and

Fig. 2. Zymograms of GPT (*top*). LDH (*middle*) and Pep B (*bottom*) comparing the fibroblast patterns of (*1*) Chinese hamster; (*2*) human; (*3*) an artificial mixture of Chinese hamster and human; (*4–6*) man—Chinese hamster somatic cell hybrids.

The intermediate band due to heteropolymeric enzyme molecules seen in GPT and LDH (channel *4–6*) are absent in Pep B. The hybrid population in channel *5* has lost the human loci for GPT B, LDH B and Pep B, while the hybrid in channel *6* has lost the human loci for GPT C and LDH A. From [16].



LDH B, while the rest have lost them together; for the GPT C-LDH A pair 26 clones were positive for both and 42 negative. In fig. 2 three different hybrid clones are presented: one having retained both loci for LDH as well as for GPT (channel *4*), one having lost the LDH B–GPT B linkage group (channel *5*) and one having lost the LDH A–GPT C linkage group (channel *6*). The zymograms for peptidase B (Pep B) also indicated in fig. 2, confirm the linkage between the loci for Pep B and LDH B [12, 13].

Chromosome studies of these hybrid clones (A Jongsma & P L Pearson. Unpublished results) in which the new chromosome identification techniques were applied, have shown that the LDH A and GPT C loci are located on chromosome 11 and the loci for LDH B, GPT B and Pep B on chromosome 12. These results confirm the assignments published by Ruddle [11] who studied mouse-man hybrids. Our finding of the linkage of LDH A to GPT C and of LDH B to GPT B and the assignment of these two linkage groups to two resembling chromosomes (C11 and C12) might support the theory that tetraploidization has occurred in the evolution of the mammalian karyogram.

The next step in these studies, which has already been made by different groups, is to determine the sequence of the genes on the chromosomes. To achieve this end, hybridization will be performed using cells from individuals bearing a chromosome translocation as one of the parental cells. The recombination of the genes as a result of the translocation and the gradual loss of the human chromosomes provide the tools for mapping the human chromosomes in this system.

Studies of Genetic Complementation by Cell Fusion

A new approach in the study of heterogeneity at the basis of a genetic disease is introduced by the application of cell fusion techniques. Cell hybridization has been successfully used in the demonstration of complementation between different genes (intergenic complementation in many different animal-human hybrid cells as well as in human-human hybrid cells) [14, 15].

Nadler and coworkers [19] have presented evidence for the occurrence of interallelic complementation by fusing galactose-1-phosphate uridyl transferase deficient cells originating from different patients having galactosemia.

A second incidence of genetic complementation after fusion of cells from different patients suffering from a genetic disease came from our studies on defective DNA repair in xeroderma pigmentosum (XP) cells [18]. We have studied complementation between different XP strains using the binuclear cells which can easily be identified after cell fusion.

Xeroderma pigmentosum is an autosomal recessive disease characterized by hypersensitivity of the skin to UV radiation resulting in severe skin lesions. DNA repair replication after UV irradiation is absent or markedly reduced in cultivated fibroblasts and lymphocytes from patients with XP, compared to normal cells [1, 3]. The synthesis of new DNA as a result of a repair process is demonstrated by means of autoradiography following UV-exposure and labelling with ^{3}H-thymidine. Under these conditions cells from normal individuals do incorporate the radioactive DNA precursor, irrespective of the stage in the cell cycle (unscheduled DNA synthesis); XP cells only incorporate ^{3}H-thymidine during the S phase of the cell cycle as a result of normal, semiconservative DNA replication. Using the excision repair mechanism, described for microorganisms as a model, evidence has been presented that XP cells are defective in the incision step of DNA repair [4].

There are some indications for a genetic heterogeneity in the basic defect of XP. Two different clinical forms have been described. In the classic form of XP the patients show skin lesion only, whereas in the De Sanctis Cacchione (DSC) syndrome, a rare variant of XP, there are severe neurological abnormalities and mental deficiency in addition. At the molecular level heterogeneity is suggested by the finding of different rates of DNA repair replication in XP fibroblasts from different patients, ranging from 0–100% of the repair found in normal cells [1].

To study genetic heterogeneity in this disease, fusions have been carried out between cells from different patients and unscheduled DNA synthesis was investigated in the binucleated cells after exposure to UV light [18]. In cell populations after fusions of classic XP cells with DSC cells, unscheduled DNA synthesis following UV exposure was demonstrated in about 30–40% of

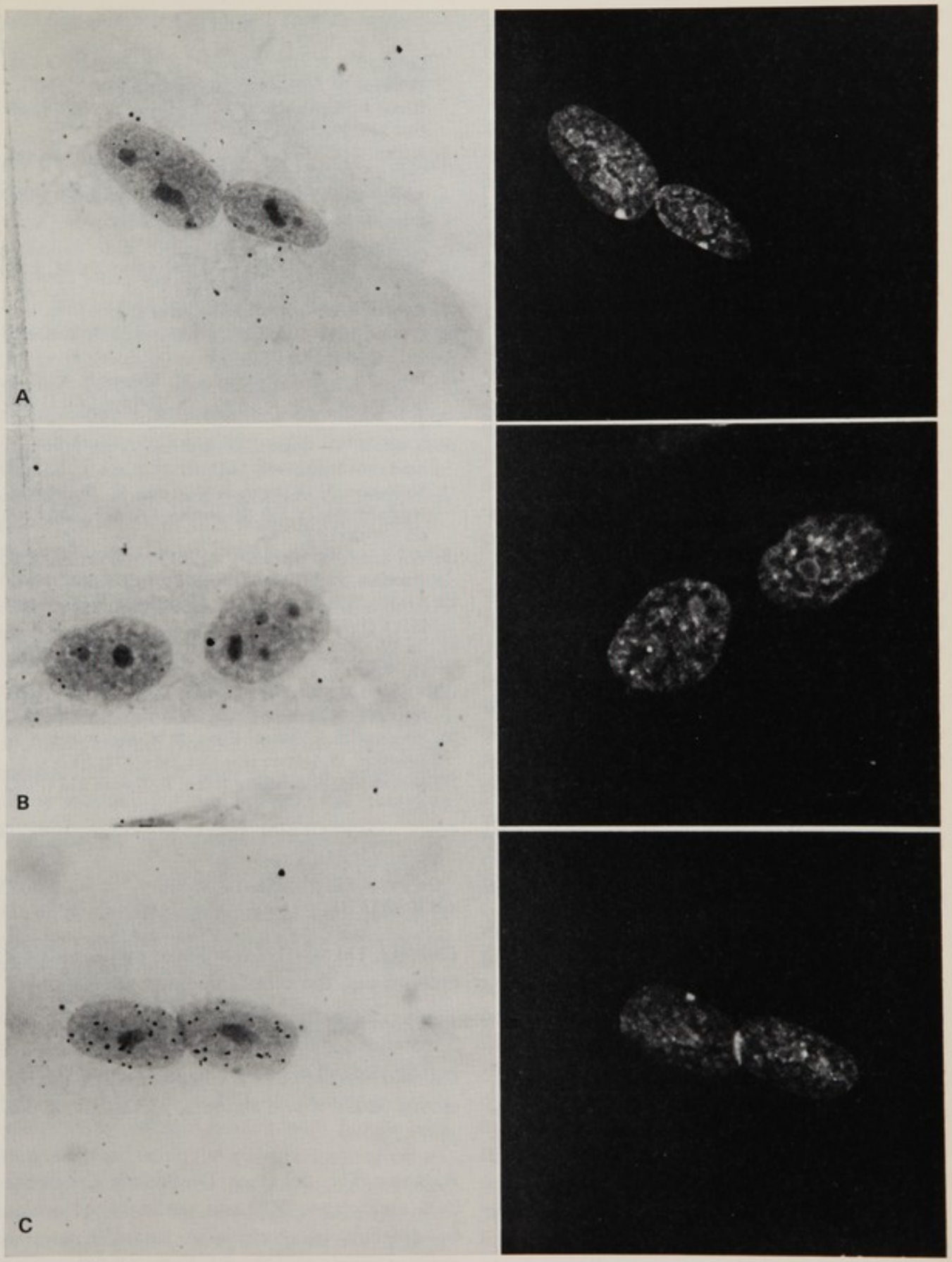


Fig. 3. Repair DNA synthesis in binucleated cells after fusion of cells from a classic XP patient (female) and cells from a De Sanctis Cacchione patient (male). Autoradiograms (*left*) and fluorescence after atebrine staining (*right*) are shown. Cells have been irradiated with 100 erg/mm² UV light (predominantly 254 nm) and have been labelled with ^{3}H-thymidine (10 μCi/ml medium, spec. act. 2 Ci/mmole).

A, binuclear cell having two nuclei with a Barr body (classic XP × classic XP); *B*, binuclear cell showing Y-chromatine in both nuclei (DSC × DSC); *C*, binuclear cell having one nucleus with a Barr body and another nucleus with Y-chromatine (classic XP × DSC).

Only the classic XP × DSC binuclear cell shows repair DNA synthesis. From [18].

the binucleated cells. Fusion of cells from either different classic XP patients or different DSC patients did not yield a significant percentage of binucleated cells with repair activity. In fusions of two different parental strains, three types of binucleated cells are present: the hybrid cell having one nucleus of each parental strain and the two types having two nuclei of only one of the parental strains. In cases where one parental strain was female and the other male, the three types of binuclear cells were distinguished by means of atebrine staining of the Barr body in the female nucleus and Y-chromatin in the male nucleus (fig. 3). The 30–40% of the binucleated cells, which show repair, almost exclusively were of the hybrid binuclear type (fig. 3*c*), the other 60–70% had two nuclei of the same parental strain (fig. 2*a*, *b*). We have interpreted these findings as being indicative for the presence of two different mutations in the two forms of xeroderma. The most simple explanation will be that even two different genes are involved which will lead to complementation and the formation of normal gene product in the hybrid binuclear cells.

Evidence for the hypothesis of different genes involved in DNA repair should come from studies of interspecific somatic cell hybrids of the type described earlier in this paper. However, those investigations require a clear distinction between human and animal repair markers. The low level of repair DNA synthesis sometimes found in rodent cell lines [17] might probably fulfill such a requirement.

Note added in Proof

In a later series of experiments involving further characterization of the GPT isozymes in the hybrids and their parental cells we got strong evidence that LDH of these cells is masquerading GPT. This is because of the fact that α-ketoglutarate, a component of the GPT reaction mixture, is utilized by LDH as its substrate analogue. Therefore, the apparent GPT-LDH association observed in the hybrid cell lines is spurious.

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Discussion

Comings: Do you know whether one gene is for excision and the other for repair replication?

Bootsma: Both seem to be involved in excision.

Comings: Perhaps this is complementation at the enzyme level of two different mutations of one gene.

Bootsma: The defect in De Sanctis Cacchione cells and classic XP cells seems as yet not to be different in biochemical assays. Using the excision repair as a model, both cell types seem to be defective in the incision step. However, the finding of XP patients having normal levels of repair replication might indicate that other repair processes than excision repair might act in mammalian cells. The possibility that in the

two forms of XP the same gene is mutated but on different sites cannot be ruled out. In that case we are dealing with interallelic complementation.

Ford: May I ask if you can discriminate between interallelic and interlocus complementation?

Bootsma: It is difficult to make the distinction between interlocus and interallelic complementation. It is known from studies of microorganisms that interallelic complementation might lead to the production of an enzyme which has altered physico-chemical characteristics. There is one example published concerning complementation in mammalian cells in which a labile enzyme was formed. I am referring to the paper by Nadler et al. [10] who found interallelic complementation after fusion of cells from different galactosemia patients. In the case that we are directly measuring enzyme activity, e.g. by microchemical analysis of multinuclear cells, we might be able to distinguish between the two types of complementation mentioned. In our case of xeroderma, not even knowing which enzyme is involved, it is difficult to make this distinction. Our grain countings suggest that we, by complementation, reached the normal level of DNA repair indicating normal enzyme activity and therefore probably interlocus complementation.

Polani: This type of fusion work requires, naturally, the demonstration of the absence of metabolic cooperation in mixed cultures, before fusion will be undertaken.

Bootsma: We have carried out these control experiments in which male xeroderma cells were mixed with cells from normal, female individuals. After UV-exposure and ^{3}H-thymidine labelling male cells in cellular contact with female cells were identified using the atebrine staining on sex chromatin. Following autoradiography these xeroderma cells were still found to be negative in terms of repair DNA synthesis excluding the possibility of metabolic cooperation. Binuclear cells originating from fusion between xeroderma cells and normal cells showed repair DNA synthesis in both nuclei. In that situation cooperation is found between the normal nucleus and the xeroderma nucleus.

Ford: Would you speculate about the applicability of cell fusion to study genetic heterogeneity in other genetic diseases?

Bootsma: The application of multinucleated cells following cell fusion in the study of genetic heterogeneity is limited if autoradiography is the technique for detection of complementation. Another example is the deficiency of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) observed in the Lesch-Nyhan syndrome. Experiments carried out in our laboratory in which we fused a number of HGPRT deficient cell lines, including Lesch-Nyhan cell strains as well as established cell lines, have shown no incidence of complementation. This technique might be useful for the characterization of mutants in the HGPRT locus isolated in mutation studies using mammalian cells.

An important extension in the applicability will be obtained if one can measure enzyme activity at the single cell level. An example might be fusion of cell strains originating from patients having a deficiency for the enzyme glucose-6-phosphate dehydrogenase (G6PD). The activity of this enzyme can be measured at the single cell level as was shown by Galjaard and collaborators in our laboratory.

Lejeune: Is this repair mechanism abnormal in other diseases which predispose to cancer, like Bloom syndrome for example?

Bootsma: We have investigated cells originating from a Bloom syndrome patient by applying different techniques to study repair (rejoining of breaks in the DNA and DNA repair replication). These cells were found not to be different from normal cells in that respect. Of great interest might be those patients showing defects in recombination events during meiosis. Dr Evans (Edinburgh) has reported on decreased levels of repair DNA synthesis in lymphocytes from an individual showing asynapsis. However, this finding has not been confirmed as yet in studies of other patients of this type.

Chromosome Identification in Chinese Hamster/Human Somatic Cell Hybrids

G. R. Douglas, Phyllis A. Gee and J. L. Hamerton

Division of Genetics (Department of Pediatrics), University of Manitoba and Department of Genetics, Winnipeg Children's Hospital, Winnipeg, Canada

The development of interspecific somatic cell hybridization as a powerful tool in the study of human gene linkage was facilitated by discoveries in three areas:

(1) The observation by Weiss & Green [19] that human chromosomes are selectively lost from mouse/human somatic cell hybrids.

(2) The development of techniques capable of detecting electrophoretic interspecific differences of homologous enzymes [16, 18].

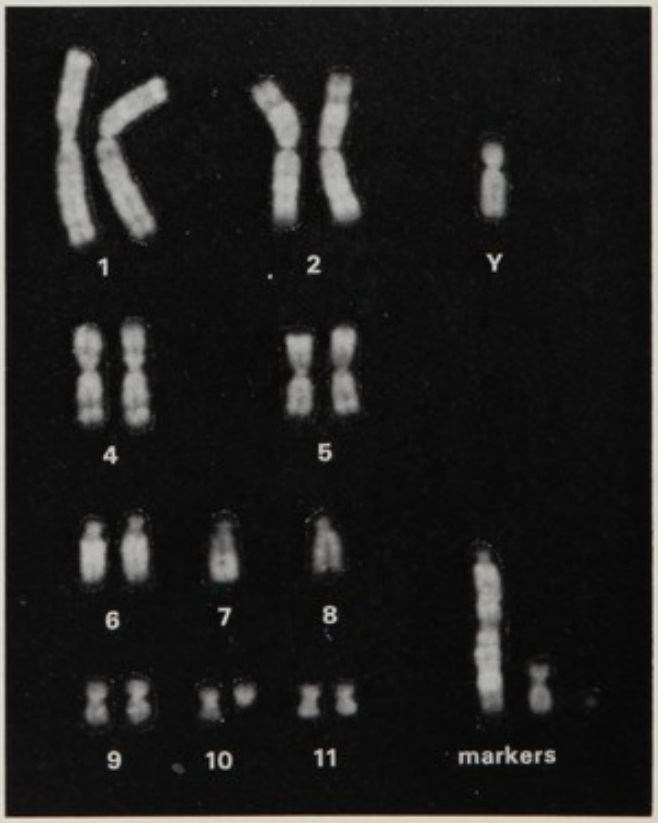
(3) The discovery that chromosomes of many mammals stained with fluorochromes exhibit specific, identifiable banding patterns, or Q-bands [2, 3, 11, 17]. Thus, by correlating the presence of detectable human gene products with the presence of human chromosomes in interspecific somatic cell hybrids, it is possible to establish the assignment of genes to human chromosomes.

The first assignment of a gene to a specific chromosome using these methods on mouse/man hybrids was the location of human thymidine kinase to chromosome 17 [10 12]. Despite the specificity of the Q-banding technique some mouse chromosomes appear similar to human chromosomes necessitating the use of additional identification procedures to distinguish all the human chromosomes [1]. The use of Chinese hamster rather than mouse cells to hybridize with human cells greatly simplifies chromosome identification. The smaller number of Chinese hamster chromosomes (22 against about 60 in mouse L cells) and their unique Q-banding patterns permits the reliable identification of each human and Chinese hamster chromosome using only one technique.

Production of Hybrids

Hybrids were made between a mutant Chinese hamster fibroblast cell line [5] (fig. 1) deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) selected from a pseudodiploid line (CHWCl) derived in this laboratory [9] and either normal human fibroblasts (fig. 2) or human leucocytes. Fusion was mediated by inactivated Sendai virus (Connaught Medical Research Laboratories, Toronto; Batch nos. 102

Fig. 1. Q-banding in the karyotype of a mutant Chinese hamster fibroblast cell deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT).



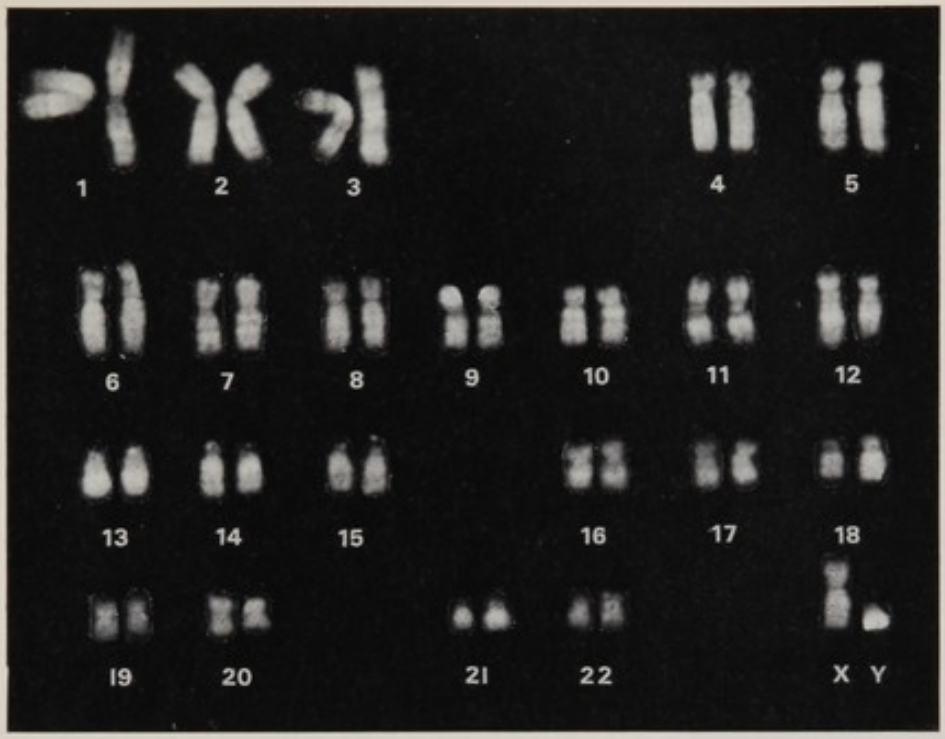
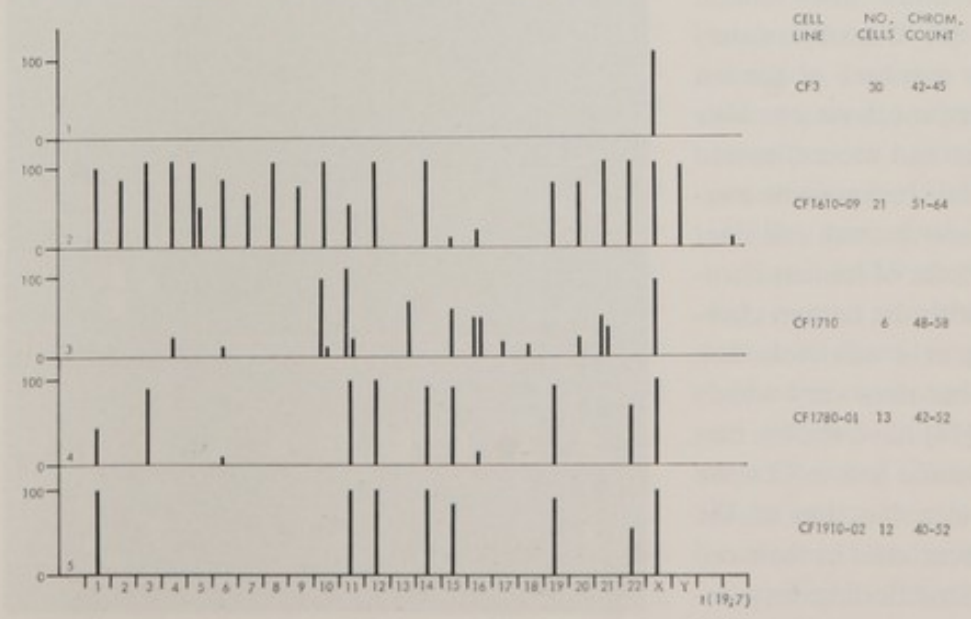


Fig. 2. Q-banding in the karyotype of a normal human fetal fibroblast cell.

and 105), and fusion products were selected in THAG medium (10^{-6} M thymidine, 10^{-5} M hypoxanthine, $3.2 \cdot 10^{-6}$ M aminopterin and 10^{-7} M glycine in Eagle's minimal essential medium with non-essential amino acids, sodium pyruvate, and 10% dialysed fetal calf serum). This medium selects for HGPRT positive hybrids. A detailed protocol for the production and maintenance of these hybrids is presented elsewhere [7].

Fig. 3. *Abscissa:* chromosome number; *ordinate:* %.
Distribution of each human chromosome present in Chinese hamster/human somatic cell hybrid lines.



Chromosome Studies

Cells were pretreated with 0.05 μg/ml Colcemid for 3 h prior to hypotonic treatment (1 part medium : 3 parts distilled water, for ½ h and fixed in 3 parts methyl alcohol : 1 part glacial acetic acid. Air-dried preparations were stained in quinacrine mustard (0.05 μg/ml, pH 7.0, 20 min) or quinacrine dihydrochloride (0.5%, pH 4.5, 15 min), rinsed and mounted in deionized water. Chromosomes were visualized using a Zeiss Photomicroscope II equipped for ultra-dark field fluorescence, and using an Ohlson KP500 or BG12 exciter filter, and a 53/44 barrier filter combination. H & W Control Ltd VTE Panchromatic film

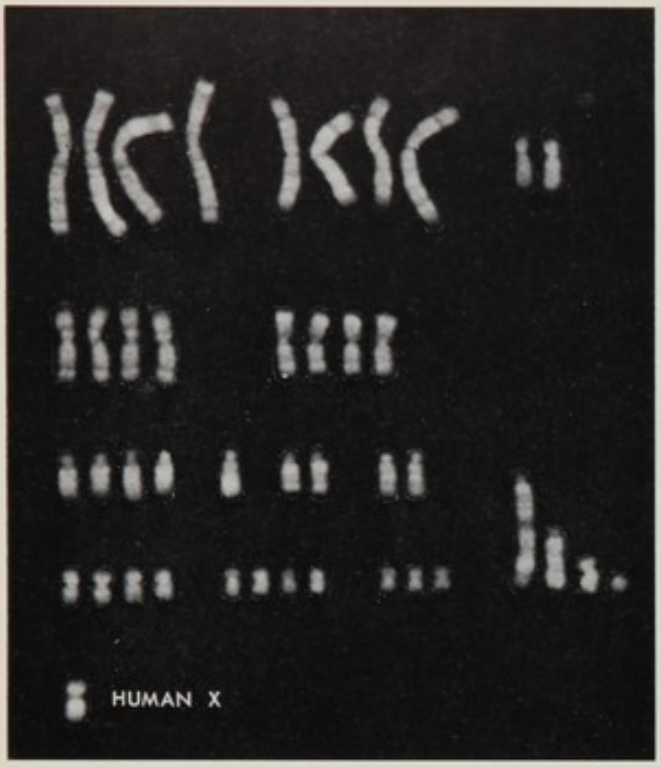


Fig. 4. Q-banding in the karyotype of a Chinese hamster/human somatic cell hybrid (line CF3).

was used for photography. Exposure times varied from 40–80 sec. The film was developed in H & W developer and printed on Kodak Polycontrast paper at contrast grades 3–4. Parallel cytological and biochemical analyses were performed on hybrid lines as soon as possible after selection.

Chromosome Loss in Hybrids

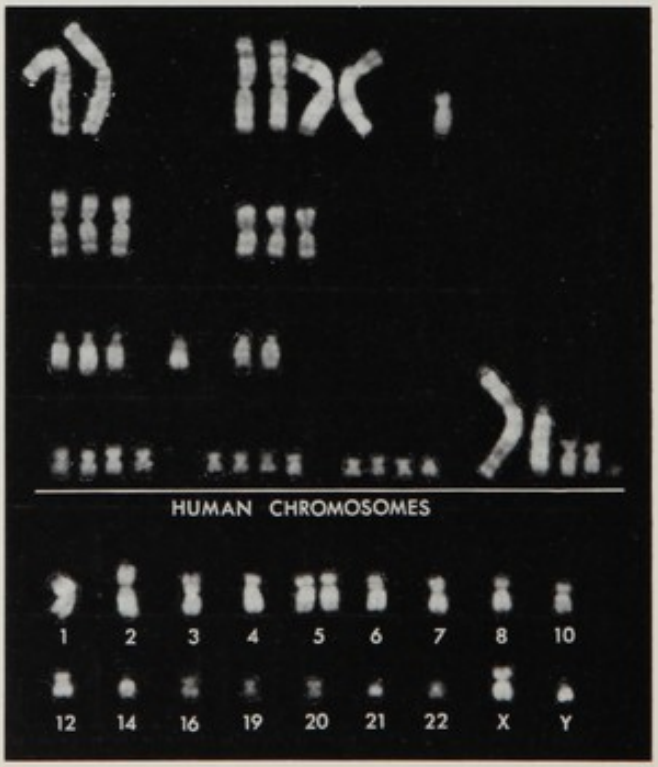
The assignment of linkage using somatic cell hybrids is simplified if fewer human chromosomes are present. Unlike mouse/human hybrids, Chinese hamster/human hybrids lose human chromosomes more rapidly [8]. Useful hamster/human hybrids, having low numbers of human chromosomes, can be obtained much sooner after they have been selected, than can mouse/human hybrids. Fig. 3 summarizes the chromosome analyses from five Chinese hamster/human cell lines obtained in our laboratory. Loss of human chromosomes is generally rapid with the human chromosome content stabilizing at a relatively low number; however, this number may vary widely (figs 4, 5). Rao & Johnson [14] have shown that the degree of human chromosome loss in Chinese hamster/HeLa cell hybrids is a function of the relative 'positions' of the parent cells in their cell cycles just prior to fusion. This finding may account for the wide variation in human chromosome content in our hybrid lines.

Unlike some Chinese hamster/human hybrids obtained in other laboratories [8, 20], our hybrids contain a basic tetraploid hamster complement the result of either fusion of a tetraploid hamster, or two diploid hamster cells with a human cell. This number is subject to variation, presumably due to the plasticity afforded by the extra hamster genome.

Initial loss of at least some human chromosomes is not random, in that certain ones are almost always present (fig. 3, chromosomes 11, 12, 14, 15, 19, 22). The human X-chromosome is always present in the lines studied here, since it contains the HGPRT locus, and is thus essential for growth in THAG medium. Since each line was chosen such that it arose from a single fusion product, the variability within each line reflects either slow but persistent chromosome loss, or stabilization of cells with slightly differing human chromosome content.

After the chromosome content of a line became relatively stable, it was cloned, and selected clones analysed. Data from karyotypic analysis of clones from two hybrid lines is summarized in figs 6 and 7. The high frequency of cells containing each chromosome in fig. 6 suggests that

Fig. 5. Q-banding in the karyotype of a Chinese hamster/human somatic cell hybrid (line CF1610-09).



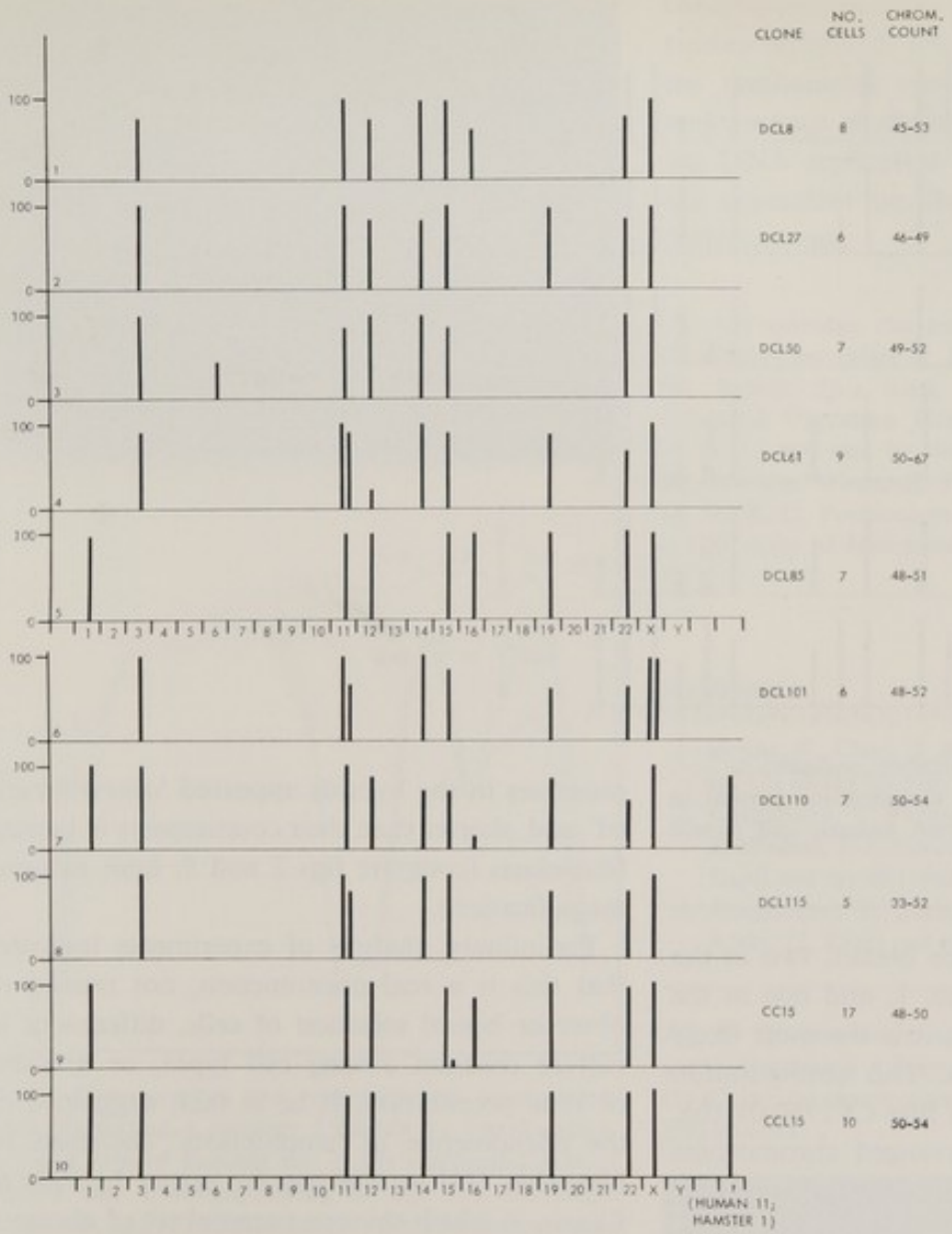


Fig. 6. *Abscissa:* chromosome number; *ordinate:* %.
Distribution of each human chromosome present in clones of Chinese hamster/human somatic cell hybrid (line CF1780-01).

in hybrid line CF1780-01 human chromosome loss has not persisted, but that variation in chromosomes among clones is the reflection of variation in the original colony. The low frequency of some chromosomes in fig. 7 indicates that human chromosomes in hybrid line CF1610-09 are still being lost after cloning. In a few instances specific chromosomes are found in duplicate. This may be due to their initial retention in the hybrid line or to non-disjunction having occurred subsequent to cloning.

Chromosome Rearrangements in Hybrids

Human chromosome rearrangements in hybrids permit the assignment of loci to chromosome segments by correlation of the presence of an identifiable human chromosome segment with the absence of an enzyme which has been previously linked to that chromosome. The reverse situation does not provide positive evidence for linkage, since a segment of the suspected chromosome may be hidden in the hybrid genome. Both pre-existing [6, 15] and spontaneous translocations [1] have been used in this manner.

Figs 8 and 9 show two rearrangements involving human chromosomes in our hybrids. To assist in identifying the origin of segments involved in rearranged chromosomes, photographs of selected chromosomes were scanned in a Joyce-Loebel Chromoscan densitometer. Fig. 8 illustrates a translocation between human chromosome 11 and another chromosome, tentatively identified as a hamster chromosome 1. While this

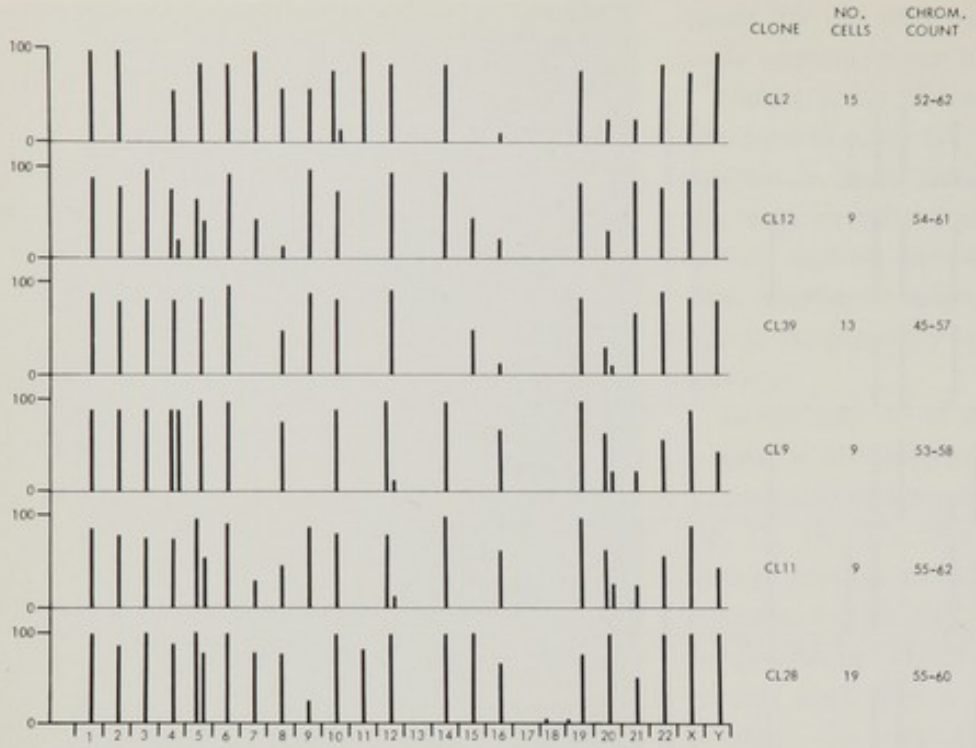


Fig. 7. *Abscissa:* chromosome number; *ordinate:* %.
Distribution of each human chromosome present in clones of Chinese hamster/human somatic cell hybrid (line CF1610-09).

rearrangement requires three breaks, two in the long arm of the hamster no. 1, and one in the long arm of human no. 11, it is the most likely based on banding patterns. This configuration was found in two clones of line CF1780-01 (fig. 6). Segregation of the rearranged chromosome containing the human chromosome 11 centromere and the enzyme LDH-A [1], as described above, will provide evidence for the location of this gene on chromosome 11. Fig. 9 describes a human t(7;19) translocation in line CF1610-09, but not yet studied by cloning.

Chromosome Contraction in Hybrids

Yerganian & Nell [21], Ephrussi & Weiss [4], and Kao & Puck [8] have noted that human chromosomes in interspecific somatic cell hybrids were more contracted than, and 'out of phase' with the chromosomes of the other parent at mitosis during early divisions after fusion. This phenomenon disappeared in a few generations. In all our hybrid lines, the human chromosomes exhibited a similar phenomenon, except that it was not transitory, but persisted as long as each line was maintained in culture. The human chromosomes in the hybrids appeared 'overcontracted' and shorter than their counterparts in human fibroblasts (compare figs 2 and 5, both at same magnification).

Preliminary analysis of experiments indicates that this is a real phenomenon, not related to observer biased selection of cells, differences in culture medium among cell types, or artifacts of slide preparation. It is, in fact, suggestive of the phenomenon of 'amphiplasty' described by Navashin [13] in interspecific sexual hybrids of *Crepis*, in which the one parental set of chromosomes displayed a total length different from that which it normally expressed in its native parental state. Navashin reasoned that this effect was mediated by the chromosomes of the other parent in the hybrid, and was not cytoplasmic, since reciprocal crosses produced the effect in the same direction.

Until a reduced Chinese hamster/human somatic cell hybrid containing a complete set of human chromosomes, and a partial Chinese hamster set, can be obtained, the mechanism of this phenomenon in our hybrids will not be completely understood.

Somatic Cell Hybrids and the Study of Chromosome Behaviour

In addition to using somatic cell hybrids to map human gene loci, they have been employed to

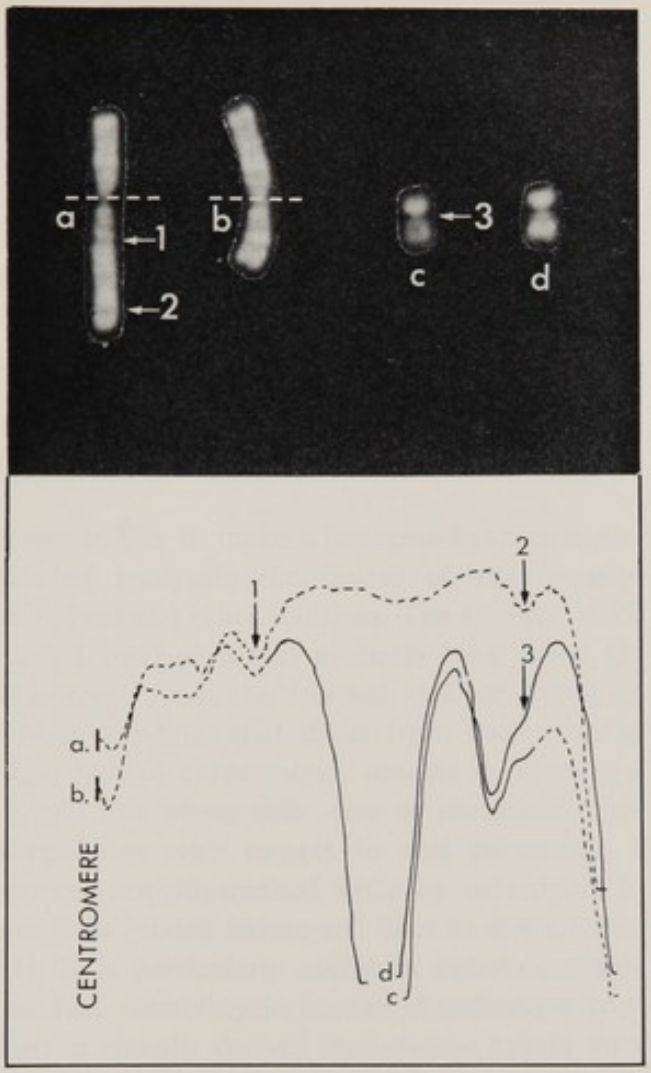


Fig. 8. Chromosomes and corresponding densitometer scans illustrating a hamster 1/human 11 translocation. (*a*) Long arm of hamster no. 1; (*b*) rearranged arm of hamster no. 1 corresponding to (*a*); (*c*) rearranged human no. 11; (*d*) human no. 11. Arrows indicate suspected breakpoints.

study the regulation of events in the cell cycle at the chromosome level [4, 14]. Human chromosomes adapt rapidly to their new environment. The degree to which human regulatory systems are operational in order to maintain viable hybrids is not always related to the number of human chromosomes present, since hybrids exist with as few as one human chromosome (fig. 3). It is possible, then, that the regulation of human chromosome behaviour can be controlled largely by the Chinese hamster cellular environment. This control is not necessarily precise, because chromosome contraction does not appear synchronous between the Chinese hamster and human complements. Somatic cell hybrids provide a unique cellular environment in which chromosome behaviour can be modified. Further studies of hybrids may provide new insights into the mechanisms controlling the initiation and maintenance of chromosome related events during DNA replication and mitosis. Such studies are dependent on the precise identification of chromosomes.

We acknowledge the technical assistance of Miss V. Niewczas-Late, Miss G. Isaacs, Miss A. Vust, and Mrs M. Riffell. This work was supported by M. R. C. (Canada) Operating Grants nos. MA-4061 and 4458 (J. L. H.) and also by the Children's Hospital Research Foundation, Winnipeg. The receipt is acknowledged of an M. R. C. Postdoctoral Fellowship to G. R. D. and a University of Manitoba Graduate Fellowship to P. A. G.

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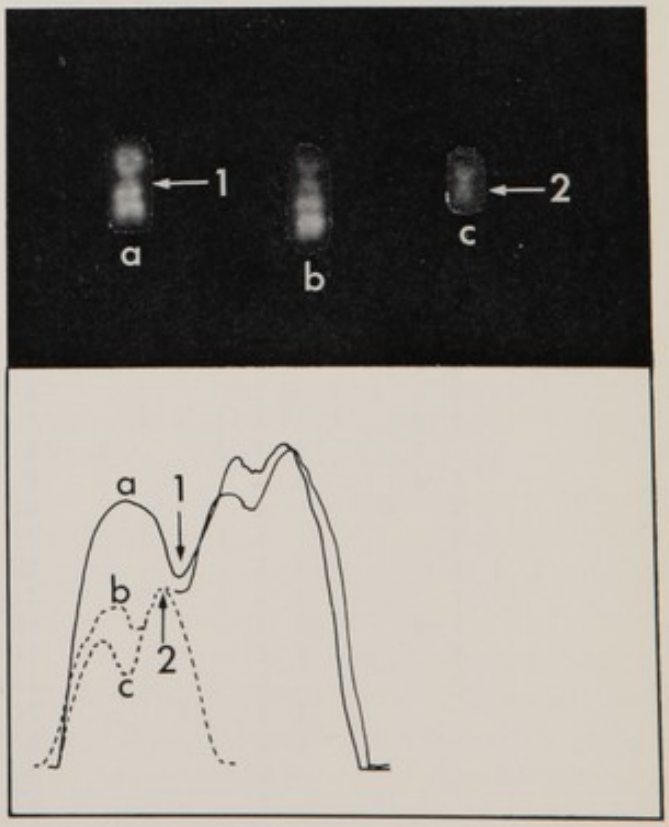
Fig. 9. Chromosomes and corresponding densitometer scans illustrating a human t(7;19). (*a*) chromosome 7; (*b*) t(7;19); (*c*) chromosome 19. Arrows indicate suspected breakpoints.



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Analysis of Heterogeneity

O. J. Miller

Department of Human Genetics and Development, and of Obstetrics and Gynecology,
College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032, USA

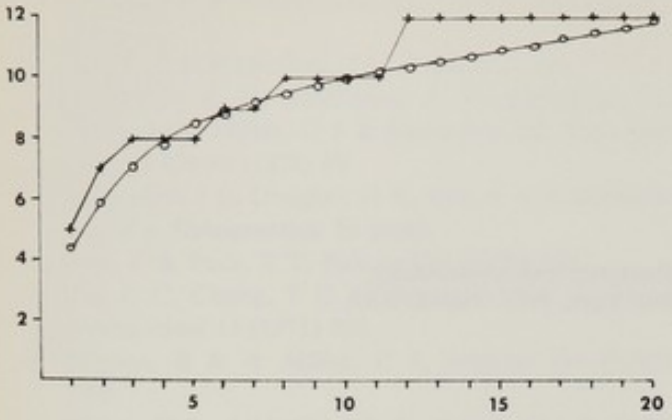
I would like to make a few remarks about methods of analysing the degree of heterogeneity in hybrid and other cell lines. The application of such a method would probably have saved Dr Hamerton from the fruitless task of trying to obtain sub-lines that differ from each other in their human chromosome content by cloning a hybrid line whose cells were so remarkably homogeneous with respect to this parameter. I worked out this method while we were trying to compare related mouse cell lines to one another [1]. It is particularly useful in detecting major residual variability in human chromosome content in clonally derived man–mouse hybrid lines [2]. Since such lines each arise from a single cell, the maximum number of structurally normal human chromosomes that can be present in the line cannot exceed the number present in that cell. However, continued loss of human chromosomes from a proportion of the cells subsequent to cloning could generate further heterogeneity, and so could subsequent structural changes of human chromosomes. Study of such lines may thus yield information on the degree of heterogeneity and the time at which it arises.

The first step in analyzing the degree of heterogeneity is to identify every human chromosome that is present in each of a small sample of cells. The results of such an analysis of 20 cells are shown in table 1. From this tabulation one can see at a glance the number of different human chromosomes present in each cell and calculate the mean number per cell. One can also determine the number of different human chromosomes that are present in the second cell examined which are not present in the first, or present in the third though not in the first or second, and so on. By considering the cells in as many different orders as there are cells, one can determine the mean value for each of these terms. One can then plot these values graphically (fig. 1) to obtain a curve whose slope provides a measure of the heterogeneity, or variation from cell to cell, in their content of different human chromosomes. A diploid cell population would, of course, have a slope of virtually zero because of the absence of significant heterogeneity.

Fig. 2 shows the heterogeneity curves we have obtained in several man-mouse hybrid cell lines. One curve is almost flat, reflecting a homogeneous population of cells, almost every one containing the same two human chromosomes, and only those two. I suspect the hybrid line Dr Hamerton

Table 1. *Human chromosomes present in 20 cells of one man-mouse somatic hybrid line* [2]

Cell no.	Number of copies of each chromosome											
	2	10	11	12	13	17	19	20	22	X	Y	3p–
1		1	1	1	2					1		
2		1		1	1		1		1	1		
3		1		1	2	1	1					
4			1	1	2	1				1		
5			1	1	2	1				1		
6		1			2	1		1		1		
7		1	1	1	1		1		1	1		
8	1			1	1	1			1			
9					2	1			1	1		
10			2		2					1		
11		1	1	1	1					1		
12					2	1	1			1	1	1
13		1		1	1	1				1		
14					1	2			1	1		
15			1		2	1				1		
16		1			2					1		
17			1		1	1						
18					2	1						
19					2							
20					1							



Figs 1, 2. *Abscissa:* no. of cells; *ordinate:* cumulation number of different human chromosomes.

Fig. 1. Heterogeneity curve, obtained by plotting the cumulative number of different human chromosomes in one man–mouse hybrid; +, considering the cells in the order shown on table 1; ○, considering the cells in 20 different orders (from [2]).

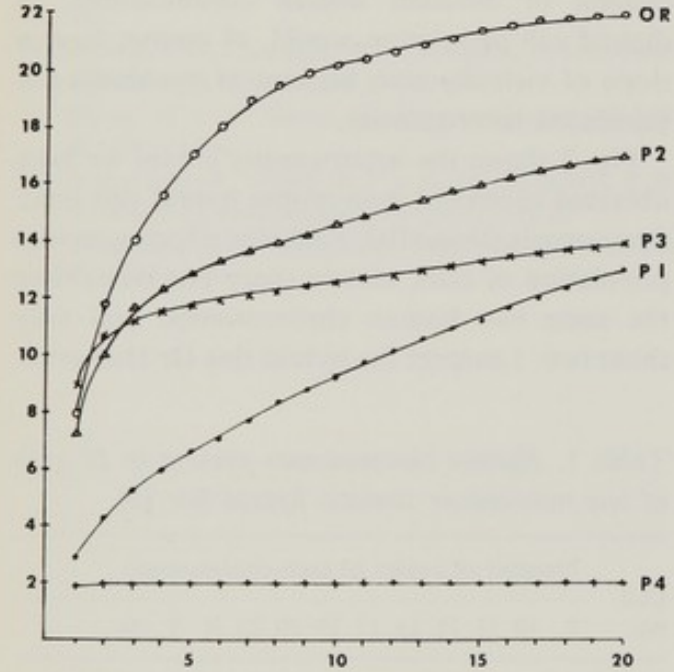


Fig. 2. Heterogeneity curves from five more man–mouse hybrid lines (from [2]).

was studying, despite its much greater content of human chromosomes, had a flat curve like this, a feature which would have been obvious after the analysis of the first 10 cells.

These heterogeneity curves may be useful in selecting hybrid lines whose clonal derivatives should have the greatest range in human chromosome content. The steeper the slope of the curve, the greater the variability, i.e. the greater the proportion of cells which contain a human chromosome not present in most of the other cells. It can be easily seen, for example, with respect to the cumulative total number of different human chromosomes, the increment in going from the next-to-last cell to the last cell examined is due solely to the presence of 'unique' chromosomes, which are present in only one cell in the sample.

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Discussion

Hamerton: I like the method you have described. Anything that saves time in these analyses is good. I am not, however, clear exactly what you are plotting, could you elucidate?

Miller: Yes. Along the vertical axis is plotted the average or mean cumulative total number of different, i.e. morphologically distinctive, human chromosomes. Along the horizontal axis is plotted the number of cells analysed. The value plotted above "1" on the horizontal axis is the mean number of different human chromosomes in a single hybrid cell. The value plotted above "2" is the sum of the first value and the mean number of different human chromosomes seen in the second cell examined which were not present in the first. And so on.

The Limits of Recognition of Human Chromosomes in Hybrid Cells

P. Pearson

Instituut voor Anthropogenetica der Rijksuniversiteit te Leiden, The Netherlands

Since the discovery that some measure of the relative genetic contributions to Sendai virus induced interspecific hybrid cells could be derived from examining the chromosomes [1], chromosomal analysis has become an integral part of the investigative procedures used. Until 1970 identification was dependent upon there being adequate morphological criteria for distinguishing the chromosomes of one species from another in terms of length, centromere position and more rarely the position of secondary constrictions [2, 3, 4]. The morphological similarity of chromosomes, both from the same and separate species, has limited the ease with which individual chromosomes have been identified in hybrid cells. Thus, in the now classical experiments of Green and his coworkers [4] in which they eventually identified the human chromosome 17 as the one carrying the thymidine kinase locus, initial observations [5] resulted in their identifying a C group chromosome as the carrier for this gene. The advent of the differential staining techniques, which permit the unequivocal identification of all individual human chromosomes [6], has vastly increased the resolution and speed of chromosomal analysis of hybrid cells [7, 8, 9].

It is the purpose of the present communication to review some of the current problems remaining in identifying human chromosomes in hybrid cells and in particular in relation to making gene assignments to individual human chromosomes. Briefly, this method of gene assignment, first used by Weiss & Green [5], depends upon the preferential loss of chromosomes of one species from a cell hybrid and then attributing an individual residual enzyme activity for that species to one of its retained chromosomes. The pattern of retention and loss of both chromosomes and enzymes in a series of independently derived hybrid cells will permit the localisation of gene loci to particular chromosomes.

An excellent review [10] clearly sets out the rapid advances that have been recently made in human gene localisation using the hybrid cell technique.

The observations described here are based on personal experience primarily gained from studying 4 human/animal hybrid cell systems, namely:

(1) Mouse Ehrlich ascites tumour / human diploid fibroblast;
(2) mouse L cell (A9 derivative) / Daudi lymphocytic line (human);
(3) Chinese hamster (various Don line derivatives) / human diploid fibroblast or lymphocyte;
(4) Syrian hamster / human diploid fibroblast.

With the exception of the Ehrlich ascites hybrids which were selected on their ability to attach to glass and overgrow the human cells, the other hybrids involved either HGPRT$^-$ or TK$^-$ animal cell lines and were selected in HAT medium.

Ehrlich Ascites Tumour/Human

The EAT line used contained a single metacentric marker and therefore human chromosomes should have been easily detected on morphology alone. Initial observations were carried out on clones grown for several months on glass and still having immunologically detectable human cell surface antigens. Chromosomal analysis of these revealed no human chromosomes but an increase in the number of biarmed mouse chromosomes produced by centric fusion. Very high initial fusion rates gave enough hybrid cells to permit studying the pattern of loss of the human chromosomes from 48 h onwards after fusion. Most hybrid cells had very high chromosome numbers, >300 (fig. 1) and the number of dividing EAT and human cells dropped rapidly. The

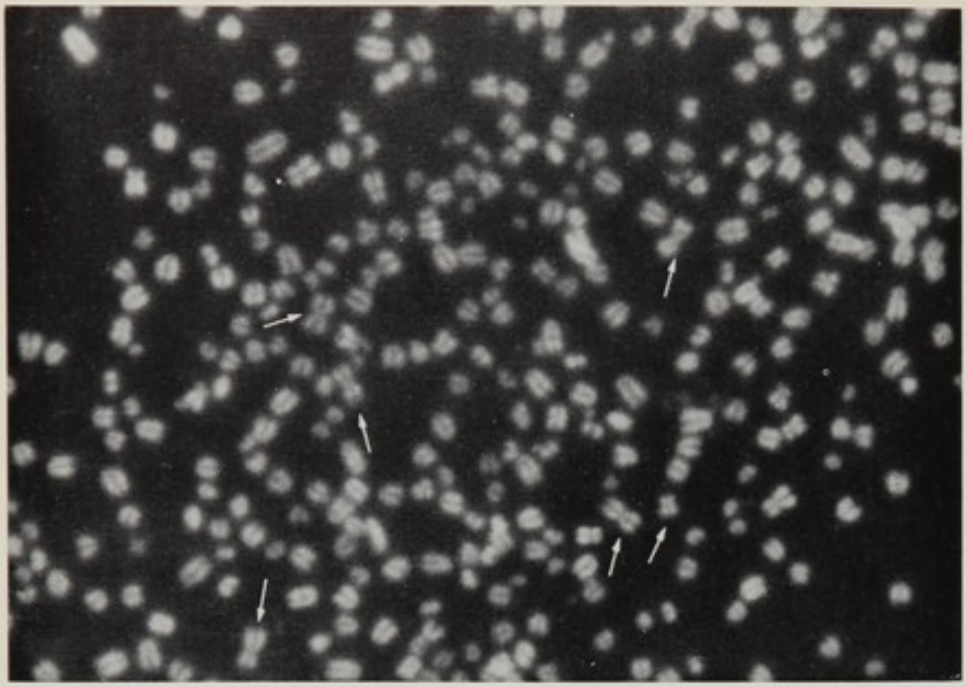
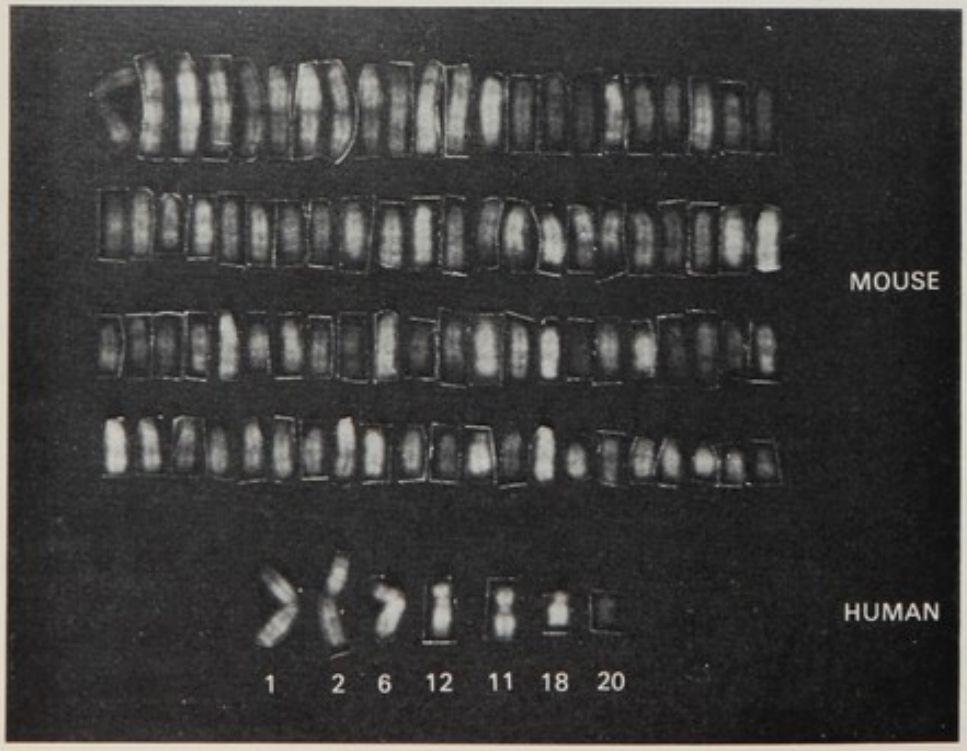


Fig. 1. Part of a chromosome spread from a single EAT/HUM hybrid cell at 48 h after fusion. Some of the human chromosomes are indicated by arrows.

total number of human chromosomes present in such cells could be calculated using a C-banding technique by virtue of the mouse chromosomes having a larger block of centric chromatin present. In 6 and 12 day cultures the majority of hybrids had chromosome numbers more amenable to karyotypic analysis, i.e. <300. From these it was concluded, that whilst most human chromosomes were still represented, there was a preponderance of particular ones (figs 2, 6). In addition the number of human chromosomes retained was related to the number of mouse chromosomes present (fig. 3). This, combined with the total reduction in chromosomes, would imply a balance being maintained between the mouse and human genotypes. In the Ehrlich ascites-human diploid fibroblast hybrids the loss of human chromosomes was rapid with most of the human chromosomes being lost in the first few divisions after fusion. Exceptions to this invariably involved large numbers of chromosomes

Fig. 2. Karyotype of a 6 day EAT/HUM hybrid cell.



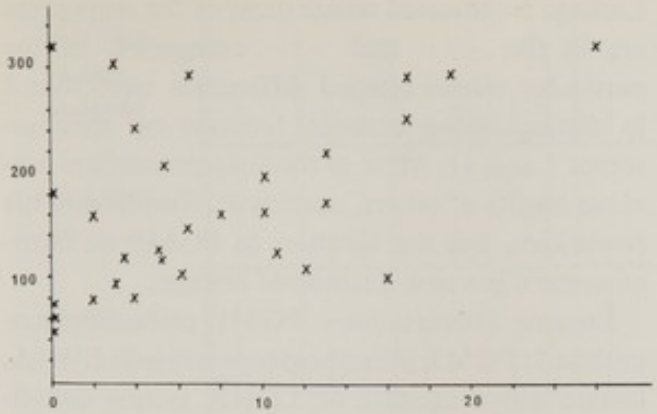


Fig. 3. *Abscissa:* No. of human chromosomes in 6 day culture; *ordinate:* total no. chromosomes present.

The numerical distribution of human chromosomes found in EAT/HUM hybrid cells with varying numbers of chromosomes.

and it was concluded that these cells had a greater tolerance to a human genetical contribution. In most instances of long term culture, although the ability of attachment to glass was retained, all identifiable human chromosomes were lost. An apparent increase in the number of metacentrics present was caused by centric fusion and/or isochromosome formation of mouse chromosomes. This was the only hybrid cell system in which there was an opportunity for examination of very early divisions of hybrid cells. The hybrid cells for this study were kindly provided by Dr S. Handmaker of the Dunn School of Pathology, Oxford.

A9/Daudi

In conjunction with Dr P. Allderdice a total of 18 clones were investigated, all grown in HAT selection medium for lengths of time varying from 6 months to over a year. Unlike the EAT hybrids grown for similar periods human chromosomes were found in all clones. Although the heterogeneous nature of some clones indicated a continuing segregation, the lack of widespread pulverisation of the type found in both the EAT and CH hybrids (fig. 4) showed that the A9 hybrids were much more stable. One possible explanation for this is that the human line used, in contradistinction to the other combinations, was a permanent cell line and this may have resulted in a higher hybrid stability. Because of several intensely fluorescent markers within an apparently normal karyotype the Daudi line should be an excellent choice for animal/human fusions in which chromosomal analysis is being considered. The detailed cytological analysis of these lines is being published elsewhere [11]. The cell lines for this study were kindly provided by Professor Henry Harris.

Chinese Hamster/Human Cells

In conjunction with groups in Rijswijk and Rotterdam, 50 clones from five fusions were examined for the presence of human chromosomes in relation to isoenzyme retention. The cytological analysis of these lines was principally carried out by A. Jongsma and myself. Karyotypes from three different clones are shown in fig. 5 and the pattern of overall chromosome retention is shown in part of fig. 6. Linkages between particular isozymes and chromosomes 1, 6, 11, 12 and 21 were found (see table 1).

Fig. 4. *a*, Pulverisation in a 4 month old Chinese hamster/human hybrid; *b*, similar chromosome damage in a 6 day Ehrlich ascites/human hybrid.

a

b

Table 1. *The segregation ratios found between particular human chromosomes and human isozymes in independent clones of Chinese hamster/human hybrids*

		+	−
Chrom. 1			
PGM1 +	+	11	2
6PGD	−	0	31
Chrom. 6			
PGM3	+	24	1
	−	2	18
Chrom. 11			
LDHA +	+	17	1
GPTC	−	1	30
Chrom. 12			
LDHB	+	15	0
	−	0	36
Chrom. 6			
PMDH	+	21	1
	−	0	16
PMDH			
PGM3	+	21	0
	−	2	16
Chrom. 1			
LDHA +	+	10	7
GPTC	−	0	29
Chrom. 21			
IPOB	+	30	1
	−	1	16

Linkage is indicated where most of the segregants are in the +/+ and −/− categories. In the particular clones studied difficulties were found in distinguishing between linkages on chromosomes 1 and 11. Most of the linkages confirm previous results of others, notably F. Ruddle and his coworkers, but the location of PGM3 on chromosome 6 is a new autosomal linkage.

Enzyme abbreviations: PGM1, phosphoglucomutase 1; PGM3, phosphoglucomutase 3; LDHA, lactate dehydrogenase A; LDHB, lactate dehydrogenase B; GPTC, glutamic pyruvic transaminase C; IPOB, indophenol oxidase B; PMDH, NADP dependent malate dehydrogenase; 6PGD, 6 phosphogluconate dehydrogenase.

In one particular clone found to be positive for the enzyme PGM1 only the short arm portions of a chromosome no. 1 could be found (fig. 7). The most likely interpretation for this is that this enzyme is located on the short arms, but as J. Hamerton has pointed out in the discussion to this paper, this matter can only be clearly resolved by further culture of the clone to see whether the enzyme activity is lost along with the putative piece of the human chromosome.

Under the circumstances of gross cytological heterogeneity found in the "clones", sampling

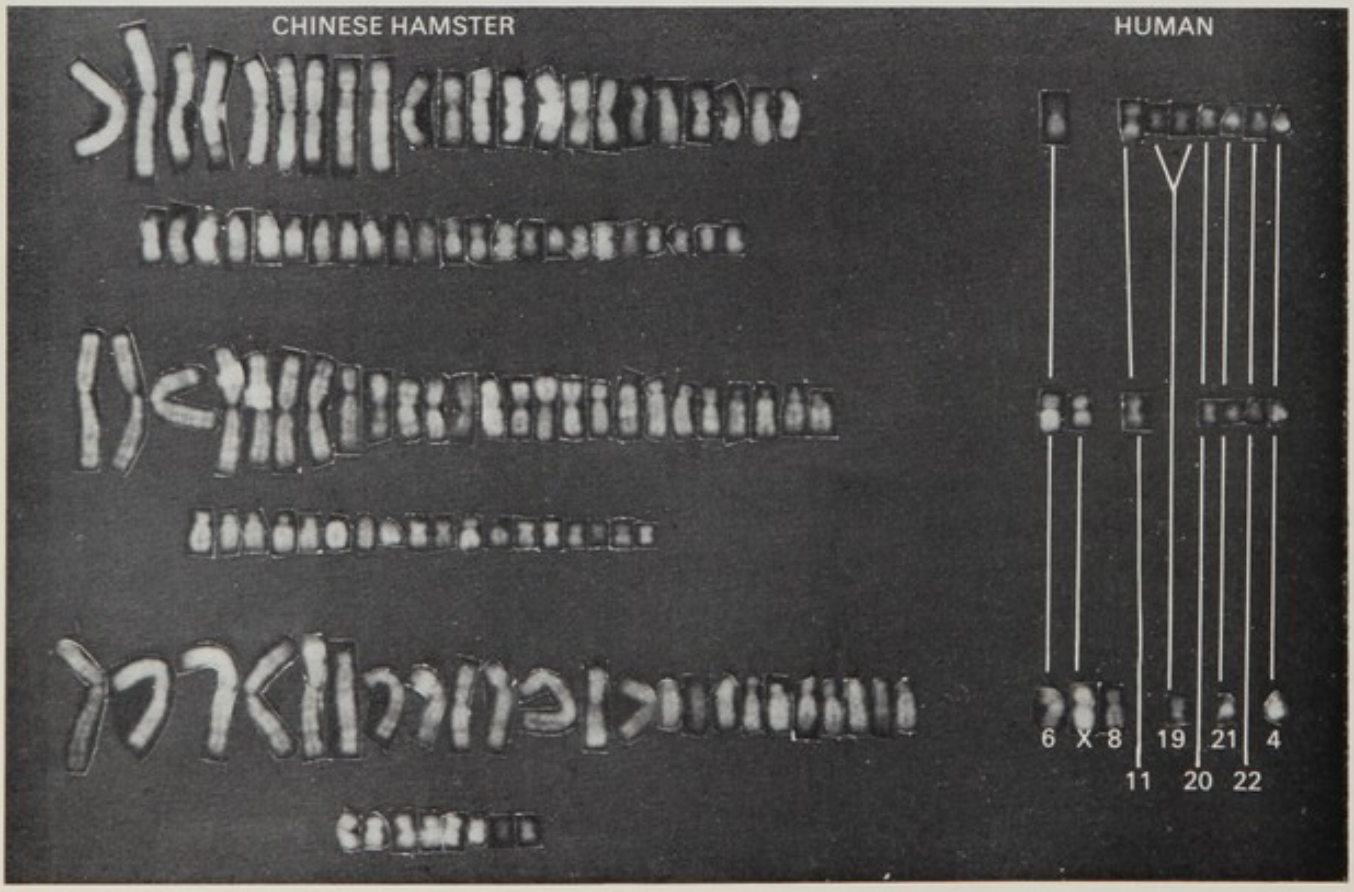


Fig. 5. Karyotypes from 3 clones of Chinese hamster/human hybrids. Note the small size of the human compared to the hamster chromosomes.

Fig. 6. The ratio of different human chromosomes retained in 5 different animal/human hybrid cell systems.

will underestimate the number of human chromosomes present and produce looser linkages than actually exist. Between 10 and 40 cells were karyotyped in each clone. Discrimination difficulties were found between the human chromosomes 16, 19 and 20 and small CH metacentric chromosomes. Comparison of prophase and metaphase chromosome groups clearly showed that the human chromosomes differentially contracted relative to the hamster chromosomes, such that in the prophasic groups they appeared to be relatively longer than in metaphase groups (fig. 8). Because of this and because the banding patterns are more clearly visible in uncontracted chromosomes, the human chromosomes could be more easily identified in uncolchicined prophasic spreads (fig. 9). In previous analyses based upon morphological criteria alone, the differential contraction found in this hybrid system has resulted in the misidentification of human chromosomes [12].

In a separate experiment, cells from a human X/3 (fig. 10), balanced translocation carrier were hybridised with Chinese hamster TK$^-$ cells. Analysis of the X linked biochemical and chromosomal segregation is shown in table 2.

The breakpoints in the translocation split the distal third of the long arm of the X from the rest of the chromosome. In table 2 the short arm and proximal portion of the long arm X chromosome is designated as X/3 and the distal long

Fig. 7. Chinese hamster/human hybrid which has a chromosome 1 with the long arms deleted (*arrow*) and also retains human PGM1 activity.

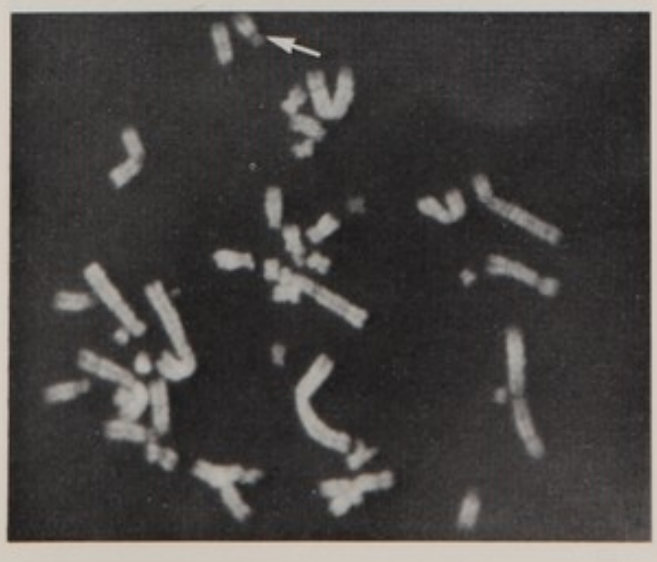
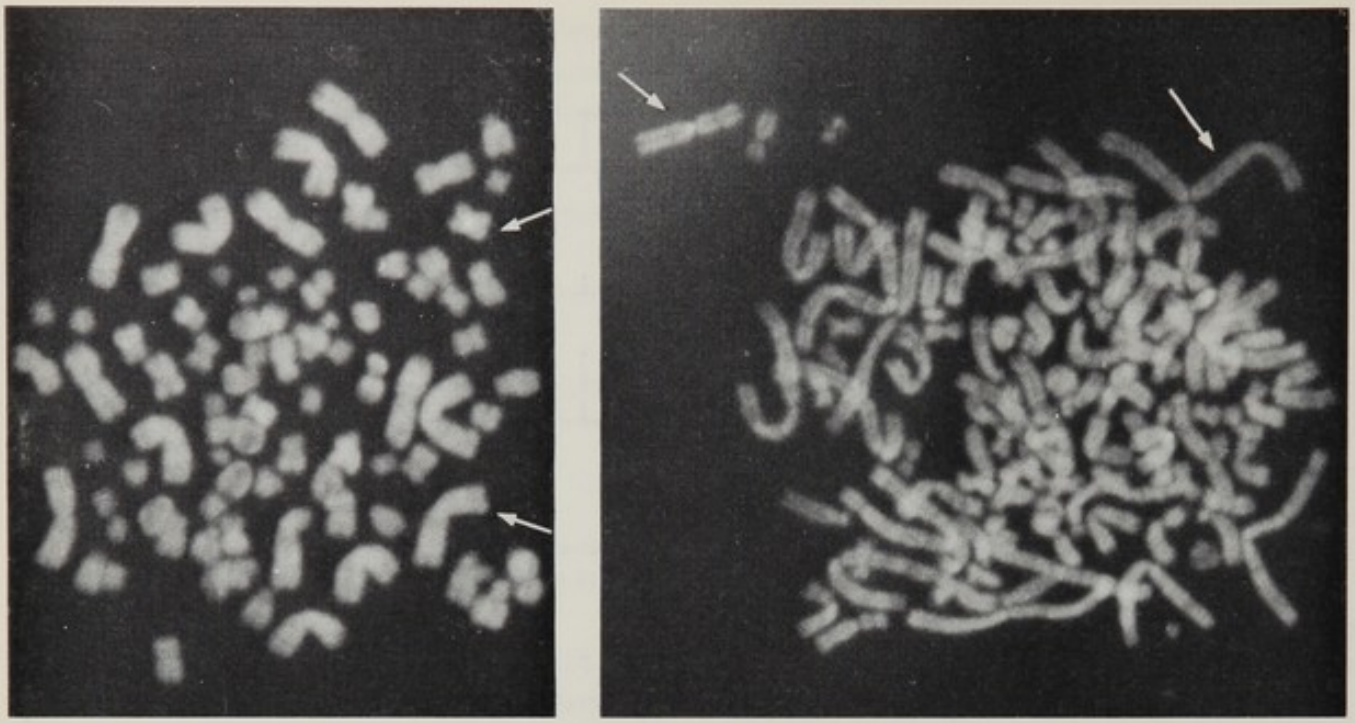


Table 2. *The segregation ratios found in separate clones between two X linked markers and two parts of the chromosomes (see text)*

	PGK	G6PD	PGK G6PD	No enzyme
X/3	3	0	0	0
3/X	0	2	0	1
X/3 + 3/x	0	0	2	0
No chromosomes	1	0	0	6



a *b*

Fig. 8. *a*, Chinese hamster/human hybrid metaphase with highly contracted chromosomes; *b*, a prophasic group from the same clone. Note how much more contracted the human 3 (arrowed and with a bright centromere band) is in *a* relative to the metacentric hamster chromosome (arrowed) than in *b*.

arm X chromosome as 3/X. In view of the conclusions of Grzeschik et al. [13] using a different X-autosome translocation, that HGPRT and G6PD were carried on the short arm, it is surprising that G6PD segregates out with the distal long arm portion.

Further studies are continuing with this and other X-autosome translocations to verify these gene localisations.

Syrian Hamster/Human Hybrids

Two clones resulting from a single hybridisation were examined and very few human chromosomes were still to be found in them. Not surprisingly,

Fig. 9. A prophasic group from a Chinese hamster/human hybrid. The banding patterns of the human X and Y are clearly visible in such a preparation.

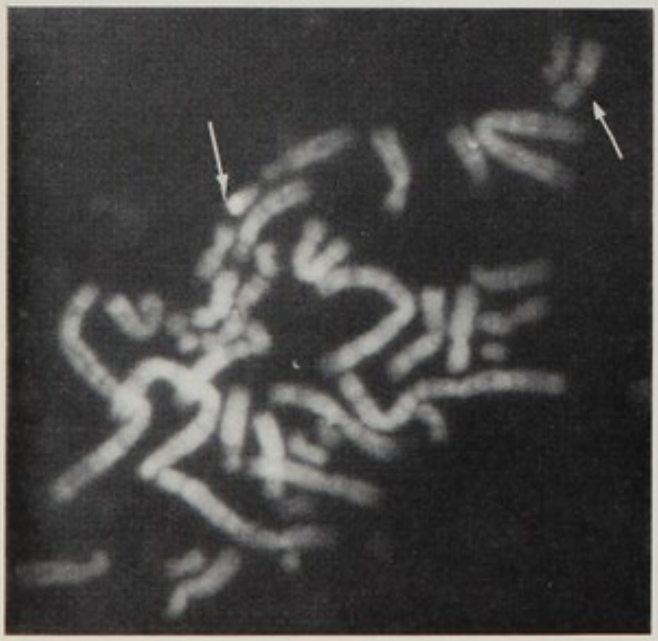
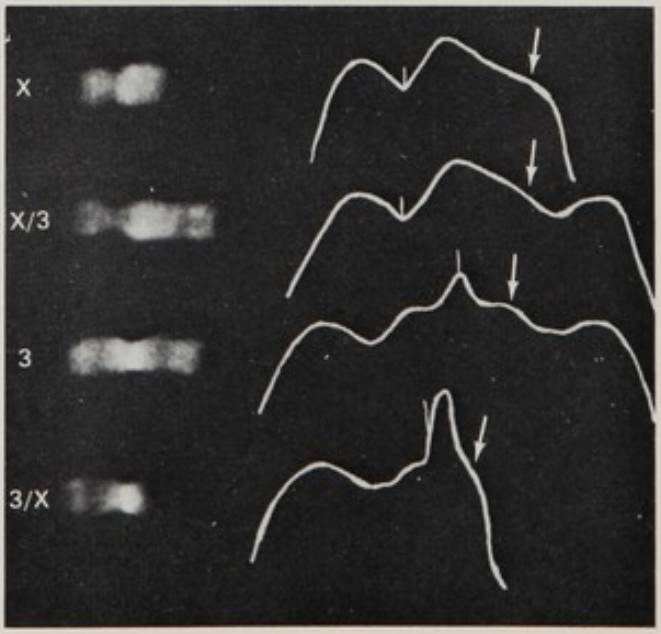


Fig. 10. A partial karyotype with density profiles to demonstrate the breakpoints in the human X/3 reciprocal translocation used in the hybrid cell studies.



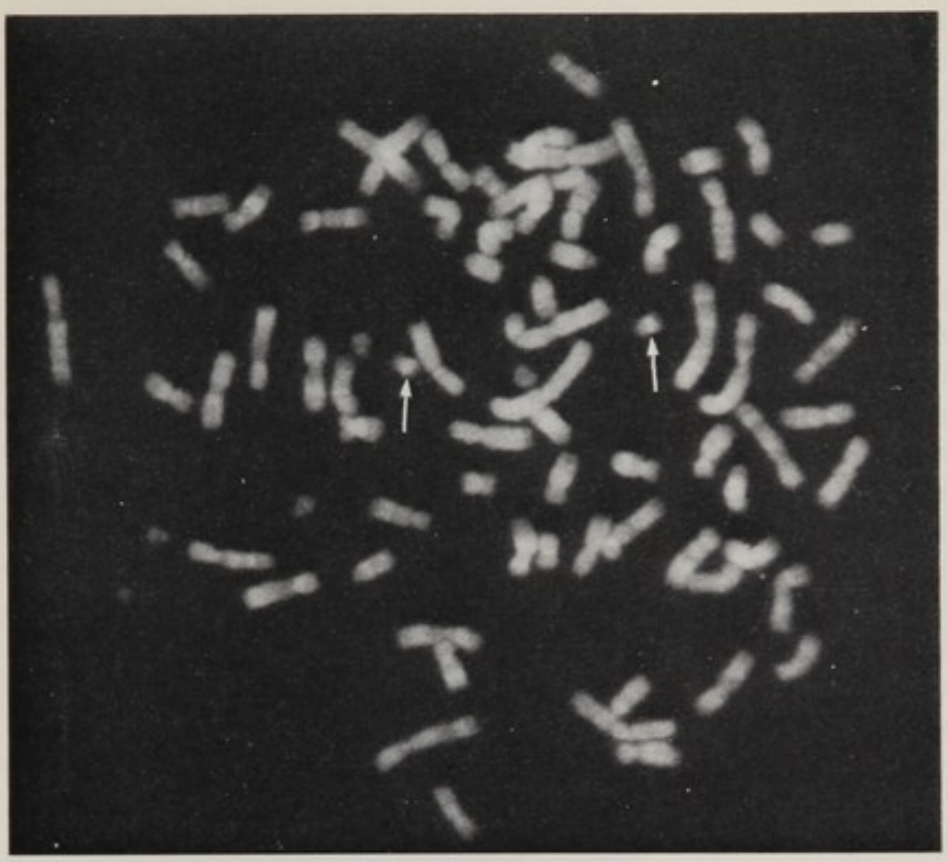


Fig. 11. Syrian hamster/human hybrid metaphase containing two human 21 chromosomes (*arrow*). Many of the hamster chromosomes are similar in size and banding pattern to the human chromosomes.

in view of the hybrids having been isolated by thymidine kinase selection, chromosome 17 was still present and in a proportion of the cells chromosome 21 also (fig. 11). In general the Syrian hamster karyotype is not as suitable as the Chinese hamster for hybrid cell studies since the size range and morphology of many of the chromosomes overlaps with that of the human karyotype.

Certain Problems of Gene Localisation Studies Using Hybrid Cells

It is clear from previous (for example see [10]) and the present investigations that gene localisation is entirely dependent upon being able to establish concordance for the isozyme activity and the presence of the chromosome carrying the isozyme locus. For the following technical reasons this is often difficult to establish and will give apparent discordance with either the chromosome or isozyme assay giving a false result dependent upon the reason for the error.

(1) The chromosome banding technique used is inadequate for identifying the material. For example, the C-banding technique is extremely useful for distinguishing mouse from human chromosomes, the quinacrine fluorescence for particularly identifying those human chromosomes which contain intensely fluorescent bands (see p. 145), the Giemsa 11 technique for distinguishing the human 9, and either quinacrine fluorescence or Giemsa banding for a general banding identification of the chromosomes. Inadequate banding will in general result in underidentification and cause the clone to be scored as positive for the enzyme and negative for the chromosome.

(2) In theory the cells studied within a clone originate from a single original fusion. However, since chromosome loss is occurring all the time, the "clone" rapidly becomes a mixed population of cells and sampling error can result in underidentification of the chromosome or overidentification if the sensitivity of the isozyme assay is rather low.

(3) The activity of isozymes present in somatic cells is normally regarded as being constitutive. That is, present in every cell in which the locus coding for the enzyme is present. There are now at least two enzymes known with variable expres-

sion, namely LDHB [14] and esterase 2. The latter which is normally expressed in mouse cells such as the Rag line, can be regulated by the presence of human controlling genes [15].

Variable isozyme expression can result in the chromosome being positive and the enzyme negative.

(4) The gene coding for the particular enzyme can be present but owing to chromosomal breakage and rearrangement an insufficiently large piece of the chromosome is left intact for it to be recognised. Here the chromosome would be scored as negative and the isozyme as positive.

(5) A chromosome can be falsely identified through a similarity of banding patterns. Thus, in certain of the human hybrids described here, a chromosome corresponding in quinacrine pattern to the human 9 was detected. However, use of the Giemsa 11 technique for detecting the human 9 [16] showed this chromosome to be non humanoid and probably a derivative of the Chinese hamster karyotype. This error will result in a positive chromosome and a negative enzyme assay.

(6) As pointed out earlier in the text, differential contraction can result in misidentification of chromosomes in which identification is based upon size and shape parameters alone. In addition the greater contraction of chromosomes from one parent over those of the other can mask banding features which require relatively uncontracted chromosomes for their full expression. The errors here can be in the directions of either underscoring or overscoring for the chromosomes concerned.

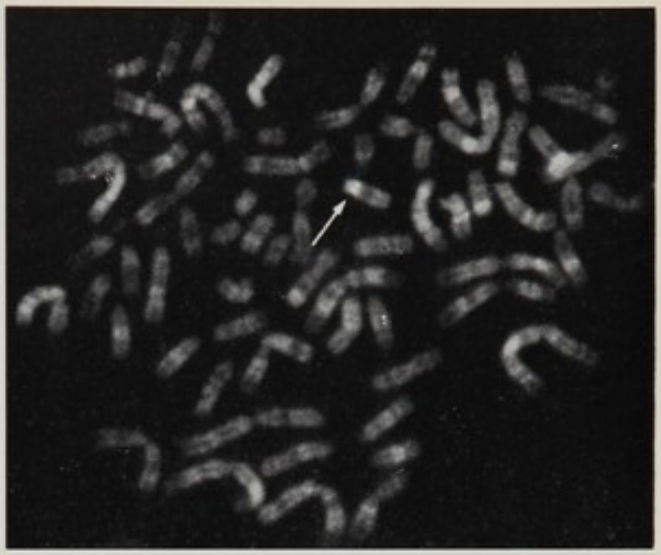
(7) The apparent patterns of loss of chromosomes are not random and are dependent upon different factors:

A, Examination of fig. 6 shows that some human chromosomes are retained much more frequently than others. That is, they make a lower contribution to hybrid instability resulting in cell death. It is patently difficult to make gene assignments to those chromosomes which are retained or lost in most clones. Ideally approximately equal numbers of both types of event are required.

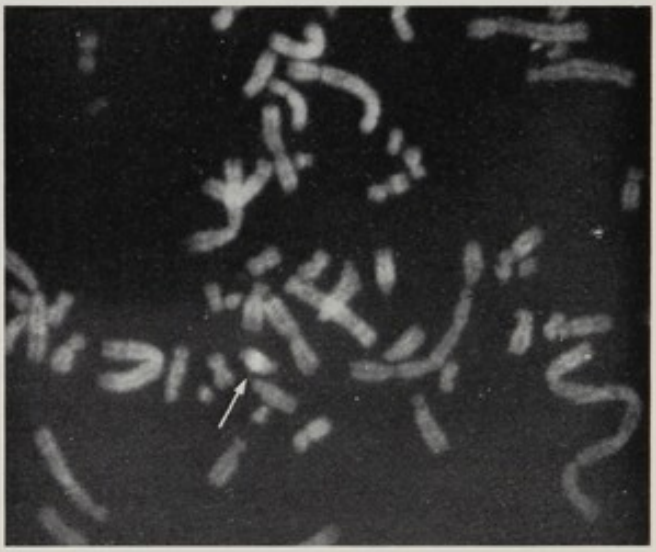
B, The growth medium can have a selective influence such that independent clones can exhibit a similar pattern of chromosome loss when treated with the same medium. Thus, in certain of the hybrids described here, growth in 8-azaguanine appears to have not only resulted in loss of the X chromosome but also in most of the autosomes with the exclusion of chromosome 21.

C, Clones which have been isolated from a single mixed population may not have had an independent origin, i.e. a relatively small number of fusions constitute the mixed population and an early non disjunction event in one of these is expressed in many of the clones.

These errors will not produce discordance for chromosome and enzyme presence but will result in the clones showing little or no independent segregation for a particular chromosome.



a



b

Fig. 12. *a*, A structural rearrangement involving the long arms of the human 3 (arrowed) in a Daudi/A9 hybrid clone; *b*, a structural rearrangement involving a human Y chromosome in a Chinese hamster/human hybrid clone. N.B. Both of these rearrangements were probably identified by having intensely fluorescent chromatin.

De Novo Chromosomal Rearrangements

Eight examples of structural rearrangements involving human chromosomes were seen in the material studied. Five of these involved chromosomes containing intensely fluorescent chromatin, see for example fig 12*A*, *B*, and the remaining three had major portions of large chromosomes.

It can be concluded that unless a large part of the banding pattern is retained then the nature of a structural rearrangement is difficult to assess in hybrid cell material. However, the presence of intensely fluorescent chromatin is an excellent indicator for particular bands found on some human chromosomes and increases the resolution in identifying parts of those chromosomes considerably.

Many rearrangements involving small pieces of chromosomes must go unnoticed.

Conclusions

(1) The differential staining techniques now permit the recognition of human chromosomes in a wide variety of hybrid cell systems.

(2) It is apparent that identification based on size and shape parameters alone is inadequate since differential contraction and changes in the proportion of biarmed to acrocentric chromosomes occurs in some hybrids.

(3) Since relatively few bands comprise a chromosome, the resolution in identifying the pieces of broken human chromosomes in hybrid cells is for the chromosomes rather low. Thus, out of 8 marker chromosomes all involved either the major portion or whole of a chromosome or a region containing an intensely fluorescent band.

The presence of human isozymes without recognizable chromosomes to attribute to them indicates the breakdown and possible incorporation of parts of human chromosomes too small to be recognized into animal chromosomes. The resolution is improved when intensely fluorescent bands are present and because of this quinacrine fluorescence and Giemsa 11 staining, which reveals the secondary constriction of chromosome 9, is the preferred general staining technique for examining animal/human fusions. For animal/animal fusions where no intensely fluorescent chromatin is involved, this advantage disappears and some form of G-banding can be used as an alternative.

(4) Segregation appears to occur not through loss of human chromosomes from hybrids per se but by differential cell death resulting from pulverisation of both human and animal chromosomes caused by a hybrid instability, itself caused by genetical instability resulting from random loss.

(5) The pattern of loss of human chromosomes from the hybrids studied and also from those already in the literature seems very similar. This has probably resulted in several laboratories making isozyme assignments to the same somatic chromosomes even though they have used different hybrid systems. An additional factor is of course that the same isozyme assays are used by different laboratories.

(6) The three smallest human chromosomes, namely 21, 22 and particularly the Y are retained more frequently than any others. The small number of genes present on them may make little contribution to hybrid instability and hence provide them with an advantage relative to larger human chromosomes. The apparently increased loss of certain larger chromosomes can be explained by their gene sequences making a greater contribution to hybrid instability.

The human material for the X/3 translocation fusions was provided by Dr Martin Bobrow of the MRC Population Genetics Unit, Oxford. The Ehrlich ascites hybrids were made by Dr S. Handmaker at the Dunn School of Pathology, Oxford. Professor Henry Harris provided the Daudi/A9 hybrids and the hamster/human hybrids were made, grown and analysed in conjunction with coworkers in Leiden, Rotterdam and Rijswijk. To these and many others, unmentioned, my deepest thanks.

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Discussion

Hamerton: I would like to make the point in relation to Dr Pearson's paper, that of the rearrangements in mapping. You showed a deleted chromosome No. 1 and drew the conclusion that because the clone was positive for PGM, that PGM had to be on the short curve. In my view, unless you have shown that PGM, in fact when the fragment is lost and vice versa in independent clones, you could make that statement and all the best you have a 50% chance of being right.

Then I think it is a pity that you make such a categorical statement.

Pearson: I quite agree in principle but not on probability.

N.B. I've clarified this point somewhat in the text to my paper.

de Grouchy: Have you considered the possibility that when you speak of differential condensation, it could be due to contamination from a different cell?

Pearson: This conclusion has been reached by looking at a large number of cells under different stages of contraction.

Cell Fusion with Enucleated Cytoplasms[1]

T. Ege, J. Zeuthen and N. R. Ringertz

Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, 104 01 Stockholm 60, Sweden

Cytochalasin B, a metabolite produced by the fungus *Helminthosporium dematioideum* was found by Carter [5] to induce enucleation in animal cells growing in tissue culture. If cells adhering to glass or plastic slides are exposed to cytochalasin, marked changes in the shape of the cell occur and frequently the nucleus is extruded into the tip of a long protrusion which only connects with the main cytoplasm through a narrow stalk. These stalks often break spontaneously causing a loss of nuclei from some cells. In order to increase the efficiency of the enucleation, the preparations can be centrifuged in such a way that the cytoplasmic stalks are broken by the centrifugal force [11, 15]. After centrifugation the enucleated cells (cytoplasms) remain on the glass slides and if returned to normal medium the enucleated cells will resume the flattened appearance of normal cells. The enucleation is often quite efficient and Prescott et al. [11] and Wright & Hayflick [15] have reported enucleation yields of better than 90 %. We have obtained similar results with HeLa cells, mouse fibroblasts, mouse macrophages and primary avian skin fibroblast cultures. In some other cases, however, we have been less successful due to unsatisfactory adhesion of the cells to the glass resulting in loss of cells during centrifugation. Enucleated cells have been found to remain viable for several days and capable of protein synthesis [10, 11]. The rate of protein synthesis, however, decays gradually and the enucleated cells therefore die after 3–5 days.

The fact that extensive enucleation can be obtained with a variety of cells raises the question whether in principle it might not be possible to reconstitute viable cells by introducing new nuclei into the enucleated cytoplasms. The fact that a variety of cells from different tissues and species can be fused with the aid of inactivated Sendai virus [7] to produce hybrid cells and the observations of Ladda & Estensen [8] and Poste & Reeve [9] that nucleated cells can be fused with enucleated cytoplasms indicate that such an approach is worth exploring. If viable cells can be formed from enucleated cytoplasms and isolated nuclei representing different genotypes or phenotypes new insights might be gained into mechanisms regulating gene expression and cell differentiation. Nuclei for such experiments would have to be undamaged by the isolation procedure and be surrounded by a layer of plasma membrane carrying receptors for Sendai virus. Nuclei obtained by the cytochalasin method may fulfil these requirements. We have therefore analysed the purity of nuclei and cytoplasms obtained by cytochalasin induced enucleation of HeLa cells and mouse fibroblasts. Furthermore we report experiments where cells have been 'reconstituted' by introducing chick erythrocyte nuclei into enucleated cells.

Enucleated Cytoplasms

The enucleation of cells attached to glass slides is illustrated schematically in fig. 1. After centri-

Table 1. *Interferometric dry mass measurements of intact L929 cells and isolated nuclei*

	Mass units ± S.E.M	No. of measurements
Intact cells	28.7 ± 1.0	50
Nuclei (cytochalasin isolated)	11.0 ± 0.6	50
Nuclei (detergent isolated)	6.1 ± 0.2	57

[1] Read by N. R. Ringertz.

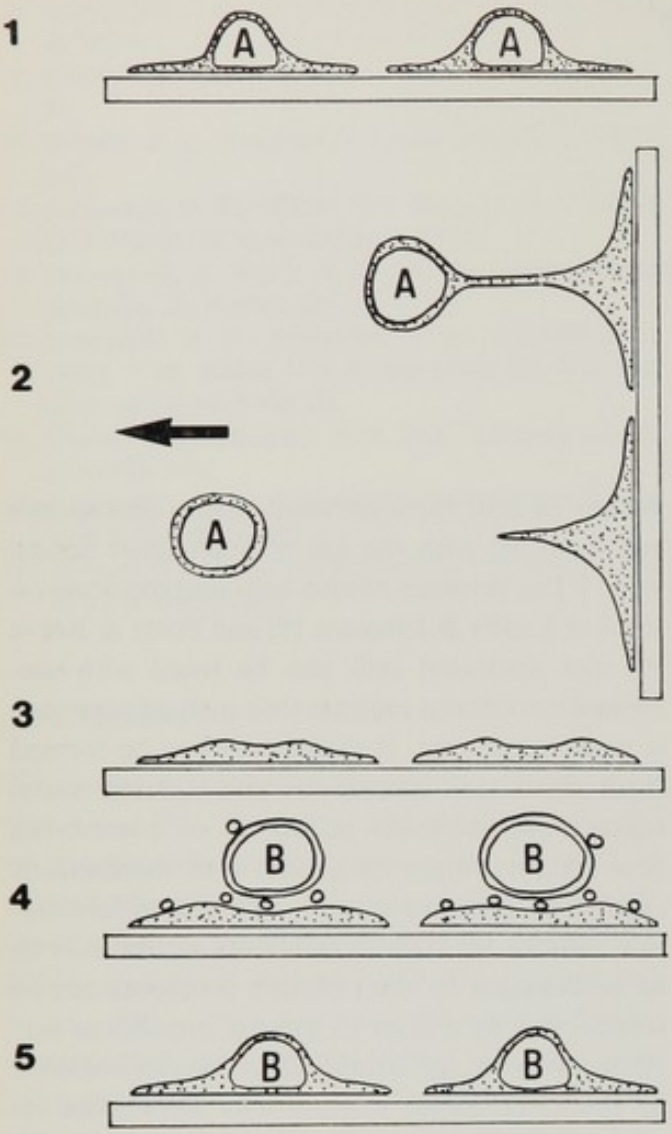
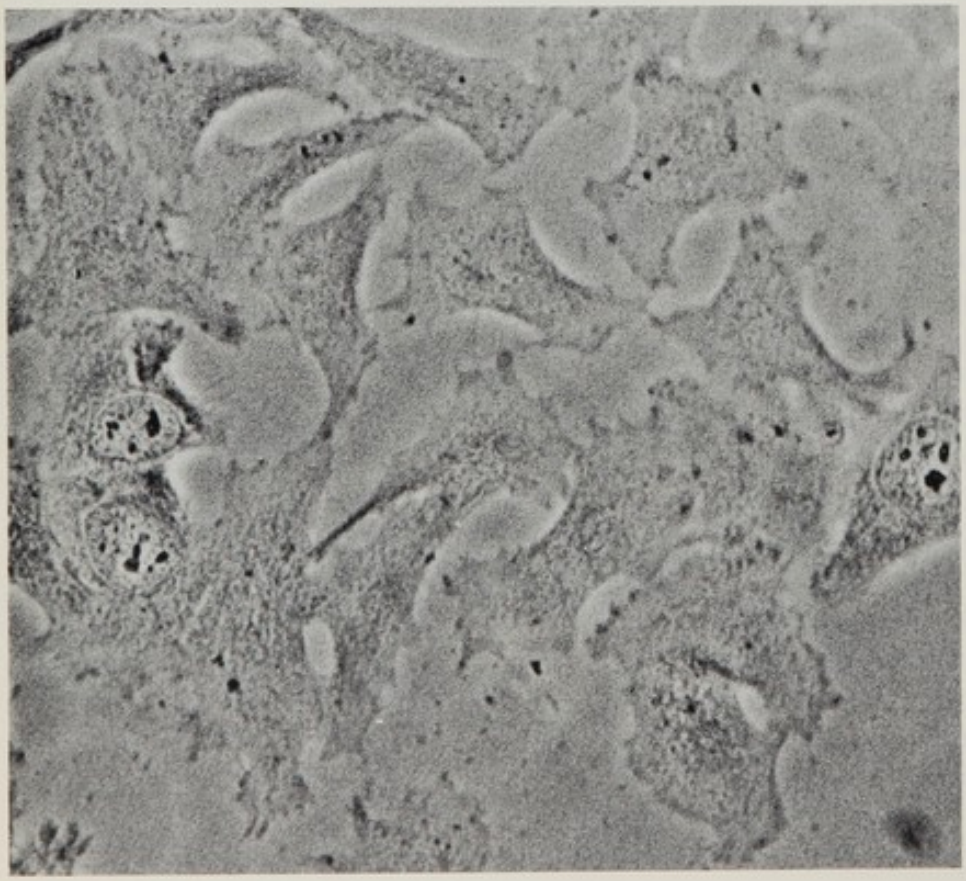


Fig. 1. Enucleation by cytochalasin treatment and centrifugation (*1*, *2*). The enucleated cells (*3*) are then fused by means of Sendai virus (Carlsson et al. 1970) with isolated nuclei or nucleated erythrocytes (*4*, *5*). Experimental conditions: Cells growing in monolayer on glass slides were placed upside down in centrifuge tubes in specially constructed holders. Centrifugation: 3 000 g for 40 min (37°C) in phosphate-buffered saline containing cytochalasin B at a concentration of 10 μg/ml.

fugation of cells in the presence of 10 μg/ml of cytochalasin B at 37°C, the slides with the enucleated cytoplasms are transferred to normal medium. The cytoplasms then resume a flattened appearance and also show limited movement indicating than the cytoplasms recover from the inhibition of motility caused by the cytochalasin. As shown in fig. 2 the cytoplasms of HeLa cells also show a form similar to that of the intact cells. The enucleated cells are viable as judged by dye exclusion tests. Shortly after enucleation the rate of protein synthesis as measured by the incorporation of ^{3}H-leucine is virtually the same as in nucleated cells. Protein synthesis continues at a gradually declining rate for 3 days. Five days after enucleation practically all the enucleated cells have rounded up and died.

Fig. 2. Phase contrast microphotograph of enucleated HeLa cells together with a few nucleated cells.



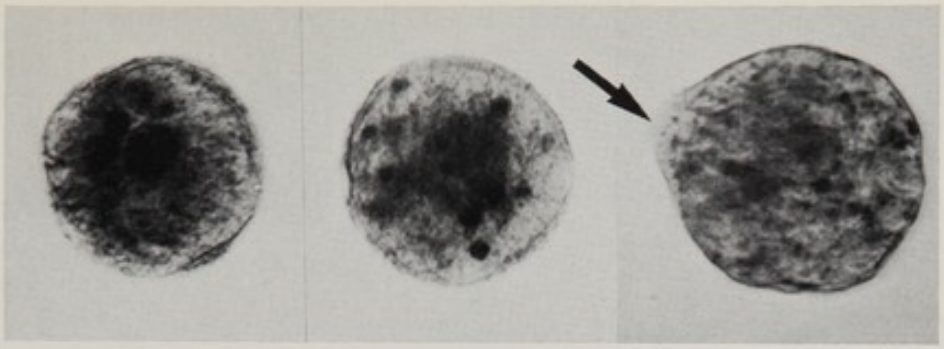


Fig. 3. UV microphotograph of A9 mouse fibroblast nuclei isolated after cytochalasin treatment and centrifugation. The nuclei appear to be surrounded by an intact plasma membrane and in some cases show small remnants of cytoplasm (*arrow*).

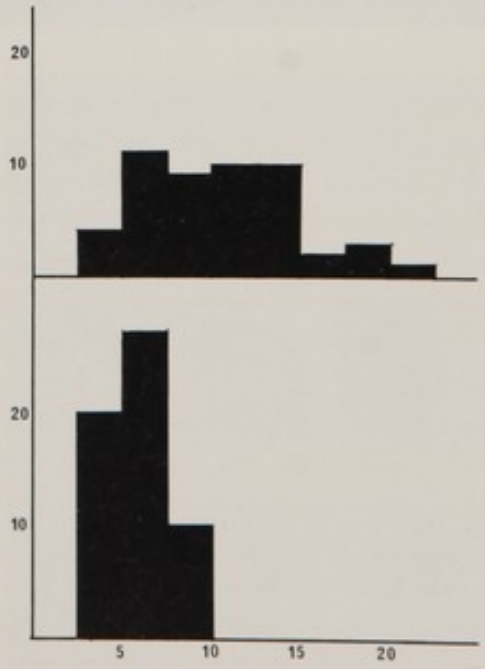
Nuclei Isolated by Cytochalasin Treatment and Centrifugation

The nuclei extruded during the enucleation procedure can be collected from the bottom of the centrifuge tubes. These nuclei appear to be intact as judged by UV-microscopy (fig. 3) and fewer than 10% of the nuclei stain with trypan blue. These results strongly suggest that the nuclei are surrounded by an intact plasma membrane. That this was the case was confirmed by electron microscopy. The extruded nuclei were seen to be surrounded by a small layer of cytoplasm and an intact plasma membrane. In some cases small remnants of the endoplasmatic reticulum and intact mitochondria could be seen to be included inside the cytoplasmic layer. The remnants of cytoplasm can also be seen in UV-microscopy (fig. 3). Dry mass measurements by microinterferometry [6] show that the dry mass of the cytochalasin nuclei is significantly higher than that of detergent isolated nuclei (table 1).

Reconstitution of Cells

UV-inactivated Sendai virus when added to a cell mixture induced aggregation and fusion of cells into multinucleated giant cells. Whether this technique can also be used to fuse cytochalasin nuclei into enucleated cells is not yet known. Previous experiments by Ladda & Estensen [8] and Poste & Reeve [9] have, however, demonstrated that nucleated chick erythrocytes can be fused with cytoplasms from mouse cells. Since chick erythrocytes lyse when exposed to Sendai virus and lose their cytoplasm before fusing with other cells [14] these experiments may be more or less equivalent to a reconstitution of a cell from a nucleus and a cytoplasm. We have, however, obtained different results which show that chick erythrocytes often fuse without prior lysis and that the chick erythrocytes contribute cytoplasmic material (hemoglobin) to heterokaryons. Undoubtedly, however, the chick erythrocyte offers special possibilities for 'reconstituting' cells and it is also a very useful type of cell for studies on gene regulatory phenomena. The chick erythrocyte nucleus is normally inactive with respect to RNA and DNA synthesis but undergoes a reactivation process in heterokaryons

Fig. 4. *Abscissa:* mass units; *ordinate:* no. of nuclei. Histograms showing the mass distribution of cytochalasin isolated L929 cell nuclei (upper frame) compared with the mass distribution of mild detergent isolated L929 cell nuclei (lower frame). Mass measurements were performed by means of interferometry according to Caspersson & Lomakka [6].



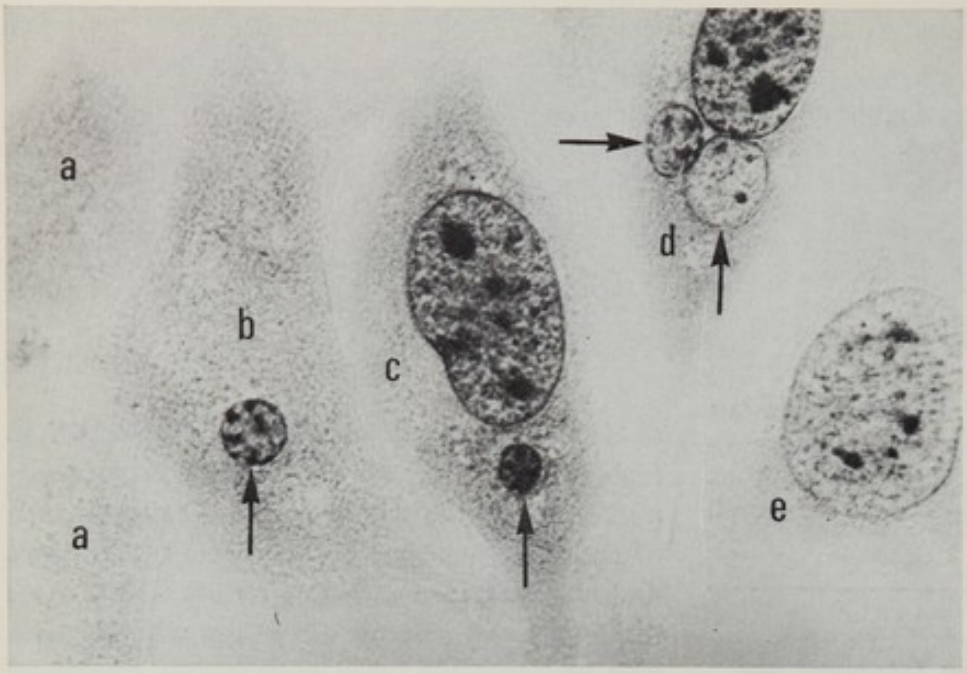


Fig. 5. Chick erythrocyte nuclei introduced into enucleated and nucleated A9 mouse fibroblasts: (*a*) enucleated mouse fibroblasts; (*b*) enucleated mouse fibroblast containing a chick erythrocyte nucleus; (*c*) mouse fibroblast × chick erythrocyte heterokaryon with one, (*d*) two erythrocyte nuclei; (*e*) normal mouse fibroblast.

formed by fusing erythrocytes with other more active cells. We have analysed the chemical composition of the chick erythrocyte nucleus at various stages of its reactivation in heterokaryons [1, 2, 12, 13]. During the first 48 h after fusion of chick erythrocytes with human tumour cells (HeLa) and before any major synthesis of chick proteins can be detected, the erythrocyte nucleus grows rapidly by taking up and selectively concentrating human nucleospecific macromolecules. It has been possible to study this process with the aid of the indirect immune fluorescence reaction using species specific antisera directed against

Fig. 6. Reactivation of 15 day chick erythrocyte nuclei in heterokaryons and in enucleated A9 cells. The sequence of morphological changes accompanying reactivation and nuclear swelling are similar in heterokaryons and in enucleated cells. *a*, 15 day chick erythrocyte; *b*, reactivation of 15 day chick erythrocyte nuclei in heterokaryons; *c*, reactivation of 15 day chick erythrocyte nuclei in enucleated cells.

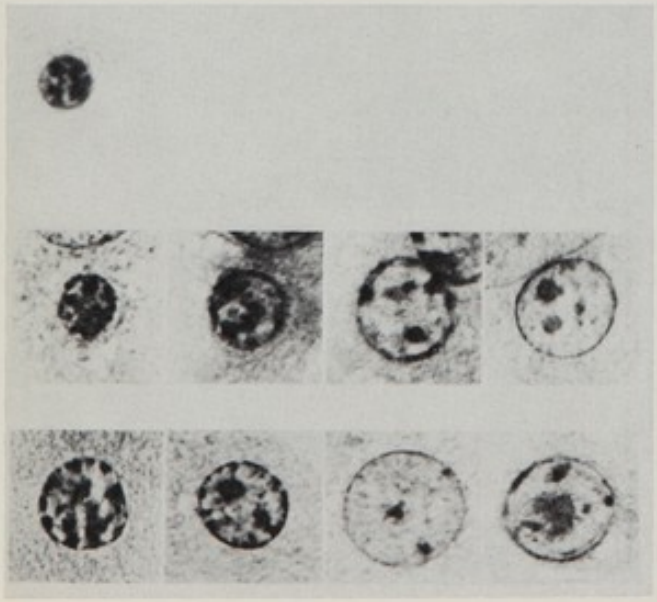
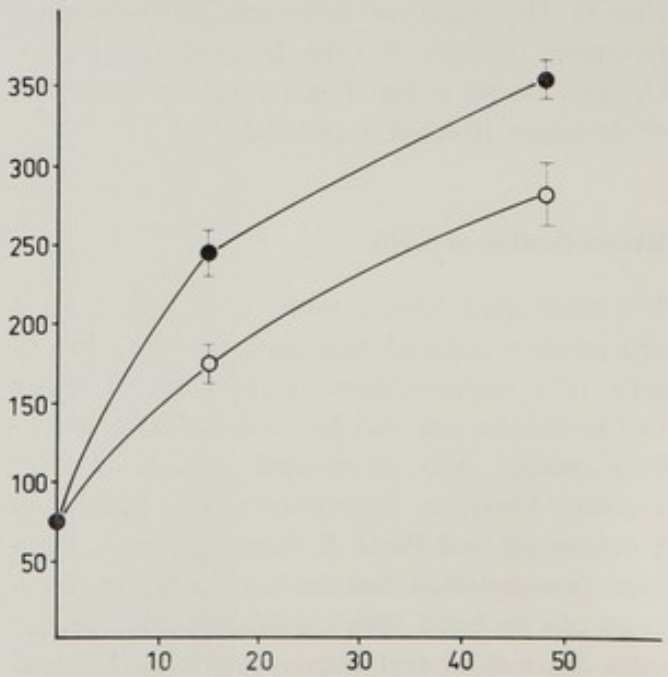


Fig. 7. *Abscissa:* time after fusion (hours); *ordinate:* mass units. Nuclear swelling as measured by interferometric dry mass measurements during reactivation of chick erythrocyte nuclei in ●, enucleated A9 cells; ○, in heterokaryons. Range bars indicate ± S.E.M.



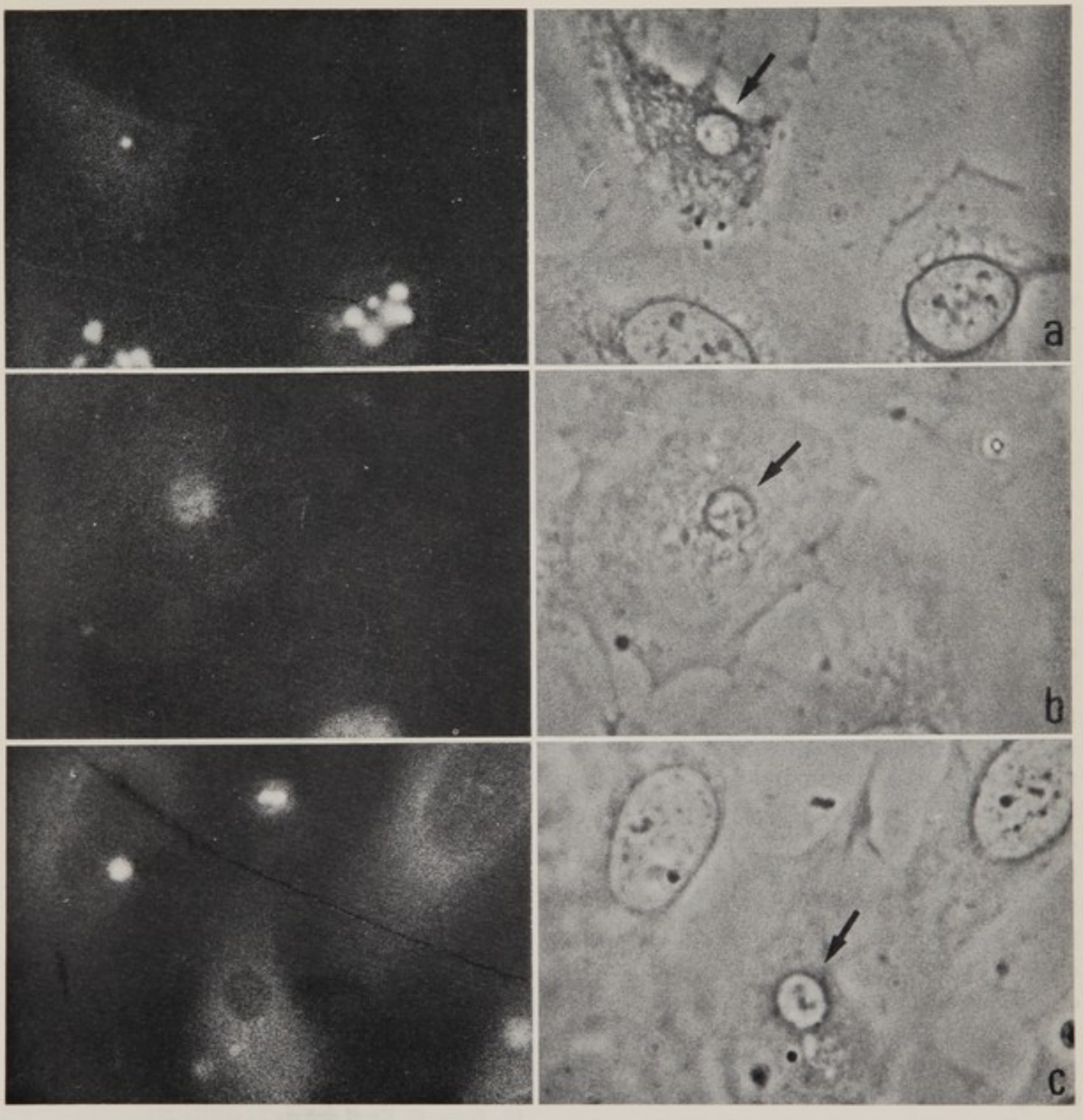


Fig. 8. Migration of human nuclear and cytoplasmic antigens during reactivation of chick erythrocyte nuclei in enucleated HeLa cells. Indirect immunofluorescence staining for: (*a*) human nucleolus specific antigens; (*b*) human nucleoplasmic antigens; (*c*) human cytoplasmic antigens. The chick erythrocyte nuclei are seen to concentrate the nucleolus specific and nucleoplasmic antigens and clearly exclude cytoplasmic antigens in the same way as previously reported for heterokaryons [12].

nucleoplasmic and nucleolar antigens [12]. Studies on the reactivation of chick erythrocyte nuclei in rat epithelial cell × chick erythrocyte heterokaryons have shown that the rate at which RNA synthesis, endogenous RNA polymerase activity and nuclear size increase are directly related to the rate at which rat nuclear antigens accumulate in the chick nucleus [4]. This suggests that the uptake of specific macromolecules from the surrounding cytoplasm or neighbouring 'host' cell nucleus may function as a signal turning on the chick genome. Since the concentration of nucleospecific molecules in the cytoplasm is very low (as judged from immune fluorescence studies of nucleospecific antigens) it appears that the chick erythrocyte nucleus may depend on the 'host' nucleus for supplying molecules of specific importance in the reactivation process.

In order to analyse whether the chick erythrocyte nucleus would undergo a normal reactivation process also in the absence of a 'host'

nucleus we have fused chick erythrocyte nuclei with enucleated HeLa cells (fig. 5). We find that the changes which take place in the erythrocyte nucleus after it has been introduced into enucleated HeLa cells closely correspond to what is seen during reactivation in chick erythrocyte × HeLa heterokaryons. The tightly condensed chromatin disperses and the nucleus grows in size (fig. 6). At late stages of the reactivation process, nucleoli form in the erythrocyte nucleus. The increase in nuclear size is not merely due to an uptake of water since micro-interferometric measurements of nuclear dry mass also show a marked increase. The rate of nuclear swelling as judged from nuclear size (fig. 6) and nuclear dry mass (fig. 7) is at least as rapid in cells reconstituted from erythrocyte nuclei and enucleated HeLa cells as it is in normal heterokaryons. We were then interested to see, if in the absence of a HeLa nucleus, the chick erythrocyte nucleus would show an accumulation of human nucleospecific antigens. As shown in fig. 8, the erythrocyte nucleus concentrated human nucleolus specific antigens into its newly formed nucleolus (fig. 8*a*), human nucleoplasmic antigens into its nucleoplasm (fig. 8*b*) and excluded human cytoplasmic antigens (fig. 8*c*) in the same way as has previously been reported for heterokaryons [12]. Thus also in the absence of a 'host nucleus' the material accumulating in the chick erythrocyte nucleus is not a representative cross section of cytoplasmic macromolecules. The cytoplasm appears to contain a pool of nucleospecific macromolecules or has the capacity to synthesize enough of such molecules to permit the reactivation of the erythrocyte nucleus. Preliminary data show that as the erythrocyte nucleus enlarges it also begins to incorporate ^{3}H-uridine into RNA. The fact that a nucleolus develops suggests that some of this RNA synthesis may be due to the synthesis of ribosomal RNA. Further details about nucleic acid synthesis and the expression of chick genes in 'reconstituted' cells will be reported elsewhere.

Conclusions

Enucleation of cells by means of cytochalasin treatment and centrifugation according to Prescott [11] produces nuclei with surprisingly little cytoplasmic contamination and cytoplasms which for some time remain viable in the enucleated state. By means of Sendai virus-induced fusion it is possible to introduce chick erythrocyte nuclei into enucleated fibroblasts and HeLa cells. The reconstituted cells are viable for at least a short period of time since the erythrocyte nucleus is activated from its dormant state, enlarges and resumes RNA synthesis. The reactivation of the chick erythrocyte nucleus in this system appears to proceed at least as rapidly as in heterokaryons showing that the presence of a 'host' nucleus is not necessary for reactivation to occur. In spite of this, the early growth of the erythrocyte nucleus is paralleled by a selective uptake of human nucleospecific antigens.

The present data also suggest that the nuclei obtained by cytochalasin enucleation of cells may be pure enough for other types of reconstitution experiments with enucleated cytoplasms.

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Discussion

Comings: Could you give some practical details about the cytochalasin enucleation technique?

Ringertz: The method for enucleation has been described by David Prescott (Exptl cell res 71 (1972) 480).

Applications of the Banding Techniques in Biology and Medicine

Medicine

The Identification of Whole Chromosomes or Parts of Chromosomes by the New Banding Techniques

Application of these techniques to certain medical problems

Karin E. Buckton

Medical Research Council, Clinical and Population Cytogenetics Unit,
Western General Hospital, Edinburgh, Scotland

Summary

The improved resolution afforded by the new chromosome banding techniques now enable a much more accurate analysis to be made of chromosome structural rearrangements in the human chromosome complement, and has revealed some hitherto unsuspected complexities. Information on the distribution of breakage and exchange sites in translocation carriers is presented. It is clear that the new techniques will considerably improve risk assessments, particularly in the case of balanced translocation carriers.

The significance of the new banding techniques to medicine can be summarised by saying that they bring a new order of precision to human cytogenetics. This can only result in more accurate identification and diagnosis of all previously recognised chromosome abnormalities, and the discovery of hitherto unrecognised chromosome aberrations. I would like to illustrate, in this paper, by reference to translocations, just one aspect of the way in which the increase in precision offered by banding techniques may be of importance to medicine.

Recently the chromosomes of the majority of individuals identified in the Medical Research Council's Clinical and Population Cytogenetics Unit as having a chromosome rearrangement from conventionally stained orcein preparations, have been reanalysed using either the fluorescent [6] and/or the acetic-saline-Giemsa [8] (ASG) techniques. We have found that among 40 reciprocal translocations ascertained through a balanced carrier, 7 translocations in 6 index cases were originally misdiagnosed (table 1). Three individuals were thought to have a deletion and two were believed to have a pericentric inversion. The misdiagnosis in each of these cases simply resulted from the fact that in orcein-stained preparations the translocation chromosome mimicked another chromosome in the human complement in its overall morphology. All that could be said of the orcein preparation of the remaining case (table 1) was that the patient lacked a chromosome 1, a chromosome 3 and a C-group chromosome and that these were replaced by three abnormal chromosomes. Her chromosome constitution was subsequently interpreted on both fluorescent and ASG preparations as being the result of two separate reciprocal translocations involving three chromosomes. One translocation was between the short arms of a chromosome 1 and the short arms of a chromosome 3,

Table 1. *Karyotypes of six individuals with reciprocal translocations unrecognised on orcein preparations*

Karyotype on orcein preparations	Karyotype on banded preparations
46,XX,Ep−	46,XX,t(8p+ ; 18p−) (fig. 1)
46,XX,Cp−	46,XX,t(8p− ; 15q+)
46,XY,Dq−	46,XX,t(1q+ ; 14q−) (fig. 2)
46,XX,inv(Bp+q−)	46,XY,t(4q− ; 9p+) (fig. 3)
46,XX,inv(2p−q+)	46,XX,t(2q− ; 10q+)
46,XX,−1,−3,−C,+B,+mar 1,+mar 2	46,XX,t(1p− ; 3p+)(3q− ; 9q+) (fig. 4)

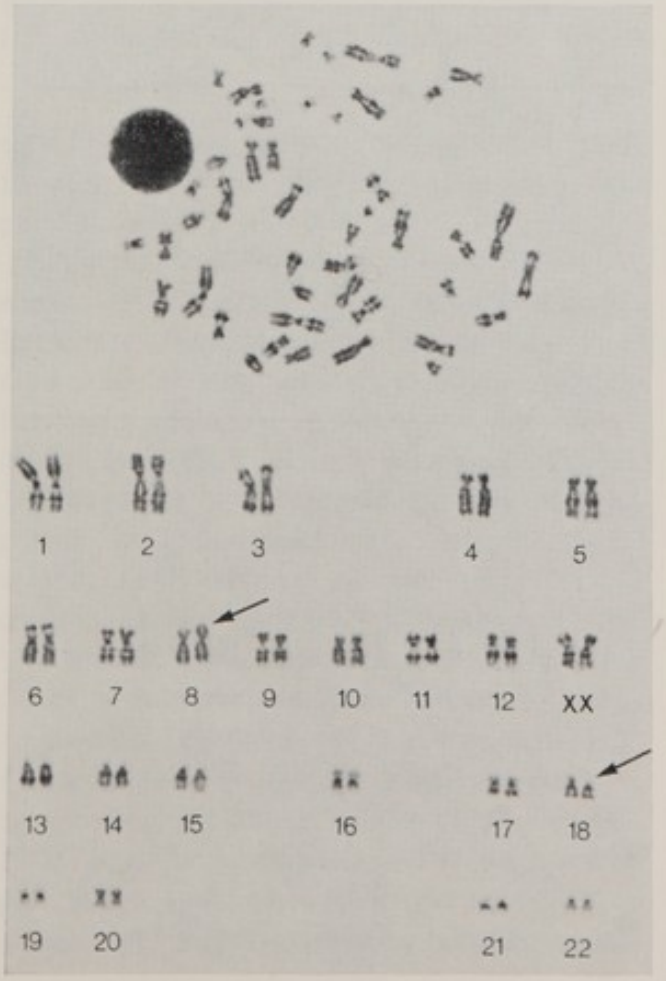
Table 2. *Five reciprocal translocations identified through an unbalanced carrier*

Karyotype of the balanced carrier[a]	Karyotype of the unbalanced carrier
46,XX,t(4;10)(q35;q23)	46,XX,−4,+der(4) mat
46,XX,t(7;19)(q32;q13)	46,XX,−19,+der(19) mat
46,XX,t(9;22)(q12;pl)	47,XY,+der(9) mat
46,XX,t(9;22)(q21;q11)	46,XY,−22,+der(22) mat
46,XY,t(9;16)(p24;q11)	46,XX,−9,+der(9) pat

[a] Nomenclature is that recommended by the Paris Conference (1971): Standardization in human cytogenetics [7].

while the other was between the long arms of the same chromosome 3 and the long arms of a chromosome 9 (fig. 4). It would appear that these two translocations are balanced, although the patient is severely mentally subnormal. The chromosomes of the parents of this patient were normal. These examples show that the higher resolution provided by the new techniques will considerably improve the identification of trans-

Fig. 1. Cell and karyotype stained by the ASG technique from patient with 46,XX,t(8p+;18p−) constitution.



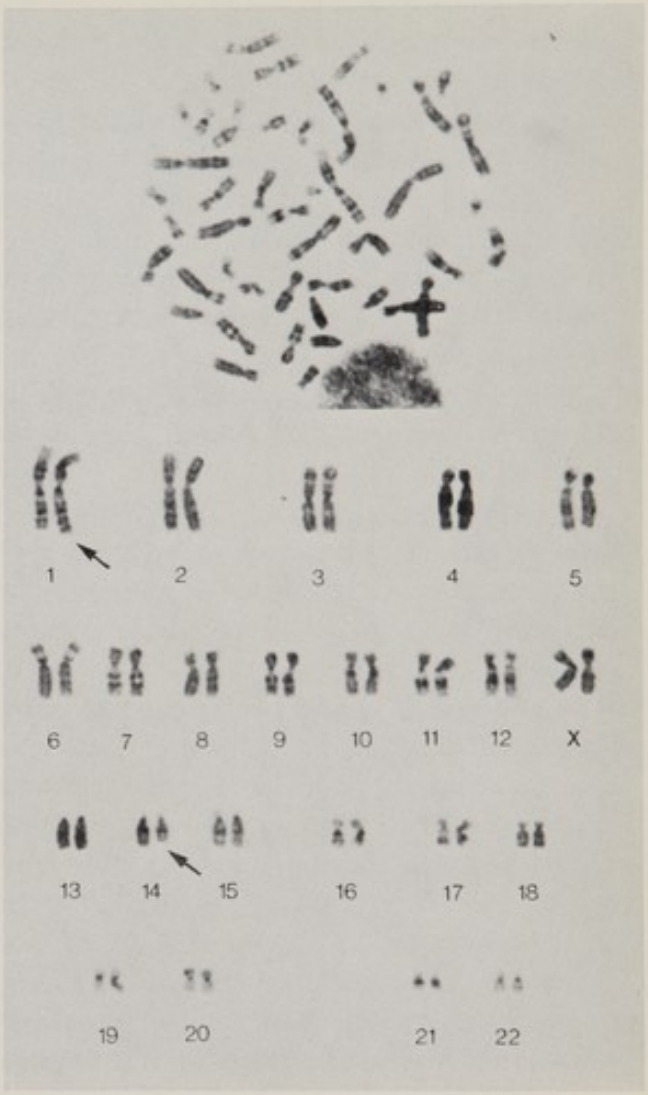


Fig. 2. Cell and karyotype stained by the ASG technique from patient with 46,XX,t(1q+;14q−) constitution.

located chromosomes and make more positive the antenatal diagnosis of a foetus with an unbalanced karyotype.

An examination was made of the distribution of the 80 break points at which an exchange of chromosome material had taken place. The expected number of breaks on a particular chromosome arm was calculated as a proportion of its length relative to the length of a complete haploid set, the length measurements being based on series 'D' in the Paris conference (1971): Standardization in human cytogenetics [7].

The total number of breaks analysed is small, but they appeared to be randomly distributed *between* chromosome arms. However, when the distribution of break points *within* chromosome arms was considered, it was found that there was an excess of break points in the centromere and

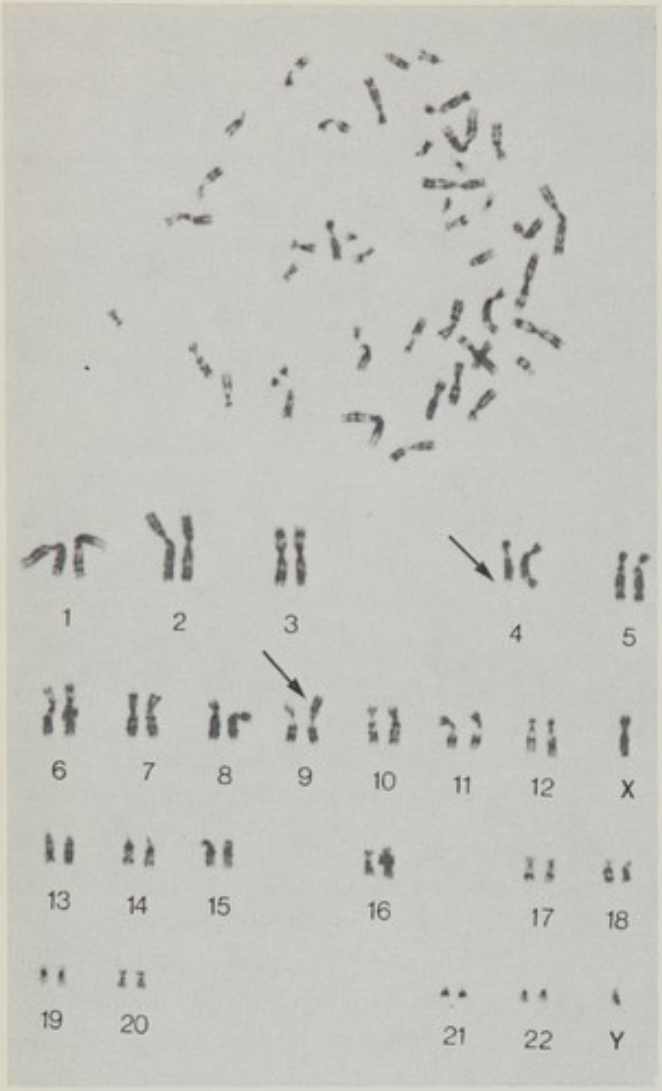


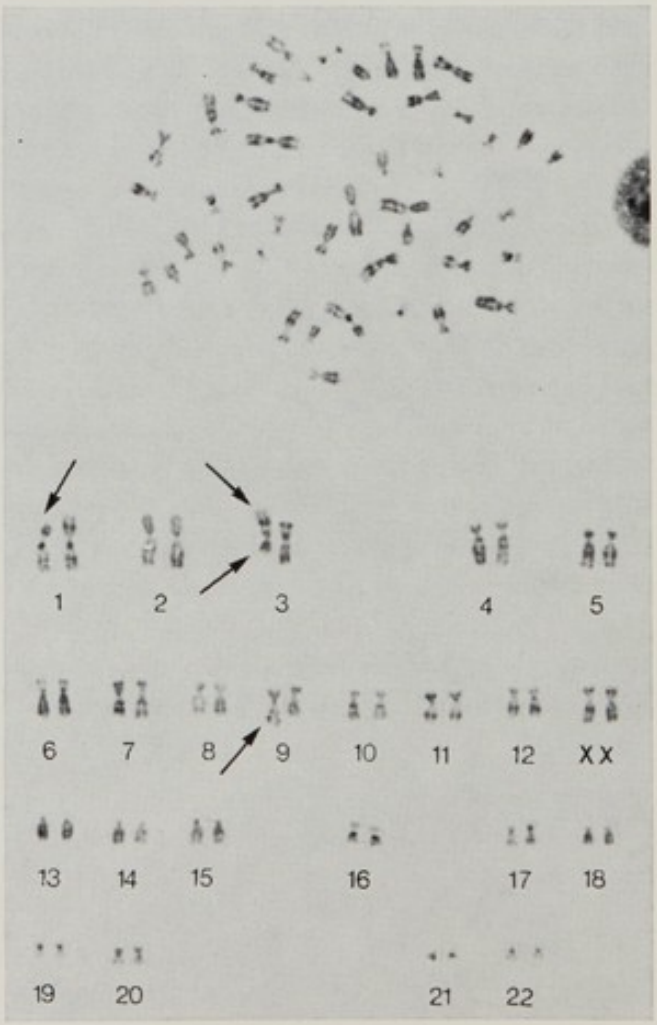
Fig. 3. Cell and karyotype stained by the ASG technique from patient with 46,XY,t(4q−;9p+) constitution.

terminal regions. It may be that the centromere and terminal regions are more liable to breakage and exchange than the rest of the chromosome. It seems more likely, however, that this is an observational bias, as all these translocations were originally identified from orcein preparations—hence favouring the detection of unequal translocations.

The 40 translocations considered above have been ascertained through a balanced carrier. If now we consider translocations ascertained through an unbalanced carrier it has been found from our own cases (table 2), and the published cases of others [1, 4], that in the majority of rearrangements so ascertained the exchange is between the terminal region on one chromosome and either the centromere or mid-arm region on the other. In the unbalanced carrier the chromosome with the minimal deficiency is present. From this, it would appear that chromosome duplication is more acceptable for foetal development than is chromosome deficiency. If the generality of this finding becomes evident with the study of further examples of unbalanced carriers, then it may be possible to delineate a group of balanced reciprocal translocation carriers who are particularly at risk to having a liveborn child with an unbalanced karyotype, i.e. individuals with reciprocal translocations where at least one of the break points is in the terminal region.

Let me now add a word of warning. The banding techniques have not yet given us all the answers. There are regions on the human chromosome complement that have no clear banding features. Therefore it is sometimes not possible to identify the origin of additional chromosome material. More worrying though is the apparent chromosome deletion in individuals who are phenotypically normal. In such instances one cannot be certain that it may not be a translocation. Take for example the patient who has a deletion of the long arm of a chromsome 21 (fig. 5). He is pheno-

Fig. 4. Cell and karyotype stained by the ASG technique from patient with 46,XX,t(1;3)(3;9) constitution.



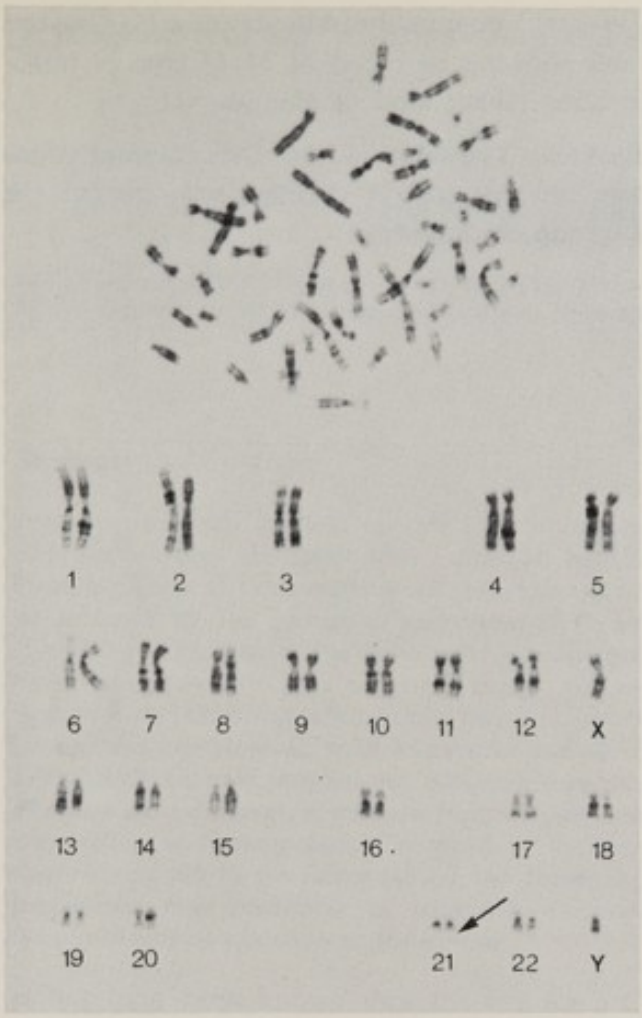


Fig. 5. Cell and karyotype stained by the ASG technique from patient with 46,XY,del(21q) constitution.

typically normal. Careful examination of ASG-banded preparations has not revealed the deleted portion. Is this a true deletion?

Since the development of the new techniques, a great deal of attention has been focused on the Robertsonian translocations (centric fusions) of the acrocentric chromosomes. If the fusion occurs between homologous chromosomes, the chance of that individual having a normal child is nil. Therefore for genetic counselling it is very important to know which chromosomes are involved. In Edinburgh we have identified the chromosomes involved in 24 D/D translocations. The probands were all phenotypically normal, except for one who had Down's syndrome having an additional chromosome 21 as well as the D/D translocation. Twenty-three centric fusions were between a chromosome 13 and 14. Of these 23, 19 are known to be transmitted through several generations often without any deleterious effect.

One D/D translocation was between a chromosome 14 and 15 and was detected in a patient who presented with a history of spontaneous abortions. As material from her relatives was unobtainable it was not possible to establish whether this chromosome abnormality had any relevance to her medical history.

A similar non-random distribution of the D group chromosomes involved in D/D translocations has been reported by others [2, 3]. Among our own group of D/D translocations there has not been an individual with a fusion between two homologues, though such have been reported [2, 3, 9].

Among the D/G translocations there has been more random involvement of D and G group chromosomes, although the D involved is more often a chromosome 14 and the G a chromosome 21 (table 3).

The fact that chromosome 21 appears to be more often involved is probably due to ascertainment bias, since 11 of these translocations were identified through an unbalanced carrier (patients with Down's syndrome). It is interesting that a number of our own, and other reported cases [2, 5], have arisen as a new mutation, the parents having apparently normal chromosomes. The family in which the 15/22 translocation is segregating is quite large, 12 individuals including the proband having the translocation. There is no indication of non-disjunction occurring in this family. It is therefore important that the nature of the D/G translocation should be established before any assessment of the risk of such an individual having abnormal offspring is made.

G/G translocations have been found to involve two chromosome 21s more often than a 21 and 22 [2, 5], this may once again be an ascertainment bias. The involvement of two chromosome 21s in a translocation carrier will mean that this individual can produce no normal offspring.

Table 3. *Chromosomes involved in D/G translocations*

	13/21	13/22	14/21	14/22	15/21	15/22
Inherited	1 (1)1		6 (4)1		1 (1)1	
Mutation			6 (5)		1	
?		1	2			

Numbers in parentheses are those cases identified through patients with Down's syndrome.

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Discussion

Pearson: Do you think that there is any evidence for the No. 9 chromosome being involved more frequently in structural rearrangements than any other C-group chromosome, which might indicate that the secondary constriction chromatin predisposes this chromosome to rearrangements? In the series of reciprocal translocations you have just described that the No. 9 chromosome was involved several times?

Buckton: There is not enough evidence yet, to say that No. 9 is more often involved in an unbalanced translocation carrier. One thing that is evident, from the various studies, is that the No. 9 chromosome is very variable.

Dutrillaux: We also found that the location of the break points was not random. The location was as follows: one-third in the centromeric regions; one third in the telomeric regions; and only one-third in the rest of the chromosome arms.

Hamerton: Your observation of the t(14;15) is interesting in relation to Dr Therkelsen's observation of trisomy 15 in spontaneous abortions, as this was your only family with any degree of reproductive loss while your other translocations did not involve 15 and showed little or no reproductive loss. This also supports Dr Lejeune's comments on the first day that different chromosomes will be found to involve rearrangements with differing levels of clinical severity.

Lejeune: You mentioned there was a bias in your table showing an excess of 21–13 type of translocation. What kind of bias was it?

Buckton: The bias in the D/G translocations was towards the 21 chromosome, not to the D-group chromosome.

Cytogenetical and Clinical Aspects of Trisomy-8 in Bone Marrow

A. de la Chapelle,[1] *J. Schröder*[1] *and P. Vuopio*[2]

[1]Folkhälsan Institute of Genetics, P.O. Box 819, 00101 Helsinki 10, and
[2]Third Department of Medicine, University of Helsinki, Finland

Summary

Bone marrow cells are less suitable for chromosome identification than are many other cell types. Reliable identification of all bone marrow cell chromosomes can be achieved by the successive application of G- and Q-banding techniques to the same cells. Three patients have been shown to have trisomy-8 in the majority of their bone marrow cells. Clinically, they had chronic intermittent pancytopenia, mild leukopenia and severe sideroblastic anaemia, respectively. Although they were clinically quite different, a common feature in all three was ineffective erythropoiesis. Trisomy-8 of the bone marrow may not be the factor causing the disease, but the disease may predispose to trisomy-8. Common symptoms may be secondary to the trisomy.

It has long been known that trisomy for a C-group chromosome occurs in the bone marrow cells of occasional individuals with haematologic diseases whose lymphocyte and fibroblast karyotypes are normal (review by de la Chapelle et al. [3]). Until recently, identification of the extra chromosome was not possible because individual chromosomes of group C could not be distinguished with certainty. Hence, the extra chromosome could be an autosome, an X chromosome, or an abnormal chromosome derived from other chromosomes, by translocation for example. Moreover, different chromosomes could be involved in different cases.

We reported recently that the extra C-group chromosome was a No. 8 in two cases [4]. In an earlier report [6] a similar example of trisomy-8 was described. The object of this paper is to discuss some aspects of chromosome identification in bone marrow cells and to evaluate the possible clinical significance of trisomy-8 in the bone marrow.

Chromosome Identification in Bone Marrow Cells

According to our modification of the method described by Tjio & Whang [12], 0.05–0.2 ml of freshly aspirated bone marrow is injected into a vial containing 15 ml Hanks' solution, 250 IU heparin and 25 μg Colcemid. The vial is immediately transferred at room temperature to the cytogenetic laboratory for further processing. Whenever possible, the next step in the procedure is undertaken 2 h after the aspiration. However, since bone marrow samples are often sent to our laboratory by air or by mail, they arrive hours or even as much as a day after aspiration. In the majority of cases such samples do also yield analysable mitoses although they are usually, but not always, inferior in quality to those obtained after 2 h treatment.

The next step is treatment with 0.075 M KCl for 15 min at room temperature. Fixation is with 3:1 methanol/acetic acid. The fixative is changed 3 or more times before the slides are prepared. One drop of bone marrow cell suspension in fixative is dropped onto a slide which has previously been washed in detergent, rinsed in water and dried thoroughly. The fixative is allowed to dry at room temperature, after which the slides are treated as described below. In some instances, the slide was heated gently from underneath.

This method most often yields a number of mitoses in which the chromosomes are sufficiently well spread to allow detailed analysis. However, there is seldom such an abundance of mitoses as there is in phytohaemagglutinin-stimulated lymphocyte cultures. Hence one difficulty is the relative scarcity of mitoses from which to choose. A more serious difficulty from the point of view of precise chromosome identification is related to the morphology of the chromosomes. Bone marrow-derived mitoses often contain short chromosomes whose chromatids are not in close apposition but are fairly wide apart. Banding patterns are difficult to evaluate in short chromosomes because the bands lie

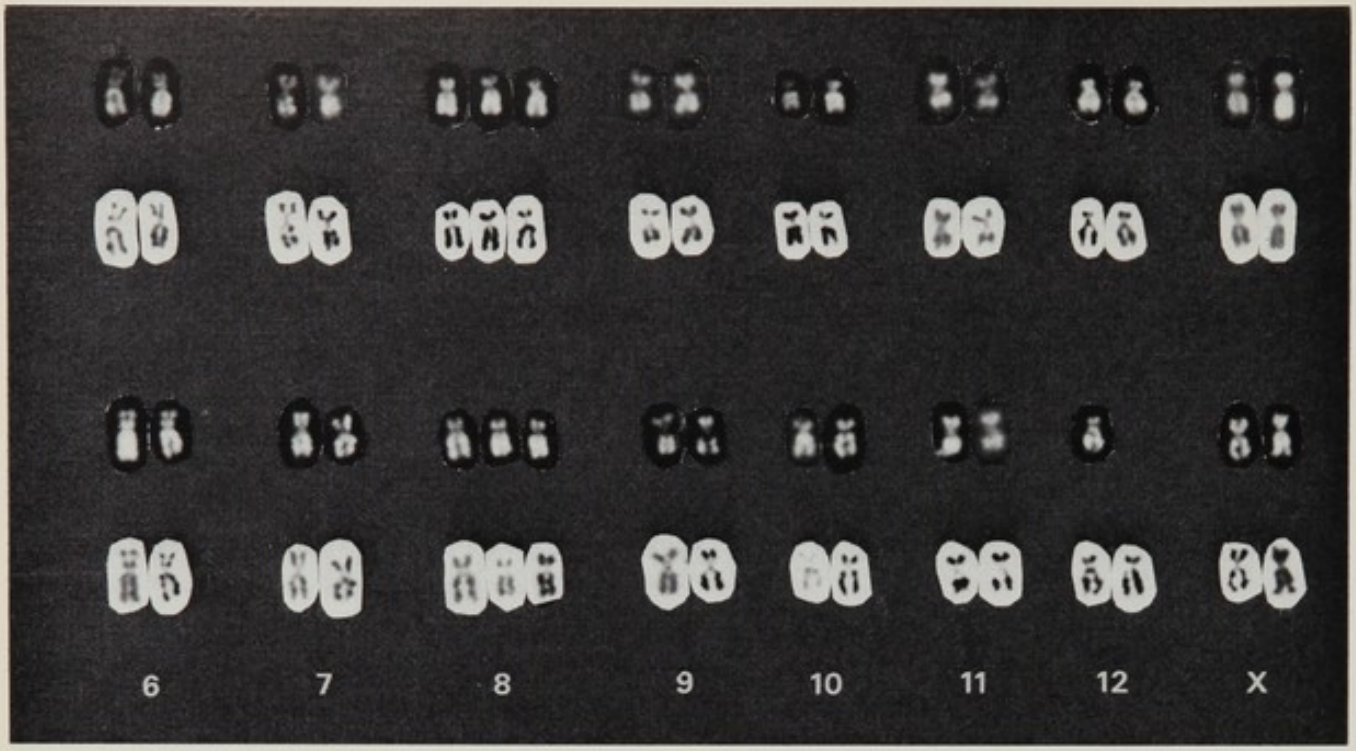

Fig. 1. Partial karyotypes of two bone marrow cells showing the C-group chromosomes successively treated according to the ASG and QM fluorescence methods. Three No. 8 chromosomes are seen in each cell. One No. 12 chromosome is missing in one of the karyotypes.

very close together or are completely obscured. When the chromatids are apart rather than in apposition, band patterns, especially with G-band techniques, may be hard to define. Hence, many bone marrow mitoses whose chromosomes are suitable for identification by groups using conventional techniques are not suitable for band pattern analysis. The peculiar morphology of bone marrow chromosomes does not appear to be the result of colchicine treatment because it is discernible even after as little as 2 h treatment. However, after many hours in the colchicine solution the contraction is still stronger.

In order to achieve reliable chromosome identification in bone marrow cells, we have used a combination of the ASG procedure [11] and quinacrine mustard (QM) fluorescence [1]. The method is as follows: slides are first treated by the ASG procedure and suitable cells are analysed and photographed. Then the slides are destained in absolute ethanol (12 h), 75% ethanol (12 h), and 50% ethanol (12 h). After staining with quinacrine mustard the same cells are rephotographed. Thus, each chromosome is depicted once with G-bands and once with Q-bands. We have succeeded in analysing bone marrow mitoses by comparing the two banding patterns of the chromosomes on such enlarged microphotographs.

As seen from fig. 1, the extra chromosome could be identified as a No. 8 in 2 of the 4 cases of C-trisomy diagnosed by us [4]. Of the remaining 2 patients, one died before banding procedures could be applied and the other had so few bone marrow mitoses that chromosome identification proved impossible. Hence, trisomy-8 was diagnosed in the only 2 patients in whom identification was possible. We are aware of only one reported instance of successful identification of an extra C chromosome by another laboratory, and it also proved to be a No. 8 [6].

Incidence

The fact that all 3 cases in which an extra C was identified had trisomy-8 appears to show that many C-trisomies of the bone marrow may prove to be 8-trisomies. This does not of course rule out the very real possibility that some or all of the other chromosomes of the C-group may be involved in other cases. Selection bias could lead to over-representation of chromosome 8 if patients with trisomy-8 have a less malignant disease. Such patients might have a longer life expectancy and therefore a better chance of being examined or they might have more mitoses suitable for chromosome identification.

Table 1. *Breakdown according to clinical diagnosis of 39 cases reported in the literature having trisomy for a C-group chromosome in their bone marrow cells, according to de la Chapelle et al.* [3]

Disorder	No. of cases
Acute myeloid leukaemia	8
Acute myelomonocytic or monocytic leukaemia	4
Chronic myeloid leukaemia (Ph^1+)	11
Eosinophilic leukaemia (Ph^1+)	1
Erythroleukaemia	3
Myelosclerosis, myelofibrosis with myeloid metaplasia	5
Atypical myeloproliferative syndrome	5
Sidero-achrestic anaemia	1
Polycythaemia vera	1
Total	39

Significance

It should be borne in mind that constitutional 8-trisomy has now been diagnosed in a number of people with congenital abnormalities [2, 5, 7]. It seems probable that constitutional trisomy for chromosome 8 leads to a clinically recognizable syndrome. The variations in phenotypic abnormalities noted by some authors could perhaps be explained as a result of normal/trisomy-8 mosaicism, whereas certain symptoms apparently occur in most or all of the patients. As far as we know, all the instances of constitutional trisomy-C in which identification was attempted proved to be 8-trisomies.

Chromosome 8 is the largest autosome to have been shown to occur in the trisomic state in living human beings. This could mean that it contains relatively few genes (structural or regulatory) that are extremely deleterious when present in triplicate. Since very little is known about the mechanisms that lead to metabolic disturbances or clinical abnormalities in trisomy, the above assumption is of course speculative. However, it apparently holds true that trisomy of certain chromosomes is better supported than others, perhaps not only on the level of the entire zygote, but also on a cellular level. If this is the case it might be postulated that cells which are trisomic for chromosome 8 may have a better chance of survival under certain conditions such as malignant bone marrow disorders. If so, cells with trisomy-8 might be able to selectively populate the bone marrow under the prevailing pathological conditions.

Clinical Implications

Trisomy for an unidentified C-group chromosome occurs in a great variety of malignant haematological disorders as shown in table 1. In fact, most known malignant diseases of the myeloid and monocytic cell lines are represented among those in which an occasional example of C-trisomy has occurred. However, in the majority of such diseases, either no chromosome abnormality occurs (as in acute myeloid leukaemia) or another abnormality is regularly present (the Ph^1 chromosome in chronic myeloid leukaemia). It is evident, therefore, that C-trisomy is clearly not associated with a uniform clinical picture. However, the lack of uniformity of clinical signs could be the result of the involvement of different C-group chromosomes in different cases.

Reliable haematological data are available on the three patients in whom trisomy-8 has been diagnosed. One of them (case 1 of de la Chapelle et al. [4]), an 18-year-old male, had *severe intermittent pancytopenia* since the age of 2. He was treated with blood transfusions and died recently from an intracerebral haemorrhage. Our second patient (case 2) is a 45-year-old female in whom *mild granulocytopenia* has been noted during the last 4 years. She is subjectively well. The patient of Hellström et al. [6] was a 57-year-old male who died of *severe sideroblastic anaemia* without any sign of leukaemia. Hence it is quite clear that trisomy-8 is not associated with a uniform clinical picture in these 3 patients. Moreover, people with constitutional trisomy-8 are haematologically normal. Nonetheless, there are some similarities in the clinical features of all 3 patients. All of them displayed signs of *ineffective erythropoiesis* at some stage of their disease. The patient of Hellström et al. [6] had outright sideroblastic anaemia while sideroblasts with and without iron granules have been observed in the two others. Other signs of the inefficiency of erythropoiesis noted in the patients are: anaemia, low reticulocyte count, presence of larger than normal amounts of foetal haemoglobin, high serum and tissue iron with normal haptoglobin levels, decreased incorporation of radioactive iron into the red cells, normal red cell life-span, increased erythropoiesis with megaloblastoid features and normal or high serum vitamin B_{12}-values. While it is impossible to prove that these features are related to the chromosomal abnormality, these symptoms

should be kept in mind and looked for when further cases of trisomy-8 are diagnosed.

Relation of Trisomy and Disease

It is formally possible that trisomy and disease are unrelated. However, this is unlikely judging by published studies of bone marrow karyotypes in normal people or patients without haematological disease showing absence of 8-trisomy [8–10]. It might be noted, however, that the Y chromosome appears to be absent in the bone marrow cells of some males without haematological disease [8].

There is at present little to indicate that trisomy of chromosome 8 causes haematological disease of the type described. If this were so, the clinical features would presumably be more similar. Furthermore, individuals with constitutional trisomy-8 are apparently haematologically normal [2, 7], and the incidence of cells with trisomy does not decrease as a result of treatment [4].

As outlined above, should the disease predispose to 8-trisomy, then this may be a question of survival of the fittest, whereby trisomic cells would have an advantage over other cells. Under certain pathological conditions, 8-trisomic cells would selectively survive and populate the bone marrow. A secondary event could be that trisomy-8 of myeloid and erythroid precursor cells would bring about some of the symptoms common to all 3 patients so far reported. The solution of these problems has to await further results.

This work was supported by grants from the Finnish National Research Council for Medical Sciences and the Sigrid Jusélius Foundation.

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Discussion

Miller: The slide you showed listed one case of erythremic leukemia as having a Ph¹ chromosome. There was a recent report in the Annals of Internal Medicine in which, by a banding technique, the deleted G-group chromosome in such a case was shown to be a deleted Y chromosome. Dorothy Warburton has recently studied a similar case at Columbia Presbyterian Medical Center and found it to have a deleted Y chromosome in the bone marrow but a normal Y in the phytohaemagglutinin-stimulated leucocytes.

de la Chapelle: One might add that there is a recent report from Britain stating that 45,X is seen quite often in the bone marrow of males without any haematological disease.

Lindsten: I would like to report that the fourth case with trisomy-8 in the majority of the bone marrow cells mentioned by Dr de la Chapelle also had the haematological picture of sideroblastic anaemia. The 3 cases with congenital trisomy-8 studied in our laboratory had a normal routine haematological picture. No detailed haematological studies have yet been made in these cases.

Pearson: Did circulating lymphocytes also show evidence of trisomy-8?

de la Chapelle: These patients have some 90–100% cells with trisomy-8 in the bone marrow. Circulating lymphocytes and skin fibroblasts have normal karyotypes.

Lejeune: I am impressed by the fact that full trisomies-8 have not obvious symptoms of leukaemias. It could very well be that trisomic-8 cells in bone marrow would be at advantage if in a diploid organism (and not in a trisomic-organism).

de Grouchy: You have not found any deletion of an F?

de la Chapelle: No.

Chromosomal Aspects of Human Male Sterility

B. Dutrillaux

Institut de Progenèse, 75 006 Paris, France

Several authors have reported that an increased frequency of chromosomal rearrangement exists among subfertile men (for references, see [3]).

In a survey of subfertile men, 271 patients have been referred to us by two infertility clinics. Most of the cases are primary sterility, with severe sperm anomaly and clinical indication for testicular biopsy. The assessment of the patients, according to their sperm anomaly, is presented in table 1. Karyotypic analysis has been performed after routine blood cells culture [5]. In more than 50% of the patients, controlled denaturation technique [2] has been applied, and a meiotic analysis has been carried out in the same proportion. The cases with Y anomaly were submitted to fluorescence technique [1].

Results

In the whole sample, 57 cases with a chromosomal peculiarity have been detected. Their assessment, whether azoospermic and oligospermic, seems to be rather proportional to their total number.

Most of the observed peculiarities are known to be compatible with normal phenotype and normal reproduction. The details of each case are set out in table 2.

Among the 4 patients with 47,XXY complement, the meiotic analysis revealed a reduced, but present, spermatogenetic activity in 3 of them. In one of them, carrying two X chromosomes in the 30 cells analysed by the denaturation technique, the testes were normally developed and an active gametogenesis was observed up to the pachytene stage.

Four patients had a metacentric Y, with intense fluorescence in one arm. Meiotic analysis of 2 of them showed an apparently normal X–Y bivalent.

Heterochromatin peculiarities were observed in 38 patients. The most common, as revealed by denaturation technique, is an excess of short arms of one of chromosome 15 (15p+).

Translocations

Seven cases of translocation were identified: Three of them are t(Dq Dq), observed in 2 azoospermic and in one oligospermic man. In the 3 cases, the analysis of primary spermatocytes in metaphase showed 21 elements, one being probably trivalent. The denaturation technique, carried out in 2 of them, revealed a t(13q 14q) (fig. 1).

A case of t(Dq Gq), without meiotic analysis,

Fig. 1. Karyotype with t(13q14q).

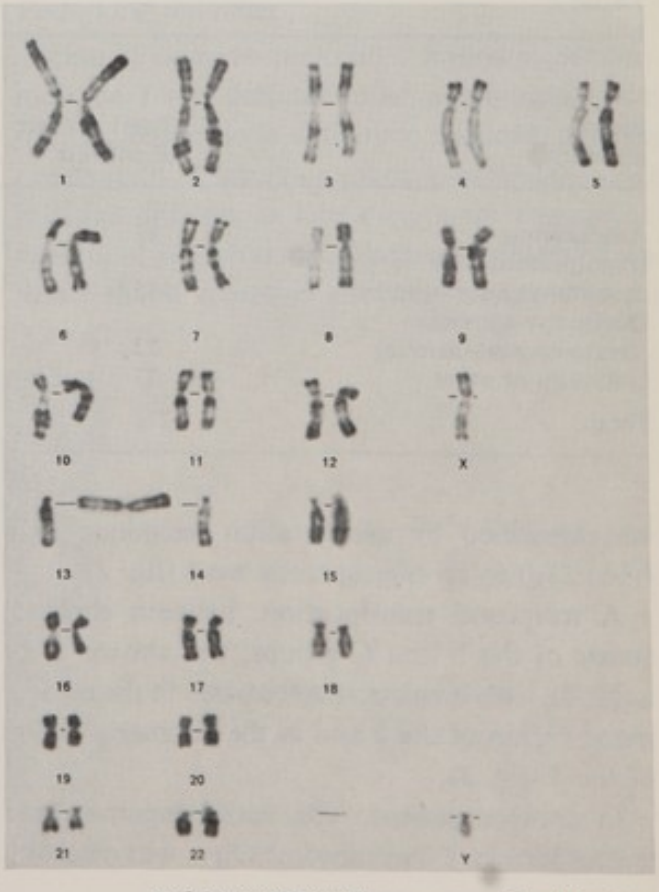


Table 1

Azoospermia (20 cases)	Oligospermia and qualitative anomaly (31 cases)	Asthenospermia and teratospermia (3 cases)	Other or unknown (3 cases)
Gonosomes anomalies			
	46, X inv (Y), 22s+ 1		
47,XXY 4	46,X inv (Y) 1		
47,X inv (Y) 1	46, XY q- ? 3		46,X inv (Y),16qh+ 1
47,X Yq- ? 1	46,XY q-?, 16 qh+ 1		
	46,XY q- ?, 22p+ 1		
	46,XY/45,X 1		
Heterochromatin anomalies			
	46,XY, Dp+ 5		
	46,XY,15p+ 2		
46,XY, Dp+ 3	46, XY, 14p+ 1		
46,XY,15p+ 6	46,XY, 13 p+ 1	46,XY, 14p+ 1	46,XY,14p+1
46,XY,21p+ 2	46,XY, Gp+ 6	46,XY, 9 qh+ 1	46,XY,11qh+ 1
	46,XY, 21p+ 1		
	46,XY, 22p+ 1		
	46,XY, 9 qh+ 1		
Autosome structural anomalies			
	45,XY, t(14q21q),22p+ 1		
	45,XY t(13q14q) 1		
45,XY,t(13q14q) 1	46,XY t(5 ; 8) 1	46,XY,t(11 ;22) 1	
45,XY,t(DqDq) 1	46,XY inv (7) 1		
	47,XY, + f 1		
Autosome–gonosome translocation			
t(1qh+ ; X) 1			

Table 2

Sperm anomaly	Total number of patients	Patients with 46,XY karyotype	Patients with abnormal karyotype
Azoospermia	81	61	20
Oligospermia, with qualitative anomalies	145	114	31
Qualitative anomalies terato-asthenospermia)	22	19	3
Unknown or other	23	20	3
Total	271	214	57

was identified by denaturation technique as a t(14q 21q) in an oligospermic man (fig. 2).

A reciprocal translocation, between chromosomes of the B and C groups, was shown to be a t(5; 8), with break points localized in the centromeric region of the 8 and in the telomeric region of the 5 (fig. 3).

In another patient, with asthenospermia and teratospermia, a chromosome Gq – was detected. Meiotic study gave no evidence of a reciprocal translocation, showing only a reduced size of the smallest bivalent. Denaturation technique revealed a t(11q+; 22q–), undectectable with classical staining (fig. 4).

A translocation t(1; X) was observed in an azoospermic patient, and was also found in his mother (fig. 5*a*) and his aunt (fig. 5*b*). This person is also sterile (fig. 5*c*). The sister of the proband

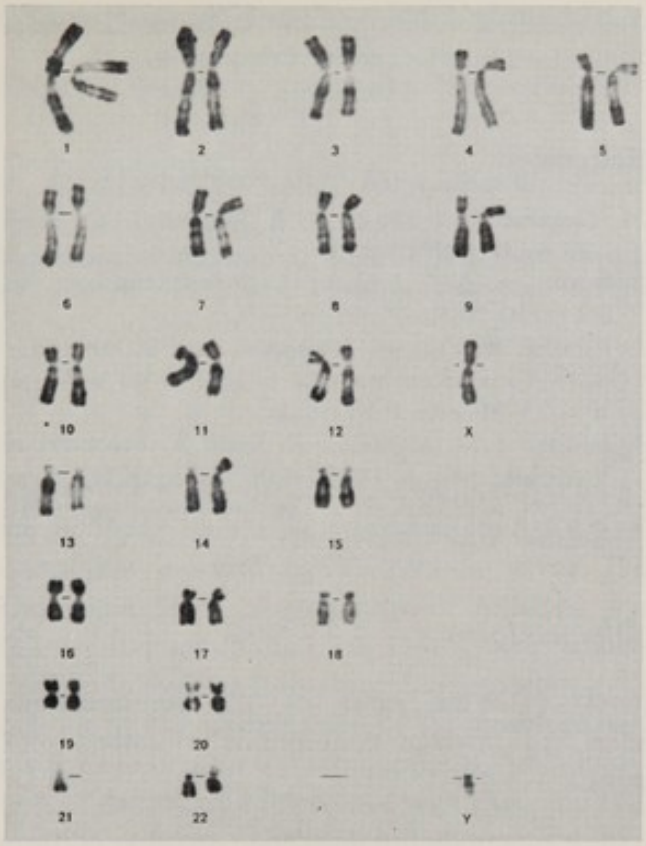


Fig. 2. Karyotype with t(14q21q).

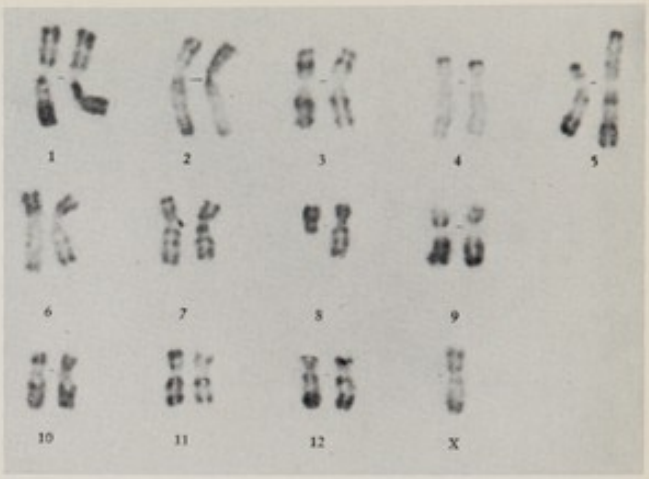


Fig. 3. Partial karyotype with t(5;8).

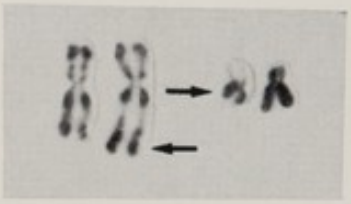


Fig. 4. Partial karyotype with t(11;22).

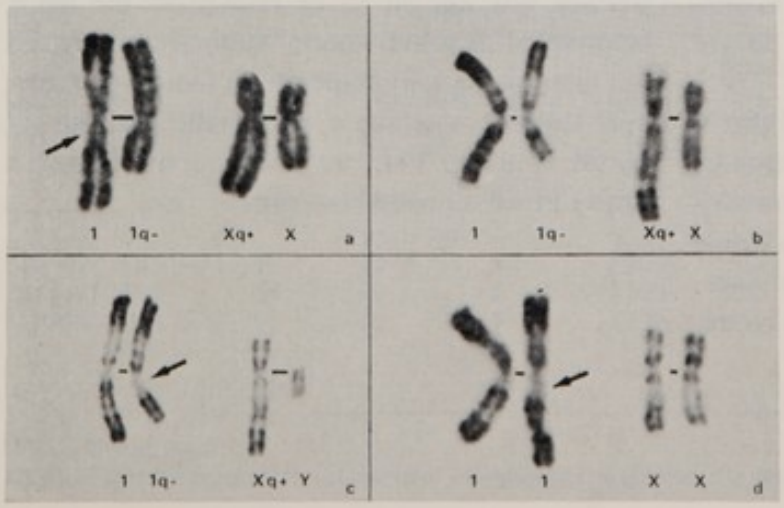
does not carry the translocation but one of her No. 1 chromosomes has an elongated secondary constriction (fig. 5*d*). The same feature is found in the normal No. 1 of the mother and in the lq – of the proband. Hence a crossover took place between the secondary constriction and the break point during the maternal meiosis.

A study of the meiosis of the proband often shows a very long element consisting of the rearranged No. 1 chromosome associated by the short arms with the normal one, itself associated by long arms with the segment translocated to the X; this latter being itself associated by its short arms to the Y chromosome.

Pericentric inversion

A case of de novo pericentric inversion of chromosome 7 was detected in an oligospermic man (fig. 6). His meiotic activity is reduced, and the observation of inversion loops in pachytene stage is rather difficult. In first metaphase the normal aspects of the bivalents, frequently observed, indicate that a crossover probably occurred in the

Fig. 5. Partial karyotype with t(1;X). (*a*) mother of the propositus; (*b*) aunt of the propositus; (*c*) propositus; (*d*) sister of the propositus.



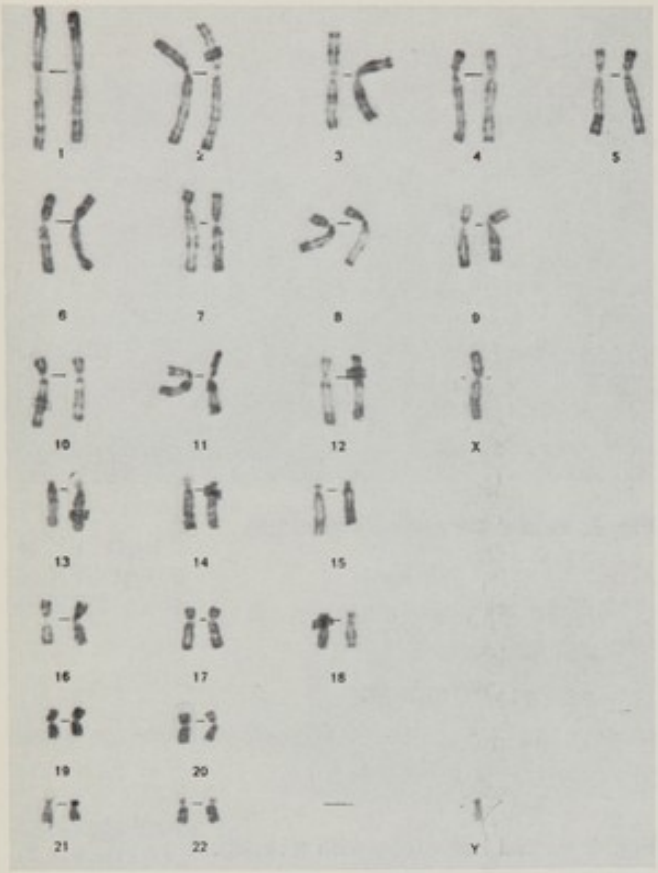


Fig. 6. Karyotype with pericentric inversion of chromosome 7.

inverted segment, inducing "aneusomies de recombinaison".

Conclusions

From this sample a clear excess of chromosomal rearrangements in subfertile men appears very likely. The frequency of 3% of major anomalies (translocations and pericentric inversions) found among the patients was around 20 to 30 times greater than in the general population.

Of the heterochromatic peculiarities, so frequently noted, it is difficult to speak of an excess, the frequency of these 'markers' being poorly known in the normal population.

With the exception of the anomalies of the X chromosome, the relationship between chromosomal anomaly and sterility is not straightforward. From our experience, it is impossible for the moment to predict from the clinical data, whether a chromosomal aberration is to be expected in a particular patient.

The author is grateful to Drs Gueguen, Le Lorier, Rotman and Salat for referral of the patients.

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Discussion

Buckton: Were most of the translocations identified through conventionally stained preparations?

Dutrillaux: Yes, the majority.

Buckton: Has autoradiography been done in the female with the autosome translocation to see whether there is selective inactivation of one of the X chromosomes?

Dutrillaux: No, unfortunately, we were not able to analyse the family a second time; they live in the countryside.

Lindsten: Previously published series of cytogenetic studies of childless couples have almost exclusively been limited to males. Do you have any information on the female partners of the males you have studied?

Dutrillaux: In this survey, the origin of the sterility was known to be related to the male, because of a grave sperm anomaly, so we did not analyse the karyotype of the female partners. We are just starting a systematic analysis of sterile women. Yet, we found a translocation t(6;X) in an amenorrhoic one.

Chromosome Banding Techniques in Clinical Cytogenetics

Report of four cases irresolvable by conventional methods

J. L. Hamerton, M. Ray and G. R. Douglas

Department of Pediatric (Division of Genetics), University of Manitoba, and Children's Hospital of Winnipeg, Winnipeg, Manitoba, Canada

The development of chromosome banding techniques using both quinacrine and its derivative quinacrine mustard by Caspersson et al. [2] and some form of pretreatment followed by Giemsa staining [5, 6, 7, 10, 13–16] have rapidly become integrated into clinical cytogenetics and provide precise identification of the chromosomes involved in structural rearrangements, the delineation of deleted and duplicated segments, and the position of break points [1]. In the present study, four cases are reported in which a complete chromosome analysis would not have been possible without the use of these techniques. Three of these were ascertained in the Newborn Study [9] (cases 1–3) and one case was referred to the Clinical Cytogenetics Laboratory, Winnipeg Children's Hospital (case 4).

Chromosome Studies

The Q-staining technique used has already been described (p. 170) and G-staining was by a modification of the trypsin digestion method of Seabright [14]. Air-dried preparations were made in the conventional way and dipped in 0.5% Bacto-trypsin Difco 1:250 (prepared from freeze-dried Bacto-trypsin—Difco Cat. 0153.59 by adding 10 ml of 0.85% NaCl to 1 vial and diluting this stock solution 1:10) for 8–10 sec. The slides were then rinsed immediately in two changes of 0.85% NaCl and stained with Leishman (diluted 1:4 with buffer, pH 6.8, G. T. Gurr) for 3–5 min. The slides were washed rapidly in buffer, dried, rinsed in xylol and mounted in a neutral mounting medium. All steps were carried out at room temperature, 22–23°C. The nomenclature used in this report is that recommended by the Paris Conference on Standardization in Human Cytogenetics [9].

Case Reports

Some clinical data about each infant is given in table 1.

AC090472 NB-8732: This full-term infant was investigated 2 days after birth when routine chromosome analysis revealed a chromosome count of 47 and only three chromosomes in the F-

Table 1. *Clinical data*

Identification ascertainment	Sex	Gestation (weeks)	Apgar @ 1 min	Birth wt (g)	Parental age		Clinical notes
					Mother	Father	
AC090472 NBS	M	40	9	2 927	41	43	Some features of Down's syndrome
H070672 NBS	F	40	9	2 835	31	39	Normal
D110472 NBS	M	40	9	2 835	29	32	Normal
TH140671 WCH	F	42	9	3 320	23	23	Some congenital malformations including microcephaly and mental and motor retardation

NBS, Newborn survey.
WCH, Referred to routine cytogenetic laboratory for cytogenetic study.

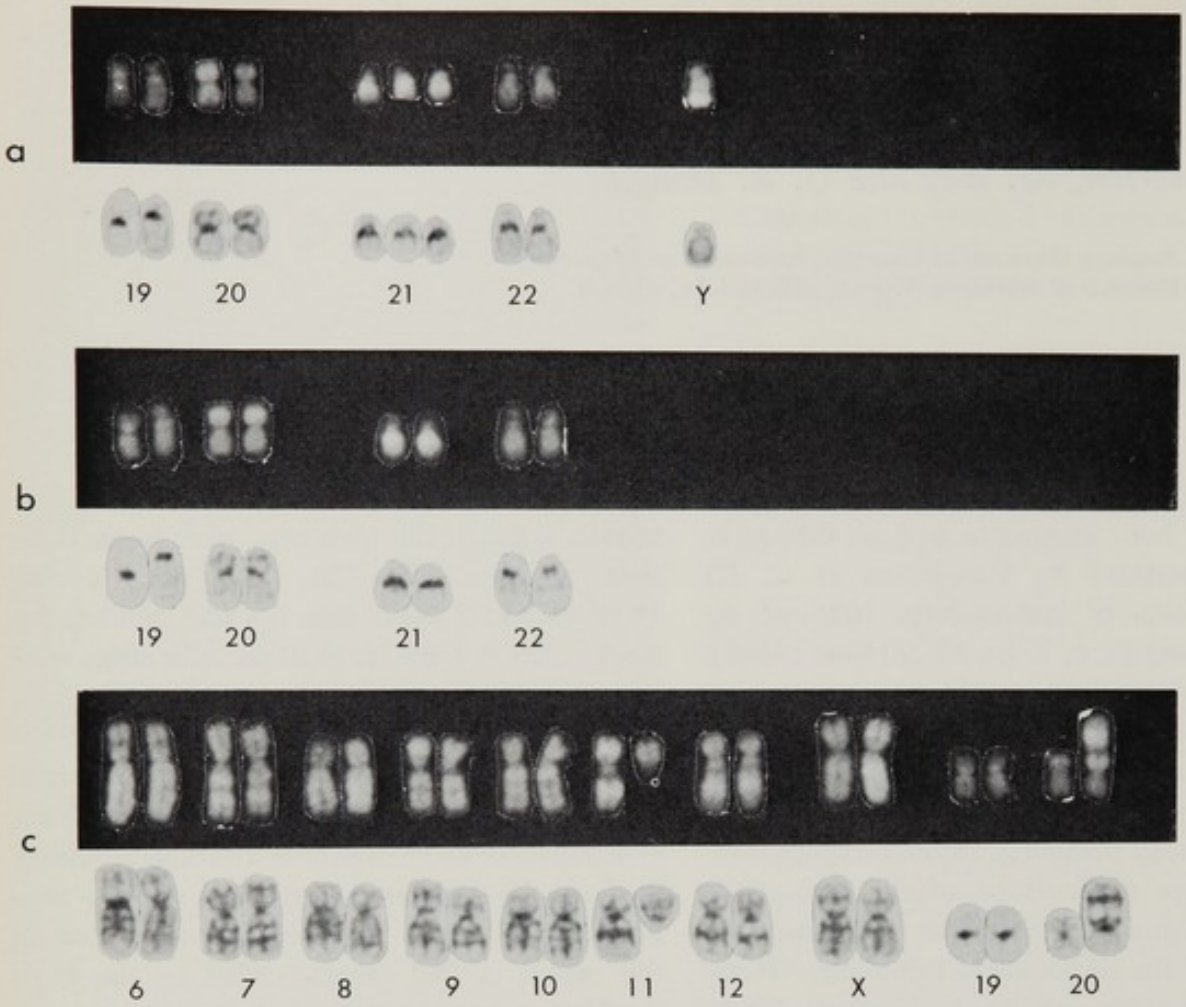

Fig. 1. Partial karyotype from three subjects with F group abnormalities. Upper line QM staining, lower line trypsin/Giemsa. (*a*) NB 8732: 47,XY,inv(19)(p13q13), +21 (*b*) NB 8732.01: 46,XX,inv(19)(p13q13). (*c*) NB 9351: 46,XX,t(11;20)(q13;p131).

group, five chromosomes in the G-group and a clear Y chromosome. There was an additional acrocentric chromosome present which approached chromosome 18 in size. This karyotype was interpreted as being most likely 47,XY, inv(Fp−q+),+G. Clinical examination of the infant at birth by the hospital staff had not revealed anything remarkable and no diagnosis of Down's syndrome was made at this time. The mother and child were discharged three days after birth and were not seen by the Genetics staff until one week later. At this time careful clinical examination revealed a hypotonic infant with right and left epicanthic folds, narrow palpebral fissures, depression of the nasal bridge with a slight degree of hypertelorism. The child had a short neck and rather small head (circ. 34 cm). The general appearance was considered to resemble Down's syndrome, although a firm diagnosis was not made. Further blood samples were taken at this time from the infant and both parents for Q- and G-banding. The father had normal chromosomes (46,XY) while the mother had 46 chromosomes and a similar and apparently inverted F group chromosome to that carried by the propositus. This karyotype was interpreted as 46,XX,inv(Fp−q+). Analysis of Q- and G-bands revealed that the propositus had a pericentric inversion of chromosome 19 and 21-trisomy 47,XY,inv(19)(p13q13),+21, thus confirming the diagnosis of Down's syndrome; his mother was a balanced inversion heterozygote 46,XX,inv(19)(p13q13), (fig. 1).

H070672 NB-9351: This was a full-term, clinically unremarkable infant. Conventional staining methods suggested a 46,XX,inv(Fp−q+) karyotype. Q- and G-banding revealed that this was, in fact, a reciprocal translocation between 11q and 20p with presumptive break points in 11q13 and 20p13. The karyotype is 46,XX,t(11;20)(q13;p13) (fig. 1). The chromosomes of the mother were normal and the father has not yet been studied.

D110472 NB-8762: This is a clinically unre-

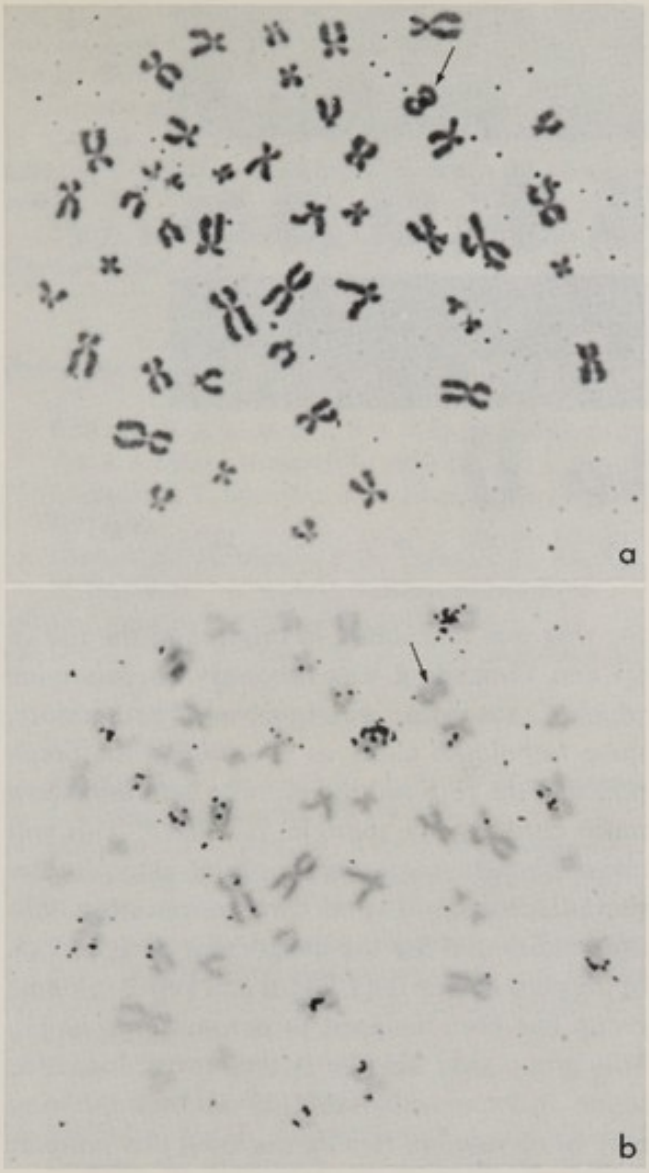

Fig. 2. Metaphase spread after autoradiography from subject with 12r chromosome abnormality (*a*) before autoradiography (*b*) after autoradiography. Note ring is not late replicating.

markable newborn infant ascertained when conventional staining methods revealed an apparent deletion of 18q (46,XY,18q −). Analysis by both Q- and G-banding however, revealed that in fact, this was a reciprocal translocation between 9p and 18q with presumptive break points in 9p22 and 18q21, 46,XY,t(9;18)(p22;q21) (fig. 3).

TH140671 WCH-5303: At the age of one year, this patient showed apparent motor and mental retardation. She had microcephaly, a broad flat face, bilateral epicanthic folds, moderately low set ears and an unusual almost transverse but double palmar creases. Chromosome analysis by conventional methods revealed a ring C chromosome (46,XX,Cr) shown to be an autosome by autoradiography (fig. 2) and subsequently identified by both Q- and G-banding as No. 12, with break points in the distal region of the long and short arms 46,XX,r(12)(p13q24) (fig. 3). The ring chromosome was present in each of 50 cells examined, and in no case was a dicentric or much enlarged ring observed. The size of the ring was relatively constant and apparently contained the very large part of chromosome 12 with only the terminal regions presumably deleted.

Discussion

Both the techniques in use here provide precise banding of the human chromosomes and thus allow their identification. Fluorescence is the simpler staining procedure, but requires complex optical equipment, fades rapidly so that slides must be examined immediately and presents certain photographic problems. Trypsin banding is simple, reliable and consistent in use. It results in permanent preparations which do not fade. It is probably therefore, the simpler to use in a routine situation. I suggest that the trypsin technique may therefore be the technique of choice for a routine laboratory. In our experience, however, flame drying ruins bands and old slides give poor results.

The 4 cases presented here illustrate the utility of these new staining techniques in clinical cytogenetics. Each case was analysed by both Q- and G-banding and give similar results with both techniques. In each case the exact chromosomal lesion could not have been determined by conventional methods. Case 1 is an interesting one in that there are at least two ways in which the unbalanced karyotype seen in the proband could have arisen from the balanced karyotype carried by the mother. The first possibility would be crossing over within the inverted segment of chromosome 19 to give a rec(19) which superficially resembled a G chromosome, followed by nondisjunction of that chromosome. The second possibility is a presumptive balanced inversion of chromosome 19 together with trisomy of 21. This is observed. A clear clinical diagnosis of Down's syndrome would have helped to differentiate these two possibilities but at least in the early stages even this was not available. Finally, in respect of this case we have no reason to suppose that the inv(19) is in any way related to the non-

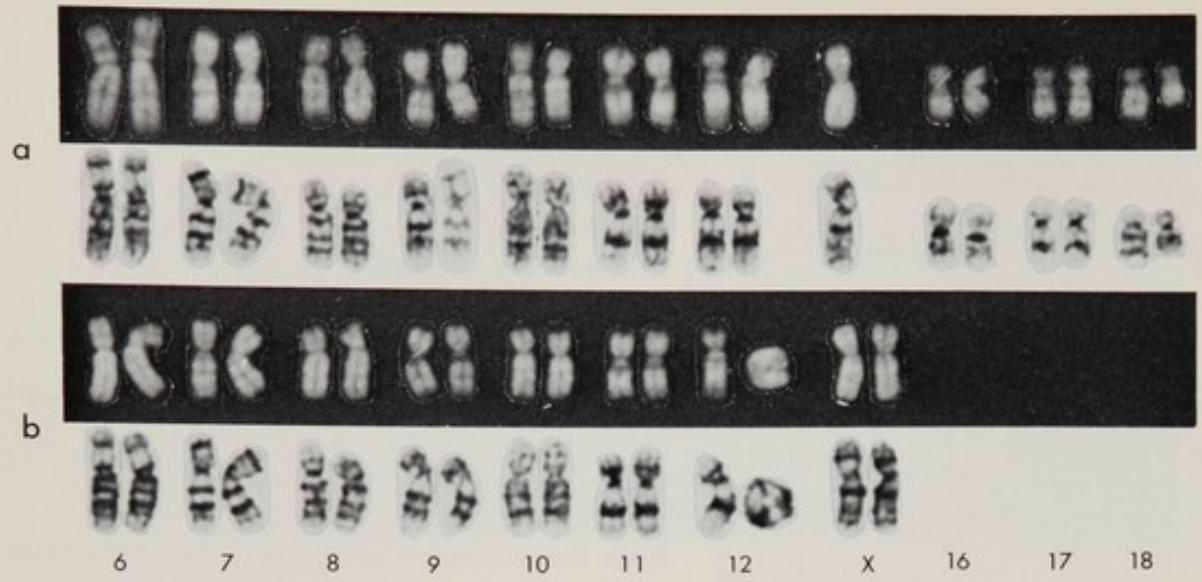

Fig. 3. (*a*) Partial karyotype from subject with 46,XX,t(9;18)(p22;q21). (*b*) Partial karyotype from patient with 46,XX,r(12)(p13q24). Upper line QM staining, lower line trypsin/Giemsa.

disjunction of 21, the most likely explanation being an inherited inv(19) and independent non-disjunction of 21 due to maternal age.

Cases 2 and 3 are presented because they illustrate the weakness of the conventional staining techniques in reaching a final cytogenetic diagnosis when certain types of rearranged chromosomes are being studied. Using conventional methods the only apparent abnormality in case 2 is a presumptive inversion of one F-group chromosome while the rest of the karyotype is normal. Using Q- and G-banding, however, it becomes obvious that there is a balanced reciprocal translocation between numbers 11 and 20. Case 3 is similar; initial conventional analysis revealed an apparent 18q – with a normal phenotype.

The phenotype associated with 18q – has been reported to be variable, but most have had some degree of clinical abnormality and an attempt has even been made at the deletion mapping of this chromosome [4]. The use of conventional methods alone therefore would have simply added one further 18q –, in this instance with a normal phenotype, thus increasing the confusion. The use of Q- and G-banding however shows this is also a balanced reciprocal translocation, thus explaining the normal phenotype.

The last case illustrates another, up to now insoluble problem, the identity of structurally abnormal C-group autosomes. Conventional staining and autoradiography revealed only that the ring was autosomal in origin and the use of Q- and G-banding was necessary to determine which C autosome was involved. Furthermore, these techniques allow us to position the break points in the ring and therefore to determine how much chromosome material is deleted. This will allow a much more precise use of clinically derived structurally altered chromosomes for linkage studies and for the assignment of gene loci. In the present case the LDH-B and Pep-B syntenic group has been assigned to chromosome 12 [3]. This group may also be syntenic with locus for serine hydroxymethylase [12] so that the ring may be of value in further mapping this syntenic group. This is at present being tested.

Concluding Remarks

It is clear from this and other papers in this symposium that no cytogenetic study on patient material can be said to be complete without the use of at least one of the banding techniques. The development of these techniques has thus increased the resolution of cytogenetics from the whole chromosome to the chromosome region and band. In turn this has not only increased the precision of clinical cytogenetics, but also allows in appropriate cases the assignment of loci, linkage, and syntenic groups to specific chromosome regions. I predict that in the next few years our knowledge of the human genome will expand as fast as our knowledge of the human chromosomes did during the descriptive phase in the sixties. The study of the human chromosomes can now be truly said to have entered a scientific era.

We are grateful for the technical assistance of Mr F. Bauder, Miss V. Niewczas-Late and Miss G. Isaacs.

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Trisomies of Chromosome No. 8

J. Lejeune and Marie-Odile Rethoré

Institut de Progenèse, 75006 Paris, France

The Full Trisomy 8

The full trisomy for chromosome 8 has been established clinically [1] and the first demonstration by chromatid fine structure analysis was given by de Grouchy et al. [2].

Recently we have reviewed 5 cases of 8-trisomy, all of them identified in our laboratory by heat controlled denaturation [3].

The full syndrome, exemplified in fig. 1, can be summarised as a general dysmorphy of the bones including koilosterny and 13 ribs, abnormalities of vertebrae and of iliac bones, brachymesophalangy, syndactyly and club foot with limitation of movements of most of the joints.

In contrast, the head is macrocephalic with a eumorphic face. On the ears there is an irregular helix and an excess of the anthelix is seen. The mental deficiency is marked but probably less profound than in 21 trisomy.

The dermatoglyphics shows an upward direction of distal flexion crease, ending between the second and the third digits and all the creases are deep, with little dimples giving an "aspect capitonné". The axial triradius is elevated, the ridges of the distal part of the palm have a cubital orientation (low index of transversality) and there is an excess of arches.

The new trisomy syndrome is thus clinically easily recognized and can be diagnosed firmly by mere inspection and secondarily confirmed by the denaturation technique.

Trisomy for Distal Part of Long Arm of Chromosome 8

The particular syndrome was first described in two brothers, mildly mentally retarded. Both of them had 47 chromosomes, the extra one being roughly of the size of a G.

According to the old techniques, both parents exhibited a normal karyotype. With denaturation, the mother showed a dark segment on the end of the long arm of No. 8 and one of her No. 22s was replaced by an acrocentric, the long arms of which were rather pale. Hence this small acrocentric consisted of the end part of long arm of No. 8 translocated on the centromere of 22. The long arm of 22 was found at the end of the rearranged 8.

The two boys were trisomics for the distal part of chromosome 8 [4].

A very similar translocation was found recently by Dr Prieur in another family, apparently unrelated, in which a boy (fig. 2) had 47 chromosomes with the same extra pseudo-G, and the father had the 22–8 balanced translocation (fig. 4*C*).

From these 3 cases we can describe the disease as a mildly dysmorphic syndrome affecting the sternum, the vertebrae, the iliac bones, the digits and the toes as in trisomy 8 but to a milder extent and with no involvement of the articulation and with normal ribs. Two of the boys were operated for ectopic testis.

The head is also macrocephalic and the mental retardation is mild (IQ in the order of 70).

Dermatoglyphics shows normal creases but an elevated end of the distal flexion crease and elevation of the axial triradius.

Trisomy for Short Arm and Middle Part of Long Arm of No. 8

In one case, analysed by Dr Rethoré, we have observed 47 chromosomes, with the extra element being recognized as a No. 8 lacking the terminal part of its long arm (fig. 4*B*).

This young girl is microcephalic with hyperte-

Table 1

	Full trisomy No. 8	Trisomy for the end part of long arm of No. 8	Trisomy for short arm and proximal part of long arm of No. 8
Phenotype			
Short neck	+	−	+
Koilosternia	+	+	+
13 ribs	+	−	+
Abnormal vertebrae	+	+	+
Narrow pelvis	+	+	+
Brachymesophalangia	+	+	−
Symphalangia	+	+	−
Club foot	+	−	+
Joint stiffness	+	−	+
Macrocephaly	+	+	Microcephaly
Dermatoglyphics			
Deep creases "plis capitonnés"	+	−	+
Distal crease ending in 11	+	+	−
Elevated axial triradius	+	+	−
Low index of transversality	+	−	+
Excess of arches	+	−	+

lorism and has a large broad nose. She has 13 ribs, abnormality of the twelfth vertebra and of the sacrum, and narrowed iliac bones. There is limitation of joint mobility and a club foot (fig. 3).

The creases are 'capitonnés' on the feet but normal on the hands. The ridges of the palms have a cubital orientation (low index of transversality).

It is obviously impossible to designate a new entity based on only one case, but apparently this girl exhibits some of the stigmata of trisomy 8 and, precisely, those which are not found in the trisomy for the end of the long arm. With the increasing amount of similar data, it seems possible that mapping of the phenotypic effects of various chromosomal segments may commence in the not-too-distant future.

Fig. 1. Trisomy 8.

Fig. 2. Trisomy for the distal part of long arm of chromosome 8 (trisomy 8q ter).

Fig. 3. Trisomy for the short arm and the proximal part of long arm of chromosome 8 (trisomy 8q−).

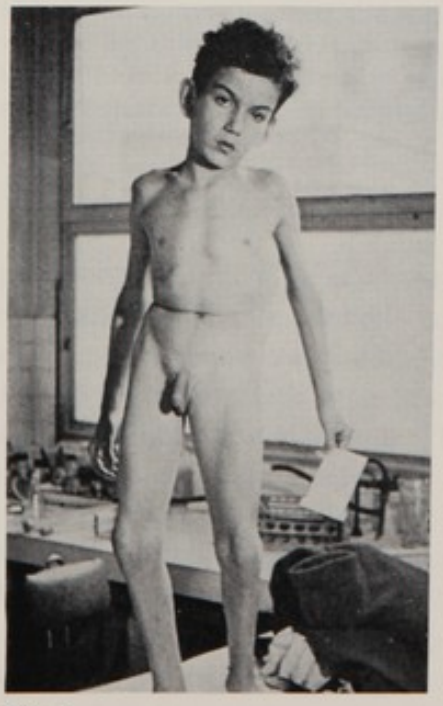
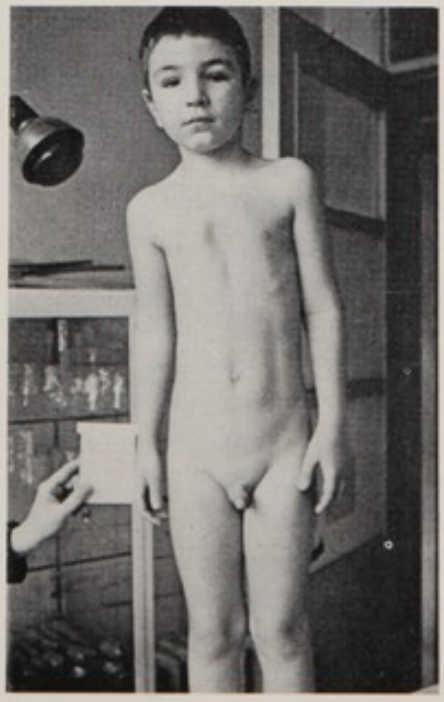


Fig. 1



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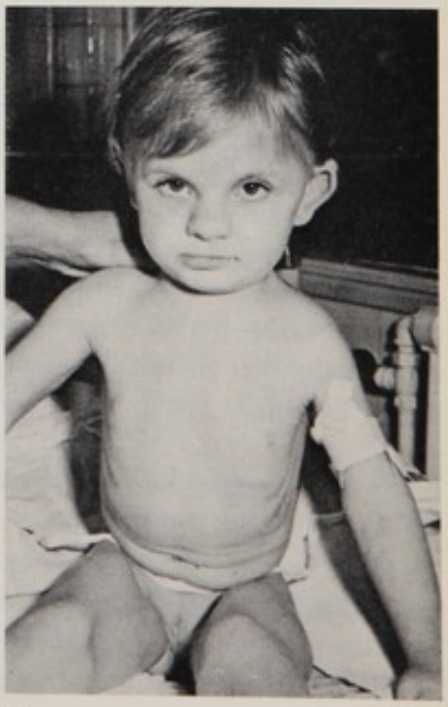

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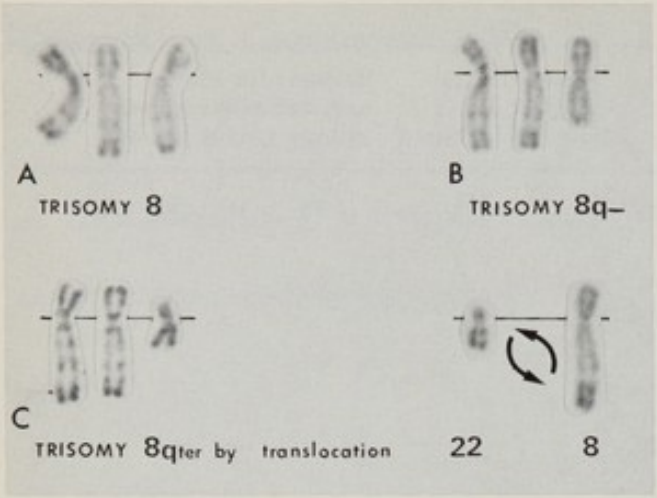

Fig. 4. (*A*) Full trisomy 8; (*B*) trisomy 8q—; (*C*) trisomy 8q ter by 22–8 translocation.

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Discussion

Gropp: It has proved to be very useful for clinical doctors to have a common name for a syndrome, such as "cri du chat" syndrome. Could you propose common names for trisomy 8, and the partial trisomy 8-syndromes?

Lejeune: Unfortunately not. To propose a useful name we need a main symptom which is quite pathognomonic. Maybe with an accumulation of new cases the clinical picture will clarify sufficiently to permit such a representative denomination.

Lindsten: Your finding of creases in the hands and feet is of interest in relation to the report by Penrose of specific dermatoglyphic features in one subject with trisomy 8. Do you have any information on the dermatoglyphic pattern in your cases with different abnormalities of chromosome 8?

Lejeune: Yes, and they are quite typical in each syndrome:

Trisomy 8 (full chromosome): Axial triradius elevated. Low index of transversality of ridges. "plis capitonnés", with the distal flexion crease ending betwen II and III (deeply engraved palmar creases); brachymesophalangy and symphalangy; arches on fingers.

Trisomy 8q terminal (trisomy for the end portion of the long arm of No. 8): Axial triradius elevated; distal flexion crease ending between II and III; brachymesophalangy; index of transversality normal; no "plis capitonnés".

Trisomy 8q (trisomy for short arm, centromere and two-thirds of long arm of No. 8): Index of transversality very low; "plis capitonnés"; excess of arches.

Chromosome Patterns in Tumours

A. Levan

Institute of Genetics, University of Lund, Sweden

Summary

Thanks to the systematic collection of chromosomal data from many individual tumours induced by the same agent in genetically uniform host animals, it has been possible to establish correlations between chromosomal pattern and histologic tumour type. Thus, chromosomal non-random patterns were found in tumours induced by Rous sarcoma virus (RSV) in 3 species of rodents. They were especially clear in the rat, in which a sequential karyotypic evolution was demonstrated, involving 3 stages of change. Sarcomas induced by 7,12-dimethylbenz(α)anthracene (DMBA) in the same tissue of the same inbred rat strain also had stemline karyotypes showing non-random sequential evolution. The DMBA-induced sarcomas, however, although indistinguishable histologically from the RSV-induced sarcomas, had a strikingly different karyotypic pattern. On the other hand, their pattern was identical with that of the DMBA-induced leukemias established by other workers. The conclusion is that the oncogenic agent exerts a specific action on the hereditary mechanism of the host cell, leading to a karyotypic evolution predetermined by the agent. Although human tumours are clearly less amenable to this type of inquiry, conclusive evidence of non-random chromosome patterns and sequential karyotypic evolution has been obtained in them too. The continued study of chromosomal patterns in tumours will profit greatly from the new banding techniques.

It has long been a challenge to the cytogeneticist to try and find understandable correlations between the chromosomal variability, ubiquitous in all forms of neoplastic growth, and the various histopathologic types of tumour. The strict correlation between the Philadelphia chromosome-carrying karyotype and chronic myelogenous leukemia (CML) still remains an exception, even though during recent years a number of unquestionable—although less obvious—correlations have been described between chromosome patterns and various malignancies. In the following I shall review certain aspects of chromosomal non-random patterns in tumours established by various members of our group, working in the Cancer Chromosome Laboratory of the Institutes of Pathology and Genetics in Lund.

Our work in this area has been stimulated by the conviction that the establishment and analysis of such non-random patterns would elucidate significant interrelations between the oncogenic agents and the hereditary apparatus of the host cell. Being geneticists, we naturally started out with the idea that chromosomal variation does not occur randomly and that not only the CML but also all natural groups of neoplasms have specific chromosome patterns, the essential features of which may, however, be elusive.

It is easy to think of factors that would interfere with the detection of chromosomal patterns: (*a*) The pattern may be vague and changeable, and only by examining many individual tumours of the same kind will the pattern become discernible. So far, few kinds of tumours have been submitted to such extensive studies. (*b*) The significant chromosomal change may be small and easily escape notice. Here, increasingly refined chromosomal technique may be helpful, including the complete analysis of many karyotypes with measurements and statistical treatment of the data. The new banding techniques with their potential for individual identification of even very small chromosome segments will offer good opportunities for progress. (*c*) Even though in CML the characteristic chromosome, the Ph[1], remains remarkably stable throughout the entire course of the disease, this may not be the case with characteristic chromosomes in other neoplasms. Significant features may become overgrown by secondary chromosome changes and the original pattern blurred. At the same time as

the tumours progress and histologically approach a common anaplastic type, their stemline karyotypes converge towards a more or less similar type. Thus, it has been found in human ascites tumours [1, 2], in malignant human tumours of different sites [3] and in tumours metastatic to the brain [4] that the stemlines tend to assume a pattern characterized by the accumulation of C chromosomes and deficits of B, D and G chromosomes. In such tumours the study of early stages, or still better, of series of consecutive stages, may help to reveal characteristic chromosome patterns. (*d*) The tumours are usually defined histologically and it is conceivable that a tumour type may be homogeneous histologically although chromosomally it is a mixture of different entities. One such instance will be discussed in this paper.

Whenever possible, our chromosome analyses were made in direct preparations from the tumours or in early in vitro passages. We also studied the chromosomes in normal host cells exposed in vitro to the oncogenic agents. Thus, we could observe the immediate chromosome response and also include human cells in our experiments. In the following. I will first review work with experimental tumours induced by Rous sarcoma virus (RSV) and 7,12-dimethylbenz(α)anthracene (DMBA), in which clear non-random chromosome patterns have been demonstrated. After that I will discuss work on human tumours and try and relate to them some of the results from the experimental tumours. Most of the work is based on conventional chromosome methods, but the new techniques for the identification of chromosomes have been utilized successfully in some cases, as will be briefly mentioned later (p. 223–225).

Rous Sarcoma Virus-induced Tumours in the Rat

According to our experience the stemline karyotype of early tumour stages is indistinguishable from the normal karyotype of the host species. We have also found, however, that even during the early stages of tumour development, cells differ from normal cells in one respect: their mitotic mechanism is unstable. This has the result that occasional cells appear with abnormal karyotype. Systematic analyses of such cells have shown that the chromosome deviations are non-random and that cells with certain specific karyotypes are found recurrently. Whether this is so because the same deviations originate de novo more often, or because these cells belong to selectively favoured clones cannot be decided yet. Probably both factors play a part. The fact that chromosome breakage induced in normal cells by the oncogenic agent—viral or chemical—has repeatedly been demonstrated to affect specific chromosome regions preferentially, speaks in favour of the former possibility [5–10]. It is especially suggestive that DMBA and related carcinogens induce preferential breakage in the same rat chromosome which most often appears as trisomic in the DMBA-induced leukemia stemlines.

In the experimental tumours it is the rule that during the first 100 to 200 days of the young primary tumour a specific karyotypic deviation appears in the population with gradually increasing frequency, first in single cells, then in a sideline (at least in 10% of the cells), and eventually in the stemline, thus in the majority of the dividing cells. It may be speculated whether these observations may be extrapolated to all malignancies, and whether consequently in CML the original malignant cells have normal karyotype. The appearance of the first cell with the Ph^1 chromosome must be associated with superior competitive capacity, since in all diagnosed cases the majority of the bone marrow cells have a Ph^1 karyotype.

Various members of our group have followed the chromosomes during early oncogenesis in a considerable number of primary RSV-induced tumours in various rodents: 91 tumours in the mouse [11, 12], 42 in the Chinese hamster [13] and 80 in the rat [14–17]. The disappearance of the normal diploid karyotype in the tumours of these 3 species is seen in fig. 1. The inverse correlation between tumour age and percentage of cells with the normal karyotype forms an S curve, indicating that once the successful karyotypic change has been established, it spreads in the population with accelerated speed. The general behaviour of the chromosomes in these tumours is concordant. Since, however, chromosome patterns are especially clear in the rat, I shall confine myself to the RSV-induced rat tumours.

To recall the appearance of the normal rat

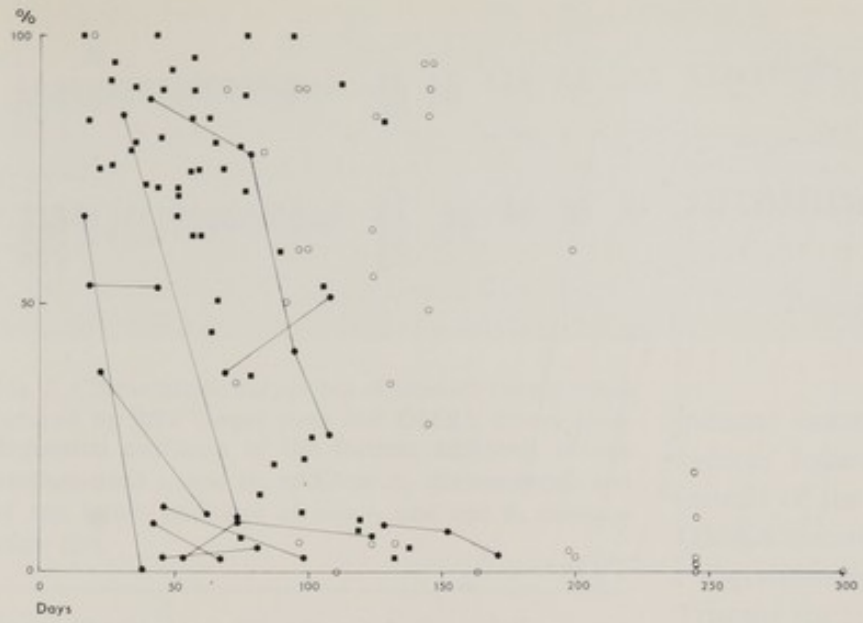


Fig. 1. *Abscissa:* tumour age (days); *ordinate:* % mitoses with normal karyotype.

Correlation between tumour age and disappearance of normal karyotype in primary RSV-induced sarcomas, ●—●, 11 mouse sarcomas, sampled 2 to 4 times [12]; ■, 50 rat sarcomas [16]; ○, 42 Chinese hamster sarcomas [13]. In the former two materials, age is calculated from the time the tumours were first palpable; in the last material, the latency period is also included.

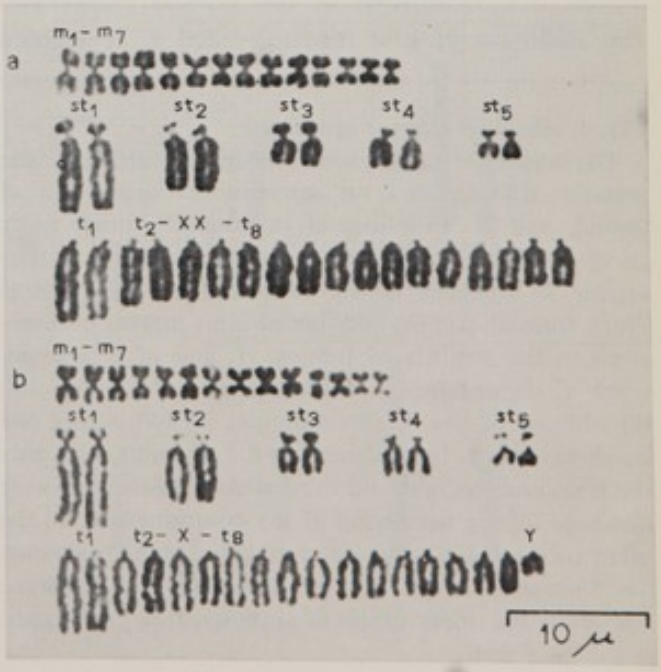
karyotype and to indicate the nomenclature used by our group, fig. 2 is given, showing one female and one male rat karyotype. Its 42 chromosomes are easily distributed into 3 groups with 7 m, 5 st and 9 t pairs. By conventional methods all the st pairs, the t_1 pair and the Y chromosome are identifiable.

After subcutaneous inoculation with RSV virus, young tumours become palpable after an average of 50 days. In the rat tumours it is the rule that the chromosomal changes appearing follow a fairly set pattern, though variations do occur. The first chromosomal change is the addition of one medium-sized t chromosome. This change is followed after some time by additions of first one st_3 and then one st_5 chromosome. In all 80 tumours analysed this karyotypic evolution involving 3 sequential steps was expressed more or less clearly.

The chromosomal events of early carcinogenesis were analysed recently by Mitelman in a highly informative experiment with 2 primary sarcomas of the rat, referred to as tumours II and IV [18]. These tumours were examined at an early stage and found to contain only normal cells with the normal diploid rat karyotype. Each tumour was then divided into several parts and carried by serial in vivo transplantation as 11 individual sublines. In all of the sublines the chromosomes were analysed at every second transfer or on an average once a month.

The disappearance of the normal diploid karyotype in these sublines is illustrated in fig. 3. The curves show the same S shape as in fig. 1. The detailed pattern of the appearance of the new karyotypes in the 11 sublines was interesting. In both series, single hyperdiploid cells began to appear after 15 to 100 days of growth, and after 100 to 250 days these cells had increased in number and formed first sidelines and then stem-

Fig. 2. The normal rat karyotype, (*a*) female; (*b*) male [16].



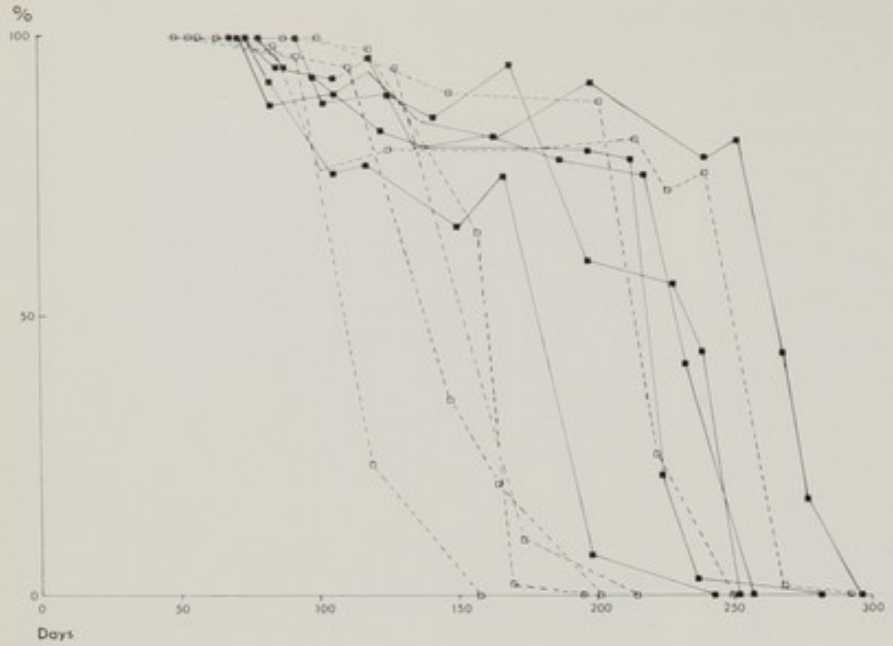


Fig. 3. *Abscissa*: tumour age (days); *ordinate:* % mitoses with normal karyotype.

Correlation between tumour age and disappearance of normal karyotype in 11 sublines of 2 primary RSV-induced rat sarcomas, each subline carried separately in serial in vivo transplantation. □---□, sublines of tumour II; ■—■, sublines of tumour IV [18].

lines. The detailed behaviour of each line is presented in the diagram of fig. 4. The 3 sequential steps (*a*, *b* and *c*) are plotted for each of the 11 sublines; the appearance of each step in a sideline is indicated by small dots and in a stemline by large dots. All sublines of each tumour exhibited the same initial change. Thus, all sublines of tumour II showed the addition of a large marker chromosome and all sublines of tumour IV the addition of 2 medium-sized t chromosomes. These are variations of the typical behaviour, the addition of one medium-sized t. It seemed very likely, according to Mitelman, that the whole or part of the critical t chromosome was contained in the marker of tumour II. Questions such as this will be solved easily by the new banding techniques, in which all chromosome pairs of the rat can be identified.

After the first change, all sublines underwent the 2 sequential changes according to schedule: the addition of one st_3 and one st_5 chromosome. In 9 of the 11 sublines both changes appeared, in the remaining 2 (tumour IV, sublines 3 and 4)

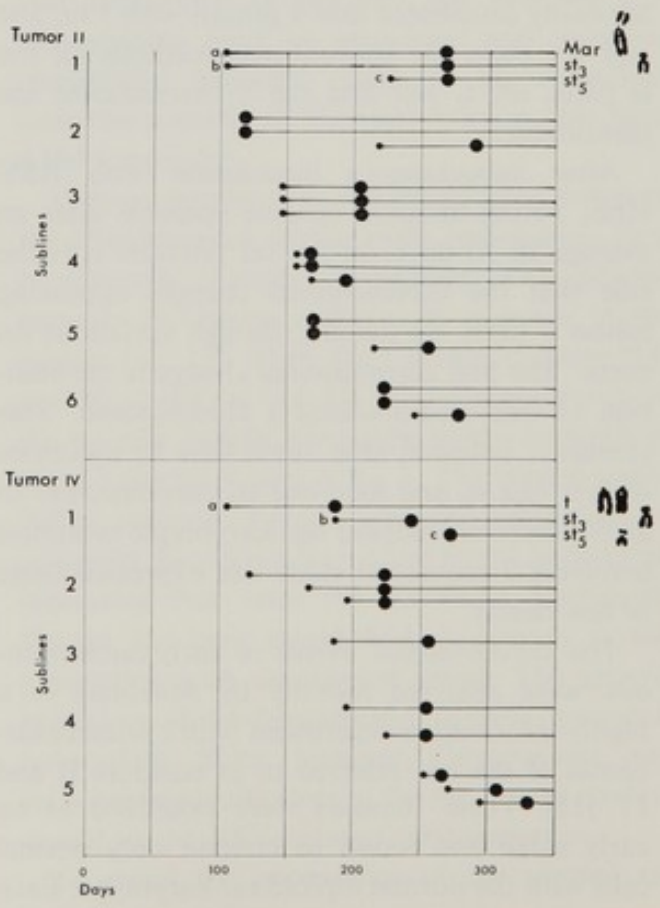


Fig. 4. *Abscissa:* tumour age (days).

The sequential karyotype evolution in 6 sublines of the primary RSV-induced rat sarcoma II (upper half of figure), and in 5 sublines of sarcoma IV (lower half). In all sublines 3 sequential steps are recorded, as they appear in sidelines (small dots) and stemlines (large dots), from above: (*a*) addition of large marker chromosome in the sublines of tumour II, and of 2 medium-sized t chromosomes in the sublines of tumour IV; (*b*) addition of one st_3 chromosome; (*c*) addition of one st_5 chromosome. In sublines 3 and 4 of tumour IV only the first, and the first and third steps, respectively, were observed during the period of the experiment, in all the other sublines, all 3 steps occurred [18]. (At the engraving, the representation of the St5 chromosome has become lost from the upper group of chromosomes to the right in the diagram).

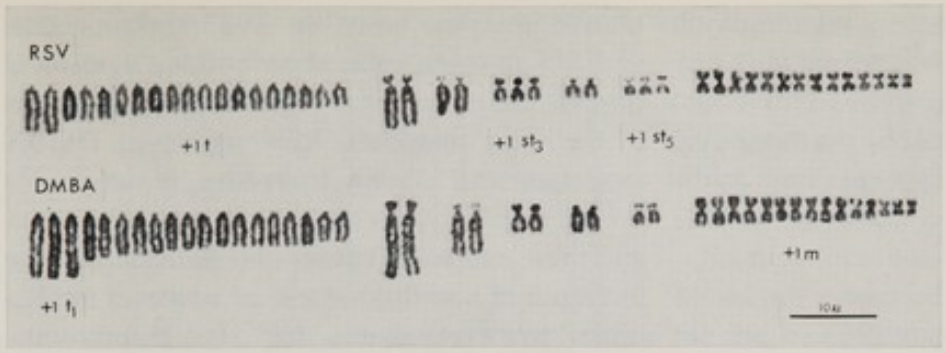


Fig. 5. Characteristic karyotypes of primary rat sarcomas induced by RSV (upper row) and DMBA (lower row). Sequential evolution of the former: additions of one medium-sized t, one st_3 and one st_5 chromosome, and of the latter: additions of one t_1 and one m chromosome [20].

one or both steps did not reach the sideline stage during the period of the experiment.

This experiment leads up to the following alternative questions: (*a*) do all the deviating cells stem from one ancestral cell originating only once in each tumour, or (*b*) do the deviating cells originate de novo in each of the 11 sublines? Mitelman's experiment does not give a conclusive answer. Even though his results indicate that the chromosomal changes originated in each of the sublines, the possibility exists that the deviating karyotypes were present in the original sample of the primary tumours in a frequency too low to allow of detection but high enough to ensure that deviating cells were transferred to all sublines. It should be possible to settle this question experimentally, for instance by performing clonings during early stages, when deviating cells are absent or rare. Such experiments are in progress in our laboratory.

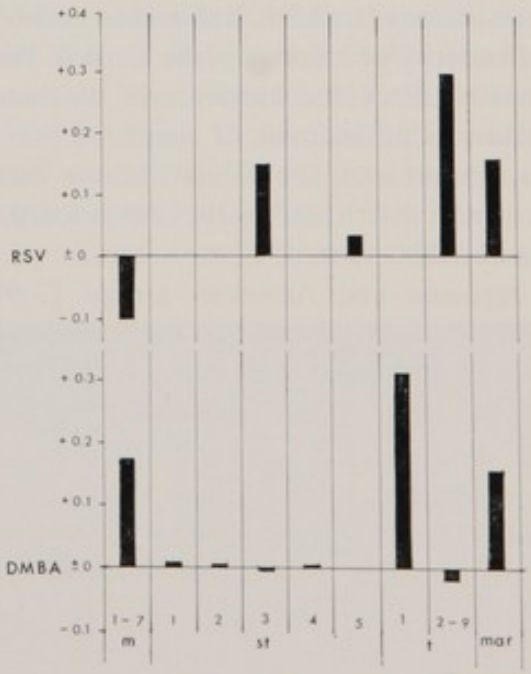
The conclusion of this part of my paper is that the primary RSV-induced sarcomas, starting out with the normal karyotype of the host species, undergo a sequence of predetermined chromosome changes.

Dimethylbenz(α)anthracene-induced Tumours in the Rat

Sarcomas have been induced by DMBA in the same tissue and the same inbred rat strain as in the work with RSV, and their chromosomes studied by Mitelman & Levan [19]. Most interestingly they exhibited a completely different chromosome pattern. In fig. 5, characteristic stemline karyotypes are compared from RSV- and DMBA-induced sarcomas [20]. It is seen that DMBA induces hyperdiploidy just as does RSV, but instead of the medium-sized t chromosome, the DMBA sarcomas have the addition of the longest t chromosome, the t_1, as their initial change. Trisomy for t_1 was found in approximately every third cell examined. The contrast between the two patterns is accentuated by the fact that in RSV tumours Mitelman scrutinized 1 075 karyotypes without once detecting a single cell with an additional t_1! Also the second sequential change in DMBA tumours, the addition of an m chromosome, is different from the RSV pattern, in which m chromosomes rather tended to become lost.

The patterns of RSV and DMBA sarcomas are compared in the diagram of fig. 6, in which

Fig. 6. *Abscissa:* identifiable chromosome pairs or groups of the rat karyotype, and marker chromosomes; *ordinate:* average gains or losses of chromosomes in 1 033 cells of 50 RSV-induced sarcomas (upper half), and in 278 cells of 12 DMBA-induced sarcomas (lower half) [16, 19].



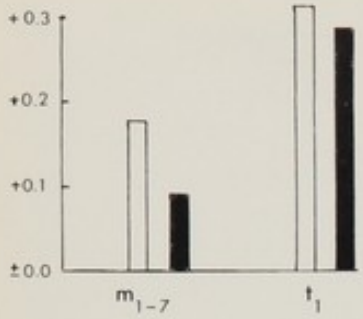


Fig. 7. *Abscissa:* chromosomes m_{1-7} and t_1 in DMBA rat sarcomas (□) and leukemias (■); *ordinate*: average gains in 278 cells of the former [19] and 3 083 cells of the latter [7].

the average incidence of chromosome changes is summarized for all cells analysed of the two sarcoma types. The zero line represents the number of chromosomes in the normal diploid cell, that is, the cell from which the evolution started out. The scale is graduated in tenths of the number of chromosomes lost or gained: a value of +0.1 means that every tenth cell has the addition of one chromosome of the type concerned. Since the general trend is towards hyperdiploidy, positive deviations predominate.

The diagram shows that most of the individually recognizable chromosomes are close to zero, and have thus taken no part in the karyotypic evolution. These are st_1, st_2 and st_4 in both tumour types, t_1 in RSV, st_3 and st_5 in DMBA. The conclusions from the direct observations are confirmed by the calculations underlying the diagram: In RSV there are additions in 3 classes; in order of falling magnitude they are: medium-sized t, st_3 and st_5, and also losses of m; in DMBA there are additions of t_1 and m. It should be noticed that the frequency of markers is the same in the two materials. Since the incidence of markers can be taken as an estimate of structural variability in the tumours, the values indicate that this parameter is determined by the host genotype rather than by the 2 tumour types.

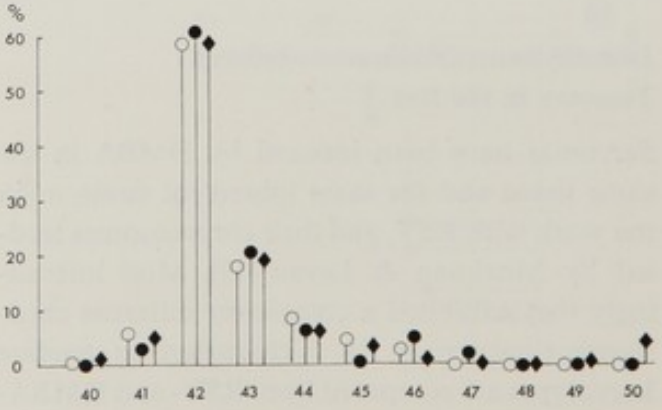
Now, Japanese and American groups [7–9] have studied great numbers of rat leukemias induced by DMBA and related compounds. This gave an opportunity to learn what effect on the karyotypic pattern the induction of malignancy by the same agent in another tissue system may have. It is extremely interesting that not only is the karyotypic pattern of the DMBA-induced rat leukemias identical in every respect with the pattern of the DMBA-induced sarcomas, but quantitative analyses, based on 3 083 leukemic cells and 278 sarcoma cells, show striking agreement (fig. 7). The coincidence in chromosome numbers in the three materials, RSV sarcomas, DMBA sarcomas, and DMBA leukemias, is rather surprising (fig. 8). As in the case of structural changes, these numerical results may indicate that the incidence of non-disjunction, or whatever mechanisms are responsible for the chromosome number variation, is determined by the rat genotype, not by the different tumours.

The conclusions of this part of my paper are that the non-random patterns of the tumour karyotypes were determined by the oncogenic agents and that the tissue specificity appeared to be without influence on the karyotypic evolution.

Human Tumours

For many reasons it is difficult to transfer experiences from experimental tumours to human tumours. In man, each tumour has a different genetic background (barring tumours in identical twins and multiple tumours in the same host); in man the etiology of a tumour is generally unknown, the same histologic type may include different etiologies; in man it is difficult to be sure about the age of an individual tumour. In addition to these points come the greater difficulties of methodology. In consequence of all this, really systematic studies of the chromosomes of human tumours have been rare, and systematic studies may be necessary to disclose non-random patterns in them. The very clear pattern of CML has proved an exception.

Fig. 8. *Abscissa:* chromosome numbers in the diploid region of primary rat sarcomas; *ordinate:* % incidence of these numbers in: ○, 1 033 cells of RSV-induced sarcomas [16]; ●, 278 cells of DMBA-induced sarcomas [19]; ◆, 3 083 cells of DMBA-induced leukemias [7].



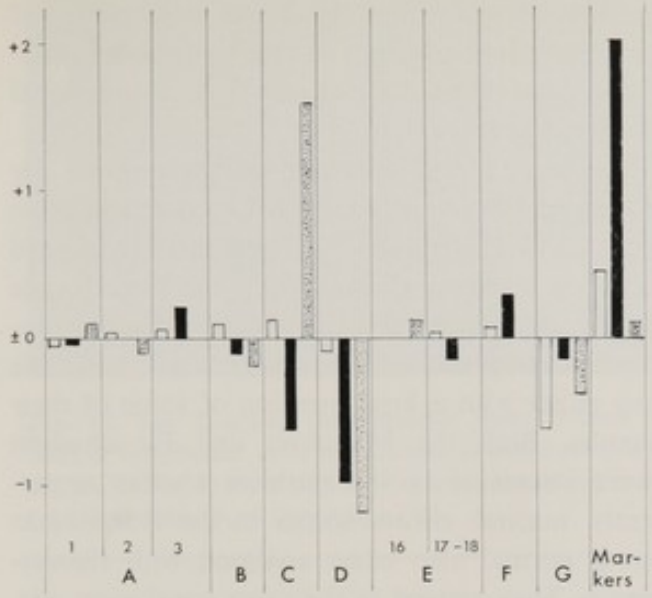


Fig. 9. *Abscissa:* identifiable chromosome pairs or groups of the human karyotype, and marker chromosomes; *ordinate:* deviations of tumour stemlines from expected random distribution, □, 104 meningomas [24]; ■, 50 gliomas [25]; stippled bars, 47 cervical carcinomas [26].

In our group the chromosomes have been analysed in 50 meningomas and 50 gliomas by Mark [18–20, 23, 24 and 25, respectively] and 47 cervical carcinomas by Granberg [26]. Each of these three materials exhibits clear non-random chromosomal patterns. The test for non-randomness involves calculations as to whether the chromosome numbers observed can have originated by random additions or subtractions of the various chromosome types. Deviations between found and expected can be submitted to ordinary *t*-tests for statistical significance. It was found that in each specific tumour the calculations resulted in small positive or negative deviations for most chromosome types and in large deviations for one or a few chromosome types. Just as in the experimental tumours, it was usually found that each human tumour had a characteristic spectrum of strongly affected chromosomes, which were responsible for the karyotypic evolution during cancer development.

Such patterns for the three tumour types mentioned are presented in fig. 9, in which the data for the meningomas in addition to Mark's tumours include 54 tumours studied by other workers, mainly the Zang group in Munich [27–29]. These data were compiled by Mark [24]. The meningomas (unfilled bars in fig. 9) show smaller amplitudes than the other two; the spectrum of the meningomas is dominated by the negative bar of the G group. The gliomas (filled bars) and the cervical carcinomas (stippled bars) exhibit much bigger amplitudes; the gliomas are characterized by losses in the G and D groups; the cervix carcinomas have still bigger losses in the D group and large additions in the C group.

In the meningomas Mark and collaborators [30, 31] and Zankl & Zang [32] have independently applied fluorescence technique to 13 and 5 meningomas, respectively. Accordingly it is possible to compare the results of conventional and Q banding techniques in the meningomas. Fig. 10 shows a karyotype of one of the meningomas in fluorescence analysis, in which all chromosome pairs were identified [24]. The immediate result, gained by both research groups, was the identification as No. 22 of the single G chromosome, the partner of which is lost more or less regularly. It was also established that the small Ph[1]-like marker seen by both groups, was a deleted No. 22 (fig. 11 sm). In this figure another marker is also pictured (M), which was interpreted by Mark as an isochromosome for the short arm of No. 22, satellites sometimes being visible in both ends of it.

By fluorescence technique Mark performed

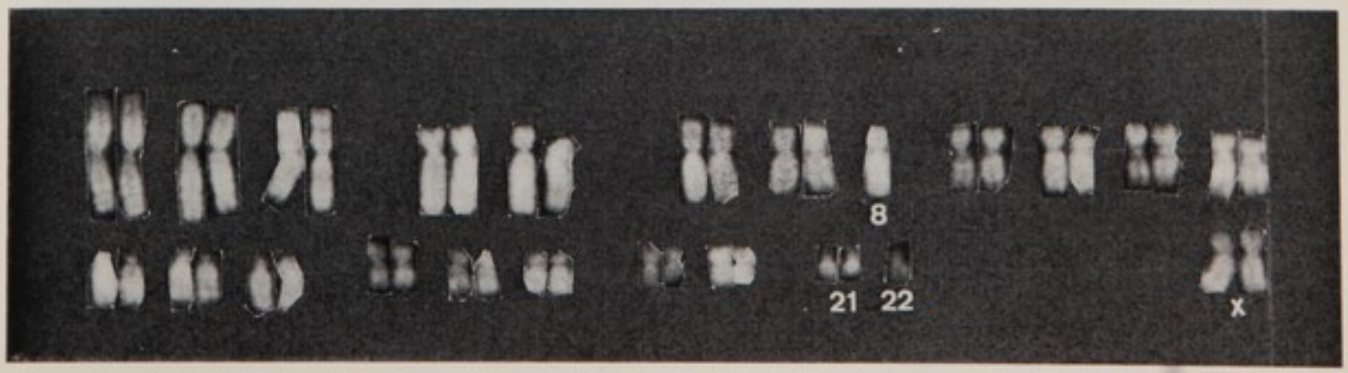


Fig. 10. Stemline karyotype of meningoma M16 in the Q-band technique, one chromosome No. 8 and one No. 22 lacking [30].

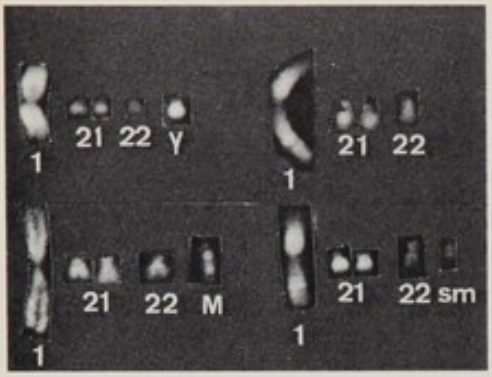


Fig. 11. Chromosome No. 1 and the G-group chromosomes from meningoma stemlines, upper row from left to right: meningomas M23 and M24 each with one No. 22 lacking, lower row from left to right: meningomas M26 and M25 with one No. 22 exchanged for a small marker, interpreted as No. 22pi (*M* in figure) and 22q− (*sm* in figure) [31].

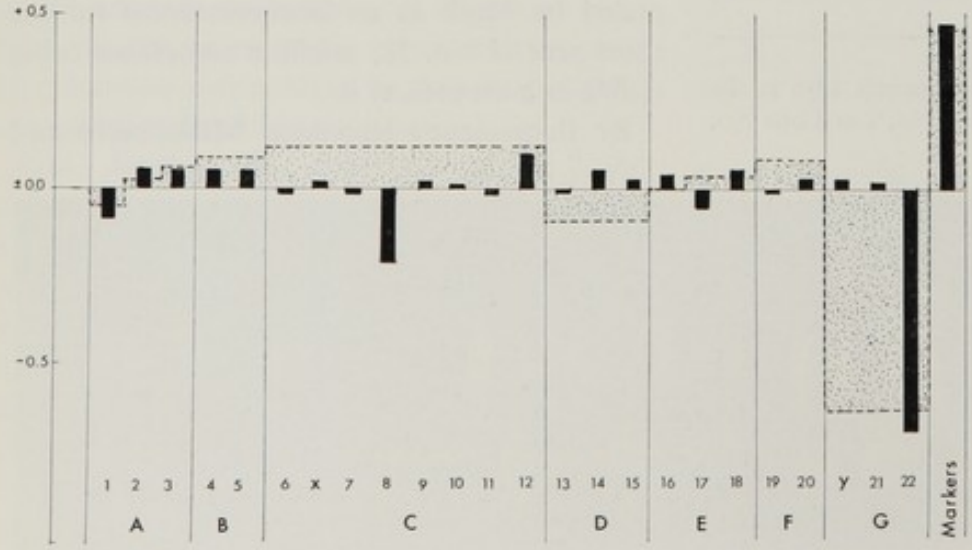
275 complete karyotype analyses in cells from 13 meningomas, and in fig. 12 the average deviations from randomness have been plotted for the individual chromosome types on a background of the stemlines of the 104 meningomas studied with conventional technique (fig. 9). This is a good illustration of the gain in exactitude achieved thanks to the fluorescence technique. The negative value of the G group is predominant, and is now revealed as due entirely to losses of No. 22, the Y chromosome and No. 21 not being affected at all. Most of the other values do not deviate much from randomness. In the C group, however, the losses of No. 8 and gains of No. 12 are interesting and may be taken as possible indications of secondary changes in a sequential karyotypic evolution.

Fig. 12. *Abscissa:* the human chromosome types and marker chromosomes; *ordinate:* deviations from expected random distribution, filled bars: 275 karyotypes from 13 meningomas studied in the Q-band technique [30, 31]; stippled histogram: distribution of 104 meningoma stemlines (= open bars in fig. 9).

During 1971, 3 guests in our laboratory, Dr George Manolov and Dr Yanka Manolova from Sofia and Dr Tamás Fleischmann from Szeged reached interesting results with the fluorescence technique in Burkitt lymphomas and other malignant lymphomas, respectively. I will conclude my paper with a brief mention of some of their results. Both the Manolovs and Fleischmann were interested in the problem whether apparently normal chromosomes in the lymphomas were normal also when analysed with fluorescence. They arrived at the same conclusion, viz. that most of the chromosomes in the lymphomas that were classed as normal by conventional methods, appeared normal also in the fluorescence analysis [33, 34].

The Manolovs, however, made the intriguing discovery [35] that 10 out of 12 Burkitt lymphoma cases thus analysed exhibited a small structural abnormality that would never have been detected by conventional chromosome techniques: Pair No.14 was heterozygous for a small additional fluorescent band near the end of the long arm. This band was stained strongly by the G-band technique (fig. 13).

In a male patient with lymphosarcoma or possibly lymphoblastic sarcoma, Fleischmann and collaborators [36] started the study with ordinary orcein slides. After the fluorescence analysis of the same material had been applied, it was found that, although not many actual mistakes had been made, the increase in precision was remarkable



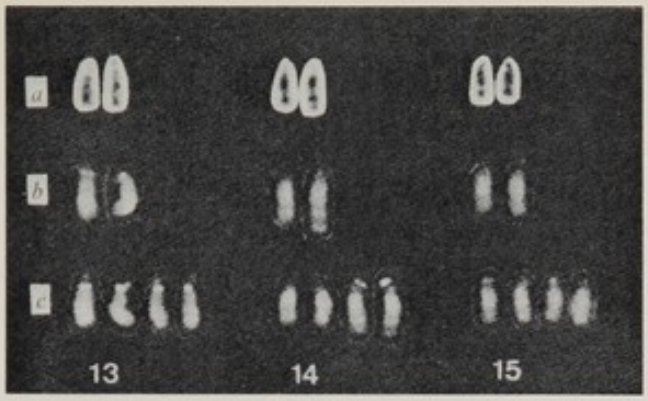


Fig. 13. The D-group chromosomes from 3 cases of Burkitt lymphoma; note extra band in one homologue of No. 14: (*a*, *b*) from diploid, (*c*) from tetraploid cells; (*a*) G-banding; (*b*, *c*) Q-banding [35].

(fig. 14). Even though the fluorescence staining of this tumour was not ideal, it was possible to identify all normal chromosome types and determine which were present in normal numbers or in monosomic or polysomic numbers. Also some of the markers were identified, one as a No. 1q –, one as a No. 3p – q –; also the Y was revealed, translocated onto another small chromosome.

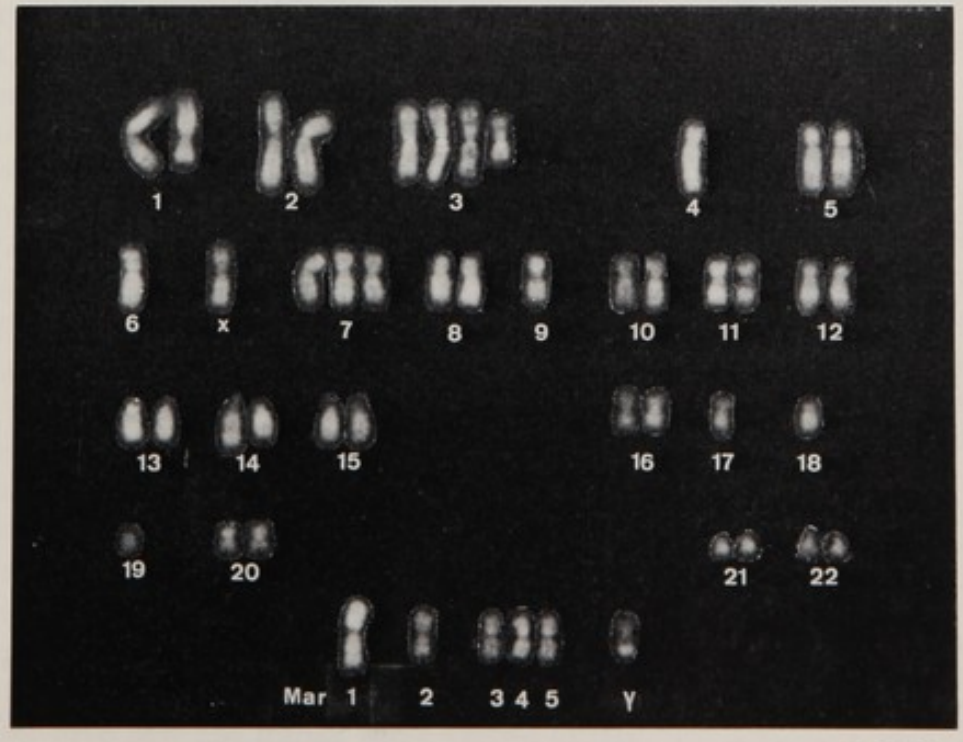
In this tumour, 3 markers were of special interest. They are numbered 3 to 5 in fig. 14 and had all the appearances of being isochromosomes. Apparently, in this stemline the same chromosome segment was repeated 6 times. Now it was especially interesting that isochromosomes of the same size and with the same fluorescent properties were present as constant members of the stemline karyotypes in 4 other malignant lymphomas. These were one lymphosarcoma, one malignant lymphoma of uncertain type, one case of Hodgkin's disease [37], and one further lymphosarcoma [38]. Representative instances of the isochromosomes are displayed in fig. 15. As seen in this figure, these 5 stemlines had one isochromosome in 3 cases, and 2 or 3 isochromosomes in one case each. Isochromosomes of similar size have been reported earlier from studies with conventional technique both from other cases of malignant lymphomas [39] and from CML [40].

In conclusion of this part of my paper it may be stated that the rules for chromosome non-random patterns found to be valid in experimental tumours also obtain in human tumours. The classical case of correlation between chromosome pattern and tumour type, the CML, is not as special as before. Thus, the meningomas exhibit a very strict pattern, including sequential karyotype evolution. All human tumours, in which sufficient material has been examined, are compatible with the same general pattern.

Fig. 14. Stemline karyotype in one case of lymphosarcoma, identification by means of Q-banding of all normal chromosomes and several marker chromosomes [36].

Concluding Remarks

The systematic collection over several years of chromosomal information from large series of



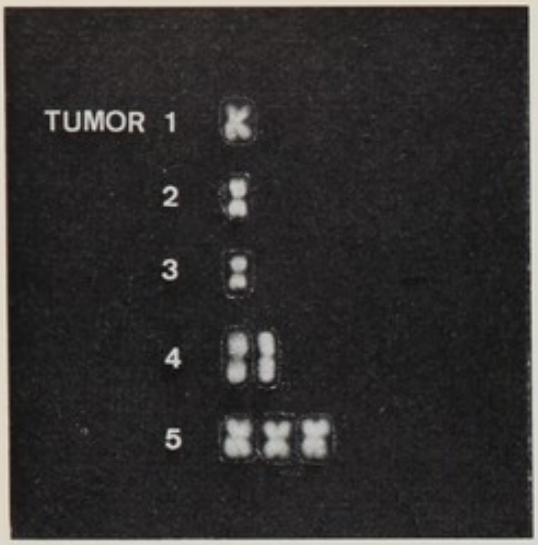


Fig. 15. Isochromosomes, representative of the stemlines of 5 malignant lymphomas, tumours 1 and 2, lymphosarcomas; 3, malignant lymphoma of uncertain type; 4, Hodgkin's disease; 5, lymphosarcoma or lymphoblastic sarcoma. Tumours 1, 3 and 4 correspond to Nos. 1, 2 and 3 in [37], tumour 2 is from an unpublished case of Dr Fleischmann [38] and tumour 5 is the same tumour as in fig. 14 [36].

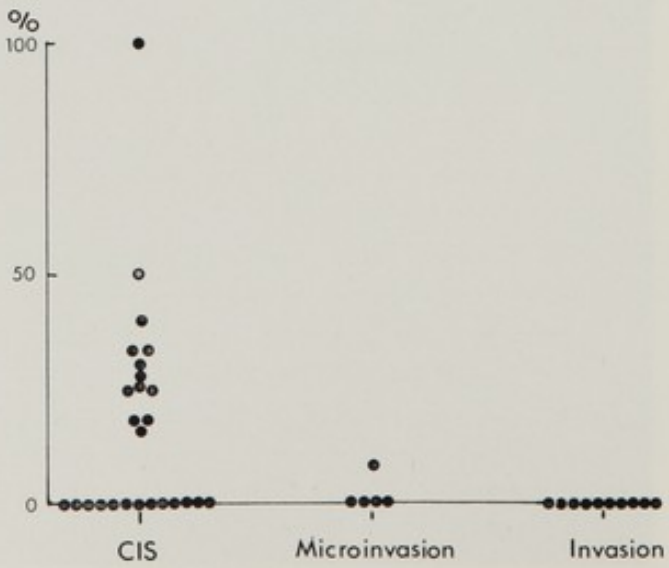
tumours has led to the establishment of clear correlations between non-random patterns of stemline karyotypes and specific kinds of tumours. These data show, especially clearly in the RSV- and DMBA-induced rat tumours that chromosomal behaviour during early tumour development is non-random and predetermined. Even though conditions are somewhat less clear in the RSV-induced tumours in mice and Chinese hamsters, enough observations have been made to show that conditions are similar in them. Even human tumours very clearly obey the same rules.

That the chromosomal evolution during tumour development is correlated with significant functional properties of the tumours has been demonstrated in several materials. Thus, Mitelman points out [41] that in RSV tumours of all 3 species investigated the disappearance of the normal diploid karyotype was associated with a clear trend to histopathologic dedifferentiation: from the highly differentiated fibrosarcomas with normal diploid stemlines, via the spindle cell sarcomas with deviating sidelines to the anaplastic sarcomas with heteroploid stemlines. A similar trend was demonstrated by Granberg [26] in the cervical tumours referred to above. In them, the disappearance of normal diploid karyotypes was correlated with the development of invasiveness. Normal cells were frequent in the 32 cases of carcinoma in situ, 64 out of the 91 cells karyotyped with 46 chromosomes being normal. In 5 carcinomas with microinvasion only 1 out of 20 diploid cells had normal karyotype and in 10 invasive carcinomas no single cell was seen with normal karyotype (fig. 16). In this tumour evidently the onset of invasiveness marks a dramatic shift in the karyotypic dynamics of the cell population.

The correlation between chromosome pattern and inducing agent is thought-provoking. Apparently each oncogenic agent has the capacity to stamp the hereditary mechanism of the host cell with its specific program for a sequence of chromosomal events. This program is then played up with only minor variations in all tumours induced by the same agent. That especially the early steps in this karyotypic evolution must be associated with tremendous gain in competitive capacity is evidenced by the accelerated speed with which the new karyotype permeates the population, outgrowing the equally malignant cells with normal karyotype.

It is premature to speculate as to the details of the genetic events underlying the chromosomal variability, or to generalize the results so far obtained. It seems that each inducing agent disturbs the chromosomal equilibrium and starts a selective pressure in favour of a new karyotype,

Fig. 16. *Abscissa:* three steps in dedifferentiation of cervical carcinomas; *ordinate:* % normal diploid karyotypes in 47 carcinomas, including 32 carcinomas in situ, 5 carcinomas with microinvasion and 10 invasive carcinomas. Among the 13 carcinomas in situ with no normal diploid karyotype, 10 had a majority of cells in the tetraploid or triploid regions [26].



specific for each agent. In tissue culture systems it has been demonstrated repeatedly that the first visible response to viral or chemical oncogens is chromosome breakage, non-randomly distributed. This suggests a model, in which different oncogenic agents affect different constellations of loci in the host karyotype, triggering the subsequent evolution.

An interesting connection between the initial chromosome lesions and the numeric changes appearing later on may be inferred from the observations that the individual chromosome types that take most active part in the early cancer development, have been shown sometimes to be the same that respond with preferential breakage to the same oncogen. As mentioned above, this was the case with the t_1 chromosome in normal bone marrow of the rat, when challenged with DMBA. Similarly, No. 22 in human meningomas is lost most often from the stemlines and is also the chromosome most often subject to structural changes.

As a result of the chromosome study in cancer, histologic tumour types can be divided into karyotypic subgroups: among the subcutaneous sarcomas in rats, two distinct subgroups were discerned, one induced by RSV and one by DMBA. These subgroups cannot be distinguished histopathologically. The chromosome study has also combined fundamentally different histologic types, such as DMBA-induced sarcomas and DMBA-induced leukemias in the rat. These two types cannot be distinguished chromosomally. Chromosomal similarities were found in human malignancies, too: Philadelphia chromosome-like modifications of chromosome No. 22 have been observed both in meningomas and in Burkitt lymphomas, the latter by Manolov & Manolova [42]. The characteristic isomarker of Fleischmann was found both in lymphosarcomas and in Hodgkin's disease. The significance of these similarities is not yet understood.

The chromosome study of cancer is at present in a very dynamic phase of development. The new findings that the karyotypic evolution during early stages is determined directly by the etiologic factors are extremely exciting. It is now urgent to do much more experimental work to test and extend the present observations. The new techniques for chromosome identification now available have only just started being applied in this area. They give excellent promise of rapid new development in the chromosome research in cancer.

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Discussion

Lejeune: First, I want to say how much I appreciated the importance of your contribution.

Question: Can you demonstrate a firm correlation between a specific chromosomal lesion and the histological type of the tumour. This would be a basic fact in the hypothesis of a "common variant".

Levan: No, in our experience the correlation is rather between inducing agent and chromosomal change. In the case of DMBA-induced sarcomas in rats we obtained the same chromosomal pattern—the addition of one t_1 chromosome—as Japanese and American workers obtained in DMBA-induced rat leukemias, whereas in the RSV-induced rat sarcomas, although indistinguishable histologically from the DMBA-induced sarcomas, we found a completely different chromosomal pattern.

Ford: May I ask Professor Levan if he would comment on the following point? When the Ph¹-chromosome was first discovered I anticipated that it would not be long before cases of chronic myeloid leukaemia were found in which, though the distal part of 22q was presumptively lost, the proximal part of 22q was involved in a rearrangement. That this has not been borne out is surprising, since the classic studies of chromosome breakage lead one to suppose that breaks in general are capable of interacting with others to generate recognisable rearrangements. Though there are inferred to be both spatial and temporal limitations on such interactions, one might reasonably expect that from time to time breaks would arise spontaneously in other chromosomes sufficiently close in time and space to the lesion that produces a Ph¹-chromosome for interaction to occur.

It seems therefore that there must be a unique feature involved in the origin of the Ph¹-chromosome: either a break (resulting in terminal deletion) with such rapid heating that interaction with other breaks is precluded; or an intra-chromosomal rearrangement (generating an interstitial deletion) that occurs in circumstances that preclude interaction with other breakage sites.

Levan: The remarkable uniformity both of the Ph¹-chromosome and of the CML stemline carrying this chromosome indicates that only a very precise chromosomal change will result in the competitive superiority of the stemline. Structural variants of the Ph¹-chromosome—including interchanges with other chromosomes—may be lacking, not because they are never formed but because they are always eliminated. It may be that hemizygosity for the deleted segment of No. 22 is necessary for the viability of the malignant stemline, as suggested recently by Susumu Ohno from other premises (Physiol rev 51 (1971) 512).

Gropp: Would you say that the loss of chromosome No. 22 and the appearance of a marker chromosome in meningomas is a semispecific or specific phenomenon? Could you imagine that in this case, material of chromosome No. 22 is translocated to another chromosome? Furthermore, how do you interpret the fact that a definitely abnormal karyotype appears in a histologically benign tumour?

Levan: In his survey of 104 meningoma stemlines, my colleague Joachim Mark showed loss of G chromosome in 70 of them. In studies with the fluorescence technique, Mark as well as Zankl & Zang demonstrated in altogether 18 cases that the G-chromosome involved in these losses was No. 22. It was also shown that the small Ph¹-like chromosome often seen in meningomas was a deleted No. 22. In my opinion this is evidence that this chromosome is specifically involved in the development of meningomas, even though the specificity is not as selective as in the case of the Ph¹-chromosome in CML. I do not think that the entire or most of No. 22 can be present translocated onto another chromosome in karyotypes lacking one No. 22, since that

would probably have been detected at the fluorescence analysis. This does not exclude that a small piece of No. 22 may be hidden, translocated to another location in the karyotype.

Meningomas are just one among many benign tumours, in which stemlines with chromosomal deviations—both numeric and structural—are found. Other instances are ependymomas, neurinomas, oligodendrogliomas, pituitary adenomas, colonic polyps, hydatidiform moles etc. It is too early to know whether any consistent chromosomal feature exists, distinguishing benign and malignant neoplasms. As mentioned in my paper, the disappearance of cells with normal karyotype marked the onset of invasiveness in the cervix tumours studied by Ingrid Granberg, and a general correlation between predominance of new stemline karyotypes and malignant progression was pointed out by Felix Mitelman in all types of RSV-induced tumours.

Application of Fluorescence Analysis of Chromosomes in Clinical Cytogenetics

J. Lindsten,[1] *M. Holmberg,*[2] *Maj Hultén,*[1] *J. Jonasson,*[1] *Gunnel Licznerski*[1] *and A. J. Therkelsen*[3]

[1]Department of Clinical Genetics, Karolinska Hospital, 104 01 Stockholm,
[2]Research Institute of National Defense, Department 4, 104 50 Stockholm, Sweden, and
[3]Department of Human Genetics, University of Aarhus, 8000 Aarhus, Denmark

The finding during the most recent years that all human chromosomes exhibit a specific and reproducible banding pattern when stained according to a number of different techniques, makes it possible to identify chromosome abnormalities in much greater detail than ever before. This has led to a rapid progress within clinical cytogenetics. Thus, extra chromosomes can be specifically identified rather than merely assigned to a group of chromosomes. Minor chromosome abnormalities are much more easily detected, and the nature of structural rearrangements can be clarified to a much greater extent than earlier. The increased resolution power of the banding techniques has made possible a more detailed study of the genotype–phenotype correlation and a more accurate genetic counselling in certain cases with chromosome abnormalities. It also offers greater possibilities to localize genes to specific chromosome regions. However, it should be pointed out that even these techniques do not always allow an evaluation of the exact nature of a structural chromosome aberration. Additional meiotic studies might be required [4], and even then some aberrations cannot be explained.

The aim of the present brief review is to introduce the session on the application of chromosome banding techniques, mainly the fluorescence technique, in clinical cytogenetics. Some aspects on the potentials of the techniques will be discussed and exemplified. Detailed presentations of other aspects on the use of the techniques will be given by other authors in this volume.

Identification of Male Meiotic Chromosomes

Soon after the introduction of the quinacrine fluorescence technique it was shown that the brightly fluorescent region on the Y chromosome can be identified not only in somatic cells but also in germ-line cells from normal males. This region has now been observed in spermatogonial metaphases, first and second spermatocytes as well as in sperms [2, 18, 19]. The X and Y chromosomes were shown to be associated by their short arms in cells in diakinesis to first metaphase [18]. However, even the more subtle fluorescent bands can be identified in meiotic chromosomes. Spermatogonial metaphases show the same fluorescence pattern as do somatic chromosomes, and a full karyotype identification can be made at least in the cell type with relatively long chromosomes [13]. Furthermore, it is often possible to identify a large number of the bivalents in diakinesis to first metaphase on the basis of their fluorescence pattern (fig. 1) [5].

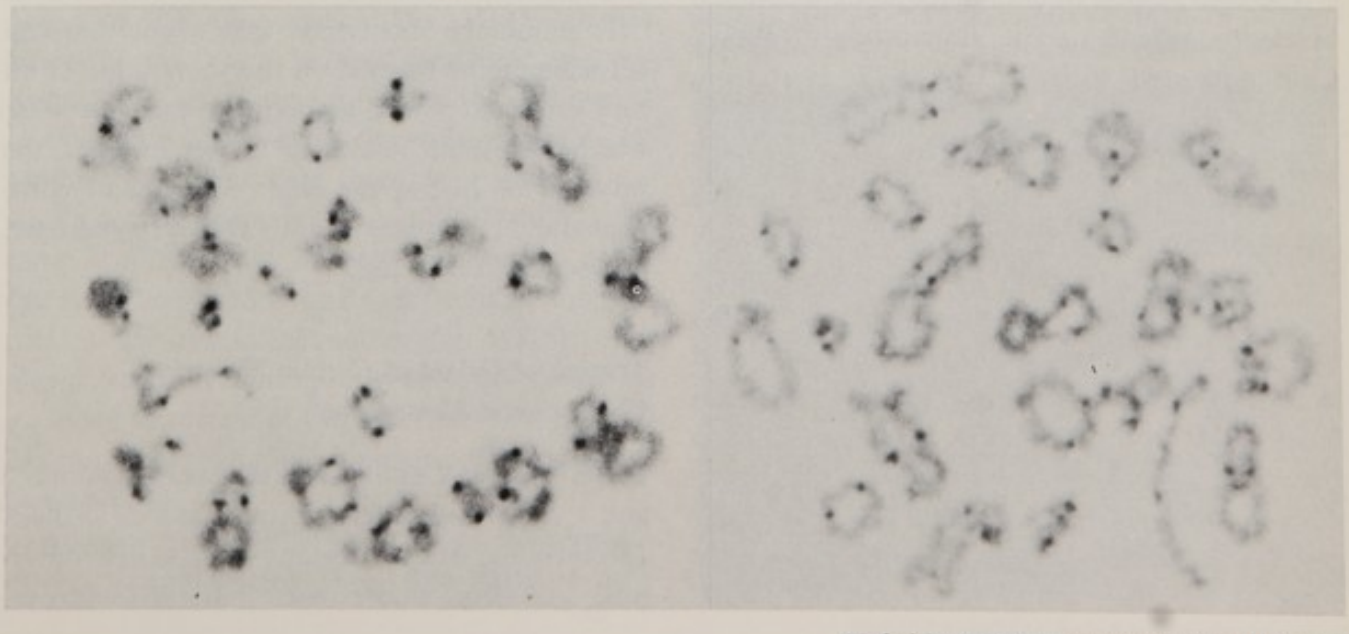
By the application of successive stainings on the same cells, i.e. for instance first quinacrine fluorescence for identification of the bivalents, followed by orcein staining for drawing and measuring of the bivalents, and finally staining for localization of the centromeres (fig. 2) [1], it is possible to obtain further information about the individual bivalents. Their relative lengths and centromere indices correspond to those of the mitotic chromosomes, and thus the bivalents do not exhibit a deviating pattern of contraction [13].

Fig. 1. Similarity in quinacrine banding pattern between mitotic and meiotic karyotypes. From Caspersson et al. [5].

On the basis of these observations it is then possible to determine the number of chiasmata on individual bivalents and their arms [13, 17].

The mean number of chiasmata per cell in diakinesis to first metaphase is about 50 in normal males [17]. Each autosomal bivalent has at least one chiasma, and the frequency of univalents is apparently very low indeed. The frequency of

Fig. 2. Cells in diakinesis to first metaphase stained for the identification of the centromere regions.



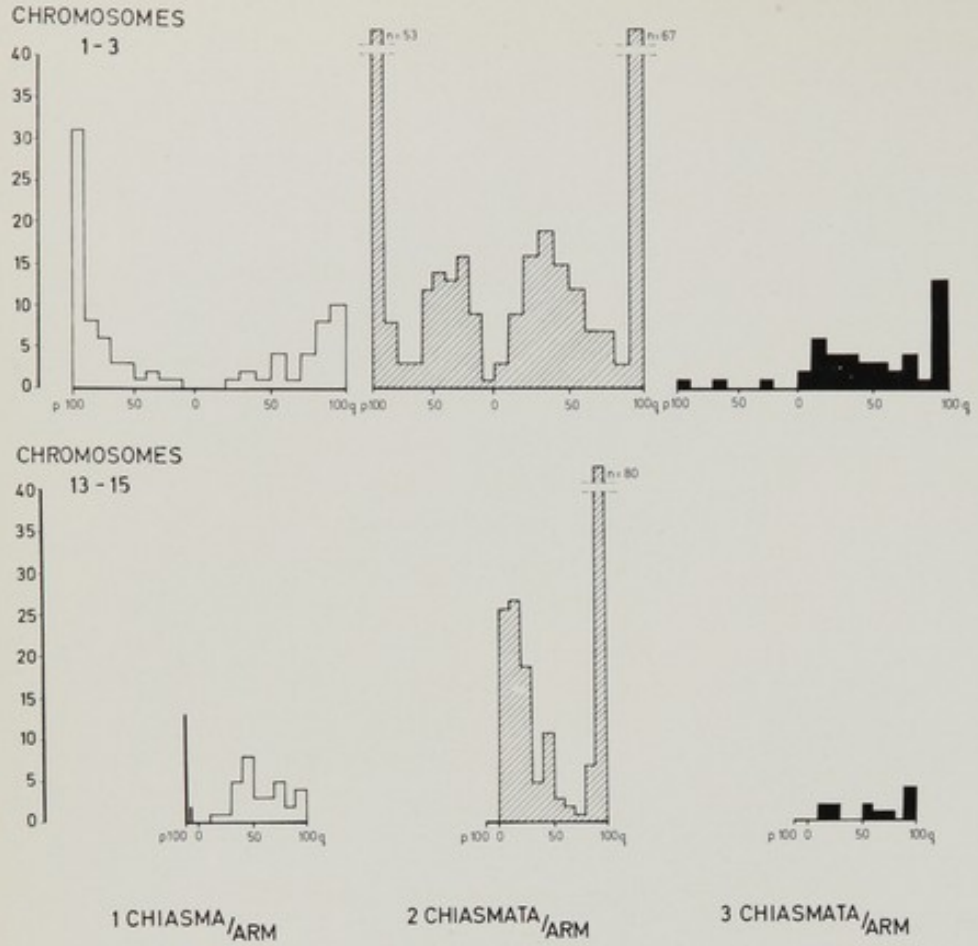


Fig. 3. Histograms showing the distribution of chiasmata on bivalents 1–3 and 13–15, respectively. The scale on the abscissa corresponds to the relative length of the chromosomes. The centromeres (0) have been orientated vertically. All chromosome arms were analysed separately. The left column shows the distribution of chiasmata on arms with only one chiasma, the middle column arms with two and the right column arms with three chiasmata.

additional chiasmata is correlated to the length of the bivalents.

The distribution of chiasmata along individual bivalents reveals some interesting features. Chiasmata seem to be located as far away from each other as possible. Thus, for instance, a D-group bivalent with two chiasmata on the long arm will have one chiasma close to the centromere and the other one close to the distal end of the arm. A submetacentric chromosome with one chiasma on the short and two on the long arm will have one chiasma at the distal end of each arm, while the third chiasma will be located at about the middle of the long arm. If a large metacentric chromosome has four chiasmata, one will be located at the end and the other at about the middle of each arm. The three loops obtained will differ somewhat in size, the middle one with the centromere being the largest, the other two being of a similar, somewhat smaller size (fig. 3). Thus, these data indicate that there is a repulsion between the chromosomes in the centromere region. Whether there is repulsion also along the whole chromosome, or whether the distribution of chiasmata merely reflects the repulsion in the centromere region transmitted towards the chromosome ends in a specific way due to the chromosome structure remains to be shown [13].

It should be mentioned that a chiasma can occasionally be formed on the short arms of all the long and short acrocentric chromosomes, which has some relevance to, for instance, the problem of tracing the origin of the extra chromosome 21 in patients with Down's syndrome, as discussed on p. 233.

Tracing of Origin of Certain Numerical Chromosome Aberrations

It has been well established that chromosomes 3 and 4, the D and G chromosomes as well as the Y chromosome exhibit regions with a heteromorphic quinacrine fluorescence pattern. In favour-

Table 1. *Age of the parents at the birth of a trisomy 21 child in families providing some information with regard to where non-disjunction might have occurred*

MI, first meiosis; MII, second meiosis.

Family No.	Meiotic non-disjunction				Mitotic non-disjunction	Age of parents (years)	
	Maternal		Paternal				
	MI	MII	MI	MII		Mother	Father
2	−	+	+	+	+	30	37
6	−	+	+	+	+	26	24
8	+	−	−	−	−	40	44
11	−	+	+	−	+	23	38
15	−	+	+	−	+	29	32
22	+	−	+	−	−	35	39
27	−	+	+	−	+	28	36
32	+	+	+	−	+	31	34
36	+	+	−	+	+	41	41
37	+	+	+	−	+	31	34
42	−	+	+	+	+	22	28
44	+	+	−	−	+	42	44

able families it should then be possible to trace the origin of an extra chromosome of any of these types [3].

Congenital trisomic conditions for the above chromosomes mainly concern chromosome number 21 (Down's syndrome). Neither of the two cases with an extra G-like chromosome but without the clinical picture of Down's syndrome which we have studied was found to have trisomy 22 [2]. In both cases the extra chromosome was a structurally altered one. However, we have observed trisomy 22 in a spontaneous abortion which was the mother's first pregnancy and abortion, but the family was not informative with regard to the origin of this abnormality. Out of 45 families having a child with Down's syndrome and trisomy 21, 12 gave some information with regard to the origin of the extra chromosome (table 1). However, in each case there were several possible alternatives for where non-disjunction could have occurred, and in only one family could the event be traced to one specific stage, the first meiotic division in the mother (fig. 4) [16]. Since non-disjunction during this division could be excluded in several of the other families, trisomy 21 can apparently originate in different ways (see also [7]).

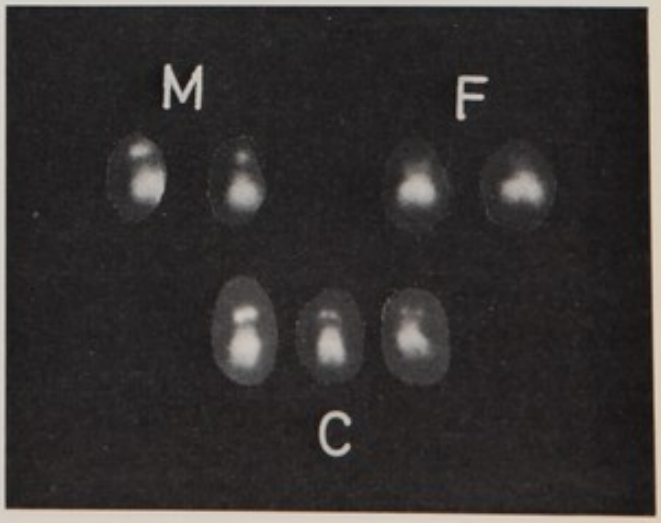
However, at the interpretation of the segregation of chromosomes 21 on the basis of their heteromorphic fluorescence satellite regions, one has to take into account that crossing-over might occur between the short arms of chromosome 21 during meiosis. If crossing-over has indeed occurred in our families, we cannot draw any definite conclusions with regard to where non-disjunction took place.

The heteromorphic fluorescence regions have also been used to trace the origin of other numerical chromosome aberrations. Out of four triploids so far reported, two were apparently due to non-reduction during the first meiotic division in the mother, while the other two could have originated by fertilization of an ovum either by two sperms or one diploid sperm [15, 22]. Furthermore, a second meiotic event in the mother was considered as the most likely mechanism of origin in a spontaneous abortion with trisomy 13 (p. 251). More data are obviously needed to settle the question of the origin(s) of trisomy 21 and other numerical chromosome aberrations.

Delineation of New Congenital Chromosome Syndromes

It was quite obvious when the banding techniques were introduced that it might be possible to detect new chromosome syndromes due to the higher resolution power of these techniques. In fact it seems as if trisomy 8 can now be considered as such a syndrome. Five such cases, most of them mosaics, have been published [5, 7],

Fig. 4. Quinacrine fluorescence pattern of chromosomes 21 from a mother (*M*), father (*F*) and their trisomy 21 child (*C*). The fluorescence pattern of the satellite regions suggests that the child has received both of the mother's chromosomes 21. From Licznerski & Lindsten [16].



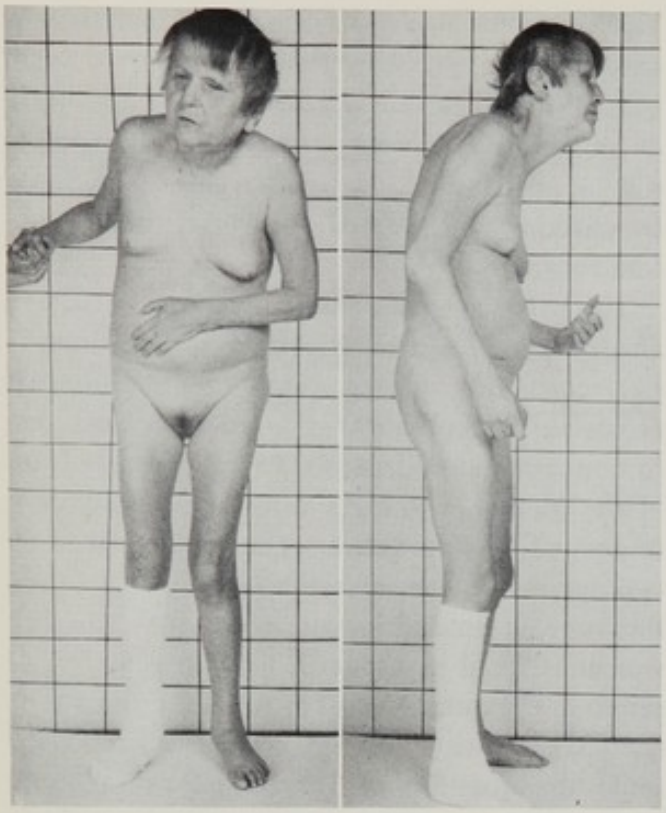


Fig. 5. The 54 year-old female with presumptive monosomy 21.

and in addition there are apparently several unpublished cases. Most of the subjects with trisomy 8 have been mentally retarded and have had different skeletal abnormalities, but it is still too early to make a comprehensive description of the phenotypic effects of the extra chromosome 8.

Monosomy 21 is another condition which in the future might turn out to be a clearly recognizable chromosome syndrome. Already since the description of the first chromosome anomaly in man, cases have been found with apparent G monosomy, and recently two such subjects were found to have presumptive monosomy 21 [1, 10]. The subjects from the latter work will be briefly discussed here. The patient, a 54 year-old female, was moderately mentally retarded (IQ about 50). Except for blindness due to congenital microphthalmia she did not have any obvious malformations (fig. 5). Only 45 chromosomes were found in cultured blood and skin cells, and karyotype analysis using the quinacrine and ASG techniques showed that there was only one chromosome 21 (fig. 6). No consistent change in any of the other chromosomes was observed, which could indicate that part of a chromosome 21 had been translocated onto another chromosome, a possibility which can never be totally excluded even when the present banding techniques are applied.

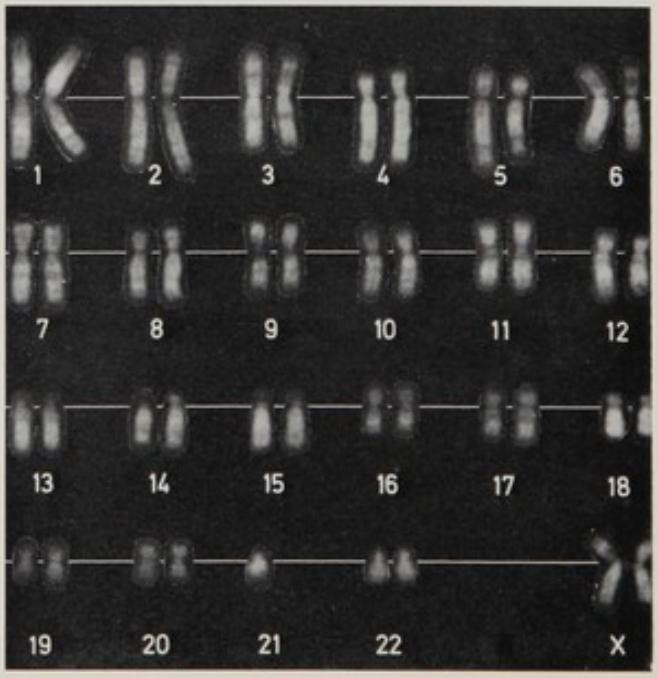
Patients like the one described here are of potential use for localizing genes to specific chromosomes and chromosome regions. However, this requires an analysis of the whole family, and in the present case both parents were dead. However, some negative information with regard to gene localization was obtained since the patient turned out to be heterozygous at 12 different loci (MN; Rh; Kell; Duffy; Gc; erythrocyte acid phosphatase, phosphoglucomutase 1 and 3, adenylate kinase, glutamate-pyruvate transaminase, HL-A and Gm). This formally excludes the possibility that any of these genes are located on chromosome 21, provided of course that the patient has indeed monosomy 21.

It seems likely that we can look forward to the delineation of several new congenital chromosome syndromes, especially involving structural alterations, in the near future. In analogy, similar progress will probably be achieved with regard to acquired chromosome abnormalities, especially in malignant disorders.

Analysis of Structural Chromosome Aberrations in Prenatal Cytogenetic Diagnosis

Prenatal cytogenetic diagnosis is today a routine procedure in many laboratories all over the world. Conventional staining techniques are in most in-

Fig. 6. Quinacrine-stained karyotype with presumptive monosomy 21.



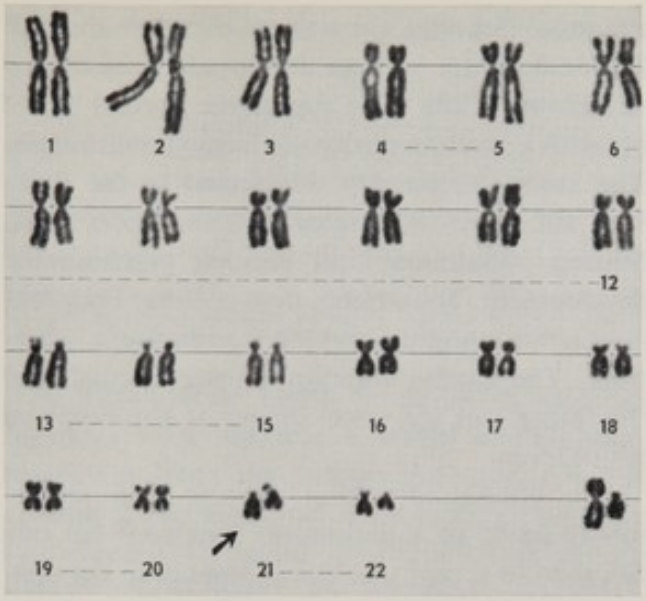


Fig. 7. Orcein-stained karyotype showing an abnormally long G chromosome.

stances sufficient for an adequate diagnosis of most chromosome abnormalities. However, it might in some cases be very difficult or even impossible to distinguish for instance a balanced carrier of a translocation from an unbalanced product of that translocation. An example will be given to illustrate the use of banding techniques in such a case.

A boy was referred for cytogenetic analysis because of severe mental retardation and an acrocephalosyndactyly-like syndrome. He turned out to have an abnormally long G chromosome (fig. 7). His brother had a similar clinical picture and also the same abnormal G chromosome. The mother had also had one spontaneous abortion. Both parents were healthy. The mother had a normal karyotype, but the father had the same abnormal G chromosome as his sons but no other obvious chromosome abnormalities. However, meiotic studies in the father revealed the presence of 20 autosomal bivalents, one quadrivalent and a normal XY bivalent (fig. 8), showing that the father was the carrier of a balanced reciprocal translocation [14]. This was later verified by fluorescence analysis which revealed a 1/22 translocation (fig. 9). Some time ago the mother became pregnant a fourth time. According to the Swedish law she would certainly have been permitted a legal abortion under the present circumstances. Amniocentesis was performed,

Fig. 8. Cell in diakinesis to first metaphase showing one quadrivalent, the interpretation of which is drawn below. The X and Y chromosomes are separated. From Hultén & Lindsten [14].

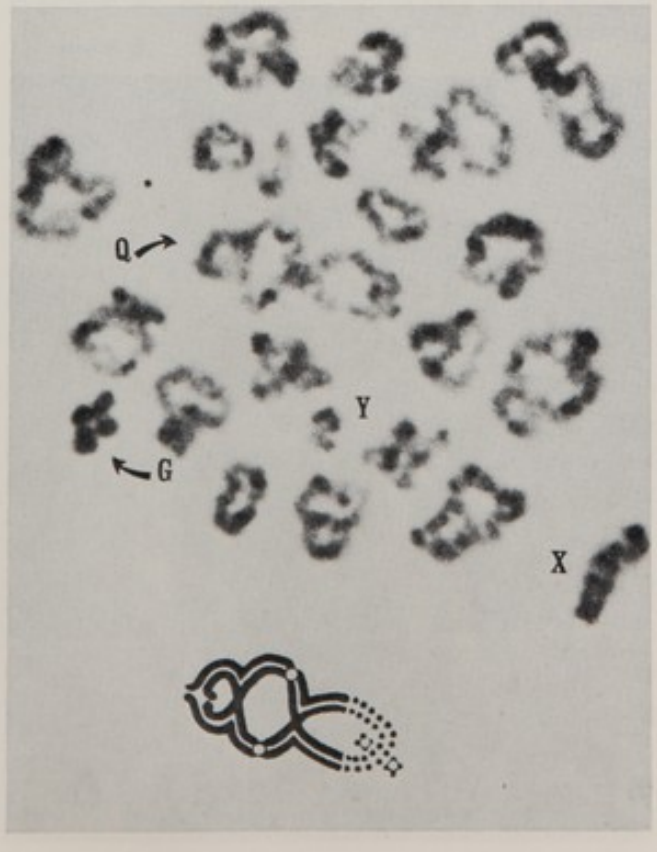
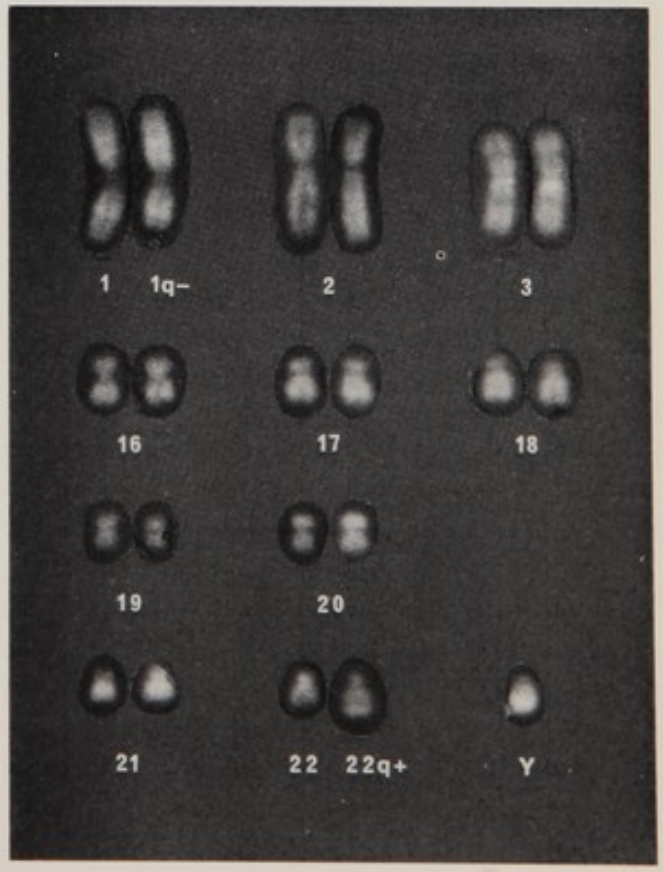


Fig. 9. Detail of quinacrine-stained karyotype showing a balanced reciprocal translocation between chromosomes 1 and 22. From Therkelsen et al. [20].



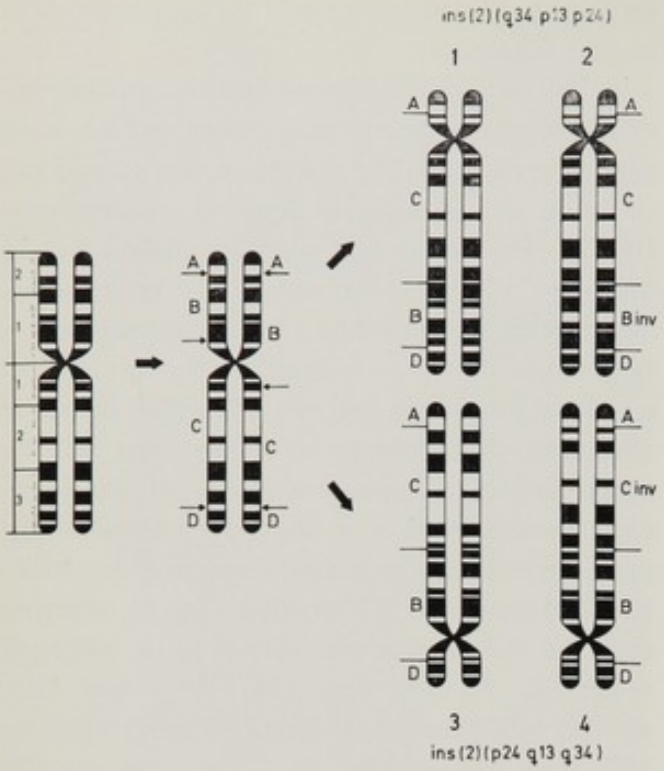


Fig. 10. Change of banding pattern of chromosome number 2 after an intrachromosomal insertion. Insertion of short arm material into the long arm above, and from the long arm into the short arm below. The banding pattern used was the one suggested by the Paris Conference (1971) [17].

and the foetus turned out to be a translocation carrier, and a healthy child was later born [20].

Thus, the banding techniques have improved the possibilities of a more detailed antenatal cytogenetic diagnosis. However, even with these techniques there remain some abnormalities, the nature of which cannot be elucidated. The family discussed in the following section from other points of view constitutes such an example.

Identification of More Complicated Structural Chromosome Rearrangements

The aim of the present section is not to make a detailed review of the possibilities of identifying structural rearrangements since this will be discussed in greater detail by other authors, but merely to give an example of how the banding techniques can help in the understanding of the nature of complex chromosome abnormalities. A more detailed description is given elsewhere [21].

A mentally retarded boy with a congenital heart malformation was found to have a structural chromosome abnormality. He had 46 chromosomes but only one normal chromosome No. 2. Instead there was an abnormal chromosome which was of the same size as the normal No. 2 but with a much more distally located centromere. The same abnormality was found in the sister who had a myelomeningocele with hydrocephalus, hearing impairment and delayed psychomotor development. The parents were healthy. They had no further children and no spontaneous abortions. The mother's karyotype was normal, but the father had the same abnormal karyotype as his children.

The present structural chromosome abnormality could be a pericentric inversion, but one has then to accept that the anomalies in the children are only due to coincidence, since it can easily be shown that it is impossible to get an unbalanced chromosome of unchanged size and arm ratio from a balanced pericentric inversion of the morphology seen in this family. It seems justified, therefore, to search for another explanation.

Fig. 11. Quinacrine fluorescence pattern of chromosomes 2 from two cells, one from the healthy father (*F*) the other from one of the malformed children (*C*) with a presumptive intrachromosomal insertion. Note the similarity in banding pattern between the proximal part of the long arm of both the normal and abnormal chromosome 2 (*left*), and between the short arm of the normal and the distal part of the long arm of the abnormal chromosome 2 (*right*).

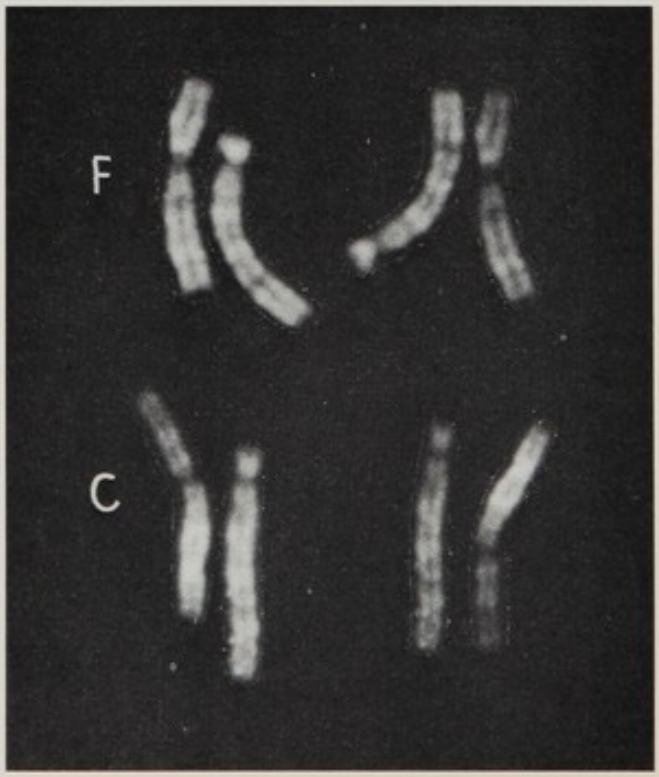


Table 2. *Types of chromosome aberration observed after X-ray irradiation*

Dose (Rad)	Number of cells analysed	Type of aberration						
		Tri-centric	Di-centric	Reciprocal translocation	Inversion	Ring chromosome	Fragment	Other
250	50	2	36	30	1	3	4	1
125	154	1	39	31	3	4	15	—

A translocation could be excluded, since 23 bivalents were regularly observed in first spermatocytes from the father. However, bivalent number 2 was abnormal when analysed with the fluorescence technique and contained several loops, which makes an intrachromosomal insertion possible. An abnormal chromosome like the one observed can thus be obtained in two ways: material from the short arm is inserted into the long arm, or vice versa. The banding pattern of the abnormal and normal No. 2 chromosomes indicated that the first alternative was the more likely (figs 10, 11). The question then arises whether the inserted segment is inverted or not. As seen from fig. 12 an unbalanced gamete, unchanged in size and shape when compared with the balanced, can only be obtained if the inserted segment is not inverted.

Thus, we would like to favour the interpretation that the present family is an example of a direct intrachromosomal insertion within chromosome 2.

Localization of X-ray Induced Chromosome Breakage

The chromosome banding techniques provide a new tool for analysing the location of induced chromosome breakage in greater detail. In order to obtain a frame of reference for such investigations it was considered of interest to restudy the effect of X-ray irradiation using the fluorescence technique. A more detailed presentation of these studies will be published elsewhere [12].

Peripheral blood was irradiated by X-rays (260 kV) at 37°C. Two different doses were used, 125 and 250 rad. Conventional lymphocyte cultures were then prepared and incubated for 48 h, after which air-dried preparations were made. Colchicine (0.125 μg/ml culture medium) was added to the cultures 2 h before harvesting. The cells were photographed on Polaroid 55 P/N film in a fluorescence microscope after staining with quinacrine mustard. The chromosome analysis was made on the photographic negatives using an illuminating box. Fifty consecutive cells with non-overlapping chromosomes (12 with 45 and 38 with 46 centromeres) exposed to 250 rad and 154 (29 with 45 and 125 with 46 centromeres) exposed to 125 rad have so far been analysed.

The different types of structural chromosome aberrations observed are shown in table 2. As seen from the table there were about the same number of reciprocal translocations as dicentric chromosomes. Furthermore, the number of inversions and ring chromosomes was about the same. The small discrepancies noted might very well be due to the fact that some translocations and inversions escape detection, even with the present technique. The distribution of dicentrics and translocations among cells is shown in table 3 for the low dose, and in table 4 for the high dose. As seen from these tables the distributions observed were in very good agreement with the Poisson distribution. The mean value for the low dose was 0.45 aberrations per cell, and for the twice as high dose 1.32 aberrations per cell. Thus, the high dose gave 2.9 times as many aberrations as the low dose, i.e. a value between a

Table 3. *Distribution of dicentrics and reciprocal translocations among 50 cells exposed to 250 rad X-rays*

	Number of aberrations per cell					
	0	1	2	3	4	5
Observed	13	16	14	6	1	0
Expected from the Poisson distribution[a]	13	18	12	5	2	0

[a] Mean = 1.32; $0.70 < P < 0.80$.

Table 4. *Distribution of dicentrics and reciprocal translocations among 154 cells exposed to 125 rad X-rays*

	Number of aberrations per cell				
	0	1	2	3	4
Observed	96	47	10	1	0
Expected from the Poisson distribution[a]	99	45	9	1	0

Mean = 0.45; $0.60 < P < 0.70$.

linear and quadratic dose–response relationship. This is in agreement with some previous findings using conventional staining techniques [9].

The distribution of all types of abnormalities among individual autosomes is given in table 4. The expected number of events was calculated on the basis of the relative mitotic length of each chromosome, and the data from cells exposed to the low and high doses were pooled at the analysis. As seen from table 4 the distribution is not significantly non-random. Three chromosomes (Nos. 2, 3 and 14) showed a considerable lack of events, while three (Nos. 9, 15 and 17) showed an excess. Data on the sex chromosomes was not included in the table because the cells analysed were obtained from one male and one female. When the cells from the different sexes were analysed separately, both the X and the Y chromosome were engaged in a proportion corresponding to their relative lengths.

The distribution of dicentrics and reciprocal translocations among different chromosomes is shown in fig. 13, and the distribution among individual chromosomes of all break points which

Table 5. *Distribution of all types of chromosome aberration among autosomes after X-ray irradiation*

Chromosome No.	Number of events: Observed	Expected from random distribution per length unit (M)	Difference (D)	D^2/M
1	30	28.61	+ 1.39	0.07
2	15	27.04	−12.04	5.36
3	15	21.89	− 6.89	2.17
4	19	20.38	− 1.38	0.09
5	17	19.50	− 2.50	0.32
6	19	19.06	− 0.06	0
7	20	17.05	+ 2.95	0.51
8	12	15.51	− 3.51	0.79
9	20	15.01	+ 4.99	1.66
10	12	15.07	− 3.07	0.63
11	11	15.13	− 4.13	1.13
12	13	14.13	− 1.13	0.09
13	14	12.15	+ 1.85	0.28
14	7	11.74	− 4.74	1.91
15	15	10.36	+ 4.64	2.08
16	12	9.86	+ 2.14	0.46
17	18	9.33	+ 8.67	8.06
18	10	8.73	+ 1.27	0.18
19	9	7.72	+ 1.28	0.21
20	8	7.07	+ 0.93	0.12
21	9	5.34	+ 3.66	2.51
22	9	5.65	+ 3.35	1.99
Sum	314			30.62

d.f. = 21
$P < 0.1$

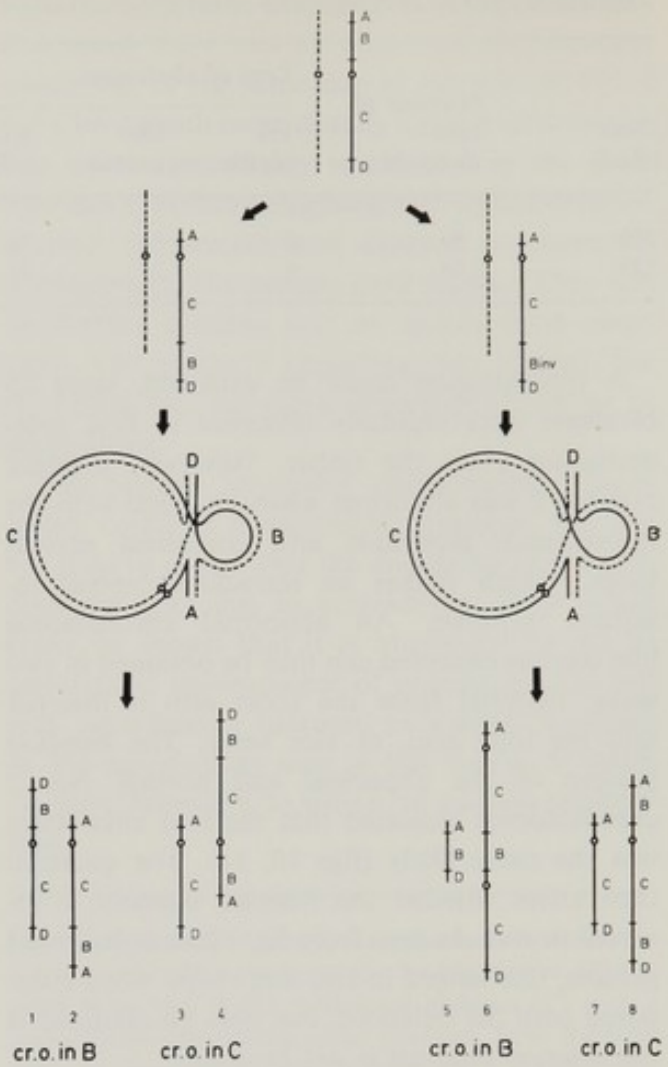


Fig. 12. Diagram illustrating the formation of gametes in an intrachromosomal insertion. Material from the short arm has been inserted into the long arm. The insertion is direct to the left and inverted to the right. Gamete 2 is the only one fitting the actual observations.

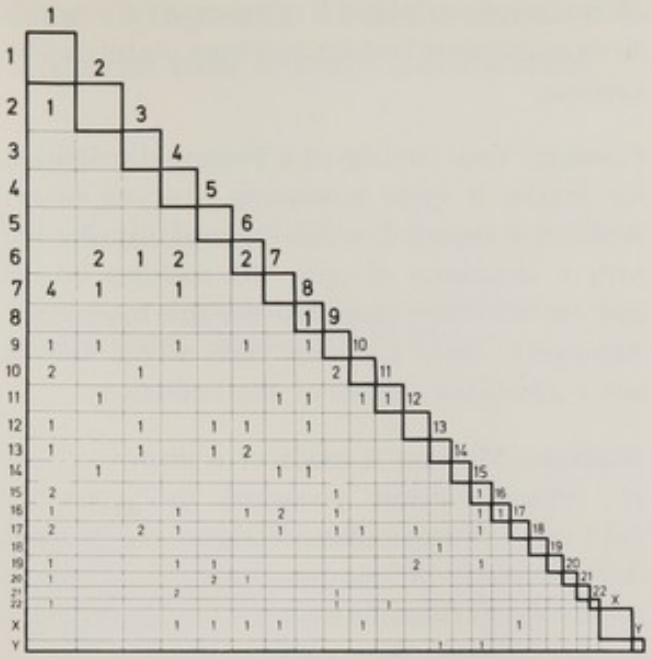


Fig. 13. Diagram illustrating the frequency with which different chromosomes were found to be involved in reciprocal translocations and dicentrics. Each square has been made proportional to the product of the relative lengths of the chromosomes involved in the rearrangements.

could be located with reasonable certainty is given in fig. 14. This information is given merely for the sake of completeness, but obviously more data must be accumulated before it is possible to draw any definite conclusions regarding the detailed pattern of the break point distribution.

Note added in proofs. The case with presumptive monosomy 21 has later been shown to have an unbalanced translocation (4q/21q) by the R band technique [10].

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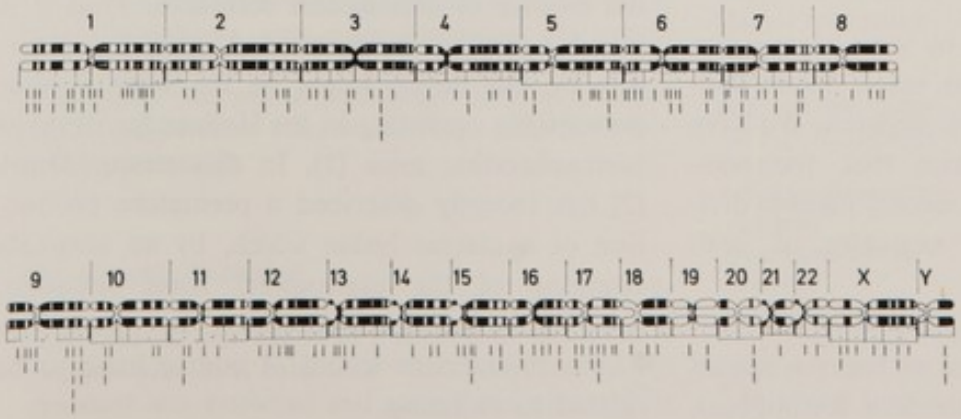
Fig. 14. Distribution of break points on individual chromosomes. The banding pattern of the Paris Conference (1971) [17] was used.



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Discussion

Ford: I have one question and would like to make one comment. First, in the metacentric bivalents the distribution of chiasmata in the one arm may be, indeed would be expected to be, influenced by chiasmata in the other. Do you have observations of the distribution of chiasmata in the long arm of B or C bivalents when no chiasma is present in the short arms?

My comment concerns the apparent non-induction of Robertsonian translocations by X-irradiation. My colleagues and I have carried out several investigations in which the testes of male mice were irradiated and the spermatocytes examined for chromosome rearrangements subsequent to recovery of fertility. Reciprocal translocation quadrivalents were frequent but we failed to detect Robertsonian translocations—though some configurations could be ambiguous. More recently Dr A. G. Searle and my group have mated irradiated males immediately after irradiation. We have examined testicular preparations from all the male progeny and although we found very many reciprocal translocations we only detected a single Robertsonian translocation—and as this was present only in about one-third of the cells examined it could not be attributed to the irradiation of the father.

Lindsten: It has not yet been possible to study the distribution of chiasmata in C-group bivalents with two chiasmata on the long arm and no chiasma on the short arm, since such bivalents are extremely rare.

de Grouchy: I was interested by your observation of a trisomic 21 proven to result from non-disjunction in the first meiotic division. We have shown in a similar instance that the non-disjunction occurred during second meiotic division. Now we thus have examples of both possibilities.

Your observation of an insertion is most valuable. In a similar instance we had postulated insertion (long before the modern techniques). Now you have proved this instance of "aneusomie de recombinaison" which had been postulated by Lejeune.

Comings: Your finding of a Poisson distribution for breaks is quite interesting. As you know, Wolff has reported a non-Poisson distribution with a deficiency of cells with multiple breaks and on this basis suggested the site hypothesis. Apparently there are some cases where there is not a significant degree of site limitation.

Wallman: Mention is made of a doctor's thesis of clinical interest presented in September 1972 in Sweden: Jan Wahlström, Prenatal determination of chromosomes—An investigation of cells from the amniotic fluid in pregnant women 35 years old or older.

Polani: One of the biological applications in man of the banding techniques, especially Q-banding, is in the study of the chimaeras. We have used this successfully in three human XX/XY chimaeras, two of them true hermaphrodites and one a normal woman. In each case we were able to detect a double autosomal contribution from one of the parents to each subject. In the second of the two hermaphrodites it was possible furthermore to be reasonably confident that double fertilization of ovum- and second-polar-body-complements (of the female pronucleus replicated before syngamy) had *not* taken place. Thus there could have been either double fertilization of two separate ova or of a two-egg follicle, in each case with subsequent zygote fusion. Or else, there might have occurred fertilization by two sperms of the ovum and its first polar body.

This conclusion for the origin of the second XX/XY chimaera was interesting because the third XX/XY chimaera, the normal female, was the mother of this second chimaera. Thus it is possible that the mechanism of familial chimaerism in this family might be the same as that presumably operating in the Hollaender strain of hermaphroditic mice [1]. In this strain, Mintz [2] has recently described a premature production of a uterine lysine which, by an untimely stripping of the blastomeric zona pellucida, would induce fusion chimaerism. In man such a fusion chimaerism would of course have to be related to twinning.

New Cytogenetic Technics Applied to a Series of Children with Mental Retardation

H. A. Lubs and Marie-Louise Lubs

Department of Pediatrics, University of Colorado Medical Center, Denver, Colo. 80220, USA

Although it is likely that routine use of the new cytogenetic technics will result in the recognition of previously undetected small abnormalities, there is still no information available on the frequency with which these will be found in various clinical groups. The purpose of the present study was to determine the number of small cytogenetic abnormalities that can be detected using a battery of new technics in a well defined group of abnormal children. A series of children referred to the Kennedy Center for evaluation of possible mental retardation were studied using conventional staining and one or more banding technics in conjunction with measurement of cells in which all chromosomes were identified.

Patient Material

Children with known trisomies or other distinct diagnoses were excluded from the study. Eighty-four children were initially included in the study, but thirty were subsequently placed in separate categories because a definite etiology was established (viral, genetic, etc.), the child was not found to be retarded, or a primary diagnosis of an atypical personality was made (table 1). The final study group, therefore, consisted of 54 children with mental retardation of unknown cause prior to cytogenetic study. These children were divided into four subgroups: developmental delay alone, 9; mental retardation alone, 9, mental retardation with major anomalies, 15; mental retardation with a definite organic central nervous system abnormality (i.e. mental retardation or developmental delay with cerebral palsy or cerebral palsy-like signs and symptoms), 21.

Cytogenetic Technics

Cells were cultured for 66–68 h at 37°C in McCoy's 5A medium with 15% fetal calf serum. Hypotonic KCl treatment was employed and metaphases prepared by blowing and heat drying for 1½–2 min on a hot plate set to maintain a beaker of water at 65°C, as described elsewhere in detail [1]. In each patient 10 cells were studied, first by conventional Giemsa staining and subsequently with quinacrine mustard fluorescence for homolog identification and study of the QM banding patterns [2]. G-banding by either the Giemsa 9 [3] or ASG [4] technics was employed routinely in place of conventional Giemsa staining later in the study. Dual karyotypes were routinely prepared for 2 cells. C-banding [5] and orcein staining were carried out as indicated when a variant was seen by other technics. The same cells (generally 6–10) were measured with an X,Y digitizer from negatives of Giemsa stained (preidentified by Q-banding) or G-banded metaphase cells essentially as reported several years ago [6]. After correction of each arm for its differential rate of contraction [7] the measurements were evaluated by an *F*-test of between homolog variation and *t*-test of the pair means. This system of quantitative evaluation is discussed in more detail under measurement studies.

Table 1. *Summary of clinical and cytogenetic findings*

Type clinical problem	*N*	Type cytogenetic findings: Abnormal	Rare variant	Inv 9qh
Developmental delay	9	0	0	1
Mental retardation	9	0	13p−	0
Mental retardation & major anomalies	15	18p+ 18q+ 8p−	0	1
M.R. or dev. delay & C.N.S. disorder	21	6q+	15p+	1
Total	54	4	2[a]	3[b]
Atypical personality (autism)	14	0	0	1
Syndromes or not retarded	16	0	0	0

[a] In 3 500 New Haven Caucasian infants: total rare variants D & G, 1/600–700: Dp−, 1/1150: Dp+, 1/350.
[b] In Grand Junction infants: inv 9qh, 5/600.

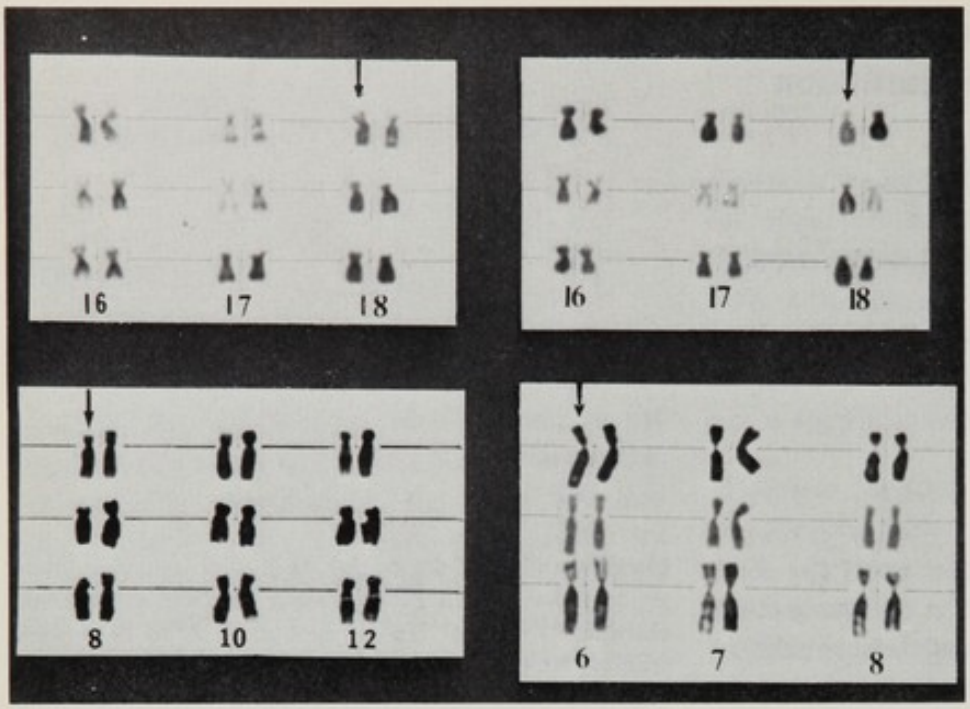
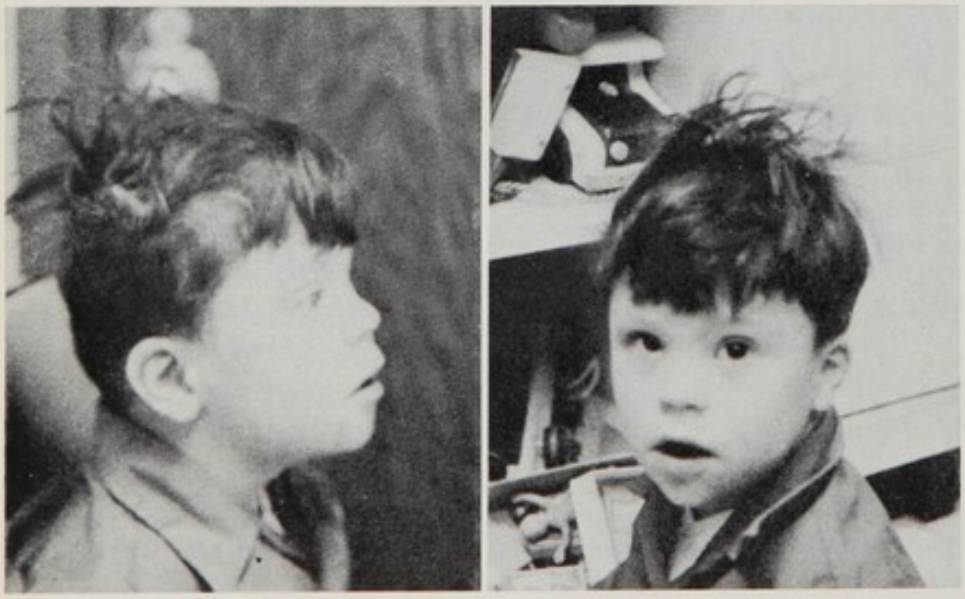


Fig. 1. Partial karyotype from three cells from each of the 4 children with small chromosomal abnormalities. Conventional Giemsa stain. In each case the chromosome identification was established by quinacrine mustard in the same cell. Without such identification, detection of the small differences in length between the homologues would be quite difficult. Patients 1, 2, 3 and 4, left to right, first and second row.

Results

Four of 54 children with mental retardation or developmental delay were found to have small but significant cytogenetic abnormalities. Before presenting the details of these patients and their cytogenetic findings it is instructive to observe the appearance of the abnormal chromosome with conventional Giemsa staining in each of these 4 children (fig. 1). These cells were also stained for Q-banding and each chromosome was identified, although only the conventional Giemsa stain is shown in fig. 1. Hence, with the aid of homolog identification, a consistent but small difference between homologs can be observed in the 3 partial karyotypes for each child. It would, however, be very difficult to detect these abnormalities without positive identification of each chromosome, since the modus operandi of karyotyping non-banded cells is to pair similar chromosomes.

Fig. 2. Patient 1. This child had many features of Down's syndrome.



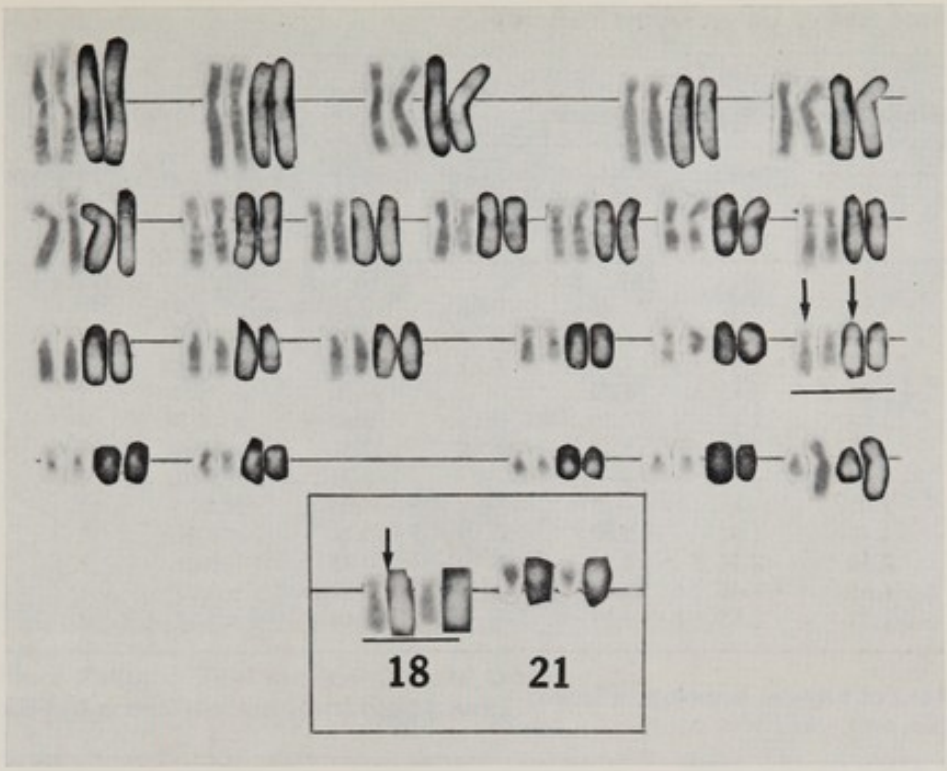


Fig. 3. Karyotype from patient 1 showing dual karyotypes, G- and Q-banding of same cell. In every cell, the short arm of one 18 homolog was slightly longer and the fluorescence pattern of the short arm was more intense than that of the normal homolog.

Patient 1

This child, aged 4, had many features of Down's syndrome but was not a classical case of Down's (fig. 2). He had previously been studied elsewhere by conventional cytogenetic technics and reported to have a normal karyotype. In each of his cells, the short arm of 18 was slightly increased in length and showed a brighter fluorescence than its homologue with quinacrine mustard (fig. 3). Although the difference between the levels of fluorescence in 18p in this illustration was somewhat obscured by the copying process, it was seen in all cells. The level of fluorescence in the abnormal 18p was identical to the brightly fluorescent part of long arm of 21. On the basis of both the clinical and cytogenetic information we have concluded that this patient has a translocation of the brightly fluorescent part of the long arm of 21 to the short arm of 18. He is trisomic for part of 21q as well as monosomic for most of 18p. This finding provides an additional explanation for cases of Down's or Down's-like children with apparently normal conventional karyotypes. The measurement data are discussed in a subsequent section; these confirmed the presence of a significant difference in length between the two 18ps (table 2). Both parents' karyotypes were normal.

Patient 2

This 6-year-old child had severe mental retardation, cerebral palsy and facial asymmetry. In all cells there was a slight difference in length between the two longs arms of 18. The karyotype showing the most marked difference is shown in fig. 4. In addition to the difference in length, the longer homolog shows three roughly equal bands, compared to two in the normal homolog. Although this was consistent with the duplication of a portion of the distal long arm of 18, present methodology does not permit us to make a positive identification of this extra chromatin material and the clinical findings were not suggestive of a particular trisomy. It is quite likely that this extra material is responsible for the child's clinical picture and that he is trisomic for part of an unidentified chormosome arm, perhaps 18q. Neither parent had this abnormality.

Patient 3

This 4½-year-old child was slightly below the third percentile in height, weight and head circumference. There was moderate mental retardation. She had epicanthal folds, high arched palate and her neck, although not webbed, was short and

Table 2. *Results of measurement studies (in corrected microns)*

Patient	Observed abnormality	Mean arm lengths Both homologs		Mean arms patients		Between homolog variation			
		Normal sample	Patient	All normal homologs	All abnormal homologs	σ_N normal sample	σ_P patient	*F*-test	Number of cells *N*
1	18p+	0.80	0.91	0.77	1.04	0.09	0.21	$p<0.01$	9
2	18q+	1.72	1.90	1.72	2.09	0.12	0.29	$p<0.01$	9
3	8p−	1.43	1.22	1.41	1.04	0.11	0.27	$p<0.01$	8
Mother	8p−	—	1.24	1.45	1.03	—	0.30	$p<0.01$	3
Mother	10q+	2.59	2.93	2.67	3.20	0.15	0.42	$p<0.01$	6
4	(6q+)	3.08	3.27	3.08	3.47	0.18	0.31	$p<0.01$	18
Father	None(6q)	—	3.12	—	—	—	0.24	N.S.	8
Mother	None(6q)	—	3.05	—	—	—	0.05	N.S.	7
5	9p+	1.45	1.47	1.32	1.62	0.13	0.26	<0.01	7
	9q−	2.63	2.52	2.74	2.30	0.19	0.35	<0.01	7
Father	9p+	—	1.65	1.46	1.84	—	0.30	<0.01	4
	9q−	—	2.32	2.65	1.99	—	0.48	<0.01	4

Between homolog variation:

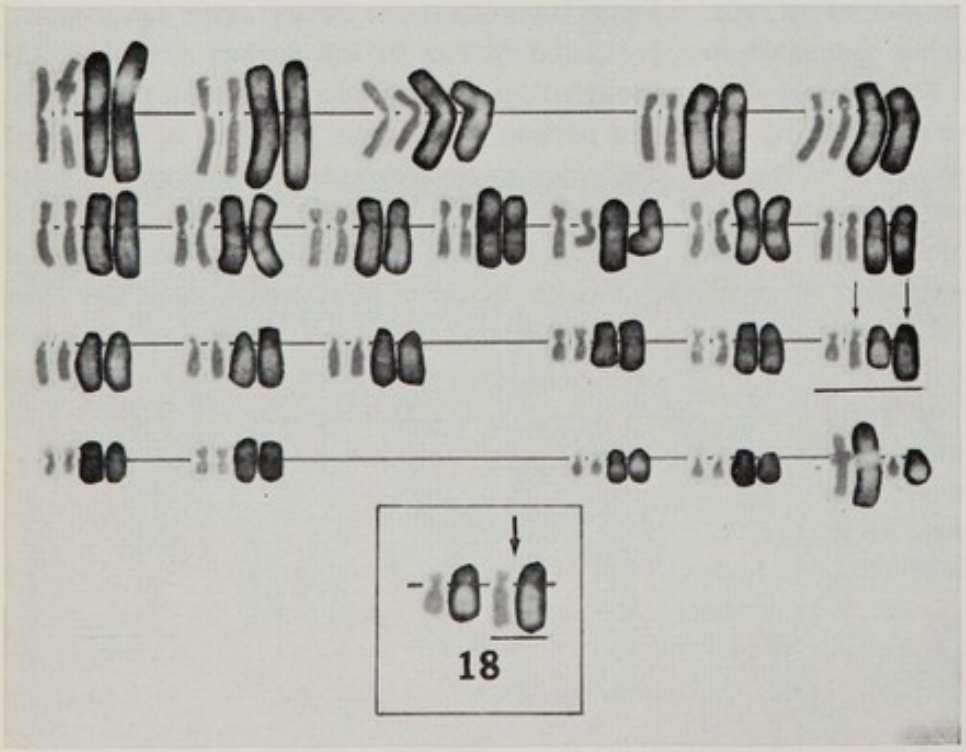
$$\sigma = \left(\frac{\sum_i \sum_j (\bar{X}i - Xij)^2}{N} \right)$$

F-test of between homolog variation:

$$F = \frac{\sigma_P^2}{\sigma_N^2} \quad \text{d.f.} = N$$

$\bar{X}_i$, Mean length of arm pair (*i*th cell)
X_{ij}, Arm length (*j*th homolog, *i*th cell)
σ_P, Between homolog variation (patient)
σ_N, Between homolog variation (normal sample)
N, Number of cells
N.S., Not significant

Fig. 4. Patient 2. Dual karyotypes, conventional Giemsa stain and Q-banding, showing increase in length of 18q in one homolog together with an extra Q-band in 18q.



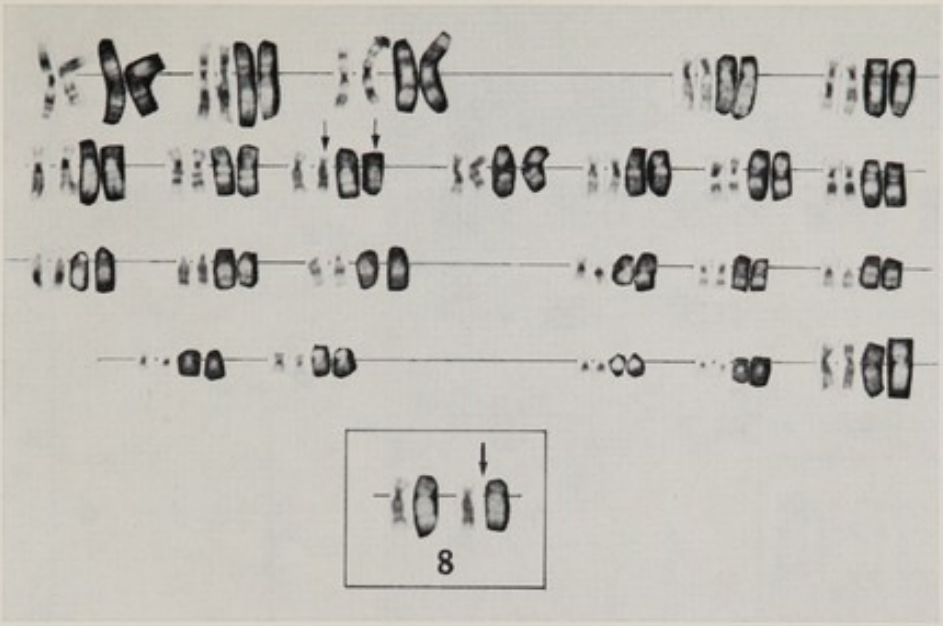
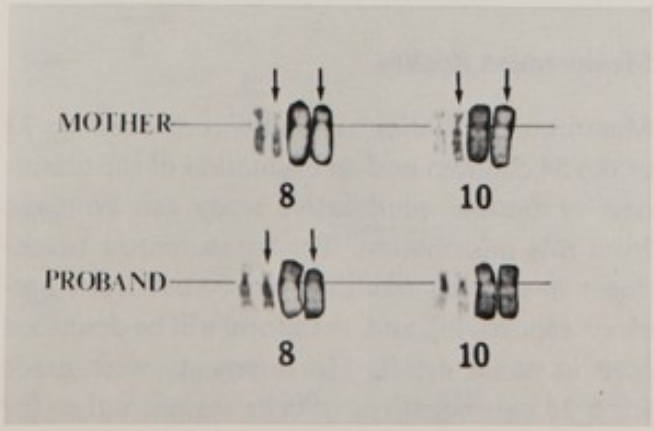


Fig. 5. Patient 3. Dual karyotype, G- and Q-banded, 8p one-half normal size with distal band missing.

thick. Her chest was broad with wideset nipples, and her back was slightly humped. She had a complex congenital heart disease consisting of atrial septal defect, ventricular septal defect and enlarged left superior vena cava. At the age of three she had been diagnosed as having Noonan's Syndrome. Her chromosomes at that time were reported as normal with conventional technics. In the present study, there was loss of about half of the short arm of chromosome 8, and both G- and Q-banded cells showed a concomitant loss of the distal 8p band. No alteration in length or banding pattern elsewhere was detected (fig. 5). Her mother's cells showed the same 8p deletion; in addition, one 10q was longer than usual and there was an extra distal 10q band. The fluorescence intensity of this extra band was comparable to that of the missing band in 8p and we have concluded, therefore, that the patient's mother had a balanced translocation between 8p and 10q, (with the principal observable change being transfer of the distal 8p region to 10q), and that the predominant abnormality in the child was deletion of the distal region in 8p. Partial karyotypes for chromosomes 8 and 10 from this mother and child are shown in fig. 6. Measurement studies confirmed both abnormalities (table 2). The child's father's chromosomes were normal. Other family studies are in progress.

Fig. 6. Comparison of chromosomes 8 and 10 in patient 3 and her mother. In the mother there was an extra distal band in 10q equivalent to absent band in 8p in size and intensity.



Patient 4

This child, aged 3, had moderate mental retardation with moderate cerebral palsy and microcephaly, but no distinctive clinical features. Inspection of cells and karyotypes with G- and Q-banding revealed no abnormality. Measurement studies, however, showed significant variation between the two long arms of 6 (table 2). These results were confirmed in a second culture and neither parent showed a similar increase in the length of 6q on measurement studies. In retrospect, a very slight increase in length of the distal dim band in the longer 6 homolog was seen (fig. 7). We believe this is the first instance in which a small cytogenetic abnormality was detected by measurement studies rather than by inspection. The slight difference in length is

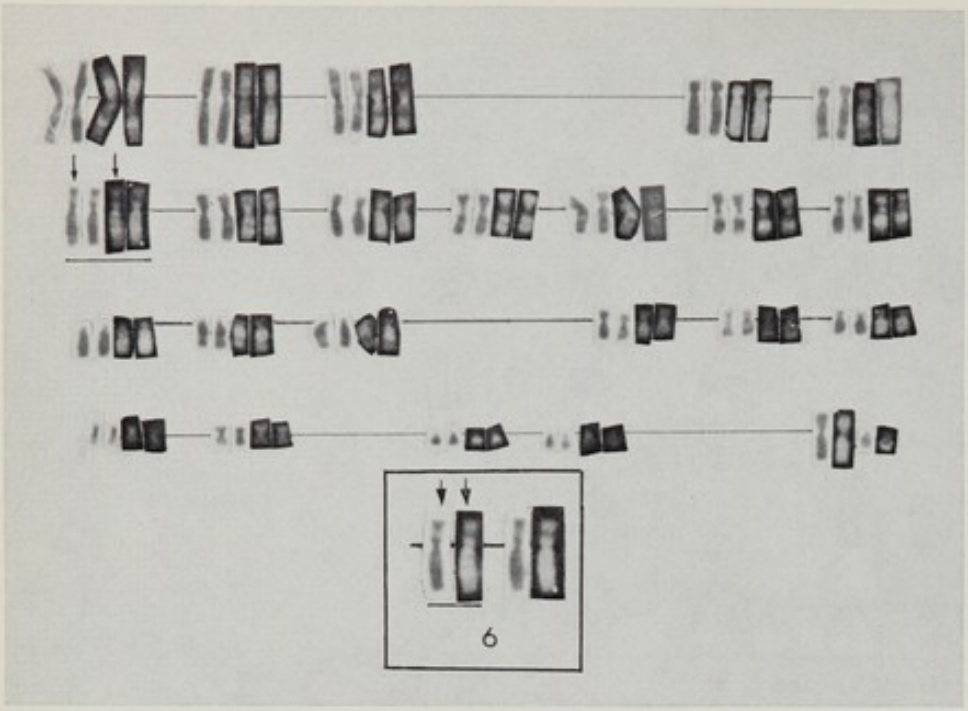


Fig. 7. Patient 4. Dual karyotypes, G- and Q-banding, showing a very slight increase in size of the distal dim region in 6q. This was seen on two separate cultures in patient but was not present in either parent. First detected from measurement studies.

somewhat more apparent with conventional Giemsa staining (fig. 1) than in fig. 7.

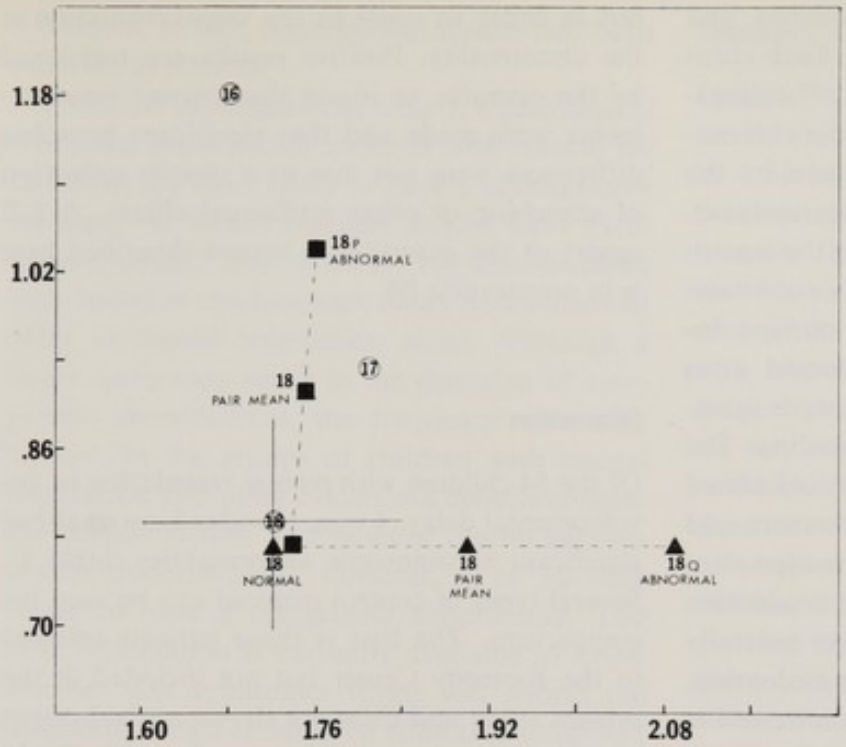
Minor variants and polymorphisms

Variations in length and in Q-banding intensity were recorded for each of these children with mental retardation. C polymorphisms were also recorded in some instances. Since the population frequency of many of Q and C variants are not yet adequately known, these results will subsequently be reported in detail when a comparison of the individual frequencies with a normal population can be made. In the interim, comparison of the frequency of certain rare length variants in these children can be made with previous population surveys. Two such rare variants were found: deletion of the short arm of 13 and a marked increase in the size of 15p (>18p). In both instances, these were confirmed by measurement studies. One child was adopted and the other is living with foster parents and family studies could not be carried out.

In addition, 3 of the 54 children with retardation, as well as one of the 14 autistic children were found to have an inversion of the secondary constriction (h) region in 9. In three of these four instances the inversion involved the entire h region and the C-band was in the short arm rather than in the long arm. In the fourth child, only a partial inversion was present since C material was found in equal amounts on both sides of the centromere. Nevertheless, the short arm was clearly too long and the long arm clearly too short as shown by the measurement values from this child in fig. 9 and table 2. Q- and C-banding showed a diminished size of the h region as well as a partial inversion. The father of the child with the partial 9qh inversion was of normal intelligence, and also had the same inversion. Family studies in the other 3 children have not yet been carried out. In each of these 4 children a similar Q-band pattern was seen in 9p: the region adjacent to the centromere showed a rounded rather than a tapered appearance. No distinctive clinical features were found in this group of children.

Measurement Studies

Measurement studies have been completed on 33 of the 54 children and an evaluation of the usefulness of routine quantitative study can be made from this information. The measurement procedures have been modified somewhat from previous reports [6], and, therefore, will be described here in some detail. Measurements were made from 35 mm negatives of cells stained either for



Figs 8, 9. *Abscissa:* long arm (normalized); *ordinate:* short arm (normalized, μ). Patients 1 and 2. Short arm vs long arm plot of E group in corrected microns. Circled numbers indicate normal mean values for each chromosome pair of group E. —, 1 S.D. of means for 18; ---, connecting 3 symbols expresses measurement values for patients 1 and 2: one symbol indicates the mean value for the pair, another the mean of all normal homolog from all cells and the third the mean of all abnormal homologs. Each value was derived from X,Y digitizer measurements. Data were normalized by the differential compaction method. Measurements were made from cells stained with conventional Giemsa stain, but preidentified with Q-banding.

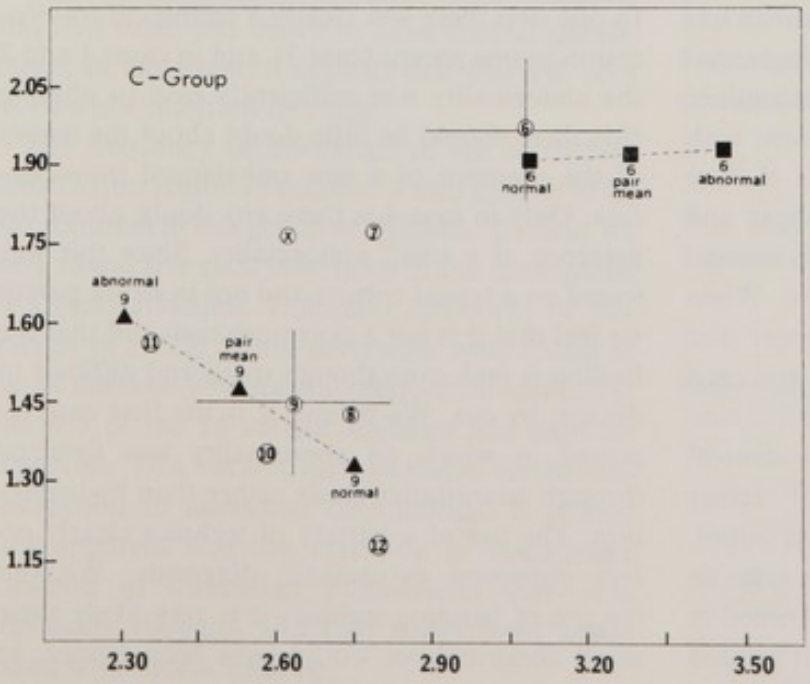


Fig. 9. Data are shown from patient 4 and a child with an unusually metacentric 9 due to partial inversion of 9qh. The latter child's father had a similar variant 9. Symbols and normalization as in previous illustration. Mean of 9s normal although *F*-test of between homolog variation was significant.

G-banding or with conventional Giemsa and projected on an X, Y digitizer table. Each chromosome was pre-identified from a 5×7 enlargement of a Q- or G-banded cell and the chromosome number was entered on the tape by the operator from a keyboard prior to measurement. The operator then placed a cursor over the centromere and ends of arms and the X, Y coordinates of these positions were also entered on tape. Interstitial points were included if curved arms were measured, but ordinarily cells were preselected so that there was minimal bending. The average long and short arms were calculated from the distances between the centromere and ends of arms. From experience with measurement of more than 400 people prior to the introduction of the banding technics 6–10 cells have generally proved adequate for the quantitative evaluation. Normal values were determined from comparable measurement studies of 35 normal adults. These values, normalized on the basis of total autosomal length, were included in the Paris Conference Report on nomenclature [8]. All values in this study were corrected for differential compaction on the basis of regression values determined from the normal sample. With this system of normalization (or correction), a specific regression correction is applied to each arm of the karyotype [7, 10]. The rate of contraction of each arm is directly proportional to the length of the arm, i.e. long arms change considerably with compaction and short arms change little. The usual normalization by total autosomal length does not correct for this differential contraction. The most useful statistical test for significant variations has proven to be an *F*-test of between homolog variation. A *t*-test of means is useful as a secondary test but must be interpreted with caution; with highly variable chromosomes, such as 9, one homolog may be long and the other short and the mean, therefore, may be well within normal limits even though the *F*-test is significant. When the *F*-test is significant (<0.01), the longer and shorter homologs from each cell are averaged over all cells as shown in table 2. Numerical output is obtained automatically as are the statistical evaluations and a plot of the short arm versus long arm of the patient against the normal values. If a significant *F*-test is obtained for an arm, an enlarged plot of the group of chromosomes in which the variant pair was found is also printed out in order to assist in the visual evaluation of the abnormality. Positive results are rechecked by the operator to insure that correct measurements were made and that significant homolog differences were not due to a chance collection of stretching or other artifactual effects. A full report of the quantitative system described here is in preparation [9].

Discussion

Of the 54 children with mental retardation or developmental delay, 4 were found to have small but significant chromosome abnormalities (table 1). Several types of control material can be used for comparison. The first is those patients referred to the Kennedy Center but not included in the present study and these are shown at the bottom of table 1: no abnormalities were found in the 14 children with atypical personality and none were found in the 16 children who were ultimately determined not to have mental retardation or whom a definite inherited syndrome diagnosis was ultimately reached. In a study now in progress, 600 consecutively born children in Grand Junction, Colorado, have been studied by Giemsa banding and *in no instance* have similar small abnormalities been found which would not have been detected by conventional cytogenetic staining technics. It is clear, therefore, that the small abnormalities, such as found in these four patients, are not common. They are likely the cause of the clinical abnormalities in the children. In one case there was clearly a reciprocal translocation in one parent (case 3), and in cases 1 and 2 the abnormality was sufficiently easy to observe that there should be little doubt about the reality of the presence of a new unbalanced translocation. Only in case 4 is there any doubt about the presence of a small abnormality. Since this was found on a repeat culture and not in either parent we feel that it is not a polymorphism and that the finding is real, even though small and difficult to discern by eye. We believe it is the first case reported in which an abnormality was detected through quantitative study rather than by inspection. The use of a battery of technics clearly offers improved cytogenetic diagnosis. Without the use of banding technics it is very likely these small abnormalities would have been missed. In

2 children, in fact, conventional studies had been reported as normal.

From the information in table 1, a preliminary statement about which children with mental retardation are most likely to have a chromosome etiology, i.e. which children should have cytogenetic studies, can be made. No abnormalities were found in children with either developmental delay or mental retardation alone. Although a larger series may result in the detection of cytogenetic abnormalities, the frequency will likely be low. In the groups of children with mental retardation and other evidence of definite congenital disease (either anomalies or central nervous system abnormalities), however, 4 of 36 were found to have a cytogenetic abnormality. Too little information is currently available to know whether new syndromes which will permit the diagnosis of such children on clinical grounds will emerge. In the interim, children falling in either of these two groups should be studied, without regard to the specific clinical findings.

It is possible that an even higher proportion of these children have a cytogenetic etiology for their mental retardation and other abnormalities. The presence of two rare variants in group D is unlikely in comparison to the frequency found in 3 500 New Haven Caucasian newborn infants in a previous study [11]. In the New Haven newborns the frequency of all such rare variants in group D was about 1 in 600. Dp – variants alone occurred in 1 in 750; comparable Dp+ variants in 1 in 350. It is possible that the banding technics will permit a better analysis of these variants, and that some will prove to have clinical significance. In any event, it appears that this will be a worthwhile question to study.

The frequency of inversions of the secondary constriction region in number 9 also appear to be too common in this group of children. To date, we have found five such inversions in the same series of Grand Junction, Colorado newborns (a frequency of 1/125). Three inversions were found in the 54 children with mental retardation and in addition 1 of the 14 autistic children had such an inversion. This variant also warrants further investigation in abnormal populations. It is also now apparent that this inversion is much more common in Caucasian populations than was previously thought, and that many went undetected with orcein staining.

Summary and Conclusions

Using a battery of the new cytogenetic technics, small, but significant abnormalities were found in 4 of 54 children with mental retardation or developmental delay. The 4 abnormalities were found in the 36 children with mental retardation and other evidence of abnormality, either major congenital defects or cerebral palsy. Children with mental retardation and other evidence of congenital disease should be studied routinely by the new technics. No abnormalities were found in the children with mental retardation or delay alone or in 14 autistic children.

In only one instance was a reciprocal translocation found in a parent, and the remaining 3 presumably represent instances of new unbalanced translocations.

One of the four abnormalities was first detected by measurement studies which showed a significant homolog difference in 6q. In the remaining three abnormalities, measurement studies, together with a statistical evaluation of between homolog differences, were quite helpful in confirming their presence.

Two rare minor variants were also found in this group of children and their frequency was considerably greater than that found in previous studies of consecutive newborns. In addition, inversion of the secondary constriction region of 9 also have occurred more frequently than expected.

Routine use of the new banding technics clearly offer improved detection of cytogenetic abnormalities and without their routine use many abnormalities will be missed. Two of the present four abnormalities were not detected by previous conventional cytogenetic study.

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Discussion

Leibold: How often does it occur that mentally retarded children have significant chromosomal abnormalities, while there is no detectable abnormality in the parents' sets of chromosomes?

Lubs: In this series of 4 abnormal karyotypes only one was inherited.

Pearson: Is it possible that the metacentric 9 chromosome you describe got a pericentric inversion which places part of the secondary constriction on each side of the centromere so that there is relatively little secondary constriction chromatin that can be detected in either arm?

Lubs: The culture was made some time ago, when our C-banding technics was not consistently good, but there appeared to be little C-band material on the metacentric 9. This chromosome, however, had no dip in fluorescence in the h region, a short arm clearly longer than normal, which did not have the constriction seen with conventional stain. It had a full, rounded appearance adjacent to the centromere rather than the tapered appearance usually seen. We feel, therefore, that it is at least clear that it is not the usual 9qhinv and probably includes deletion of much of 9qh.

Studies on Spontaneous Abortions

A. J. Therkelsen,[1] *O. Myhre Jensen,*[2] *J. Jonasson,*[5] *L. U. Lamm,*[4] *J. G. Lauritsen,*[3] *J. Lindsten*[5] *and G. Bruun Petersen*[1]

[1]Institute of Human Genetics, University of Aarhus, Departments of
[2]Pathology and [3]Surgery, Aarhus County Hospital and
[4]Tissue Typing Laboratory, Aarhus Kommunehospital, Aarhus, Denmark, and
[5]Department of Clinical Genetics, Karolinska Hospital, Stockholm, Sweden

Quite a large number of studies of the cytogenetics of spontaneous abortuses have been performed during the last few years, and the results have been reviewed recently by Carr [5] and by Hamerton [8]. The results seem very variable from study to study. In the 22 studies reviewed by Hamerton the frequency of chromosomally abnormal foetuses varied between 2 and 64%.

The reason for this large variation has to be sought first of all in the different methods of selecting the material for study; but even in studies which seem comparable as to the method of selection, large variations are seen and may be caused by

(i) Variations in the frequency of undetected induced abortions;

(ii) Selection related to the age of the abortuses studied;

(iii) Geographical or local factors;

(iv) The variation in success rate for the investigations which in the 22 studies reviewed by Hamerton was between 24 and 78%.

When we started our investigation a little more than a year ago, our main aim was of course not to add another frequency to the collection, but first of all to contribute to the exact description of the large number of chromosome abnormalities found in spontaneous abortions by using the new banding methods introduced by Caspersson and his group [6]. The exact identification of supernumerary and missing chromosomes and of structural aberrations also offered new possibilities for locating genes to specific chromosomes. In particular, loci such as the very polymorphic HL-A loci could possibly be located, if the abortuses could be HL-A typed and investigated cytogenetically at the same time. The still very controversial subject of a possible selective effect on abortion caused by ABO and HL-A incompatibility was also a main object for our study.

The main laboratory investigations which we have done or intend to do in the abortuses appear from table 1. The brackets show the things which we have not been able to do so far, either because of technical difficulties or lack of capacity in the laboratories. The ABO and HL-A typing especially has given us a lot of trouble, and we are unable to present any results, but we feel certain that the typing will be possible.

The investigations done in the parents are shown in table 2. Apart from the investigation of karyotypes and the various genetical markers we also test for the presence of various antibodies. So far the selection of antibodies has been somewhat determined by the techniques which are available at the moment in our laboratory, but the list will be extended to other antibodies also.

During the most recent investigations, mixed lymphocyte cultures have also been done, since the frequency of transformed cells by such investigations has been shown to be higher in couples with abortions and hydatiform moles.

The preliminary results of our investigations

Table 1. *Foetal tissues*

Karyotype	Orcein
	QM
X Chromatin (Feulgen–light green)	
[ABO Type]	
[HL-A Type]	
PGM_1, PGM_3, [ADA], [GPT]	
Histology	all samples cultured
	all intact foetuses

Table 2. *Parents*

Karyotype	Orcein [QM]
Genetic markers	
Blood types	
Serum types (also C'3)	
Enzyme types (PGM_1, PGM_2, PGM_3, GPT, AcP, ADA, AK)	
HL-A types	
Antibodies	
Thyroid auto-antibodies	
Sperm antibodies	
Antibodies against mycoplasmas	
Antibodies against cytomegalovirus	
Mixed lymphocyte cultures	

appear in table 3. The total number of specimens investigated so far is 233, 72 out of which were excluded because histologic examination showed the tissue to be either purely maternal or a mixture of foetal and maternal cells. Out of the remaining 161 specimens, a little more than 90% grew; and out of these, 77 had a grossly abnormal karyotype.

The karyotypes also appear in table 3. We shall not go into detail concerning all the karyotypes, but only point out that out of the 9 D trisomies, 8 were +15 and one a +13 trisomy. As liveborn babies with Patau's syndrome seem to be 13 trisomies, one may ask where all the 14 trisomies have gone. They may either be lost among the very early abortuses which we do not catch, or chromosome No. 14 may be more resistant to non-disjunction than the other chromosomes of the D-group.

Of the four G trisomies, two were +21, but in the two remaining cases no preparations were available for fluorescence analysis. As a curiosity we can mention that one of these abortions was the result of an artificial insemination. A later successful artificial insemination of the same woman terminated in a new abortion, where the abortus was shown to be a +15 trisomy.

The results, compared with the results of a number of other studies of unselected spontaneous abortions, appear in table 4. Our incidence of heteroploidy is higher than in any of the other studies, although very close to the one from Hawaii of Arakaki et al. [1]; but it must be stressed also that our success rate is higher than in any of the other studies, and especially much higher than that in Arakaki's study, which was only 35%. The first three investigations shown in table 4 had success rates around 60%.

The distribution of the abnormalities compares reasonably well with the other studies.

The distribution by gestational age in the same studies appears in table 5, which may give one explanation for our relatively high frequency of abnormalities. Most (i.e. 73.6%) of our abortions are in the first trimester, and it is a well known fact that the frequency of chromosome abnormalities is much lower in the second trimester. The decrease after the first trimester also appears in our material, as shown in table 6. This table shows that the frequency of chromosome abnormality is 57.3% in abortuses with a menstrual age of 50–90 days, but only 43.8% in the relatively few abortuses with a menstrual age of 91–119 days.

The maternal ages are shown in table 7. The

Table 3. *Spontaneous abortions*

Number of specimens excluded as maternal		72	30.9%
Number of failures		15 (9.3%)	
Number of specimens with normal karyotype			
46,XY	32	69 (42.9%)	
46,XX	37		
Number of specimens with abnormal karyotype			
92,XXXX	5		
92,XXYY	1		
69,XXX	4		
69,XXY	3		69.1%
48,X inv(Y),+16,+18	1		
47,XXY	1		
47,XXX	3		
47,XY,+2,Dp+,Gp−	1		
47,XX,+B	1		
47,XY,+8	1		
47,XX,+13	1		
47,XX,+15	3		
47,XY,+15	5		
47,XX,+16	7	77 (47.8%)	
47,XY,+16	3		
47,XY,+18	1		
47,XY,+E_{17-18}	2		
47,XY,+E	1		
47,XY,+21	2		
47,XY,+G	1		
47,XX,+G	1		
47,XXX/46,XX	1		
47,XXY or 47,XY,+G	1		
46,XY,−F,+C or +E_{17}	1		
46,XX,−D,t(13q14q) pat+	1		
45,X	25		
Total		233	

Table 4. *Incidence of heteroploidy in spontaneous abortions*

	Total	Abnormal	Abnormal %	Trisomic %	45,X %	Polyploid %	Other %
Dhadial et al. [7]	423	101	24	46	31	17	7
Carr [4]	227	50	22	52	24	22	2
Boué et al. [3]	132	50	37	54	16	22	8
Arakaki et al. [1]	127	63	49.5	30	28.5	13	28.5
Stenchever et al. [11]	101	8	8	12.5	25	37.5	25
Present material	146	77	52.7	45.5	32.5	16.9	5.2

Table 5. *Distribution of abortuses by gestational age*

	Specimens for which data available	Heteroploid %	% in 1st trimester	Mean gestational age of abortuses (weeks)	"End point" (weeks)
Dhadial et al. [7]	390	24	33.6	14.7	24
Carr [4]	213	22	45	12.5	22
Boué et al. [3]	132	37	—	—	—
Arakaki et al. [1]	124	49.5	—	10.1	17
Stenchever et al. [11]	101	8	39	—	20
Present material	121	53.7	73.6	11.8	16

Table 6

Menstrual age	Karyotype of abortus: Normal	Karyotype of abortus: Abnormal	Total successful	Frequency of failures
50–90 days	38	51 (57.3 %)	89	10 (10.1 %)
91–119 days	18	14 (43.8 %)	32	5 (13.5 %)
	56	*65* (53.7 %)	*121*[a]	*15*[a]

[a] Menstrual age unknown in 25 cases.

average is 27.0 years, but higher for D and G trisomies. It is not higher in the 16 trisomies as found by Arakaki et al. [1] but not by others [10]; and the mean maternal age for tetraploidies is not lower than the average, which was suggested by others [5].

One of the problems we had hoped to solve by HL-A typing was the mechanism of the origin of triploidy, but because of the problems with the HL-A typing it has not been possible so far. Unexpectedly it showed possible to elucidate the problem by aid of the QM-staining technique.

All the three first triploidies investigated proved to be informative, and those are the only cases investigated so far. In the first case non-reduction undoubtedly took place in the first meiosis of the mother, whereas in the other two cases non-reduction in the first meiosis of the mother was the most likely in one case, but in the other only non-reduction in the first meiosis of the mother and in the second of the father could be excluded, leaving the other possibilities open. It is unnecessary to go into detail with these cases, as they have been published recently by Jonasson et al. [9]. It should also be mentioned that in our only case of 13 trisomy non-disjunction in the second meiosis of the mother was likely, as she had one chromosome No. 13 with strongly fluorescent short arm regions, whereas the abortus had two such chromosomes No. 13.

We have also tried to extract as much information as possible from our material concerning the selective effect of ABO and HL-A incompatibility. The amount of information would of course

Table 7. *Maternal age*

	Number	Mean maternal age
Karyotype of abortus unknown	15	25.7
Karyotype of abortus normal	69	27.3
Karyotype of abortus abnormal	77	27.1
	161	27.0
Triploidy	7	26.4
Tetraploidy	6	26.8
Trisomy 15	8	34.5
Trisomy 16	10	25.7
Trisomy E_{17-18}	3	24.7
Trisomy G	4	30.0

have been much greater if the foetuses had been typed, but also without these types some information can be extracted. The results concerning ABO incompatibility appear from table 8. One could expect the frequency of incompatible matings to be higher among the parents of chromosomally normal matings, but as can be seen from this table no difference at all exists. The sample seemed to be a random one, when expected frequencies of matings were calculated from the gene frequencies known from other sources.

Table 9 shows the results of the only analysis done so far with respect to HL-A incompatibility. The frequency of couples with no antigens in common is not statistically significantly different among parents of chromosomally normal and abnormal foetuses. It is especially worth noting that with respect to the Four locus there is no difference. This locus seems to be more closely linked to the genes determining histocompatibility than the LA-locus [2].

We hope that our future investigations will help elucidating the very controversial problems concerning the significance of ABO and HL-A incompatibility in the etiology of abortions, though this may be more than we can achieve. The most

Table 8. *ABO compatibility*

	Karyotype of abortus		
Parents	Normal obs (exp)	Abnormal obs (exp)	Total
Compatible	15 (15.4)	22 (21.6)	37
Incompatible	12 (11.6)	16 (16.4)	28
	27	*38*	*65*

$\chi_1^2 = 0.035 \quad 0.80 < P < 0.90.$

Table 9. *HL-A incompatibility*

Number of antigens in common	Karyotype of abortus		
	Normal	Abnormal	Total
I *LA-series*			
0	14 (56.0%)	24 (40.7%)	38
1 or 2	11	35	46
	25	59	84
$\chi_1^2 = 1.103 \quad 0.20 < P < 0.30$			
II. *FOUR-series*			
0	19 (76.0%)	46 (78.0%)	65
1 or 2	6	13	19
	25	59	84

$\chi_1^2 = 0.008 \quad 0.90 < P < 0.95.$

important information we have had so far is the information concerning the frequency of 15 trisomy among abortuses and the information concerning the etiology of triploidy. In both cases the information could not have been obtained without the banding technique introduced by Caspersson et al. [6].

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Discussion

Lejeune: It is particularly interesting that the D trisomies you recorded were 15 and not 13 trisomies, and that trisomies in abortions can be different from the types found in liveborn children.

Lubs: Dr Margery Shaw has suggested that the C polymorphisms might be related to the frequency of non-disjunction. Determination of the frequency of C-polymorphisms in this sample, following identification of homologs by Q- or G-banding, might answer this question. It would probably work out better to proceed from G- to C-banding rather than from Q to C, since we have noticed that cells photographed by UV light do not subsequently C-band well (adjacent cells that were just out of the photographic field do band). Apparently the UV exposure altered the C-region chromatin.

Pearson: Your high rate of aneuploidy is presumably due to meiotic non-disjunction. This is interesting in view of the high rate of both Y and 9 non-disjunction in human male meiosis, where our own calculations place this in the order of 2%. If all chromosomes behave similarly then 37% of all spermatozoa will be aneuploid in one way or another.

Therkelsen: I think that different frequencies of non-disjunction for different chromosomes constitutes the most likely explanation for our finding.

Background for the Banding Patterns

Chemical composition of chromosomes

The Chemistry of the Eukaryote Chromosome

C. H. Ockey

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, UK

The study of the major components of the chromosomes at metaphase and the chromatin in interphase was essentially pioneered in the 1940–50's by various groups utilizing new techniques in microscopy and biochemistry. Amongst these were the critical qualitative and quantitative photometric studies of Caspersson and his colleagues [15, 16]; the extraction procedures and enzyme treatments of cells and nuclei [52, 42, 80] and the staining of cells with specific nucleic acid and protein stains [82].

Although some of these methods had certain disadvantages, considerable knowledge of the chemistry of the chromosome was accumulated. The chromosome was found to consist of four major components, DNA, RNA, histones and non-histone proteins. DNA was found to be associated with histone in a more or less constant ratio of 1.2:1 as a nucleo-histone complex. It was suggested that the non-histone (acidic or residual) proteins termed chromosomin by Stedman & Stedman [80] were concerned primarily with the structure of the chromosome while the histones (basic proteins) were involved with the inactivation of the gene [80]. With the discovery of the chemical structure of DNA by Watson & Crick [86], emphasis shifted towards a more molecular approach to the problems of gene translation and expression, particularly in prokaryotes. The relative stability and ease of extraction of DNA gave a great impetus to the chemical investigation of its structure in eukaryotic cells at the expense of studies of the other components. These have been increasingly investigated only over the last 10 years. The discovery of new, but frequently little understood, techniques has resulted in a great increase in our knowledge concerning histones and RNA. With this knowledge, however, there has been little parallel increase in the understanding of their function and chemical relationship to DNA in the cells. The role of each of the major constituents, their chemical interactions and their importance in controlling chromosome condensation and transcription, still remains somewhat of a mystery.

With advances in tissue culture techniques and methods of cell synchronization, large quantities of metaphase cells became available in relatively pure form from monolayer cultures [81] and many analyses of extracted chromosomes were carried out from different cell lines using a wide range of techniques of preparation. Each method, however, had its difficulties [28]. Basically, they all involved homogenizing the metaphase cells in hypotonic buffer solutions of varying pH, either by continuous agitation, pipetting or by pressure homogenization to release the chromosomes from the cells. These are then separated from the debris by centrifugation or filtration and analysed for their components.

Dr T. D. Allen has examined chromosomes extracted by different methods in our laboratories with the electron microscope. At low pH the condensed form is maintained and compares favourably with those seen in fixed metaphase cells [30]. The fibres range from 270–280 Å in diameter and chromosomes show little contamination (fig. 1). However, extraction at this pH is known to remove a considerable amount of histone and is close to the pH allowing some protein denaturation to occur. At neutral pH the chromosomes appeared less condensed with fibres increased in diameter (370–460 Å) and frequently the chromosomes are contaminated with membrane fragments and other debris, and sometimes show differential condensation along their length (fig. 2). Their histone content is, however, higher at neutral pH.

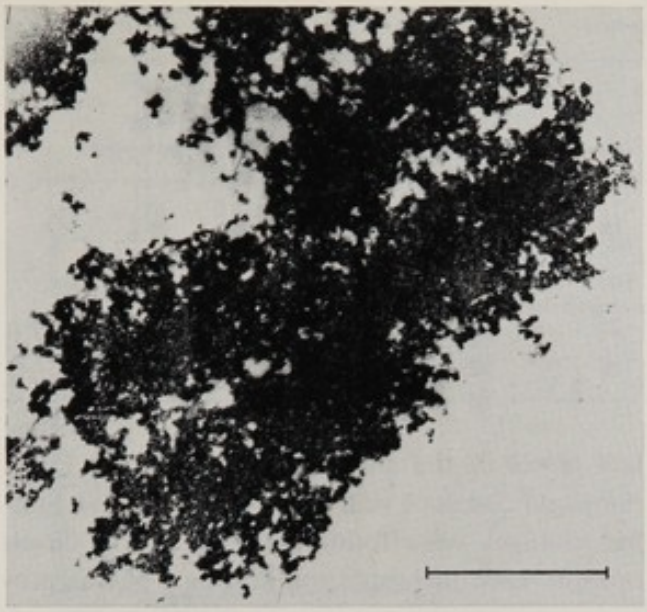

Fig. 1. Thin section of Chinese hamster chromosome extracted at pH 3.2 by the method of Skinner & Ockey [76]. Fixation in glutaraldehyde followed by osmium tetroxide. Stain: uranyl acetate and lead citrate. Scale, 1 μm.

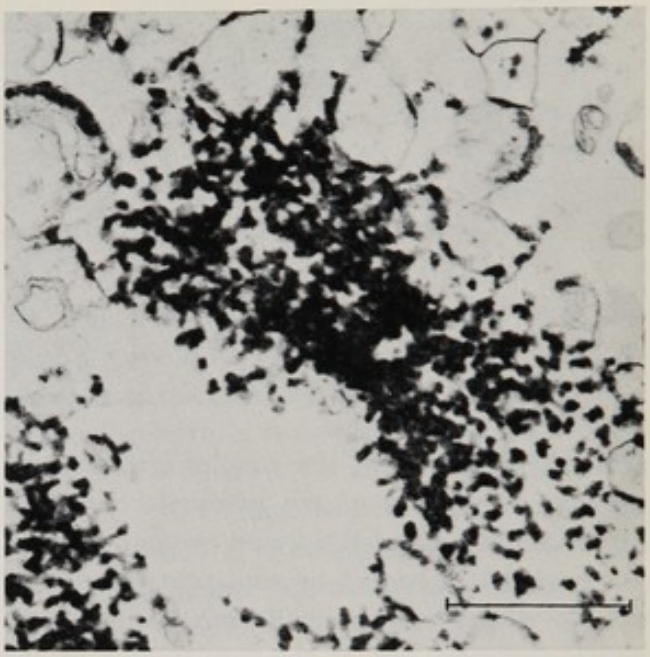

Fig. 2. Thin section of Chinese hamster chromosome extracted at pH 6.5 by the method of Wray & Stubblefield [88]. Fixation in glutaraldehyde followed by osmium tetroxide. Stain: uranyl acetate and lead citrate. Scale, 1 μm.

The composition of chromosomes extracted by different techniques is shown in table 1, where this reduction in the histones (expressed as acid-soluble proteins) can be seen with the low pH extractions. However, even with different extraction procedures, results of the analyses are remarkably similar. The two exceptions are the absence of RNA in one extraction of Chinese hamster cells [88] and the difference we have observed between the ratios of acid soluble:acid insoluble protein between Chinese hamster (1.61) and *Microtus agrestis* (0.57) using the same extraction procedure. Our own experience in this field [76] indicates that no ideal extraction procedure has yet been designed for all cell types and each type may require different modifications.

Gradient separation of chromosomes into size classes has been carried out by several groups of workers, two examples of which are shown in

Table 1. *Chemical composition of metaphase chromosomes from different species, using different extraction procedures*

Authors' results have been related to a normalized DNA content

Reference	Source	Extract pH	DNA	RNA	Total protein	Acid-soluble (0.2 M HCl)	Acid-insoluble
[88]	Chinese hamster chromosomes	6.5	100	0	209	—	—
			100	17	220	—	—
[76]	Chinese hamster chromosomes (7 samples)	3.2	100	44.9±2.6	196±20.4	121±15.1	75±8.6
[50]	Chinese hamster chromosomes	7.0	100	97	439	334	105
[73]	HeLa chromosomes	3.0	100	70	330	85	245
[31]	HeLa chromosomes	3.0	100	66	470	200	270
[72]	HeLa chromosomes	7.0	100	67	444	252	192
		3.0	100	66	470	200	270
[50]	Hela chromosomes	7.0	100	71	439	254	185
[76]	*Microtus agrestis* chromosomes (8 samples)	3.2	100	81	226	82	144
		6.5	100	68	169	85	84

Table 2. *Chemical content of fractionated chromosomes*

Reference	Source	Chromosome Size	DNA	RNA	Total protein	Acid-soluble (0.2 M HCl)	Acid-insoluble
[51]	Chinese hamster	Large	100	18	240	—	—
		Medium	100	79	540	—	—
		Small	100	140	840	—	—
[76]	*Microtus agrestis*	Large X	100	59	232	54	178
		Medium	100	73	180	68	112
		Small	100	116	329	172	157

table 2. In all cases, the smaller chromosome fraction at the top of the gradient contains a higher percentage of RNA and protein. This, in our material, is caused by contamination of the sample with ribosomes and cytoplasmic debris which separates out at the top regions of the gradient, the protein of which also contains some non-histones soluble in 0.2 N HCl. Chromosomes, however, do not necessarily contain the same amounts of protein in relation to DNA even within the same organism. Pierce's investigations with *Viola* plants showed that by lowering the phosphate content of the nutrient solution used to grow the plants, chromosome size and nuclear volume could be reduced [64]. This work was extended by Bennett & Rees [6] in *Allium* and rye. Here the doubling in chromosome size of plants grown in phosphate nutrient over no phosphate was found to be due to increased protein levels in the chromosome while the DNA remained constant at a diploid value (fig. 3). Similar changes were found to occur during development and differentiation and part of this protein excess was due to increases in histone (Rees & Kirk, personal communication). Others have described increases in the histone fractions rich in lysine during development in plant nuclei [25] and also in non-histone proteins in animal cells during increased metabolic activity [53]. Such differences in protein content in mammalian chromosomes presumably will also occur as size differences occur regularly in chromosomes from different tissues. In interphase chromatin, Frenster [27] observed increases in the non-histone proteins in euchromatin when compared to heterochromatin. We have, however, investigated the composition of gradient fractionated chromosomes of *Microtus agrestis*, when the large X chromosomes rich in constitutive heterochromatin could be separated from the smaller euchromatic chromosomes. Here we found the reverse situation, the large heterochromatic X chromosomes contained a higher proportion of non-histone protein (table 2). Both Sadgopal & Bonner [72] and ourselves [76] have found that there is a large increase in the amounts of RNA and non-histone proteins in metaphase chromosomes compared to interphase chromatin. This would suggest that there is a considerable danger in equating results obtained from chromatin analysis with those from chromosomes.

Fig. 3. Root tip metaphase chromosomes of *Allium cepa* bulbs after 10 days in solutions with (*a*) high phosphate; (*b*) no phosphate. From Bennett & Rees [6].

Chromosomal RNA

The RNA content of extracted metaphase chromosomes has frequently been found to be rRNA of 28S and 18S. This has led to the conclusion that chromosomal RNA represents contamina-

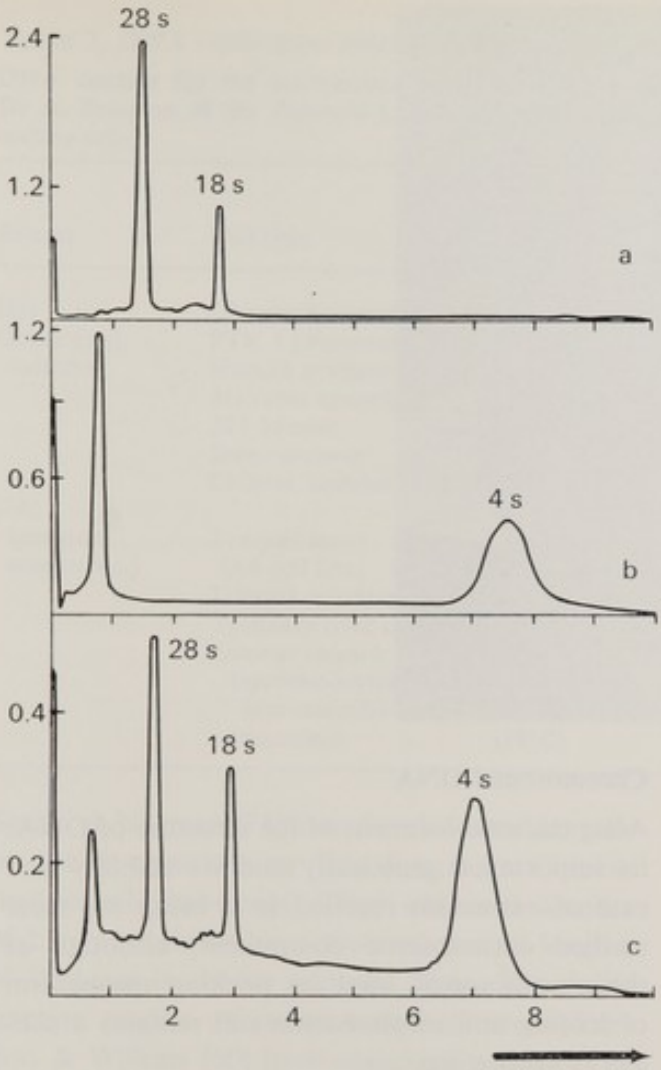

Fig. 4. *Abscissa:* distance travelled in cm; *ordinate:* A_{265}.
Electrophoresis of chromosomal RNA on polyacrylamide gels (2.4% w/v acrylamide, 2 h at 5 mA). (*a*) Rat liver ribosomal RNA; (*b*) RNA extracted from isolated Chinese hamster chromosomes with added yeast tRNA (4S) carrier; (*c*) mixture of Chinese hamster chromosomal RNA (with yeast tRNA as carrier) and rat liver rRNA.

tion from attached ribosomes and cytoplasmic debris [31, 50, 73] and Wray & Stubblefield [88] have obtained at least one sample of Chinese hamster chromosomes lacking any RNA (table 1). The significance of the RNA present in extracted chromosomes remains, therefore, undecided. However, L. G. Skinner of these laboratories has observed little variation in the RNA content in seven samples of Chinese hamster cells originally containing different percentages of metaphase cells (table 1); this was consistent with our previous observations on *Microtus* chromosomes. These results suggest to us a regular component as one would not expect this constancy following different extractions if contamination was the cause. He extracted RNA from isolated Chinese hamster chromosomes and subjected it to electrophoresis on polyacrylamide gels by the method of Loening [47]. The analysis revealed the presence of a single, slow moving homogenous component (fig. 4*b*).

Comparison of this pattern with that of liver ribosomal RNA extracted by the same cold phenol procedure (fig. 4*a*) shows the complete absence of the 28S and 18S rRNA components. When the mixture is applied to the gel together with the 4S yeast tRNA added as carrier to the chromosomal extracts to permit recovery of the small amounts of RNA present (fig. 4*c*), it is possible to deduce the molecular weight by the method of Loening [48]. This appears to be of the order of 4.0×10^6 D, consistent with the estimate made by Weinberg et al. [87] for 45S RNA. The presence of small amounts of 45S together with 28S and 18S rRNA has previously been detected by sucrose gradient analysis of chromosomal RNA extractions [31]. RNA of high molecular weight has also been described in salivary gland chromosomes, where in nucleoli a 38S component was found [22] or in HeLa cells [49], where a 45S component was found. It would appear that the RNA we have observed is therefore unlikely to be due to contamination. In chromosomes labelled with ^{3}H-uridine, Prescott & Bender [66] were able to show that RNA has a random distribution along the chromosome with no evidence of semi-conservative replication but a general halving of the labelled RNA at the first mitosis. This RNA was lost rapidly from the chromosomes in later mitoses.

A further indication that RNA in the metaphase chromosome is not an artefact of extraction comes from some studies by Dr T. D. Allen in our laboratories. Sections of metaphase cells were treated by the EDTA method of Bernhard [7] which reveals ribonucleoprotein in the cells by bleaching out the stained deoxyribonucleoprotein of chromatin. In fig. 5, the RNP appears to coat the chromosomes with some distributed internally. It is likely that this RNA represents the nucleolar RNA deposited on the metaphase chromosomes when the nucleolus has disappeared and they undergo condensation and is consistent with the "chromosomal RNA cycle" originally described by Kaufman et al. [42].

Fig. 5. Thin section of Chinese hamster metaphase cell fixed in glutaraldehyde and treated by the method of Bernhard [7]. RNA structures in the chromosomes stain with heavy contrast compared with DNA. Scale, 1 μm.

Fig. 6. Autoradiograms of DNA from three synchronized mammalian cell lines after labelling with 20 μCi/ml ^{3}H-thymidine (spec. act. 23 Ci/mmol) for 30 min, followed by a chase period of 45 min. Scale, 20 μm. (*a*, *b*) Human primary embryonic lung fibroblasts; (*c*) *Sorex araneus* fibroblasts (90th passage); (*d*) Chinese hamster ovary fibroblasts (CHO). (*a*) (lower strand) replicons with tails; (upper strand) replicated and split; (*b*) (lower strand) replicon with tails; (upper strand) replication commenced before pulse introduced; (*c*) replication fork with no tails to replicons; (*d*) replicons with no tails (initiation sites arrowed).

Chromosomal DNA

After the establishment of the structure of DNA, its importance genetically and its stability and ease of extraction resulted in it being the most studied chromosome component, although at the chromosomal level its packing mechanism of folding and condensation still remains a subject of conjecture.

DNA exists as a helix of approx. 20 Å diameter and when complexed with histone it forms a supercoil of 100–230 Å [21]. These workers estimated that the medium sized human chromosome of the C-group contained 4–4.7 cm of DNA helix condensed to a chromosome length of <10 μm.

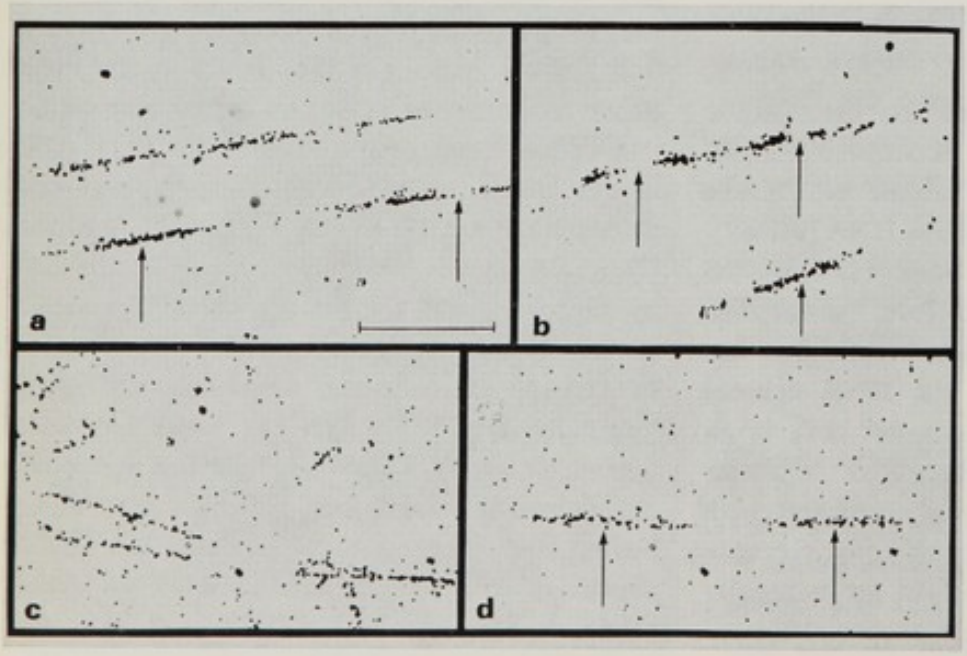

Table 3. *DNA replication rates in eukaryote chromosomes calculated from autoradiograms*

DNA content for the mammalian cells calculated by the techniques of Munro & Fleck [54] and Burton [10] by Dr A. Peterson of the Paterson Laboratories. Mammalian DNA contents based on total cells rather than on cycling cells

Source	Cell type		DNA (pg/ haploid)	S period (hours)	Replic. rate (μm/min, one way)	Interreplicon distance (μm)
[32]	Chinese hamster (B14FAF)		—	—	0.5–2.5	7–30
Ockey (cell cultures)	PTK 1 (*Potorous tridactylis*)		13.3	13	0.19	50.7 (27–76)
	Human primary		4.8	8.5	0.22	56.2 (27–108)
	Microtus agrestis		—	5.6	0.18	37.0 (23–76)
	3T3 Mouse		11.8	9.5	0.19	35.7 (26–56)
	Sorex araneus		5.3	8	0.21	50.9 (30–97)
	Chinese hamster (CHO)		7.3	8	0.17	33.4 (26–46)
[14] (personal communic.)	*Xenopus laevis* (A6 cell line)	(25°C)	3	13	0.15	57.0 (20–124)
	Triturus cristatus (primary liver culture)	(25°C)	29	48	0.33	150–200
	Triturus vulgaris (spermatocytes)					
	(pre-meiotic)	(18°C)	33	216–240	0.2	~2000
	(neurulae)	(18°C)	33	<48	0.1	43.0 (20–90)

Sasaki & Norman [74] have demonstrated lengths of DNA approaching this figure in Chinese hamster chromosomes. They observed lengths of 2.2 cm, while we have observed shorter lengths of 0.94 cm in similar material. From X-ray diffraction studies on isolated nucleo-histone, Pardon & Wilkins [60] have suggested a model for nucleo-histone supercoils with a pitch of 120 Å and a radius of 50 Å. When they studied the X-ray diffraction patterns of separated metaphase chromosomes they found a similar structure to nucleo-histone with no evidence of regular tertiary arrangements in the chromosome at this level [59].

Since Taylor's classic experiments with ^{3}H-thymidine labelling of *Vicia faba* chromosomes, where the semi-conservative nature of chromosome replication was demonstrated [83], the whole nature of chromosome and DNA replication has been extensively studied. The chromosome before replication acts as a two stranded chromatid analogous to a double helix with each new strand being synthesized on the outside of the old strands [29, 84]. These facts together with other evidence suggest that the DNA in a metaphase chromatid exists as a double helix packed in some yet undetermined way. Cairns [13] showed that DNA replicates in separate segments along this strand. Replication proceeds at the same rate in both directions from an initiation point in each segment or replicon [32] as shown in fig. 6. This can be shown by first labelling cells for a certain period with a heavy ^{3}H-thymidine pulse, followed by a chase period in unlabelled thymidine, and finally preparing autoradiograms of the DNA extracted in Millipore chambers. Each replicon will have a central initiation point with a dense autoradiogram on either side and beyond this lighter tails of grains (fig. 6*a*, *b*). However, if the replicon has already commenced replication prior to the introduction of the labelled pulse an unlabelled region will be present in the centre of the replicon (fig. 6*b*). Replication forks are formed when duplicated replicons meet and the newly replicated strands separate (fig. 6*c*). The "tails" in a pulse-chase experiment may not always be visible, this being due primarily to the different thymidine precursor pools in the cells. Human primary cells (fig. 6*a*, *b*) probably contain lower pools than the cells of either Sorex or CHO lines (fig. 6*c*, *d*) where grain gradients are less frequently observed. Callan [14] was able to show in *Triturus* and *Xenopus* DNA that by comparing DNA content, replication rate, and inter-replicon distances the length of the DNA synthesis period was governed, not so much by rate of replication, but by interreplicon distances or distance between initiation points and DNA content (table 3). Some evidence of this can be seen from our data for mammalian cells where replication rate is relatively constant and DNA content and inter-replicon distance

together relate more to S period length. Callan also observed that inter-replicon distance varied between different tissues of the same organism. He estimated that the number of initiation points in the haploid genome of *Triturus* pre-meiotic spermatocytes was remarkably similar to the number of chromomeres in the lampbrush chromosome and the packaging of the chromomeres may therefore restrict the number of initiation sites. These sites are probably controlled either by different recognition regions to DNA polymerases with greater variety in somatic cells or by limitation in the number of enzyme molecules per cell with uniform enzymes and recognition sites.

In cytological terms interphase chromatin exists in three forms: (1) The euchromatic state, where DNA is available for transcription in the cell, remains decondensed in interphase and replicates early in the S period; (2) the facultative heterochromatic form, where DNA may be condensed, inactive in transcription and late replicating or the same DNA may be present in the euchromatic state. One of the X chromosomes in most female mammals shows this behaviour; (3) the obligatory or constitutive heterochromatic form, where it always remains condensed, inactive in transcription and late replicating. Such DNA is usually present in both homologous chromosomes in regions associated with centromeres and telomeres, but can also be inter-calary, as whole chromosome arms (*Microtus agrestis*) or as whole chromosomes (grasshopper B chromosomes), and in mammals parts of the Y chromosome are usually of a constitutive heterochromatic nature.

Britten & Kohne [8] in 1968 demonstrated by denaturation and renaturation kinetics of mouse DNA that some DNA consists of families of repeated sequences. 10% of the DNA was found to be of a highly repetitive nature (1×10^6 copies), 15% consisted of families of related sequences (1 000 to 100 000 copies) and 70% of the genome was made up of unique sequences. If mouse DNA was extracted and run in a caesium chloride or sulphate gradient the repetitive sequences appeared as a light satellite peak rich in AT regions and separated from the heavier main peak DNA. These satellite sequences were shown, by in situ DNA/RNA hybridization, to be present in chromosome regions adjacent to the centromere in all chromosomes except the Y [39, 61]. However, the presence of such peaks of repeated sequence DNA in other species will depend on whether they are rich enough either in AT or GC regions to separate them from the main peak DNA. Arrighi et al. [4] found satellites in 48 out of the 93 species they investigated. In most cases this repetitive DNA is equivalent to the constitutive heterochromatic regions of the chromosome demonstrated by the C band technique of Arrighi & Hsu [5]. However, Arrighi et al. [3] suggest that caution should be used in equating the two terms. This is particularly true since the human Y chromosome constitutive heterochromatic regions have not yet been found to be repetitive DNA, and similarly in grasshopper, the heterochromatic B chromosomes are known to contain as much as 72% of their DNA as unique sequences [27*a*]. Only the basic units of the satellite DNA of the mouse and the guinea pig have been sequenced and found to consist of TTTTTC and CCCTAA respectively [77, 85]. Certain repetitive sequences, including those responsible for the production of 18S and 28S ribosomal RNA, are active in transcription and here the basic sequence complexities are found to be similar for such diverse rRNAs as *Xenopus*, rabbit and HeLa cells [67]. These rRNA species form single families of RNA molecules and have, due to their functional importance, not measurably diverged in evolution although encoded by highly redundant genes. Brown and his colleagues [9] using the partial denaturation methods of Inman & Schnos [35] demonstrated that in two closely interbreeding species, *Xenopus laevis* and *Xenopus mulleri*, that the rRNA 18 and 28S regions were essentially similar in sequence while the spacer regions between each rRNA sequence were entirely different. These rRNA sequences are usually less repeated than some of the satellite DNAs, for example in *Drosophila* there are 130 copies while in *Xenopus* somatic cells 400–450. Considerable differences occur between satellite sequences from closely related species and even within the same species. This latter was borne out by the studies of Gibson & Hewitt [27*a*] on B chromosome satellite DNA taken from grasshoppers selected from different areas. Here they observed distinct differences between the content of repeated sequences of the satellites.

In man, several repetitive satellite sequences

Table 4. *Amino acid analysis for the five main calf thymus histones from Butler, Johns & Phillips* [12]
The highest values found in all the fractions are given in bold face type and the lowest values in italics

	f1	f2(b)	f2(a)2	f2(a)1	f3
Aspartic acid A	*2.5*	5.0	6.6	5.2	4.2
Threonine	5.6	6.4	*3.9*	6.3	6.8
Serine	5.6	**10.4**	3.4	*2.2*	3.6
Glutamic acid A	*3.7*	8.7	9.8	6.9	**11.5**
Proline	**9.2**	4.9	4.1	*1.5*	4.6
Glycine	7.2	5.9	10.8	**14.9**	*5.4*
Alanine	**24.3**	10.8	12.9	*7.7*	13.3
Valine	5.4	7.5	6.3	**8.2**	*4.4*
Cystine/2	0.0	trace	trace	trace	1.0
Methionine	*0.0*	**1.5**	(trace)	1.0	1.1
Isoleucine	*1.5*	5.1	3.9	**5.7**	5.3
Leucine	*4.5*	4.9	**12.4**	8.2	9.1
Tyrosine	*0.9*	4.0	2.2	3.8	2.2
Phenylalanine	*0.9*	1.6	0.9	2.1	**3.1**
Lysine	**26.8**	14.1	10.2	10.2	*9.0*
Q-*N*-Methyllysine	0.0	0.0	0.0	1.2	1.0
Histidine	(trace)	2.3	**3.1**	2.2	1.7
Arginine	*1.8*	6.9	9.4	12.8	**13.0**

have been found [18] and the chromosome location of some of these demonstrated by in situ DNA/RNA hybridization [40, 75]. It would appear that with improved techniques many more families of repetitive sequences will be found, some of which have already been shown to be common to the same chromosome regions. Speculations as to the origin, evolution and purpose of these redundant sequences has been the subject of several publications [85, 89].

Chromosomal Proteins

Histone proteins

The increased knowledge obtained over the past few years about chromosomal DNA has not been paralleled by a similar insight into the structure and role of the histones. The histones have, however, received more attention than the non-histone proteins principally because techniques for their extraction and characterization were easier and more reproducible and partly because it was first thought that they played an important role in gene control in translation. This latter view is now no longer popular and this role has been attributed to the non-histone proteins.

The chromosomal histones are relatively small polypeptides with molecular weights of between 10 000 and 20 000 D. They are composed of >22% basic amino acids and are extractable from chromosomes with 0.2 N HCl. Their separation is not necessarily complete and small amounts of non-histone proteins are liable to be extracted with them [28]. The extracted histones have a net positive charge. They are characterized into fractions by the analysis of their terminal groups, presence and absence of cysteine and their behaviour in gel electrophoresis [37]. In table 4, the composition of the five major histone fractions are shown. Briefly, f1 is characterized by its rich lysine content and its high proportion of basic amino acids, f2 (b) its high serine content and its *N*-terminal proline, f2(a)2 its high leucine, f2(a)1 its high arginine content and f3 its high arginine content as well as being the only fraction containing cysteine.

The amino acid sequences of the f2(b) (125 amino acids) and the f2(a)1 (102 amino acids) have been worked out independently and in the f2(a)1 fraction, the sequences have been found to be similar for both pea and calf chromatin [19] suggesting an importance of this fraction in a structural role in chromosome and chromatin rather than a transcriptional role. In contrast to the histones, the protamines, which replace them in sperm, are relatively small some having only 30 amino acid residues.

Both Sadgopal & Bonner [72] and ourselves [76] have observed a reduction in the f1 fraction in metaphase chromosomes. The explanation of a loss of f1, however, is questionable, since the recent work of Lake et al. [45] indicates that the f1 fraction becomes phosphorylated at metaphase and thus moves only slowly on polyacrylamide gel electrophoresis. They could obtain the normal f1 proportion if cells were first treated with alkaline phosphatase prior to gel electrophoresis.

The histone fractions appear to be present in the same proportions in chromosomes which contain mainly unique copies of DNA as in those composed almost entirely of repetitive DNA (table 5) [76]. This also applies to heterochromatin and euchromatin in interphase and is independent of whether the heterochromatin is facultative or constitutive [17].

When the demonstration of Huang & Bonner [34] that histones added to DNA repress tran-

Table 5. *Histone fractions, as per cent total histone, from fractionated metaphase chromosomes of a fibroblast culture of* Microtus agrestis *isolated at pH 3.2*

From Skinner & Ockey [76]

Histone fraction	Fractionated metaphase chromosomes isolated at pH 3.2		
	Large X	Medium	Small
f1	9.0	8.9	8.1
f2(b)+f3	48.9	46.0	49.4
f2(a)2	33.1	29.2	28.3
f2(a)1	9.0	16.0	14.1

scription was made and that this mixture exhibited the characteristic X-ray diffraction pattern of native nucleo-histone [91], histones were proposed as the primary constituents repressing transcription. Although this theory is now not generally accepted in toto, due to the similarity of fractions between different species and tissues and the general lack of diversity among the histones, the effects of the various fractions on structure or repression were important. The arginine-rich fractions are required for the superstructure of nucleo-histone complex, while the f1 lysine-rich fraction is not apparently involved [70]. It is also not involved in transcription since its removal from nucleo-histone does not increase this function [79]. It has been suggested that it may be concerned with the condensation of chromosomes at metaphase [38, 46] and the phosphorylation of this fraction could well be concerned in this role. Several pieces of evidence indicate that the different histone fractions appear to be bound to the DNA with different degrees of attachment. Ohlenbusch and colleagues [57] were able to show that f1 was removed by sodium chloride extraction at 0.4–0.5 M while the arginine-rich fractions required molarities of 0.8–1.6 to dissociate them. Murray [55, 56] also obtained differential extraction of the f1 with acid at 0°C. Here the order of release was similar: pH 1.7, f1 removed and at pH 0.2, f3 and f2(a)1 were dissociated. F1 also appears to be the most easily removed by proteolysis [69]. These factors are of interest since they may be possible explanations in the techniques used for chromosome banding where ionic strengths or pH are also important. In this connection, Roberts [71] obtained differential contraction and puffing of specific bands in polytene chromosomes with ionic strength and pH changes, while Kato & Moriwaki [41] have stressed the importance of pH in the Giemsa banding technique.

It would appear from the results of Itzhaki [36], where she found that 40–50% of the phosphate groups of DNA in chromatin were accessible to polylysine in several types of chromatin, that fairly long stretches of DNA must lie unbound beneath the histone; she indicates that histones, therefore, are unlikely to act as repressors simply by "blocking" the DNA.

The interaction of the polyamino acids with DNA shows specific affinities for AT-rich regions in the case of polylysine and GC for polyarginine. However, it is not necessarily correct to equate the lysine rich f1 with the behaviour of polylysine. These reactions have been dealt with in some detail elsewhere [26], but there is no experimental evidence that f1 has any specific affinity for any specific base sequences. The f1 histone is also different from the other fractions in that it has the fastest turnover rate in the cell [33] and has been further fractionated with the result that it is the only fraction that shows any species specificity [11]. One of several possible structures of the DNA–histone complex has been proposed by Fredericq [26] which fits closely to the known facts. He suggests that the strongly bound histone fractions (with high or moderate arginine) would have their basic sequences wrapped around the DNA double helix in the small groove, in close contact with the phosphates. They would be in extended configuration, the non-polar residues would protrude outside, forming α-helical chains which could lie either in one groove (very arginine-rich), or outside the double helix running parallel to its axis (moderately arginine-rich). The loosely bound lysine-rich fractions would have only intermittent contacts with the phosphate groups; their chains in extended conformation would establish links between different DNA molecules or between different parts of the superhelix. A participation of acidic proteins in such cross-links is also likely.

Modifications of the histones must also play a considerable part in their function in the nucleus. The processes of de-condensation of chromatin and its subsequent transcription have been linked with the phosphorylation, methylation, acetyla-

Table 6. *Amino acid composition of various nuclear non-histone proteins*

Amino acid	*a*	*b*	*c*	*d*	*e*	*f*	*g*
Lysine	6.7	6.6	3.4	4.2	6.9	6.2	9.5
Histidine	3.1	2.2	2.5	1.7	1.4	1.5	1.3
Arginine	6.7	5.6	5.6	5.3	3.2	5.6	5.2
Aspartic acid	9.2	10.2	16.9	12.1	10.1	9.5	9.5
Threonine	4.9	4.8	7.6	5.5	4.9	5.5	5.0
Serine	6.1	5.8	7.0	12.5	16.9	7.5	7.6
Glutamic acid	13.5	13.9	11.0	14.4	17.6	12.4	14.0
Proline	5.5	5.6	6.0	10.0	4.3	5.0	4.7
Glycine	8.0	8.6	6.6	N.D.	12.5	7.5	9.4
Alanine	7.4	8.1	7.0	9.7	7.7	7.6	9.6
Half cystine	0.6	N.D.	N.D.	N.D.	N.D.	1.3	N.D.
Valine	6.1	6.3	6.5	5.3	3.6	5.6	7.3
Methionine	1.8	1.5	1.7	1.8	0.8	1.9	1.8
Isoleucine	4.3	4.0	4.1	2.8	2.4	4.3	3.4
Leucine	8.0	9.5	7.6	6.7	4.0	9.9	7.2
Tyrosine	3.7	3.1	2.4	3.2	0.8	2.6	1.9
Phenylalanine	4.9	4.1	4.4	3.2	1.7	3.9	2.4
Tryptophan	N.D.	N.D.	N.D.	N.D.	N.D.	2.0	N.D.

All values represent per cent of total moles of amino acids recovered.
N.D., not determined.
A, mouse spleen chromatin; *b*, rat liver chromatin, phenol-soluble; *c*, early blastula of sea urchin, alkali-soluble nuclear protein; *d*, Ehrlich ascites dehistonized nuclei; *e*, Novikoff hepatoma chromatin phospho-protein; *f*, rat liver non-histone protein fraction; *g*, rat liver chromatin (single electrophoretic band).
From review by Spelsberg, Wilhelm & Hnilica [78].

tion or thiolation of histones [1]. Phytohaemagglutinin stimulated lymphocytes show both increased phosphorylation [44] and acetylation [65] coincident with chromatin de-condensation. An increased binding of acridine to stimulated lymphocytes was also observed early in the process by Killander & Rigler [43]. However, it must be again stressed that events connected with interphase chromatin condensation do not necessarily reflect those involved in metaphase chromosome condensation.

Non-histone proteins

Of all the investigations into the chemistry of the chromosome components, those concerned with the non-histone proteins have been the most neglected. At the present time very little is known about either their structure, diversity or their interactions with other chromosome components. This group has been ignored principally because of the difficulties encountered in characterizing them, extracting them without problems of contamination and their general tendency to aggregate during separation. The problems involved have been reviewed recently by Spelsberg, Wilhelm & Hnilica [78].

Non-histone proteins form a heterogeneous group in chromatin and are, as previously stated, regarded as having a functional role in gene transcription. They present a mixed collection including phospho-proteins, together with the polymerase, nuclease and protease enzymes of the nucleus. They aggregate readily which may account in part for their high molecular weights of 5 000 to 100 000 D. The amino acid content is primarily acidic with high levels of glutamic and aspartic acid. Analysis of these proteins by various workers from different chromatin sources (table 6) shows similarities in their composition.

Because of their tendency to bind to nucleohistone on extraction and isolation [38] the figures shown in table 1 must be treated with some caution. Further evidence of this binding was observed in extracted chromosomes stained for non-histones both before and after extraction from metaphases [90].

Two conflicting pieces of evidence concern the association of non-histone proteins with chromatin. Mirsky & Ris [53] showed an increase in non-histone proteins in cells showing increased metabolic activity, and also Frenster [27] demonstrated an increase of amount in actively transcribing euchromatin in contrast to the inactive heterochromatin. This would suggest that

non-histone proteins are concerned with the decondensation of chromatin and with gene translation. On the other hand, we have the evidence discussed earlier of increases in non-histone proteins in metaphase chromosomes compared to the state of chromatin in interphase nuclei. Our own metaphase chromosome study [76] also shows an increase in the non-histone proteins of heterochromatic chromosomes over euchromatic ones. This disagreement must await solution when techniques for the characterization of the individual non-histones have been established.

Some advances have been made in this direction. With modifications in gel electrophoresis, Elgin & Bonner [23, 24] and Arnold & Young [2] have shown that upwards of 13–15 reproducible bands can be visualized, with some evidence of species specificity. Paul & Gilmour [63] have evidence of organ specificity also following reconstitution experiments with DNA-histone and non-histone proteins from various sources. The presence of the latter was found to increase transcription in vitro over the presence of histones alone. These authors suggest a major role of non-histone proteins in the control of gene transcription.

Sadgopal & Bonner [72] showed increases in the disulphide groups in both non-histones and the f3 histone containing cysteine in metaphase chromosomes. At interphase, they observed a higher frequency of thiol groups in these components in chromatin compared with metaphase chromosomes. It would appear that the increase of oxidized thiol groups may be associated with the condensed state of chromatin and such suggestions have been made in the literature [20, 58]. It was suggested that the proteins were polymerized in metaphase chromosomes through disulphide bridges between the individual DNA-bound proteins.

In conclusion, it is disappointing to find that from the wealth of experimental data present in the literature, the understanding of the relationships between the various chemical components of the chromosome is so limited, although attempts at a general theory have been put forward [62]. It is, however, certain that the protein components change considerably from their chemical nature in interphase chromatin to that in metaphase chromosome, so that much of the data from chromatin studies may not be relevant when one considers chromosomes.

The binding behaviour of histones to non-histone proteins and their relationship to DNA of different base sequence is not yet clear. Similarly, the binding of these protein components to DNA of repetitive or single copy nature remains uncertain. Further studies on separated chromosomes of the *Microtus agrestis* type with an analysis of the non-histone fractions may reveal specific differences in this last respect, while analysis of chromosomes in birds where microchromosomes, rich in GC pairs, could be separated, would give information as to the histones and non-histones in DNA of different base sequence.

Chromosome banding techniques give a new impetus to the investigation of chromosome chemistry, although here we are dealing with the complex treated with hypotonic solutions, acid fixation and dehydration. However, even with these alterations, valuable data on structure and component relationships should be forthcoming.

Addendum: The replication rates calculated from the autoradiograms for the 6 mammals (table 3) although similar were approximately half those described for certain mammals by other workers. We have recently found in later experiments with CHO cells that a considerable number of half replicons (fig. 6*b*, upper autoradiogram) have been mis-interpreted as whole ones when they had initiated replication some minutes before the introduction of the pulse of ^{3}H-thymidine. These replicons were present even after cells had been synchronised at the beginning of S by DNA inhibitors. Due to the presence of varying but considerable pools of thymidine precursors in these cell lines, the resulting autoradiograms frequently produced a grain gradient of increasing intensity after the introduction of the pulse. This gradient was similar to that produced by the chase period with unlabelled thymidine where the precursor pool was slowly depleted of labelled molecules. Half replicon tracks, therefore, in a typical pulse-chase experiment could appear similar to the whole track of the replicon with gradients at either end but be half as long. The figures shown in table 3, col. 5, would therefore be approximately twice the value shown and the inter-replicon distances will also be affected. These six species are now under re-investigation.

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Polytene Chromosomes

J.-E. Edström

Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden

Interphase chromosomes usually appear in the form of chromatin, and as such are not amenable for study as chromosomal entities. In the polytene chromosome, because of the lateral magnification of the original post-mitotic chromatid, study is possible not only on a chromosomal level but even down to a level represented by presumed genetic units of function. It is obvious that the polytene chromosome is a genetically active chromosome since cells possessing them may increase the size of the protein synthesizing machinery several thousand times during development.

Is the Chromosome Band a Unit of Function?

Studies on polytene chromosomes are of interest mainly insofar as they can be referred to subchromosomal entities, in particular if these represent units of function. The most striking feature of these chromosomes, apart from their size, is their pattern of DNA-rich bands alternating with DNA-poor interbands. The mere fact that most of the DNA is in the bands and that the number of bands, in *Drosophila* around 5 000, seems suitably large for a full complement of genes, prompts the question whether the band is indeed a unit of function. The question has long been debated. In a recent paper Judd et al. [1] in a study of the zeste-white segment of the X-chromosome of *Drosophila* found about 120 mutants split into 14 groups of allelic mutants. Members of the same group do not complement each other, in contrast to members of different groups and therefore, presumably, originate in the same gene. The number of groups, 14, is similar to, or identical with, the number of bands or interbands in the segment in question. Members of the same group, furthermore, affect the same function and the groups form a linear map. Judd et al. have concluded that a band and/or one of its associated interbands determines only one essential function. Their conclusions regarding a one-to-one correspondence between bands and complementation groups have received support in similar studies on other parts of the *Drosophila* genome by Hochman [2] and by Lifschytz & Falk [3, 4]. Also, mapping studies have consistently indicated that mutants, the location of which can be delimited to single bands and/or associated interbands, affect the same function [5].

The relation between primary and secondary gene products, i.e. RNA and proteins on the one hand and an observable character in *Drosophila* on the other is, however, unknown in studies of this kind and what the band and/or its associated interband represents in operational terms remains a secret. As pointed out by Beermann [5], the interband is an alternative to the band as a gene locus, or is functionally cooperating with the band. None of the cytogenetic studies specifically exclude that it is the interbands rather than the bands that are the seat of genetic activity. Even if the DNA concentration in bands is of the order of at least 20 times that of the interbands [6], there may still be enough DNA in interbands for a single gene or a small number of genes. Since bands and interbands occupy, on the average, about equal parts of the total length of the chromosome set [5], it follows that the band should contain DNA in amounts sufficient for a large number of genes. Rudkin [7] has measured, in *Drosophila*, the X-chromosome DNA contents per band and single chromatid in the range of 5 000–100 000 base pairs and values within this range have also been obtained by others in this

[8] or other dipteran material [9]. An average sized gene would be expected to require a number of base pairs one or two orders of magnitude less.

In view of the apparent functional simplicity of the band the significance of the high DNA content becomes enigmatic and a number of alternative explanations have been considered. Thus it has been suggested several times that the band may be constructed of repeating templates, but most of the DNA from Diptera has been found to behave in DNA reannealing tests like unique DNA [10, 11]. In view of possible unknown complexities of polynucleotide interactions in the eukaryotes it may, however, be too early to dismiss this possibility. Another alternative is that the band DNA does indeed constitute a large number of templates for structural genes either involved in the same function or largely not elicited with available cytogenetic methods. A final alternative is that much of the band DNA does not represent structural genes and is either concerned with gene regulation, or else constitutes genetic nonsense.

Is a Puff a Manifestation of Genetic Activity?

The chromosome bands or, on the single chromatid level, the chromomeres, may appear in any one of two alternative states. They may be compact or they may be swollen, forming so-called puffs. Puffing has been portrayed as a decondensation and looping-out of the DNP filament normally packed within the chromomere. The main basis for this hypothetical picture, apart from electronmicroscopic observations of DNP filaments in the puffs, is the observation on what is probably an analogous formation, the lampbrush chromosome loops extending from chromomeres and in all likelihood created by a decondensation of a continuous DNP filament. Puffs contain in contrast to non-puffed bands both RNA and non-histone protein, although RNA synthesis can be observed in bands not obviously puffed [12]. If one makes the assumption that all non-ribosomal RNA synthesis is accompanied by puffing, although sometimes below the detection limit, it is clear that puffing must, at least to a certain extent, be an expression of genetic activity. Studies of puffing patterns by a number of workers have given data in agreement with this expectation. Puffing patterns have been found to be specific both for type of tissue and developmental stage [13, 14]. If puffs represent genetic activity it would be expected that a large number of puffs would be common to several tissues and that some would be tissue-specific. This is also generally the result. Thus Berendes [15], comparing different tissues in *Drosophila* hydei, finds a few of the puffs to be tissue-specific, the majority common for the tissues investigated. The fact that RNA synthesis need not express itself in visible puffing detracts of course from some of the interest in correlations between puffing patterns and genetic activities. Studies of this kind are naturally made even more complex by the possible existence of long-lived messengers and post-transcriptional controls. Some direct positive evidence exists, however, in the case of certain large, tissue-specific puffs, called Balbiani rings, for an involvement of puffs in gene expression due to the work of Beermann [16] and Grossbach [17]. The midge *Chironomus pallidivittatus* has a salivary gland with a main lobe and a side lobe. The cells in the side lobe differ from those in the main lobe by the presence of special secretory granules. In the main lobe there are altogether three Balbiani rings in the fourth chromosome; in the side lobe there is also a fourth one. The sibling species *Chironomus tentans* has a salivary gland of similar design but the side lobe lacks granules. In this species the fourth Balbiani ring is lacking altogether. In hybrids between the two species different types of genomes could be obtained with different contributions from *Chironomus tentans* and *Chironomus pallidivittatus*. Beermann [16] found that the ability to form granules was positively correlated to the presence in the genome of a small chromosome segment carrying the fourth Balbiani ring. Grossbach [17], in studies with a similar design, subsequently identified a protein fraction correlated to the presence of the fourth Balbiani ring and made an allocation of another protein to another chromosome segment with another ring. These studies suggest a relation between puffs and the production of specific proteins although they do not tell anything about the nature of this relation. It does not in any case appear likely that there is a one-to-one relationship between rings and proteins. Wobus et al. [18] compared chironomids with 1–4 Balbiani rings which all contained

about the same number of secretory protein fractions. Furthermore, a particular strain of *Chironomus thummi* was detected which had an extra ring, seemingly having arisen de novo. This ring was not accompanied by any detectable additional protein [19].

What is the Nature of Chromosomal RNA?

The evidence that the band may be a relatively simple unit of function and that the puff is an expression of genetic activity motivates an interest in puff RNA as a possible carrier of genetic information. There are, however, many questions to be answered before such a role can be assessed. Here I will only discuss the question of the origin of puff RNA and evidence for its export to the cytoplasm.

To investigate the presence of RNA of local origin it is labelled with radioactive precursors for brief periods, since RNA of local origin is likely to show rapid uptake of precursor label. The rapidly labelled RNA of polytene chromosomes is localized predominantly over puffs [12]. The labelling of high molecular weight RNA over puffs but not nucleoli is inhibited by alfa-amanitin which inhibits polymerase II with a known localization to the chromosomes [20]. The rapidly labelled puff RNA is therefore synthesized by a polymerase characteristic of the chromosomes. By means of sedimentation analysis, or gel electrophoresis it is possible to characterize this RNA with regard to turnover and size [21, 22]. It is a heterogeneous, high molecular weight fraction which is also characteristic of the nuclei of higher cells in general. A local origin has been demonstrated down to the level of the individual puff by Lambert [23] who has extracted the RNA from isolated Balbiani rings and used it for in situ hybridizations. He has found that the RNA will mainly hybridize the DNA in the ring from which it was extracted. Such a relation is not unexpected in view of results obtained by Miller & Bakken [24] on what is probably a formation analogous to the puffs, the loops of the lampbrush chromosomes. Here, RNP filaments can be demonstrated increasing in size from one end of the loop towards the other, a picture indicating a continuous transcription of tens of microns of DNA. RNP granules can also be seen in Balbiani rings close to DNP filaments. Structures that have been interpreted as attachment stalks of the granules to DNA have also been described [25, 26], suggesting that the situation in the lampbrush chromosomes may be of general application. If the chromomere produces one main transcript as studies on lampbrush chromosomes suggest, a single puff would be expected to give a simpler distribution of RNA molecules than a whole set. Furthermore, such a distribution should be skewed towards the low molecular weight side. A distribution of this type is indeed obtained in separations of RNA from single Balbiani rings [22]. Much of the RNA, therefore, may be derived from a single transcript or a series of transcripts of similar size. These data do not tell whether all band DNA is engaged in transcriptory activity or only a part. The RNA is, however, of such a size that it could very well account for an involvement of an appreciable part of the DNA. To summarize, at least a large part of the rapidly labelled puff RNA has properties suggesting a local origin.

Is there also RNA of non-local origin in the puff? By using a microelectrophoretic procedure, Ringborg (unpublished) has found, when analysing the optical density profile of RNA from isolated Balbiani rings, that the RNA does not migrate as might be expected from the labelling profiles, but that a large part of it forms two peaks at about the position of the ribosomal RNA markers. This result suggests that there may also be RNA present which is not of local origin. Analyses performed after prolongation of the labelling periods showed that this surmise is correct and that much of the puff RNA is in fact preribosomal RNA originating from the nucleolus. The ribosomal RNA formation starts in the salivary gland cells with the formation of a 38S RNA precursor in the nucleolus. This is subsequently converted to a 30S and 23S component [21, 27]. Shortly after the appearance of these fractions in the nucleolus they can be discovered also in the chromosomes [28, 29]. This is a specific association and does not represent contamination by nuclear sap, since the components become detectable in the chromosomes before there are measurable amounts in the surrounding sap. Furthermore this RNA is localized to the puffs since the fourth chromosome with the large Balbiani rings may contain up to half of the label in the light component although it represents

only 10% of the genome [29]. An origin outside the chromosomes (chromosome = non-nucleolar regions) is shown by the lag period in appearance in the puffs, and is confirmed by the fact that one of the components will appear also when the labelling of high molecular weight RNA is suppressed pharmacologically, excluding the possibility that it is a product of such RNA [29].

The presence of preribosomal RNA in the chromosomes is of special interest in view of the fact that ribosomes are attached to the growing messenger of bacterial chromosomes. There is, however, no convincing evidence for any role for preribosomal RNA in protein synthesis. A function for preribosomal RNA in chromosomal RNA metabolism may nevertheless be a general one. In *Sciara coprophila*, another Diptera, ribosomal RNA is formed in a large number of nucleolar-like bodies spread all along the chromosomes [30, 31]. Preribosomal RNA is also present in the chromosomes of other chironomids outside the nucleolar organizer region [32]. It is of some interest that Deak et al. [33], in studies of gene expression in cell hybrids, found that the maintenance of an enzyme activity specific for one of the parental cells is dependent on the integrity of the nucleoli or some structure close to the nucleolus in the parental cell in question, indicating the possibility that ribosomal RNA may be involved in the regulation of the expression of chromosomal messages.

For the sake of completeness it should be mentioned that low molecular weight RNA with properties of transfer RNA is present in considerable quantities in the chromosomes. This RNA appears as a precursor and shows with increasing age a more mature profile in gel separations [34]. At present it is unknown whether it is localized to puffs or not and the functional significance of the maturation process is obscure.

A cardinal question concerns the possible relation between the puff products and messenger RNA. In this connexion studies of the export of puff products become of interest. Evidence pertinent to this question is still scarce and concerns mainly the Balbiani rings of the chironomids. These large puffs are characterized by large RNP granules which can also be seen in the nuclear sap and at the nuclear envelope. Here they are often present in the nuclear pores and may assume shapes that have been interpreted as were they in the process of being transferred to the cytoplasm. RNA hybridizing with the Balbiani rings has recently been found in the salivary gland cell cytoplasm by Lambert [35] suggesting that an export of Balbiani ring RNA may actually take place. Cytogenetic evidence implicates the Balbiani rings in the production of secretory proteins in the salivary glands. The messengers for this protein synthesis are stable during RNA synthesis inhibitation [36, 37]. It is of interest in this connexion that a cytoplasmic RNA fraction of high stability can be demonstrated which, like the Balbiani rings, is tissue-specific [38, 39]. It remains to be proven, however, whether puff RNA may serve as a messenger RNA and if so what fraction of it. The relation between puff RNA and messenger RNA may be complex since messenger RNA on the whole behaves as a much smaller molecule than the nuclear heterogeneous RNA or its counterpart in the puffs. Studies on the white-Notch region in the *Drosophila* X-chromosome by Green, Lefevre and others, recently summarized by Beermann [5] provide evidence that only a small initial segment of the band may provide a structural template and that much of the band DNA may be superfluous, at least during the test conditions of the laboratory. The idea that only a part of the band DNA provides structural messenger is the basis of a number of recent gene models. For the evaluation of such models, however, considerably more has to be learned about the transcription products. Such work will require an awareness of the possibility that different types of puff may show important differences in construction and mode of operation.

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Background for the Banding Patterns

General aspects on the banding techniques

On the Mechanisms for Certain Chromosome Aberrations

G. F. Bahr

Armed Forces Institute of Pathology, Washington, D.C. 20305, USA

The new banding techniques have provided a fresh impetus for more detailed analysis of translocations, even of small sections of chromatids, as has been demonstrated during this Symposium. There are no difficulties in explaining translocations with reference to recently gained insight into the fine structure of chromosomes. Breakage and rejoining occur organizationally at the fiber level, i.e., along the long single chromatin fiber consisting of a single supercoiled DNA molecule and 70 to 85% protein, now strongly believed to be the principal element of chromatid structure [1]. The model mechanism for the translocation of a section from chromosome A to B is illustrated in fig. 1.

Inversions are a different matter, since difficulties will be encountered with a simple scheme such as that of fig. 1 in that the handedness of the double helix would be of opposite direction at both points of rejoinder (fig. 2). The body of knowledge on molecular properties of DNA makes this solution highly unlikely.

A significantly more complex mechanism for inversions is possible in which no problems of principle of molecular incompatibility arise. Let us consider the concept that chromomeres appear to be conformational modifications of a long chromatin fiber, and that they are separated by lengths of longitudinally folding fibers of various lengths. The proposed mechanism involves the loss of two lengths of longitudinal fiber at interphase prior to DNA replication (fig. 3). DNA in longitudinal fibers is likely to be structural DNA, i.e., serving as a spacer and having no coding function. The lengths of longitudinal fiber to be eliminated would equal the length of the inversion measured on an early metaphasic chromosome. In the illustrations matters are presented only in two dimensions; in reality the

Fig. 1. Model illustrations for the mechanism of translocating a section from one chromosome to the other. (*a*) Explanation of events at the chromosomal level (After Lejeune, p. 16); (*b*) explanation of events at the chromatin fiber level at interphase. *Arrows* indicate handedness of DNA double helix.

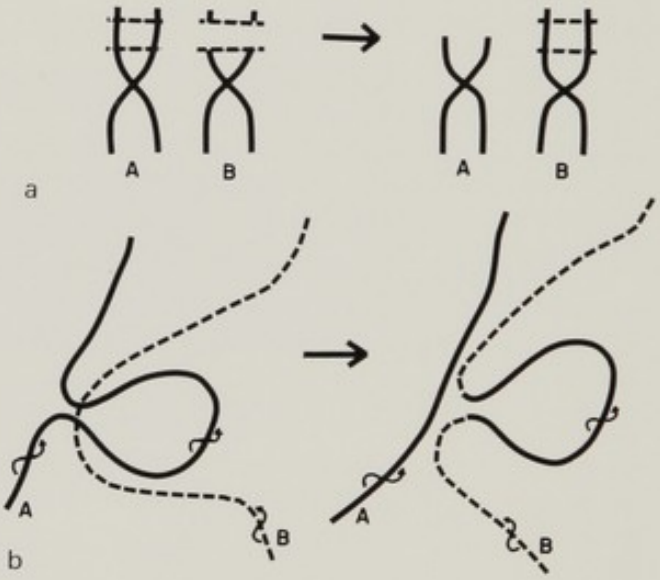
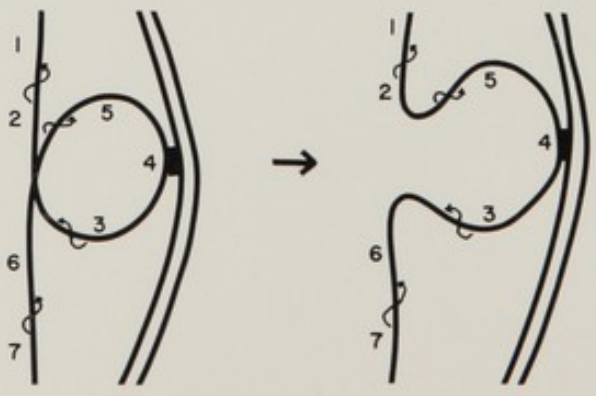
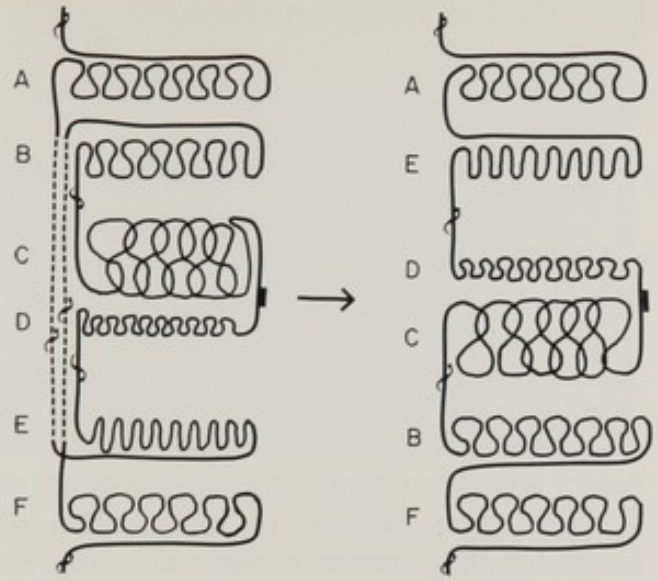
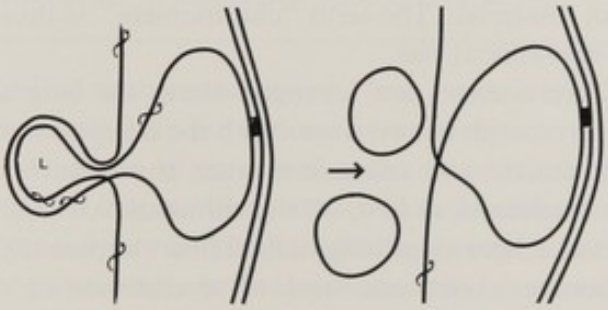


Fig. 2. Unlikely mechanism for an inversion, illustrated at the fiber level before DNA replication. The block at point 4 symbolizes hypothetical attachment of the centromere to the inner nuclear membrane. Fiber ends containing the open end of a double helix of supercoiled DNA are unlikely to join if their sense of spiralization has an opposite direction.





a



b

Fig. 3. Model explaining possible mechanism of inversion, in this instance a central inversion, ■, location of the centromere. (*Upper left*) chromomeres (bands) A-F with their respective longitudinal fibers. The sense of DNA spiralization is indicated by arrows. After inversion (*a*, right) two stretches of longitudinal fibers (---) have been lost; (*b*) events are explained for interphase chromatin fibers prior to replication of DNA. It is likely that the narrow area in which four fibers come together is twisted during actual strand exchange, and that this proximity facilitates the process, possibly induces it.

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double loop (*L*) is probably twisted around in the neck portion. A model for the origin of dicentric chromosomes after DNA replication has been proposed by DuPraw [1].

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Correlates of Chromosomal Banding at the Level of Ultrastructure

G. F. Bahr, U. Mikel and W. F. Engler

Armed Forces Institute of Pathology, Washington, D.C. 20305, USA

Principal Features of Chromosome Structure

In the course of studying, with the aid of electron microscopy, the human karyotype for quantitative aspects of its structure and composition, it became apparent that the principal constituent of chromatids, the chromatin fiber, is arranged in two structural forms.

In one of these forms, chromatin fibers are more or less oriented in parallel to an imaginary axis of the chromatid (figs 1, 2). By quantitative measurement and by count, there are on the average from 12 to 18 such longitudinal fibers per chromatid cross section in prophasic chromosomes.

The other of the structural forms of chromatin fibers is a whorl of folded, looping fibers intimately associated with longitudinal fibers (figs 3, 4). Because these whorls are quite comparable to the "chromomeres" of classic light microscopy, they will be called chromomeres hereafter.

Chromomere fibers are arranged in tortuous, somewhat kinky loops, having diameters, measured from fiber center to center corresponding to at least three times the diameter of a single fiber (fig. 5). This general configuration holds for loops not in contact with the supporting substrate and not affected by the forces stretching chromosomes during preparation [1]. Such loops are considered to be relaxed. Diameters of chromomere fibers are preferably determined from relaxed sections of fibers.

Loosely looping fibers are found in varying quantities along the entire chromatid; thus a chromomere is not a sharply delineated structural entity but a space in which looping fibers are assembled at increased density. In the two-dimensional aspect of an electron micrograph, fiber density appears to increase towards the center of a chromomere because of superposition of fibers along the direction of observation (fig. 3). Stereopairs reveal, consequently, that the center of a chromomere is geometric in nature rather than material. The term "chromomere" is thus an operational one.

Chromomeres are arranged along the length of chromatids in two ways. With the exception of centromeric and telocentric sites, chromomeres are condensed, at first, off the chromatid axis (fig. 3). At a later stage longitudinal fibers apparently penetrate about one-third of a chromomere's volume (fig. 4). It is common to find two chromomeres juxtaposed on opposite sides but at different cross-sectional levels of the chromatid, often imposing the impression of a spiral on the appearance of chromatids. Because of the three-dimensional preservation of whole-mounted chromosomes after critical-point drying, chromomeres are located to the sides, on top of, and below the longitudinal fibers, a fact not readily apparent in the two dimensions of regular transmission electron micrographs.

Chromatin fibers are divided into longitudinal and chromomere fibers because of different conformation. Mass per unit length, diameter, and unevenness or bumpiness are nearly the same for both longitudinal and chromomere fibers. Chromomere fibers are kinky; i.e., they are characterized by short stretches of relatively straight fibers framed by bends or kinks, usually of wide angle, rendering an irregular loop. One may see chromomere fibers emerge from the longitudinal bundle or, conversely, becoming part of it. Fibers can at times be followed from one chromomere to the other, constituting—in the semantics of this description—longitudinal fibers between chromomeres (fig. 3). It is not surprising

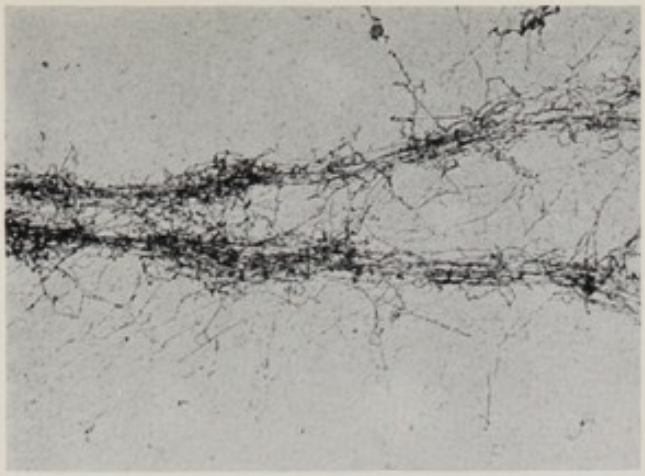


Fig. 1. A length of chromosome in which longitudinal fibers dominate and chromomeres are small. Chromomeres defined by looping kinky fibers are at some places not much more than a few loops of fiber. The course of individual fibers can be analysed only in stereopairs. The number of longitudinal fibers is 11. × 28 000.

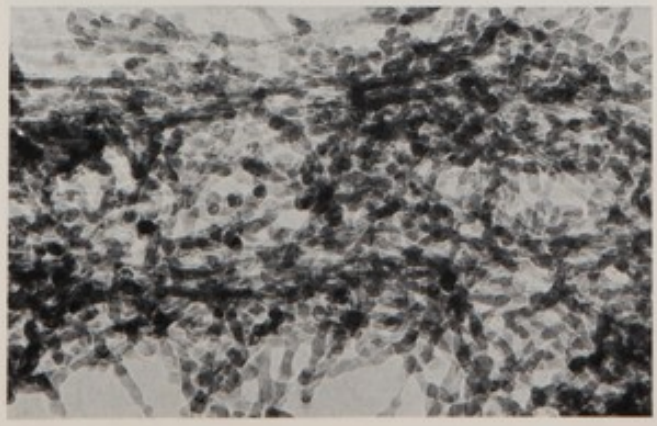
to find such continuity of fibers, since it is to be expected from the concept of uninemy.

There is often a well-centered, much larger mass of looping fibers at each end of a chromatid. Not only are longitudinal fibers looping back, but a prominent chromomere complex is usually located here (fig. 4).

Close to the centromere, two prominent and often two less prominent small, rather dense chromomeres are situated in such a manner as to form a square or rectangle with analogous chromomeres on sister chromatids (fig. 6). Fibrous connections between sister chromatids occur regularly here.

Chromosomes in transition from interphase to metaphase are characterized under the electron

Fig. 2. High magnification of chromosomal fibers. Longitudinal elements can clearly be recognized despite presence of numerous chromomeric fibers. Note contrast increased in areas where fibers are piled up. × 96 000.



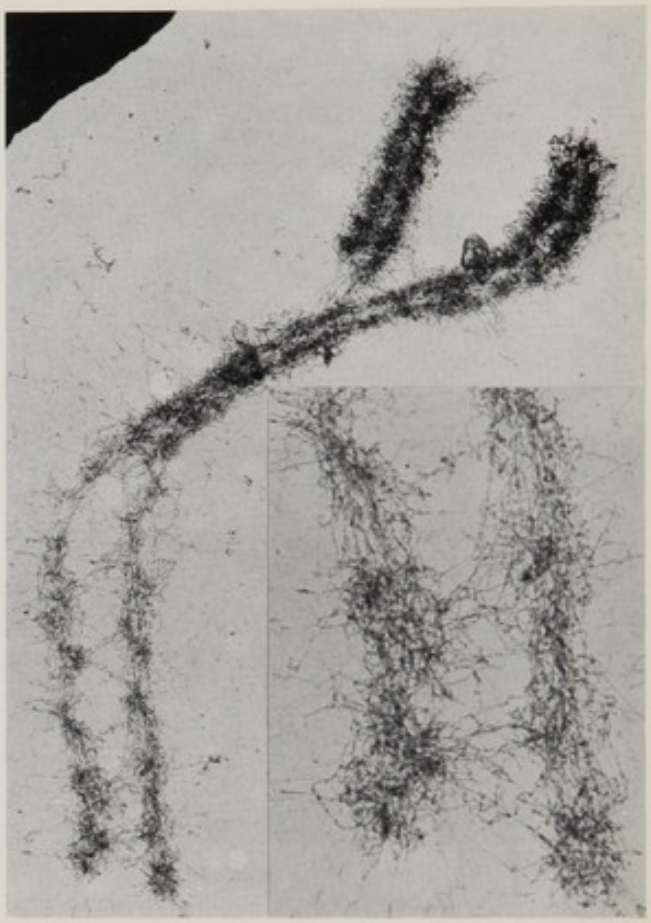


Fig. 3. One almost-condensed E-group chromosome attached by numerous fibers to an unevenly condensed B group. In the latter chromosome, condensation decreases from the short-arm end to the long-arm. The insert shows the terminal chromomere masses of the long arm, and especially the very intimate association of longitudinal fibers with chromomere fibers, and underlines the fact that our division of fiber types into longitudinal and chromomere fibers is arbitrary. Because of the many loops radiating from uncondensed chromomere areas there is extensive entanglement of chromatids. Oblique or off-axis location of chromomeres is evident. × 11 000; *insert* × 30 000.

microscope by numerous chromatin fibers radiating from the body of each chromatid (fig. 4). Upon closer inspection one finds that these fibers are actually extended loops of fiber portions that have not made or only partially made the transition from the interphase fibers which are about 100 Å thick to the more highly coiled chromosome fiber of about 230 Å [2]. These loops originate more often at chromomere regions than at in between areas. Since there is structural evidence that defined stretches in these loops are uncoiled to 40 Å and further to the limits of resolving small unstained masses, it is likely that genetically active sections of the genome are prominent in loops that are the last to be supercoiled or condensed

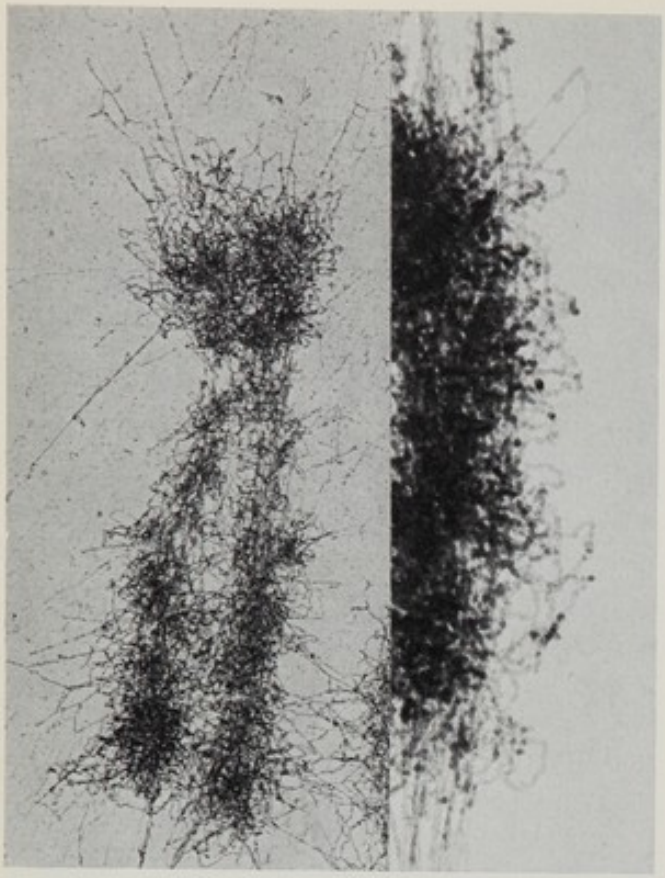


Fig. 4

Fig. 5

Fig. 4. A chromosome at late prophase-early metaphase. Condensation of chromatin fibers and aggregation into chromomeres is progressing. There is good agreement between chromomeres of both chromatids. The end of the left chromatid arm is more condensed and fusion of two chromomeres still discernible in the right chromatid is shown. Fiber loops extending from the chromosome originate at chromomeres. × 23 000.

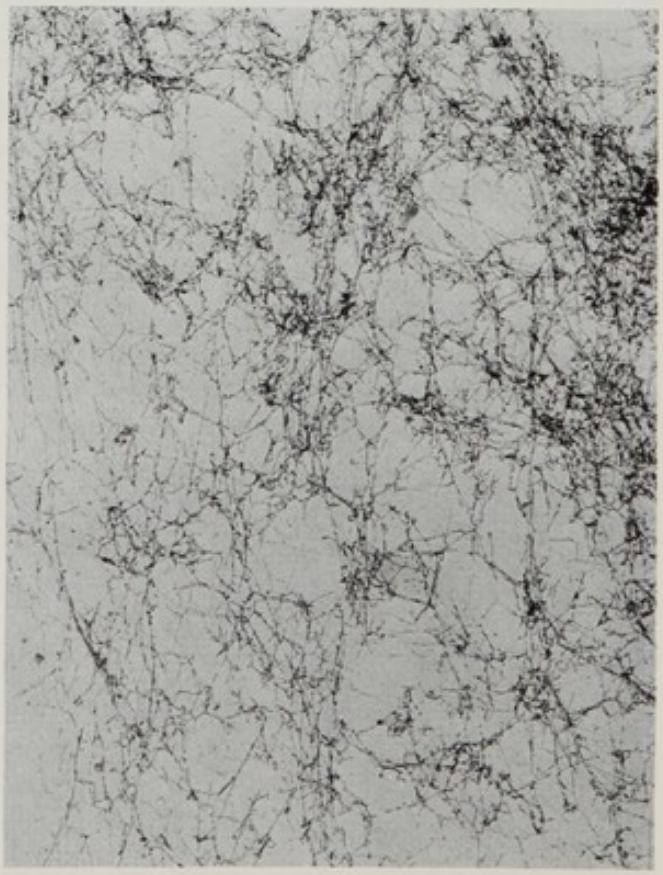
Fig. 5. Edge of a nearly fully condensed chromosome. Chromatid fibers originate from chromomere area. At this stage of condensation, loops are close to the body of the chromatid, and only a few extend into the surrounding or to other chromosomes. Although loops are seen in this micrograph only at one side of a chromatid, they do extend equally in the direction of the observer, because three dimensionality is well preserved by critical point drying of the specimen. Superposition of fibers in the direction of observation (which is synonymous with the direction of the illuminating electron beam) produces contrast in proportion to the quantity of fibers. × 59 000.

into their chromomere (fig. 5) and ultimately into the almost solid-appearing metaphase chromosome [1]. Thus some chromomeres at least are implicated in being loci of formerly genetically active chromatin.

Chromomeres lend a beaded appearance, rather than the impression of banding, to a chromatid. Since banding patterns in chromosomes are a main topic of this Symposium, however,

Fig. 6. Almost fully condensed chromosome with two prominent, condensed centromeric fiber masses on the short arms and two minor condensations on the long arms. × 25 700.

Fig. 7. Area of a spread nucleus at interphase. The meshwork of 100–150 Å chromatin fibers shows no prominent signs of organization, although some fiber aggregations occur, which may be organizational foci for chromosomes. × 21 000.



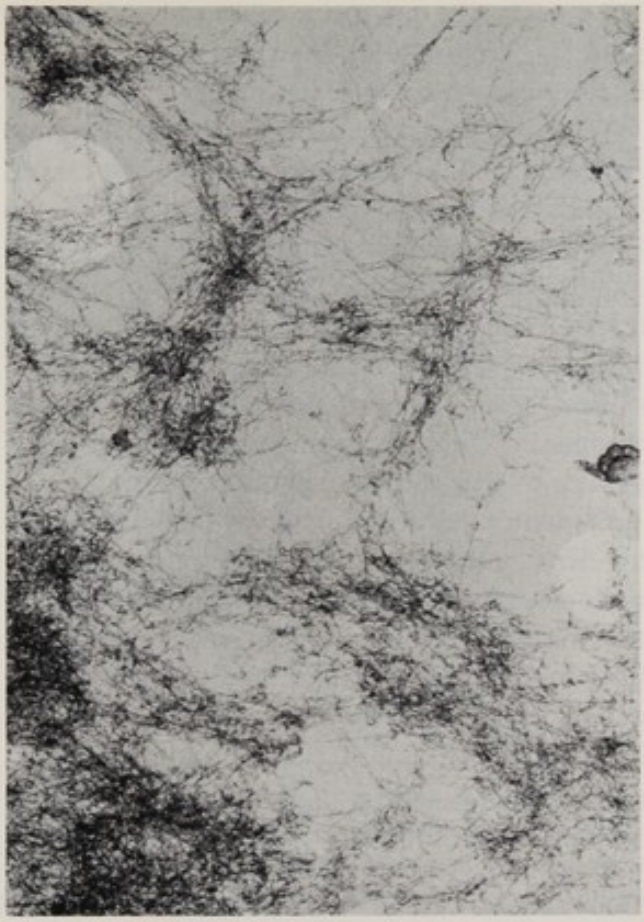


Fig. 8. Condensation of chromatin progresses and the onset of chromosome formation can be discerned. This micrograph is an enlarged area of the nucleus shown in full in fig. 9. × 16 000.

this term will also be used in describing the chromomeres of electron microscopy.

From among the almost 5 000 chromosomes studied by us under the electron microscope, we have selected sets of suitable electron micrographs for densitometric scanning. Two to seven individual scans have been evaluated so far for each of the 23 chromosomes in the haploid female karyotype plus several Y chromosomes. This report deals with the analysis of only a portion of available material and is thus neither complete nor definitive. There is nevertheless enough information to enable us to begin to compare electron microscopy with light microscopy.

Chromosomes Condensing from Interphase

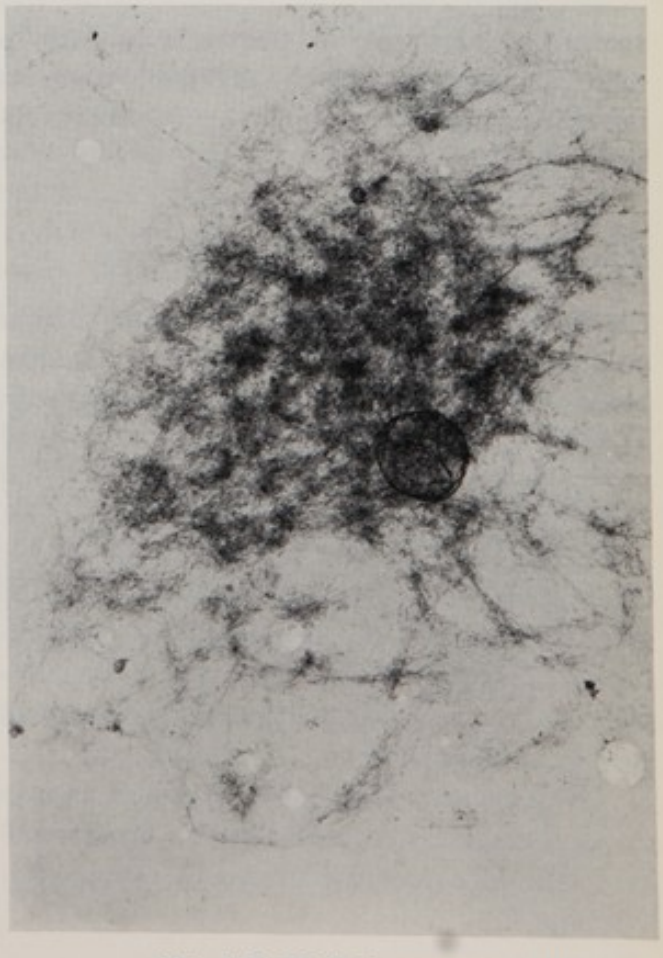
Chromosome condensation proceeds from the apparently unstructured meshwork of chromatin fibers at interphase (fig. 7). We see aggregates forming in which both longitudinal and chromomeric fibers are involved from the very start (figs 8, 9).

In the next phase, extended, slender chromosomes of middle prophase appear with numerous chromomeres at identical locations on both chromatids, producing a railroad track appearance (fig. 10). Upon further condensation a phase is reached at which the chromomeric structure of the chromatid is most prominent and can easily be appreciated on electron micrographs (fig. 11). Condensation progresses until individual chromomeres can be discerned only with difficulty or not at all.

Often chromosomes do not appear to condense in good synchrony. In our preparations a mix of variously condensed chromosomes is found, the sequential order of which is subject for future study.

There is often also a notable difference in the condensation of the short chromosome arms

Fig. 9. Early prophasic condensation of chromatin in a spread nucleus. Wisps of aggregated chromatin fibers are prominent in the periphery, accompanied by early condensations of chromomeres. A mass of condensed chromatin occupies the center of the spread. A remnant of nuclear membrane is lodged on top of this condensation. Light circles are holes in the support film. × 5 200.



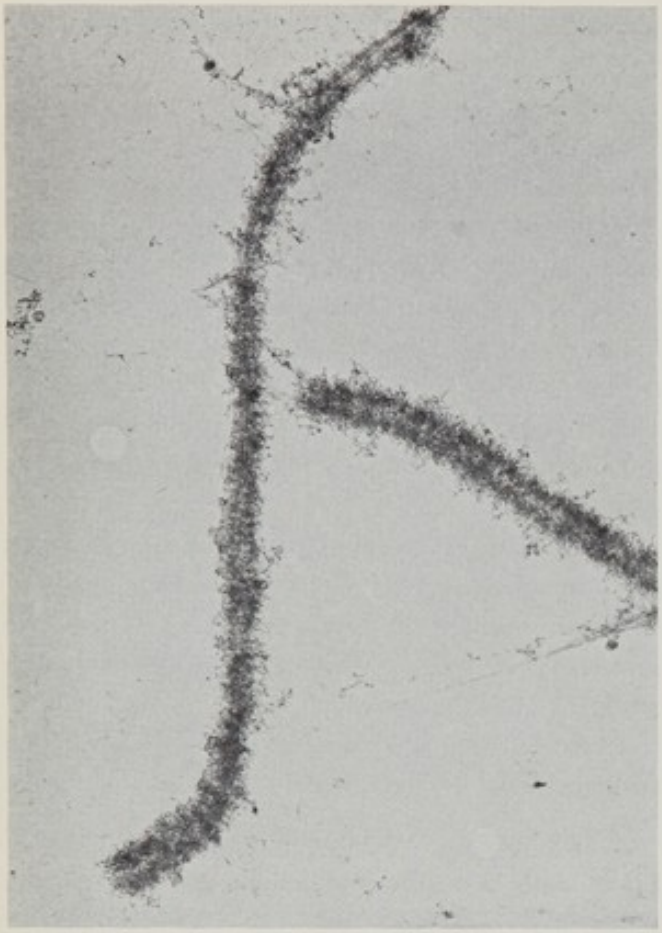


Fig. 10. Railroad-track appearance of two chromosomes at end of prophase. Chromomeres are single and somewhat difficult to discern individually. × 12 200.

versus the long arms or vice versa, possibly a relic from the time when two differently condensing chromosomes were joined by acrocentric fusion.

Recording Chromosomal Banding Patterns

Electron micrographs of whole-mounted critical point-dried chromosomes from cultures of human blood lymphocytes were used for densitometry. The photographic material had been exposed and processed as required for quantitative evaluation [3, 4]; i.e., contrast was proportional to dry mass.

Single chromatids were scanned with an evenly illuminated slit aperture covering the entire width of the chromatid, but not more and carefully following eventual bends. The photomultiplier current was amplified and fed to a small strip chart recorder.

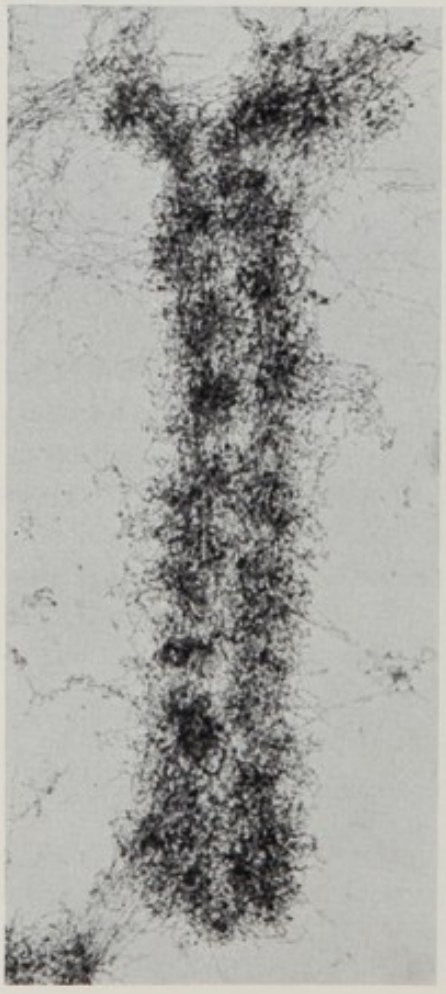
This same scanning system was also used in scanning transmission in negatives of Dr Schnedl' karyotype as a representative of a Giemsa (G) technique [5].

Recordings of quinacrine mustard banding patterns (Q) are available from the publications of Caspersson and his colleagues [6].

There is a good general agreement between the appearence of specimens prepared by the chosen Giemsa technique (G) and principal features seen in quinacrine mustard bandings (figs 12–14). Our tracings from electron micrographs represent nothing but the distribution of dry matter along chromatids and carry no direct chemical information. It is apparent that the distribution of chromatin fibers in chromomeres is fairly comparable to that seen by light microscopy.

Electron microscopy deserves interest because the densitometric traces of whole-mounted chromosomes exhibit a wealth of additional information. Each band seen by light microscopy is in fact further structured by major and minor discontinuities of mass in the chromatid. The fact that

Fig. 11. An acrocentric chromosome exhibiting the beady quality of chromomeres rather than band-like appearance. Chromomeres appear as rounded bodies, more prominent in one than the other chromatid. There is gradual transition from one chromomere to the next and fuzzy delineation. Many loose loops can still be seen at the periphery. × 54 000.



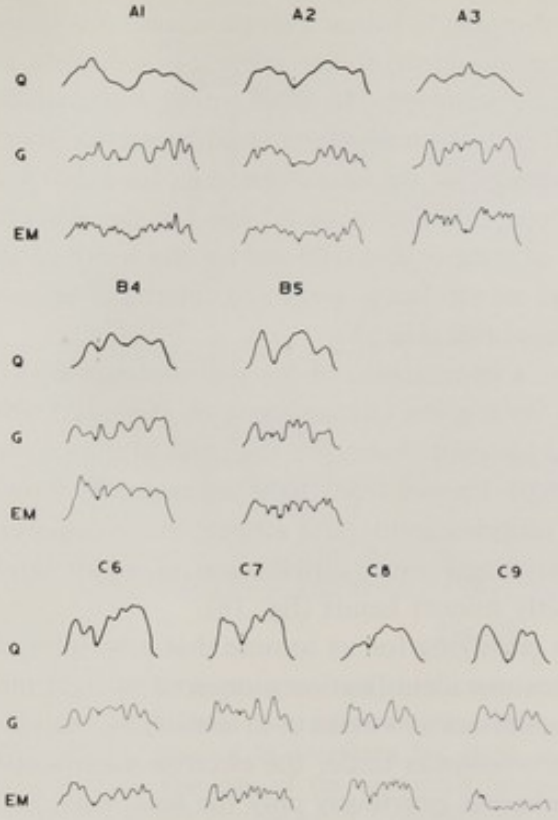


Fig. 12

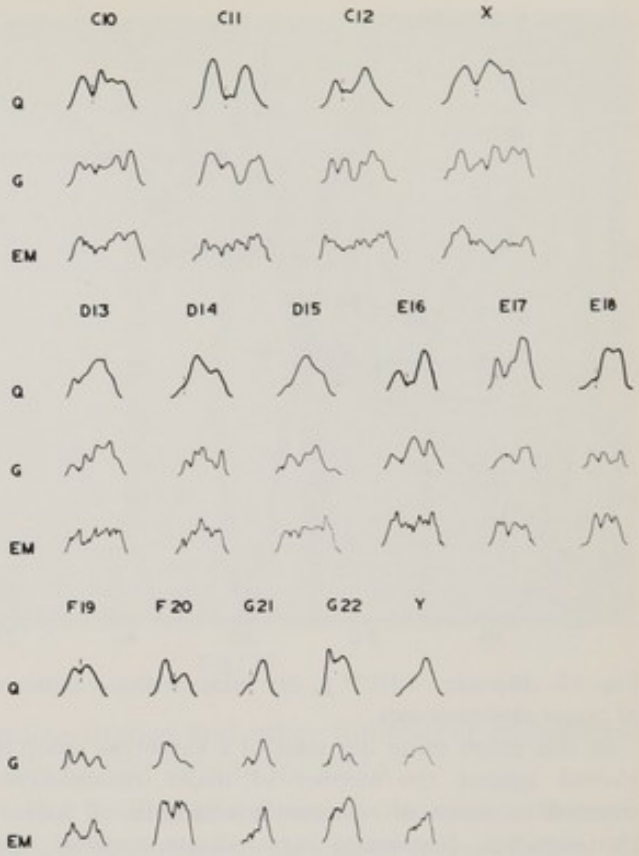


Fig. 13

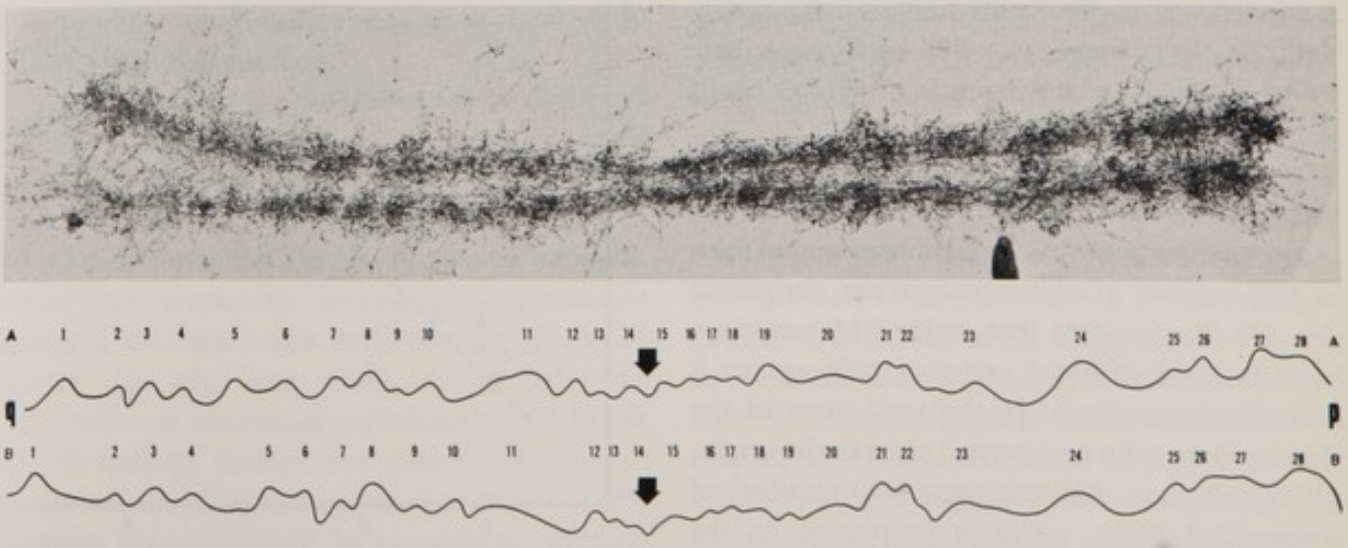
Figs 12, 13. Comparison of scans from quinacrine mustard-stained fluorescing human chromosomes [6] *Q*; from Giemsa-stained G-banded chromosomes [5] and from electron micrographs reflecting mass distribution directly (*EM*).

such detail of chromosomal structure is not a reflection of recorder noise or other spurious influences is evident from comparisons of independent scans of each of the chromatids in an A-group chromosome. Structural detail is essentially alike and differs only in its relative location because of different stretching of the chromatids (fig. 14).

While there is reason to expect only small changes in the mapping of major bands as further material is being analysed, assignment of minor bands will have to be clarified on more extensive material.

After quinacrine mustard staining nearly 126 bands for the haploid karyotype including X and Y can be discriminated in digitally filtered curves.

Fig. 14. An A-group chromosome has been scanned along each chromatid. Upon close inspection one finds that structural detail in both chromatids is highly comparable.



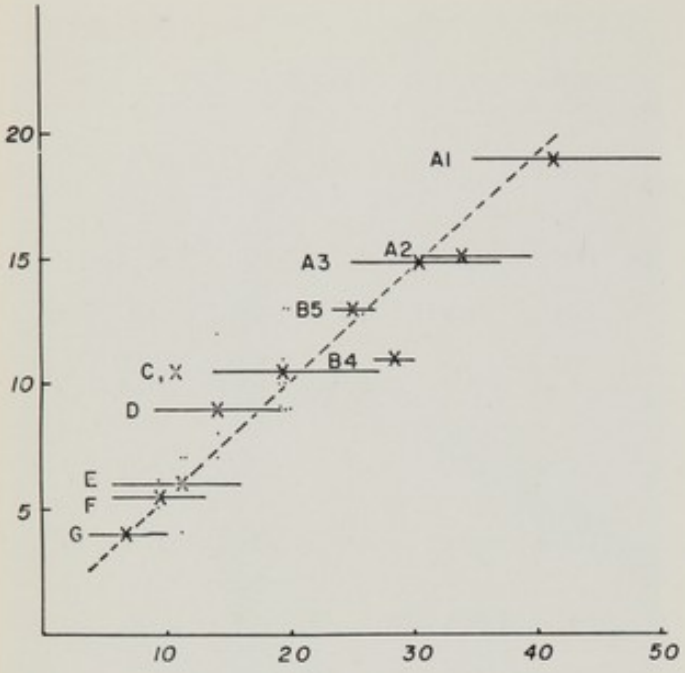


Fig. 15. *Abscissa:* $\times 10^{-13}$ g, dry mass; *ordinate:* number of major chromosomes.

In this graph mean dry mass of a karyotype group is plotted against the number of major chromomeres counted in scans of electron micrographs of human chromosomes. Horizontal bars indicate maximal and minimal mass values for each group (from Bahr & Golomb [14]). Points in horizontal direction give chromomere numbers encountered in groups C, D, E and F.

In the one type of Giemsa preparation (24 XY) scanned, 134 bands were clearly identified. Dr Schnedl reported to this Symposium that 82 major bands and about 250 minor bands have been counted by him for the human haploid karyotype.

For the distribution of chromatin mass (24 XY) we have found 225 bands that we consider major (EM) and 453 minor (em). Although the ratio of major to minor bands is an overall 1:2, there is no absolute correspondence to this ratio for individual major bands (table 1). It appears that, on the average, two EM bands contribute to one Q band, while the ratio of Q to G bands is 1:1.07 in our assessment.

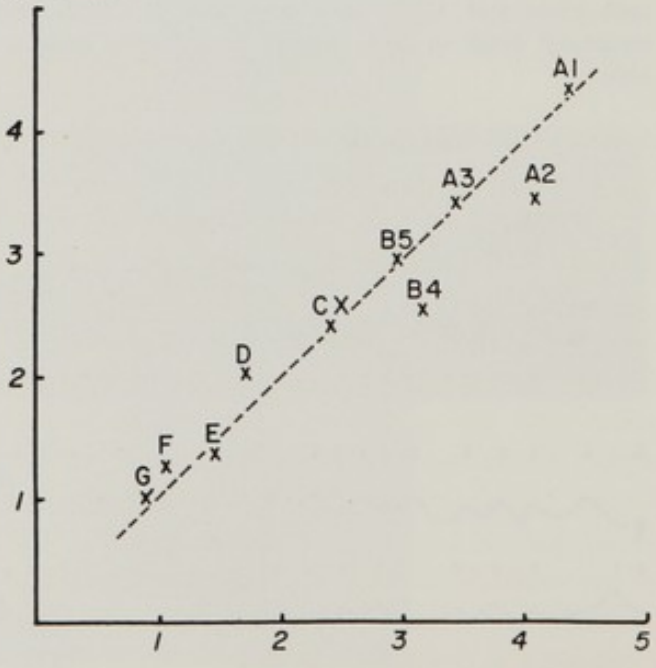
In order to answer the question of how evenly these bands are distributed throughout the human karyotype, we compared total chromosomal mass to the number of chromomeres per chromatid (fig. 15). It is evident that major EM bands are quite evenly distributed and that their number is generally proportional to the total mass of the chromosome, with an intersect close to the origin, which is to say that the mass (not number) of longitudinal fibers varies in proportion to the number of chromomeres. Since there is a 1:2 ratio of major to minor chromomeres, it follows that these considerations are applicable also to minor chromomeres. In other words, the smallest type of chromomere occurs at fairly regular intervals throughout the karyotype; e.g., for a 200 μm karyotype this would be 0.2 μm for the average width of a major and 0.05 μm for the width of an average minor band, assuming interband spaces of comparable size.

Also, a comparison of the percentage of major bands for a given chromosome or class with the percentage contribution to the total length of the karyotype known from light microscopy shows good proportionality and affirms the impression of a relatively even distribution of major (and implicitly minor) bands (fig. 16).

It is gratifying for us to note that principles of chromosome identification pioneered by light microscopists can now assist us in identifying individual chromosomes under the electron microscope also, whereas previously only the total mass and the mass ratio of short to long arm were available, which was really no better than classical cytogenetic techniques [7]. Group assignment through mass determination can now serve the same presorting purpose as grouping does for banded chromosomes under the light microscope (p. 315).

Fig. 16. *Abscissa:* % of length of karyotype (46 XY); *ordinate:* % of total chromosomes.

A good correlation is seen between the number of major and minor chromomeres per chromosome group with the percentage contribution to the total length of the karyotype is found.



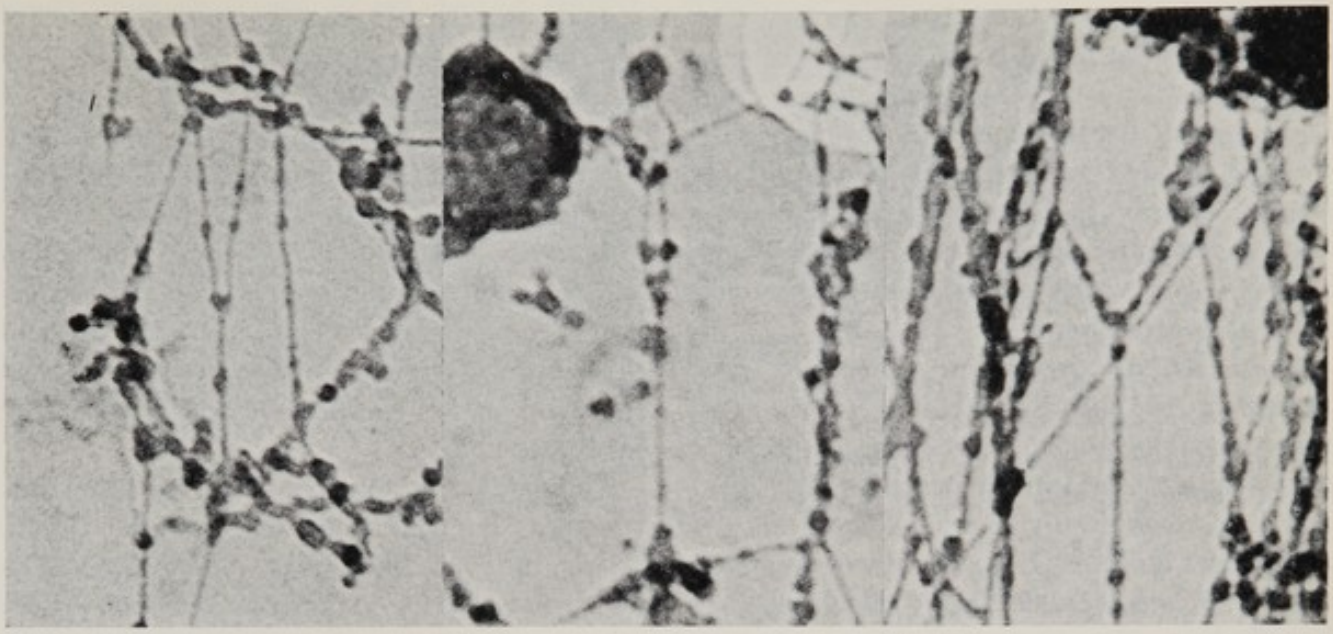


Fig. 17 Fig. 18 Fig. 19

Figs 17, 18, 19. Replication forks in nucleus of rapidly growing cell. These are enlargements from survey micrographs required in searching for good examples of forks. The diameter of the apparently smooth fiber part near the fork is close to 35 Å. Replication forks are characterized by a large mass at the fork proper. Daughter fibers emerge from this mass at 40–50 Å distance. There are lumps on the fibers before and after replication. The location of lumps on daughter fibers at closely comparable distances from the fork is the chief criterion in recognizing replication. Fibers retain not only association with non-DNA components of chromatin in a ratio of 2:5, but whatever special conformation or components occur along them are precisely reproduced in daughter fibers. × 180 000.

Discussion

There remains to be discussed the relationship of banding patterns seen under the electron microscope to those made visible by various methods for light microscopy.

It is clear from the presentations at this Symposium that molecular composition along a chromatid varies in demonstrable quantities. Not only the earlier findings by Hsu & Arrighi of C-band material but the elucidation of enhancement and quenching processes of quinacrine mustard and quinacrine by Weisblum [8] and by Rigler (p. 43); and—most convincingly—the localization of nucleotide antibodies by O. J. Miller (p. 43) indicate strongly that major chromosomal molecules are discontinuously distributed. These findings are underlined by experiments with acridine orange and other fluorescent compounds with known affinity for DNA.

Our findings of a distinctly discontinuous distribution of chromosomal matter along chromatids suggest a basis for all banding observations. Accordingly chemical differentiations are superimposed on this basic pattern. The very fact that chromatin fibers occur both as fairly straight structures and as precisely located looping portions presupposes chemical differences. A distribution of AT/GC that neither enhances nor quenches GC-bound quinacrine would thus render existing bands fluorescent in prophasic chromosomes but would not yield the strong fluorescence of 3, Y and other chromosomes.

Quinacrine mustard-stained chromosomes, mounted on object carriers for electron microscopy but viewed first in a fluorescence microscope with glycerin as a mounting medium, exhibit fluorescence with precisely the same chromosomal detail as under subsequent electron microscopy. Precise analysis of the contributions of mass and chemical composition respectively, to intensity of fluorescence is difficult but possible and will be carried out.

We have determined the number of longitudinal chromatin fibers to be about 15, according to principles described in [2]. Regardless of whether or not the overall thickness of chromosomal fibers has been increased by adsorbed proteins, this relative approach renders the correct number of fibers in a cross-sectional scan. Comparing total chromosomal mass with the mass of longitudinal

fibers, again on a relative basis, we find 25% of the total mass in the latter, i.e., 75% of chromosomal structure is in chromomeres. With knowledge of the length of each particular chromosome studied and its DNA content (DNA length), it is easy to calculate that a chromosome like the one shown in fig. 5 has about 75 lengths of DNA per length of chromatin fiber, most likely arranged in super coils [1, 2, 3].

Since Miller's experiments indicate a good agreement of adenosine-rich chromatin bands with quinacrine bands, we suggest that electron microscopically identifiable bands are rich in AT and that consequently interband areas are GC rich. Could it be that GC prevalence is a characteristic of longitudinal fibers? This is open to investigation. Thus it is likely that underlying differences in DNA composition prompt differences in the composition of the four to five parts of chromosomal proteins associated with each part of DNA. This conclusion agrees with Coming's observations (p. 293) that DNA-histone interactions play a major role in banding.

A further variable, then, is the degree of AT or GC enrichment producing a gradient of banding options from the multitude of Giemsa bands of Dr Schnedl to C-bands [5].

The facts that eukaryotic DNA has never been shown to occur as the largely naked double helix of prokaryotic cells (or of test tubes) and that resolvable differentiations of structure have already been shown to occur at a level of one order of supercoiling of chromatin attests both to the significance and ubiquity of chromosomal proteins in the coiling and folding of DNA. Figs 17, 18 and 19 show replication forks in fibers in which DNA is associated with protein and possibly supercoiled.

From this view it appears understandable that commonly employed preparatory steps such as acetic acid-alcohol treatment, or heating in air or in liquid media produce rearrangement of chromomeres in the direction of prevalent fiber connections through various degrees of denaturation. Such effects can already be observed on ordinary unstained chromosome spreads mounted in ethylene dichloride (for optimal difference of refractive index between object and medium) and viewed with high-contrast phase optics. Major chromosome bands become visible. Also, the electron microscope shows this differentiating effect.

It can now be said that, from findings on chromosomes analyzed so far, a detailed description of chromomere positions at the 0.2 to 0.05 μm level is possible and that characteristic features can be extracted for computer-assisted identification by principles of pattern recognition.

It seems likely that such detailed description will be useful in the mapping of the human genome, in defining new cytogenetic markers and in identifying very small marker chromosomes in cancer as well as chromosomal fragments or microchromosomes.

Comparable chromomere patterns have been observed, but not fully analysed, for *Gorilla gorilla*, *Muntjak muntjakus*, *Bos taurus*. It is likely that electron microscopy can follow light microscopy and throw light on chromomere patterns of many other species, with the possible option also of tracing the phylogeny of parts of chromosomal fine structure, as suggested by the work of de Grouchy (p. 124).

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Discussion

Rigler: Dr Bahr pointed out that possibly part of the banding pattern in chromosomes might already be caused by the heterogeneous mass distribution in chromosomes that he is able to show by quantitative electron microscopy. Having the picture of the QM-bands in mind we certainly have to be careful to equalize every QM-band with AT-rich regions. In principle, higher fluorescence intensity of QM giving rise to

bands could be caused by (*a*) AT-rich regions leading to increased quantum yield; (*b*) increased amount of DNA binding stoichiometric amounts of QM; (*c*) changed protein–DNA interaction allowing more QM to be bound to DNA. This third possibility can in principle be distinguished quite easily by measuring how dye and DNA are distributed along the chromosome. In those instances where this has been done (chromosomes of *Vicia faba*) the distribution of proflavine (giving the same banding as QM) follows closely the DNA distribution. In the QM-band regions, however, the amount of dye is not increased, which means that in this material the dye is really fluorescing with a higher quantum yield than in AT-rich DNA in in vitro experiments.

Comings: I would like to play the devil's advocate for a minute. Two things bother me. (1) The normal coiling of the chromosome may play a role and produce pseudobands. (2) In the matching of Q-band curves and EM curves a certain amount of selection might be involved. To control this you should also try to match the EM curves to the curves from the R-band techniques.

Bahr: If you refer to the type of coiling revealed by the technique of Onuki, in which the chromatids are zig-zagging, but are called coil, our slit scanning of Onuki's micrographs with a slit wider than the width of a chromatid did not render anything comparable to Q-, G- or EM-banding. As a matter of fact the curves were rather flat. Yes, there is selection involved in matching EM-bands to Q- or G-bands. First we categorize the group as Dr Lubs suggested, by mass determination in our case. Then we proceed as light microscopists do and narrow down the choice within each group guided by banding features. The procedures are quite comparable as far as major bands are concerned. When I talk about minor bands, that is terra nova.

Miller: The electron micrographs shown by Dr Bahr and Dr Comings suggest that the banding patterns produced after various treatments, e.g., NaOH or trypsin, are due to distortion following disruption of the structural integrity of the chromosomes, perhaps specific parts of them, and this is just what one would expect if there were a deficiency of the divalent cations, Ca^{2+} and Mg^{2+}. The importance of these ions in maintaining chromosome structure is well known. We have several lines of evidence indicating that they do play an important role in Giemsa banding. (1) EDTA pretreatment will produce G-banding, but not in the presence of excess Ca^{2+}; (2) trypsin itself may produce G-banding in part by acting as a divalent cation binder. It works mainly through its proteolytic action, however. My associate Dr Warburton has shown that the extent of G-banding that is produced in the presence of trypsin inhibitors is proportional to the remaining proteolytic activity of the trypsin.

Bahr: We deal with two kinds of "banding", namely, firstly, that which is due to the principal organization of chromosomal matter; and secondly, with the effects of various methods influencing and rearranging this basic organization. I fully agree with Dr Miller as to the significance of the divalent ions in maintaining structure or, conversely, giving rise to material rearrangements when lost by chelation.

Sequential Denaturation and Reassociation of Chromosomal DNA in Situ

A. de la Chapelle, J. Schröder and R.-K. Selander

Folkhälsan Institute of Genetics, 00101 Helsingfors 10, Finland

Summary

Acridine orange (AO) fluorescence was used to determine the strandedness of chromosomal DNA in situ. The DNA in the secondary constrictions in human chromosomes 1, 9 and 16 denatures at lower NaOH concentrations, and the DNA in the distal half of the Y chromosome at higher concentrations of NaOH, than is the case in other regions. Chromosomes treated with alkali dissolved in salt solution instead of water have a banded appearance, with green and red regions alternating. Alkali-denatured chromosomal DNA quickly reassociates after incubation in a salt solution. After prolonged incubation, many regions (e.g. all mouse, *Microtus agrestis* and human centromeres) fluoresce more brightly than the rest of the chromosomes with AO and quinacrine mustard (QM). Tentative interpretations are: AT-rich DNA denatures easily. The DNA of the human Y chromosome may be both very AT-rich and highly repetitious, hence it reassociates so rapidly that it appears not to denature. In situ DNA reassociates extremely rapidly due to high local concentrations. All centromeric regions in man and *Microtus agrestis* contain repetitious DNA which fluoresces brightly with AO and QM after denaturation-reassociation.

The fluorochrome acridine orange (AO) is useful in the study of chromosomal DNA in situ. When slides are inspected in ultraviolet light after staining with AO, the fluorescence is green with double-stranded DNA and red with single-stranded DNA and RNA [2, 9]. We have used this property in an attempt to determine under what experimental conditions the DNA of mitotic mammalian chromosomes is denatured and reassociated. Some of the results reported here have been published previously [2, 3].

Denaturation

The colour of the AO fluorescence of mitotic chromosomes is consistently green or yellow-green on slides prepared by air drying. This presumably signifies that the DNA is double-stranded. The chromosomal DNA remains undenatured even after treatment with RNase (20 μg/ml for 30 min at room temperature) and with acids (e.g. 1 N HCl for 30 min at room temperature). However, heating of the slide during drying leads to a change in the colour of the fluorescence from green to red, indicating denaturation of chromosomal DNA. The extent of the denaturation depends on the temperature and the other conditions during treatment. Moreover, even on the same slides there are considerable variations in the extent of denaturation between cells or groups of cells. When heated with a flame from below, it proved impossible to standardize the procedure so as to produce slides with either undenatured or completely denatured chromosomal DNA. Hence, we have consistently used the following air-drying method which yields chromosomes with green or yellow-green fluorescence: A drop of cell suspension is pipetted on to a clean, dry slide and allowed to dry at room temperature or at a maximum temperature of 40°C.

Denaturation is achieved by immersing the slides for 2 min in sodium hydroxide before staining with AO. Some distortion of the morphology of the chromosomes is inevitable, but the effect is minimized if the sodium hydroxide is dissolved in physiologic NaCl rather than in water.

Increasing concentrations of alkali produce an increasing degree of denaturation, as is indicated by the shift from green to yellow, orange, and finally red fluorescence with AO. Denaturation is uniform along the chromosomes in mouse and *Microtus agrestis* cells when NaOH in water is used. Under similar conditions human chromo-

somes show a sequence of denaturation whereby the secondary constrictions of chromosomes 1, 9 and 16 are the first, and the distal half of the Y chromosome is the last to become denatured when the concentration of NaOH is raised. Details of these findings, indicating the influence of the pretreatment of the cells and the concentrations of NaOH, have appeared elsewhere [3].

Denaturation of human chromosomal DNA with NaOH in physiologic NaCl produces a somewhat different picture. With lower concentrations of NaOH, such as 0.005–0.07 M, not only the secondary constrictions in chromosomes 1, 9 and 16, but also a number of other centromeric regions are the first to become red. In addition, the chromosomes often have a banded appearance, with reddish and greenish bands alternating along the chromosomes. Regions with bright fluorescence after quinacrine mustard (QM) staining of untreated cells are mostly red, whereas the areas with dull QM fluorescence are green. With high concentrations of NaOH, such as 0.5 M, all chromosome regions become red.

Reassociation

The colour of the AO fluorescence of the chromosomes can be reversed from red to green by immersing NaOH-treated slides in 0.9 M NaCl, 0.09 M tri-sodium citrate, pH 7.0 at 67°C. This presumably signifies reassociation of denatured chromosomal DNA. The reversal is rapid and uniform. All regions of mouse, *Microtus agrestis*, and human chromosomes, become green within 30 sec of incubation. When incubation is prolonged, the green fluorescence with AO becomes brighter in some areas than in others. After 16 h of incubation, the following chromosomal regions have distinctly brighter fluorescence than the others: in the mouse, all centromeric regions; in *Microtus agrestis*, all autosomal centromeres plus the constitutive heterochromatin in the X and Y chromosomes; in man, all centromeres, the secondary constrictions in chromosomes 1, 9 and 16, all the autosomal regions displaying polymorphic bright fluorescence with QM, and the distal half of the Y chromosome.

The chromosomal regions listed above displaying bright fluorescence with AO after denaturation-reassociation also show bright fluorescence with QM after the procedure. Microphotographs showing these findings have been published [2, 3].

It is worth pointing out that the overall chromosomal fluorescence with AO and QM is much weaker after treatment with alkali and salt than before. The decrease in fluorescence intensity is so marked that photography of the cells is occasionally quite difficult after the treatment.

Discussion

The secondary constrictions in human chromosomes 1, 9 and 16 contain repetitious satellite DNA [6] which is rich in AT [4]. They may be the first regions to denature because of their high AT-content [5]. At present, there is little evidence to suggest that repetitious DNA as such denatures more easily than non-repetitious DNA. Different classes of repetitious DNA fractionated by their Cot values display considerable heterogeneity of their heat denaturation properties [10].

In our system the distal half of the Y chromosome is the last human chromosomal region whose DNA is denatured when the concentration of alkali is raised. Weisblum & de Haseth [11] and Pachmann & Rigler [7] have shown that bright QM fluorescence is associated with a high AT-content of the DNA. Hence the Y chromosome is presumably extremely rich in AT. If this is so, rapid rather than slow denaturation should be expected. There are at least two possible explanations for this apparent discrepancy. First, the DNA in the Y chromosome may indeed be easily denatured, but it reassociates so rapidly (for instance in the buffer solution used for AO staining) that denaturation cannot be detected. The fact that it appears denatured after treatment with very strong alkali might be due to the disruption or alteration of chromosomal components other than DNA, such as acid proteins, with resulting impairment of the reassociation of the DNA. This theory implies that the Y chromosome contains DNA whose degree of repetition is greater than any other known human DNA fraction. Secondly, the exceptionally bright fluorescence of the Y chromosome might be due to a factor other than richness in AT, such as a special protein or unusual DNA-protein binding. In this case, the relative resistance to denaturation of the DNA in the Y chromosome could be more easily explained by another mechanism.

Numerous chromosomal regions show bright fluorescence with both AO and QM after denaturation-reassociation treatment. Many of these are already known to contain repetitious DNA, notably the mouse centromeres [8], the constitutively heterochromatic regions in the X and Y of *Microtus agrestis* [1], and the secondary constrictions in human chromosomes 1, 9 and 16 [6]. It is proposed that the other regions shown by us to have strong fluorescence, e.g. all autosomal centromeres in *Microtus agrestis* and man, also contain repetitious DNA. The molecular mechanism leading to the bright fluorescence in these regions after denaturation-reassociation is not known at present. One possibility is that more DNA becomes extracted from other regions than from those containing repetitious DNA.

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Discussion

de la Chapelle: (Response to Dr Rigler.) It has been shown by Corneo & Jones and others that the centromeric regions or paracentromeric constrictions in chromosomes 1, 9 and 16 do contain satellite DNA which is highly repetitious. Incidentally, it is also rich in AT. The reason why we suggest that repeated DNA denatures easily is not backed by chemical experiments but by our own cytological results.

Ockey: The mouse has high AT regions round the centromeres which stain red. In *Microtus*, however, the repetitive DNA lies under the main peak of DNA in $CsCl_2$ gradients and is not particularly AT-rich, but still stains red. In man the satellites are probably fairly high in AT. I would agree with Dr de la Chapelle that it seems questionable whether high AT is the main reason for the red staining of those regions with acridine orange.

Comings: In my presentation I will show that the important factor is selective removal by NaOH of the non-C-band material. Thus the C-band regions stain well with quinacrine and acridine orange because there is more DNA there.

Comings: One thing should be emphasized about the acridine orange staining. Dr Lubs and I were able to obtain differential staining after heat and alkali denaturation. However, Bobrow has apparently been able to obtain similar results after treatment with trypsin alone, which would not denature the DNA. This would favor the idea that the dye is intercalating.

Biochemical Mechanisms of Chromosome Banding and Color Banding with Acridine Orange

D. E. Comings

Department of Medical Genetics, City of Hope National Medical Center, Duarte, Calif. 91010, USA

Summary

Constitutive heterochromatin can be divided into a centromeric type which occurs in C-bands and an intercalary type which occurs in the G-bands. They both show condensation during interphase, decreased RNA synthesis, late DNA replication, and association with the nuclear membrane. The centromeric type is frequently but not always enriched in highly repetitious satellite DNA, which may be AT-rich, GC-rich or neutral in base composition. DNA intercalary heterochromatin is AT-rich, non-repetitive, and poorly methylated DNA. SDS-gel electrophoresis indicates there are some non-histone proteins present in constitutive heterochromatin which are not present in euchromatin.

Various experimental observations suggest that protein-DNA interactions are important in all of the banding procedures. For Q-banding the base composition (AT-richness) appears to play a major role but this is modified in some regions by proteins. In C-banding the selective extraction of non-band DNA plays a major role but the intensity of staining by Giemsa is enhanced by DNA-protein interactions and the DNA in the C-bands is presumably resistant to extraction because of the proteins associated with it. Differential renaturation of repetitious DNA is not involved. In G-bands, base composition may be involved but DNA-protein interactions seem to play the major role. The same is true for R-banding.

The possible variables involved in these DNA-protein interactions in terms of qualitative or quantitative differences in non-histone proteins, variations and tightness of binding to DNA, and occurrence of sulphydryl and phosphorus groups are discussed.

A number of different techniques have recently become available for the production of chromosome banding. Since the C- and G-bands generally correlate well with regions of late replicating heterochromatin, a knowledge of the biochemical differences between heterochromatin and euchromatin is essential to an understanding of how the banding techniques may work. Up until several years ago heterochromatin was defined primarily as chromatin which is condensed throughout interphase [31], genetically inactive [45], and late replicating [40]. If it occurs on homologous portions of homologous chromosomes it is termed constitutive heterochromatin [10]. In recent years additional biochemical features that help to distinguish euchromatin from heterochromatin have been delineated, and it has become clear that there are at least two general types of constitutive heterochromatin.

Centromeric versus Intercalary Heterochromatin

Constitutive heterochromatin can be divided into centromeric and intercalary heterochromatin [14, 16]. Some of the biochemical characteristics of the two types are listed in table 1. A major clue that there must be at least two types comes from comparison of patterns of late DNA replication in human chromosomes [44] with banding patterns by the C-band technique [3]. Autoradiography shows late replicating DNA in the arms of many chromosomes, while the heterochromatin detected by C-banding is primarily at the centromeric regions. However, the heterochromatin that is missed by the C-banding can be detected by Q- and G-banding which show a good correlation with late replicating regions in the arms [30]. Studies in other species [2, 49] show that C-band material is also late replicating. This allows us to distinguish two general types of constitutive heterochromatin, the centromeric type which is detected by the C-band technique and usually, but not always, occurs at the centro-

Table 1. *Comparison of centromeric and intercalary heterochromatin*

	Centromeric heterochromatin	Intercalary heterochromatin
Occurrence	In C-bands	In most Q- and G-bands
Location	Usually near centromeres	In chromosome arms
Condensation during interphase	+	+
Decreased RNA synthesis	+	+
Late DNA replication	+	+
Condensed onto nuclear membrane or nucleolus	+	+
Synchronized replicons	+	Probably
Satellite DNA	Frequently present	−
AT-rich DNA	±	+
Repetitious DNA	+ and −	Probably no more repetitious than euchromatin
DNA methylation	Variable	Relatively decreased
Proteins	Probably contain some specific non-histone proteins	Probably contain some specific non-histone proteins

meric regions, and intercalary heterochromatin which occurs in the arms. EM autoradiography using ^{3}H-thymidine [21] indicates that both types are condensed during interphase and occur in intimate association with the inner surface of the nuclear membrane or around the nucleolus [20, 24, 43].

Light and EM autoradiography with ^{3}H-uridine show this condensed chromatin is deficient in RNA synthesis [33, 40, 50] and relatively genetically inactive. The use of quantitative autoradiography indicates that the rate of chromosome replication in the centromeric type of heterochromatin [13] and in facultative heterochromatin [12] is greater than in euchromatin. While the possibility of an increased rate of chain growth has not been completely ruled out, this apparent increase in rate is probably due to a synchronization of the individual replicons [13]. This is presumably the case for intercalary heterochromatin as well although this has yet to be demonstrated.

Whole mount [21, 22, 28, 38] and thin section electron microscopy [24] show that interphase chromatin is attached to the nuclear membrane at many sites. Several chromatin fibers appear to come together at these sites of membrane attachment [21]. Well spread preparations frequently show the retention of these regions of converging fibers even in metaphase chromosomes. Since late replicating but not early replicating chromatin is nuclear membrane associated [24, 32, 53] it is possible that the density of the nuclear membrane attachment sites may be greater in heterochromatin than in euchromatin [16]. This could form the basis for the chromomere-like regions that can be seen in whole mount electron micrographs of metaphase chromosomes (p. 280) [18]. Thus, some of the features which these two types of heterochromatin have in common, and which distinguish them from euchromatin, include (*a*) generally positive staining with the different banding techniques; (*b*) condensation throughout interphase; (*c*) decreased RNA synthesis; (*d*) late DNA replication; (*e*) synchronization of replicons; (*f*) attachment to the nuclear membrane and the periphery of nucleolus; (*g*) probably distinct patterns of chromatin fiber folding which may be dictated by a different density of nuclear membrane attachment sites, or by specific proteins.

DNA of Heterochromatin

One of the more important differences between these two types of heterochromatin is the type of DNA they contain. Utilizing in situ hybridization Pardue & Gall [46] demonstrated that highly repetitious satellite DNA was localized to the centromeric heterochromatin in the mouse. Similar studies in other species also show satellite DNA to be localized to the C-band regions [2, 9, 34, 48]. This can also be demonstrated by studies of the DNA extracted from heterochromatin isolated from disrupted nuclei [20, 42, 55].

The emphasis on the presence of highly repetitious DNA in C-band heterochromatin makes it imperative to point out that a significant amount

of non-repetitious DNA is also present in these regions. For example, in the Chinese hamster, even though there are negligible amounts of satellite DNA [20], about 15–20% of the genome stains with the C-band technique. In vitro renaturation shows that heterochromatic DNA in this species has approximately the same content of repetitious DNA as the euchromatic DNA [19, 20], and in situ hybridization, with RNA transcribed from repetitious Chinese hamster DNA does not hybridize to the large amount of centromeric type heterochromatin on the X and Y chromosomes (Hsu. Personal communication), although it does hybridize to the centromeric heterochromatin of many of the autosomes. Also, in the mouse, 50% of the DNA of isolated heterochromatin, containing predominantly the centromeric-type heterochromatin, is AT-rich non-repetitious DNA [20, 43]. The existence of C-bands which do not contain highly repetitious DNA suggests differential renaturation of the DNA is not the basis for C-banding, a conclusion which is verified with acridine orange studies described later.

The intercalary heterochromatin is enriched in AT-rich, non-repetitious DNA. The evidence for this is as follows.

(1) In all the mammals in which it has been examined early replicating DNA is GC-rich and late replicating DNA is AT-rich [6, 7, 8, 15, 51]. Since in the Chinese hamster this AT-rich DNA is non-satellite DNA, and is no more repetitious than early replicating DNA [19], and constitutes over 20% of the total nuclear DNA, it must belong predominantly to the intercalary heterochromatin.

(2) Independent evidence for the AT-richness of the DNA of intercalary heterochromatin comes from studies of Miller and co-workers (p. 43), using base specific antinucleoside antibodies which have shown that the Q- and G-staining bands are relatively AT-rich.

Cesium chloride ultracentrifugation of ^{14}C-methylmethionine labeled Chinese hamster DNA also suggests that the late replicating DNA is less methylated than early replicating DNA [18]. This is substantiated by analysis of absolute amounts of methylcytosine using thin layer chromatography [11]. The same is also true for late replicating mouse DNA [1]. These two features of AT-richness and decreased amounts of methylcytosine may be directly related to each other in that removal of the amino group from methylcytosine would convert it to thymidine. Thus, if a segment of euchromatin were genetically inactivated and removed from selective pressures, methylcytosine-to-thymidine mutations could occur. This would slowly change well methylated, GC-rich DNA to undermethylated AT-rich DNA [17].

In summary, there is good evidence for two general types of constitutive heterochromatin, a centromeric type occurring in the C-bands and an intercalary type occurring in the Q- and G-bands. Further biochemical features and subclassifications are discussed in detail elsewhere [17].

Mechanisms of Chromosome Banding

With these characteristics in mind we can ask which of these features, if any, is responsible for chromosome banding. A series of biochemical and ultrastructural experiments examining these factors has been reported elsewhere [18] and only the conclusions will be outlined here.

(1) Chinese hamster cells were labeled with ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine and labeled metaphase cells isolated by mitotic selection, fixed, and air-dried on slides. These slides were then exposed to the various treatments involved in C- and G-banding and the amount of DNA, RNA or protein extracted was determined. These studies indicated that (*a*) 0.07 N NaOH for 30 to 180 sec removes 16–81% of the DNA from the chromosomes. (*b*) 6×SSC at 65°C overnight also removes about 30% of the chromosomal DNA. (*c*) On the average the complete C-band technique removes 60% of the chromosomal DNA. (*d*) All of the G-band techniques (with the exception of that of Schnedl which uses sodium hydroxide) remove less than 5% of the chromosomal DNA. (*e*) All treatments which involved exposure of chromosomes to sodium hydroxide removed large amounts of RNA. (*f*) The trypsin treatments for G-banding remove little ^{3}H-leucine labeled protein suggesting that if these proteins are involved in the banding it is due to an alteration of the proteins more than to their extraction.

(2) Since the C-band techniques remove large amounts of chromosomal DNA the banding could be largely explained if the DNA was pre-

ferentially removed from the non-band regions. Several different experiments, including extraction of isolated labeled and fixed heterochromatin, Feulgen staining and microdensitometry of C-banded mouse chromosomes, analytical ultracentrifugation of extracted and non-extracted mouse chromosomal DNA, and examination of treated chromosomes by whole mount electron-microscopy, indicated this was the case. Prolonged treatment with trypsin also causes preferential dispersion of non C-band regions.

(3) Differential extraction of non-band DNA does not completely explain the C-banding since far greater differential staining is obtained with Giemsa than with Feulgen. The enhanced effect with Giemsa suggests DNA-protein interactions are also playing an important role. It is probably the presence of certain C-band specific proteins which are responsible for the resistance of this DNA to sodium hydroxide and prolonged trypsin treatment, and the enhanced staining with Giemsa.

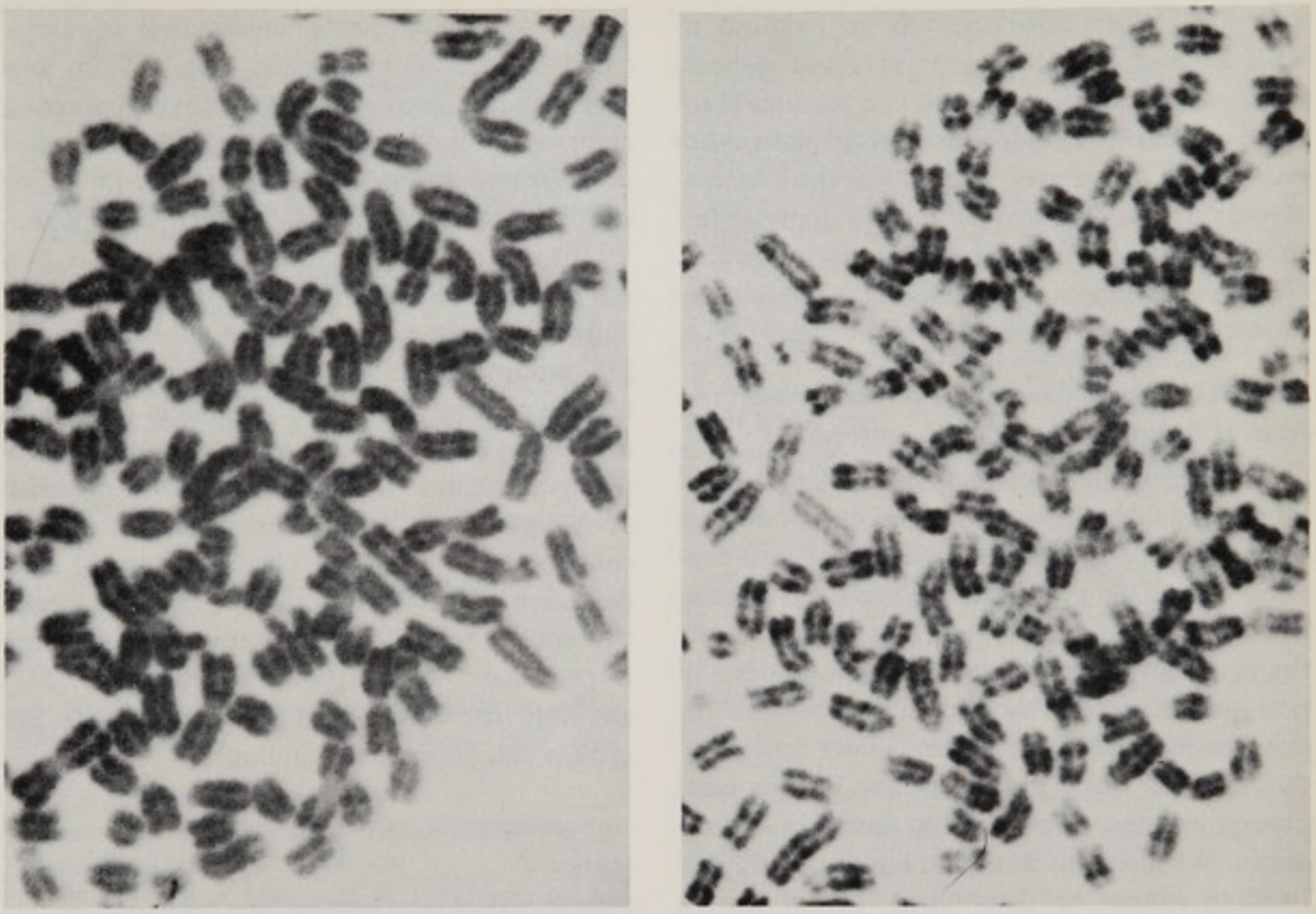
(4) Microdensitometry tracings of Feulgen stained untreated chromosomes show no significant variations in the concentration of DNA along the length of the chromatids. When the chromosomes are treated with trypsin and stained with Feulgen, densitometry tracings still show no variation in the amount of DNA. However, when stained with Giemsa, the bands by visual inspection and densitometry are very striking. This indicates that in contrast to C-banding, the G-banding is not due to the production of significant variations in DNA content along the chromosomes. This agrees well with the radioisotope studies indicating that most of the G-band techniques extract very little DNA from the chromosomes. This also indicates that the Giemsa stain itself is playing an important role in producing or markedly enhancing the bands.

(5) To examine the role of DNA denaturation and renaturation, mouse chromosomes fixed on slides were treated in various ways and then stained with acridine orange to determine whether DNA in the satellite-rich C-band regions or in the arms contains single or double stranded DNA. They were also stained with Giemsa to determine whether C- or G-banding had been produced. These experiments indicated the following: (*a*) When chromosomes are fixed in cold, methanol-acetic acid, air-dried, and the RNA removed with RNase, the chromosomes are all green indicating they contain double stranded DNA. (*b*) When these chromosomes are treated with alkali or heat, and formaldehyde added *before* removing the slides from denaturing conditions, all the chromosomal DNA is red or single stranded. (*c*) If the slides are removed from denaturing conditions (simply cooled or placed in $2\times$SSC at 60°C) for even a few seconds and *then* fixed in formaldehyde, the repetitious centromeric DNA has renatured (green or orange) while the non-repetitious DNA in the arms is still red. (*d*) If the chromosomes are removed from denaturing conditions and placed in $2\times$SSC at 60°C for 3–5 min, both the repetitious centromeric DNA and the non-repetitious DNA in the arms is now renatured. (*e*) 2 or $6\times$SSC at 60°C for overnight actually denatures rather than renatures chromosomal DNA. (*f*) Good C-banding can be obtained with both the centromere and arm DNA denatured, with both native or renatured, with centromeric DNA renatured and arm DNA denatured, or with centromeric DNA denatured and arm DNA native. (*g*) G-banding can be produced without denaturing any chromosomal DNA. These studies indicate that the denaturation and preferential renaturation of repetitious DNA plays no role in the production of either C- or G-banding.

Color Banding with Acridine Orange

During these studies with acridine orange we noted that mouse chromosomes that were placed in 0.07 N NaOH containing 4% formaldehyde, at room temperature, for 2 min, then washed in 95% ethanol and stained with acridine orange [18], showed bright red centromeres and red or orange-red banding in the arms (fig. 1). Similar treatment of human chromosomes also showed banding patterns in which the red bands correlated with the G-bands [55]. Lubs (p. 315) has been able to produce similar acridine orange color banding by heat denaturation of human chromosomes.

The most likely explanation for the acridine orange results is that the red bands represent AT-rich regions (mouse centromeres and G-band DNA) and the orange regions represent GC-rich DNA. Bobrow [5] has reported that human chromosomes treated with trypsin and then



a *b*

Fig. 1. Acridine orange banding of mouse L cell chromosomes. (*a*) Printed through a green filter; (*b*) through a red filter. (See Castleman, p. 77, for further details on the processing of the photographs.)

stained with acridine orange may also show color banding. We have found, however, that it is possible to get color banding simply by allowing the slides to age on the shelf. Thus, if fresh slides are stained with acridine orange the chromosomes are green. With old slides they are frequently red, and with slides only a couple of weeks old red-green color banding can be produced without the use of either NaOH or trypsin [54]. Presumably the DNA of acid fixed, air dried chromosomes undergoes slow denaturation with time.

Q-banding

Studies of Weisblum & deHaseth [52] have shown that with increasing concentrations of poly-G there is progressive quenching of the fluorescence due to quinacrine. This is not true for poly-dAT. This, plus the bright green fluorescence of an AT-rich chromosomal region in *Samoaia leonensis* [29] indicate that much of the effect of quinacrine banding is the result of binding to AT-rich regions. This, of course, agrees with the demonstration by nucleoside antibodies that these bands are AT-rich.

The Role of protein-DNA Interactions

There are many different observations which emphasize the role of protein or protein-DNA interactions in chromosome banding. Some have been mentioned above and others are reviewed in more detail elsewhere [16]. Several questions can be asked about the nature of these interactions.

Are the proteins involved histones or non-histones?

Perhaps the major piece of evidence that non-histones are most important is the fact that Q-banding [14] and G-banding [20] are relatively unaffected by the prior removal of histones with 0.2 N HCl. This does not entirely rule out the role of histones for the following reasons: (*a*) Small amounts of arginine-rich histones may not be completely extracted. (*b*) Condensed metaphase chromatin shows RS–SR linkages in his-

tone III, while these tend to be oxidized to RSH in interphase chromatin [47] and reagents which affect sulphydryl groups can produce chromosome banding (p. 346). (*c*) SDS-gel electrophoresis of the proteins of isolated and fixed heterochromatin and euchromatin show some differences in the content of lysine-rich histones [26]. However, until proven otherwise, the non-histone proteins seem the more likely candidate.

Are there any differerences in non-histone proteins of heterochromatin and euchromatin?

To investigate this, Comings & Tack [26] isolated mouse and Chinese hamster heterochromatin and euchromatin and examined the non-histone proteins by SDS gel electrophoresis. The non-histone protein-to-DNA ratio was approximately the same for both fractions and the pattern of non-histone proteins showed many more similarities than differences. However, under some conditions there was a prominent non-histone band present in heterochromatin but absent in euchromatin. Whether this has anything to do with the banding cannot be determined, but at least a distinct difference can be demonstrated. Proteins that bind to specific sequences and then bind to each other [25] could be involved.

Is the dye interacting with protein alone or is it responding to protein-DNA?

The intercalating properties of metachromatic dyes would seem to favor the possibility that the dye may be intercalating between the DNA and protein. To determine if the Giemsa can interact with proteins alone, Chinese hamster liver cytoplasmic proteins were electrophoresed in urea-acetic acid and stained with either Coomassie blue or Giemsa [26]. Some bands showed some preferential Giemsa staining indicating a primary interaction with the protein is at least possible. Studies to see if there are specific Giemsa staining proteins in heterochromatin are in progress.

Are variations in the tightness of binding of protein to DNA involved?

In several respects this is an attractive hypothesis. The presence in the C-band regions of a protein that is bound tightly to DNA could explain the decreased quinacrine fluorescence of the AT-rich mouse C-bands, and the resistance of the C-bands to NaOH and trypsin. For G-bands, alterations in the tightness of binding to DNA could be induced by trypsin, heat, sodium ions [35] chelating agents [27] or sulphydryl reacting compounds (p. 346), thus allowing either increased or decreased access of Giemsa to the DNA, or to the space between the DNA and the protein.

Are variations in SH groups involved?

Sadgopal & Bonner [47] have shown that the SH groups in histone III and in some non-histone proteins occur as SS bonds in condensed metaphase chromatin and as SH groups in interphase chromatin. This suggests that the presence of SS bonds or an increased amount of SH containing protein might play a role in the condensation of heterochromatin. Further evidence in favor of a possible role in chromosome banding is provided by the studies of Utakoji (p. 346) showing that compounds which react with SH groups can produce G-banding.

Are variations in protein phosphorylation involved?

Phosphorylation of non-histone protein may play a role in the genetic activation of lymphocytes stimulated by phytohemagglutinin [37] and when chromatin is fractionated the euchromatin appears to be enriched in phosphoproteins [36]. More direct evidence for the role of phosphorylation in chromosome condensation comes from the studies of Lake et al. [39] who found that the lysine-rich histones of metaphase chromosomes were well phosphorylated and upon entering G1 they were dephosphorylated. The lysine-rich histones are also more phosphorylated in rapidly dividing hepatoma cells than in non-dividing cells [4]. In an opposite direction Louie & Dixon [42] have suggested that dephosphorylation of protamine may be important in the condensation of trout spermatid chromatin.

Thus variations in protein phosphorylation could be playing a role in the differential condensation of heterochromatin and thus in the mechanisms of chromosome banding. The interactions of methyl groups of methylene blue (a component of Giemsa) with the phosphate groups of DNA was emphasized by Modest (p. 327). The presence of protein phosphorylation could allow this interaction to be extended to protein.

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Fluorescence Chromosome Banding Patterns Produced by a Benzimidazole Derivative

A. Gropp,[1] *Ingeborg Hilwig*[2] *and P. K. Seth*[1*]

[1]Institut für Pathologie, Medizinische Akademie Lübeck, 24 Lübeck, and
[2]Farbwerke Hoechst, 623 Frankfurt a. M. Hoechst, BRD

In view of the staining properties of the derivatives of the quinacrine series, fluorescent studies with an alkaline dibenzimidazole derivative 'Hoechst 33258' (fig. 1) belonging to a new class of fluorochromes seemed to be of interest, because it similarly produces a staining pattern of longitudinal differentiation along chromosome arms. Earlier reports by Lämmler & Schütze [9] and Hilwig [6] refer to the ability of this substance to bind to DNA and it was first used by Herzog & Schütze [5] for the staining of mammalian chromosomes. A first account of its capacity to visualize banding patterns and, particularly, segments of constitutive heterochromatin in the chromosome of the mouse and of the hedgehog has been recently given by Hilwig & Gropp [7]. It is the objective of the present report to summarize both the previously known and the newly obtained information about *Mus* species, and to give a brief survey of the observations in selected species belonging to different mammalian orders, insectivores, rodents and artiodactyla. So far the results obtained with 'Hoechst 33258' in human chromosomes are in too early a stage to be discussed at the present time.

Method

Slides with spreads of metaphase chromosomes obtained according to usual air-dry techniques are directly stained with a freshly prepared solution of the fluorochrome '33258 Hoechst' as described elsewhere [7, 16]. They may be stored for some days up to a couple of weeks in a refrigerator prior to the observation in the fluorescence microscope. The staining procedure does not involve denaturation or DNA reassociation pretreatments.

*Permanent address: Department of Anthropology, University of Delhi, Delhi 7, India.

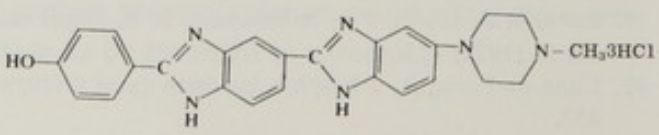
Observations

Chromosomes of the mouse (genus Mus)

The fluorochrome '33258 Hoechst' produces intense fluorescence in localized regions of pericentric heterochromatin and a diffuse strong fluorescence of the Y-chromosome in *M. musculus* (fig. 2) and *M. poschiavinus* (fig. 3). The latter has a diploid chromosome number of 26 with seven pairs of metacentrics derived from Robertsonian centric fusions. According to this origin, the metacentrics display a double set of brightly fluorescent pericentric blocks which sometimes fuse and thus form a larger mass. It is noteworthy, however, that the size of this mass may vary between the individual acrocentric and metacentric chromosomes.

Accordingly, the centromeric regions show bright fluorescence in the corresponding chromocentres of the interphase nucleus (fig. 4) and also in diakinesis and meiotic metaphase. In the meiotic XY-bivalent the brightly fluorescing blob corresponding to the centromeric region of the X-chromosome is opposite to the site of the association with the Y-chromosome. This fluorescent staining property of the chromosomes of the laboratory and the tobacco mouse (*M. poschiavinus*) is similar to that observed with the C-banding techniques of Arrighi & Hsu [1] and Yunis et al. [20].

Fig. 1. 2-[2-(4-hydroxyphenol)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol-trihydrochloride.



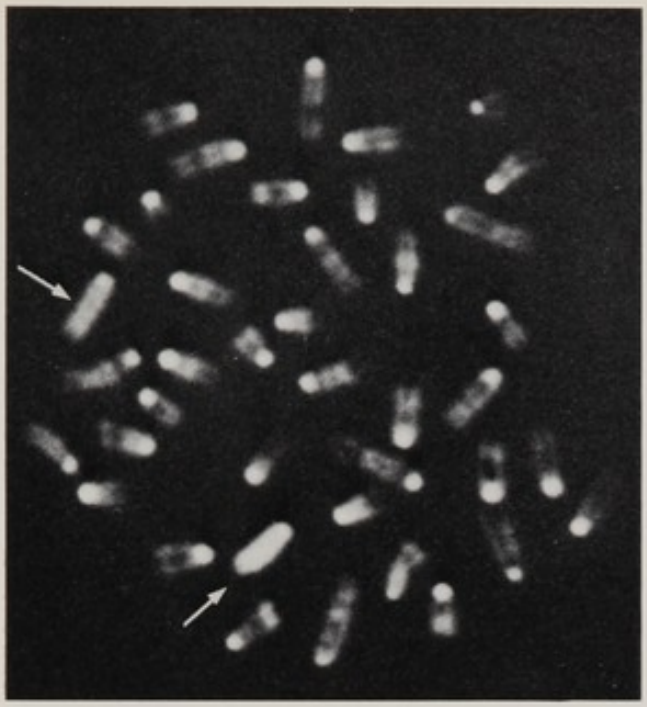


Fig. 2. Metaphase. Female laboratory mouse, *M. musculus*. Fluorescence staining with the benzimidazole derivative '33258 Hoechst', revealing C- and Q-type banding. →X chromosomes.

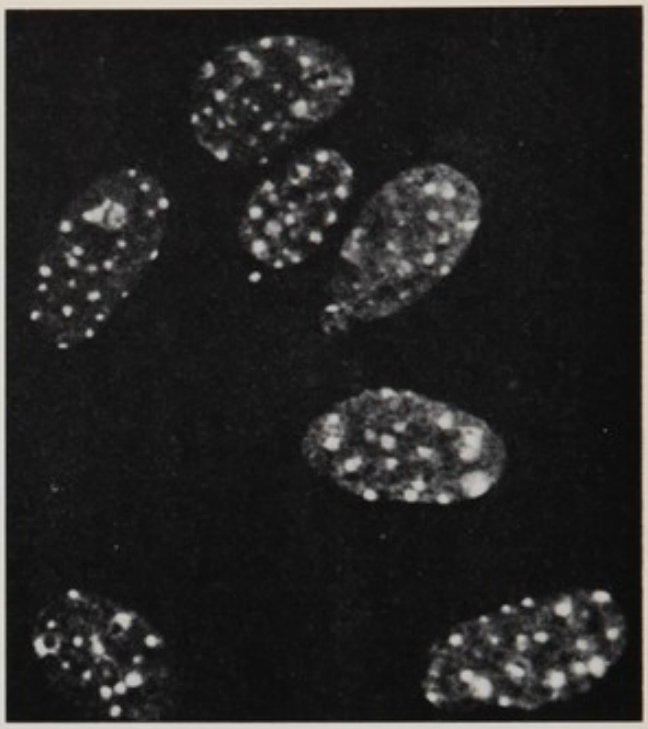
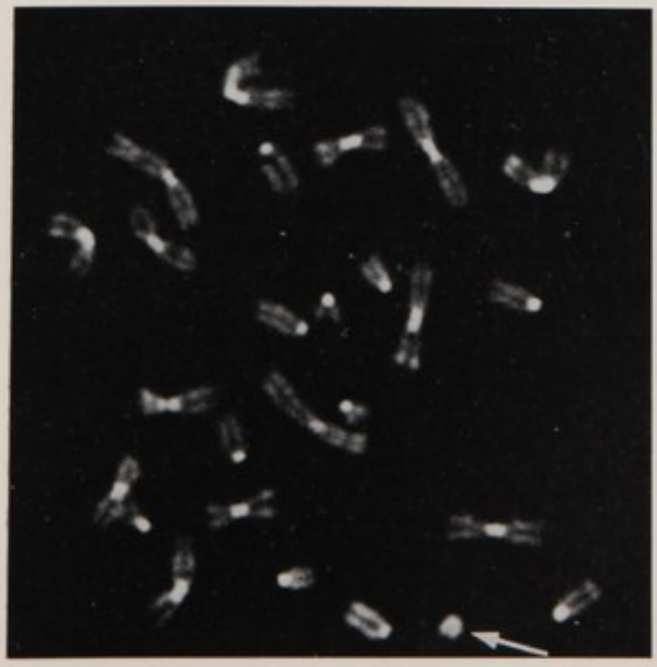


Fig. 4. Interphase nuclei of (*M. musculus* × *M. poschiavinus*) F_1 hybrid. Bright fluorescence of perinucleolar and nucleolus-independent chromocentres [11] after staining with the benzimidazole derivative.

In addition, regular as well as shortened staining periods reveal in mouse chromosomes a banding pattern along the chromatids similar to Q- or G-banding patterns (figs 2, 5). In view of the simultaneous staining of the pericentric chromosome regions, however, the pattern bears a greater likeness to the result of G-banding techniques in the mouse [2, 14].

Fig. 3. Metaphase. Male tobacco mouse, *M. poschiavinus* ($2n = 26$; N.F. = 40). Benzimidazole staining. The brightly fluorescing centromeric blobs tend to fuse. Their size is different in the individual "fusion"-metacentrics.→Y chromosome.



In contrast, most of the chromosomes of *M. pahari*, a spiny mouse from Thailand possessing a diploid complement of 48 acrocentrics, lack the bright fluorescence of the centromeric regions (fig. 6). Yet there is a banding pattern which at least in some chromosomes bears similarities to that of *M. musculus*.

Other rodent species

It has been possible to study *Apodemus agrarius* and *Microtus agrestis*. (Slides with chromosome preparations of *M. agrestis* were contributed by Dr F. Pera, Bonn). *A. agrarius* has 48 chromosomes, 40 acrocentrics and 8 very small metacentrics, as reported by Kral [8]. As in *M. pahari*, most of the acrocentrics fluoresce only faintly in the centromeric regions with the exception of two pairs which display bright staining of this segment (fig. 7). The staining behaviour of the Y-chromosome resembles that of *M. musculus* which allows the identification of the Y-chromosome in this species and also, for instance, in *Apodemus sylvaticus*. Since a faint

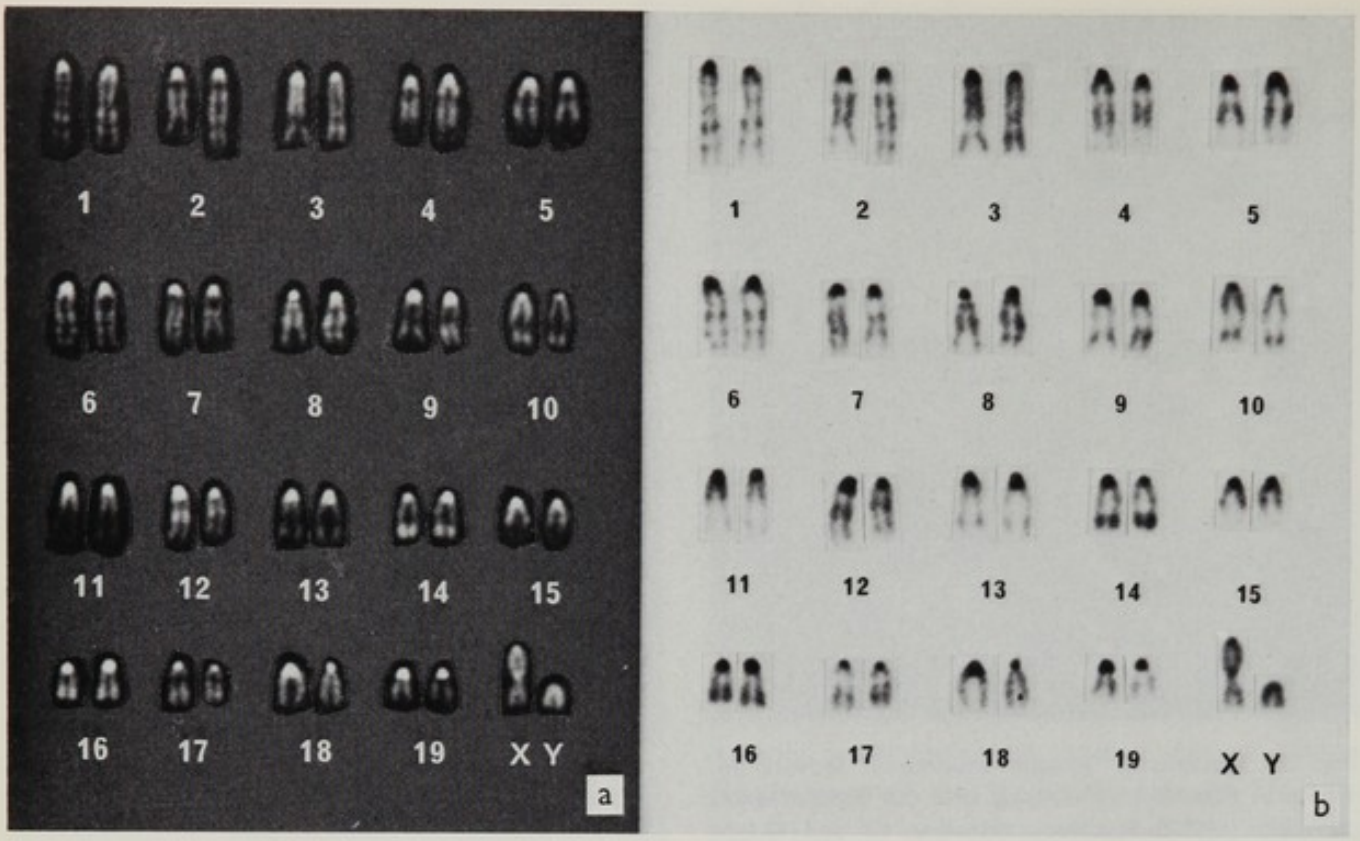


Fig. 5. Karyographic arrangement of a metaphase of a male laboratory mouse. (*a*) Fluorescence banding after benzimidazole staining. (*b*) Reverse photographic print of the same karyotype.

though distinguishable banding of the chromosome arms is produced by the '33258' staining, there may be a good chance of making use of this technique for a comparative search for distinctive patterns in different *Apodemus* species.

Staining of the chromosomes of *M. agrestis* with the '33258 Hoechst' substance revealed the following features (fig. 8):

(*a*) Diffuse bright fluorescence of the entire long arm of the X chromosome and of the Y. Sometimes the fluorescence of the distal third of the long arm of the X-chromosomes seems to be more intense than that of its remaining part.

Fig. 6. Metaphase. Male *M. pahari*. Fluorescence after benzimidazole staining. The animal was contributed by Dr J. Marshall, Bangkok.

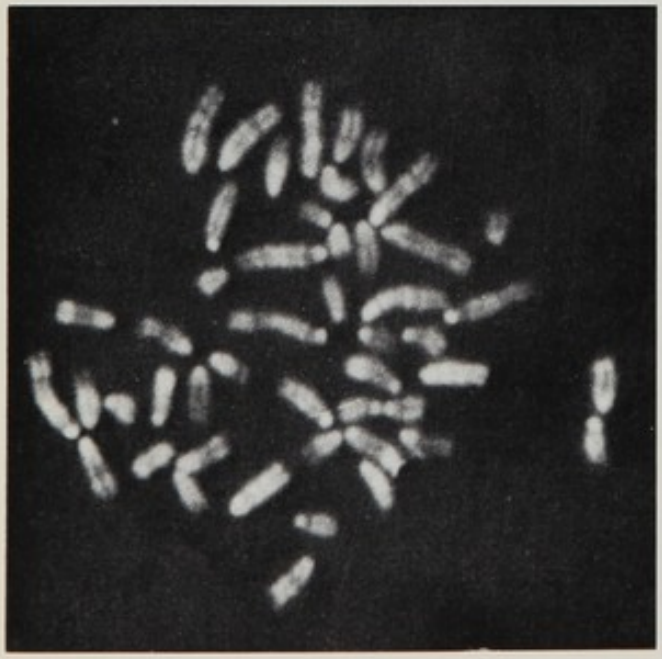
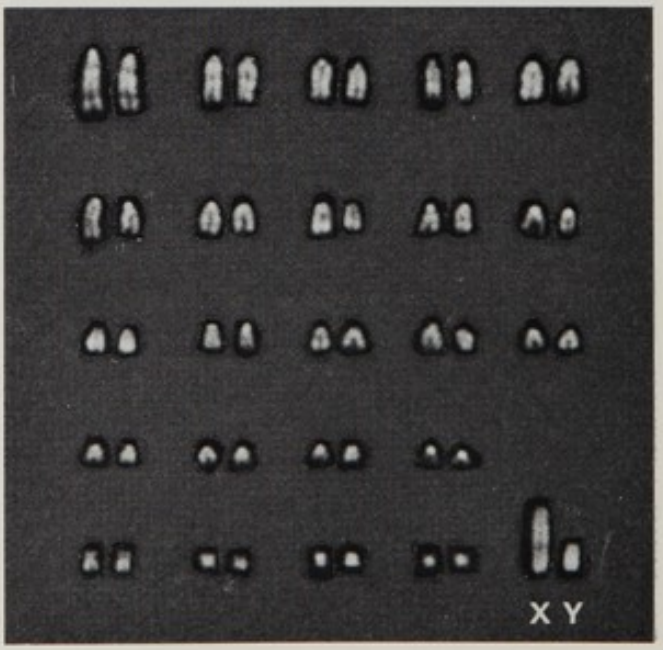


Fig. 7. Karyotype of a male *Apodemus agrarius* ($2n = 48$). Benzimidazole fluorescence. Bright centromeric fluorescence of pairs 10 and 19.



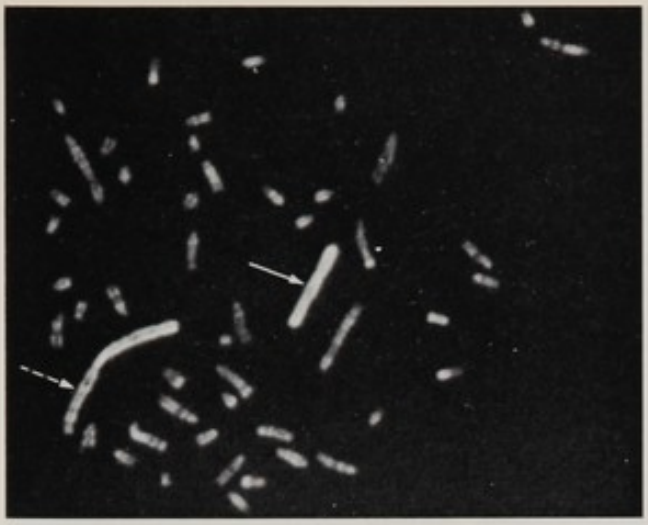


Fig. 8. Metaphase. Male *Microtus agrestis*. Benzimidazole staining. Bright fluorescence of the Y (→) and of the long arm of the X chromosome (→). The short arm of the X chromosome and the autosomes exhibit a banding pattern.

(*b*) In contrast to the results obtained with QM [11] or with methods demonstrating constitutive heterochromatin [13], there is no distinct bright fluorescence of a proximal segment of the short arm of the X chromosome. The entire short arm shows a banding which, in the female sex, is similarly arranged in both X-chromosomes, i.e. the euchromatic and the facultatively heterochromatic part.

(*c*) All autosomes are characterized by distinctive banding patterns closely resembling those revealed by Q-staining [11] or by methods using Giemsa staining after denaturation and reassociation of the DNA [13].

Fig. 9. Karyotype of a female common shrew (*Sorex gemellus*). Benzimidazole fluorescence banding pattern.

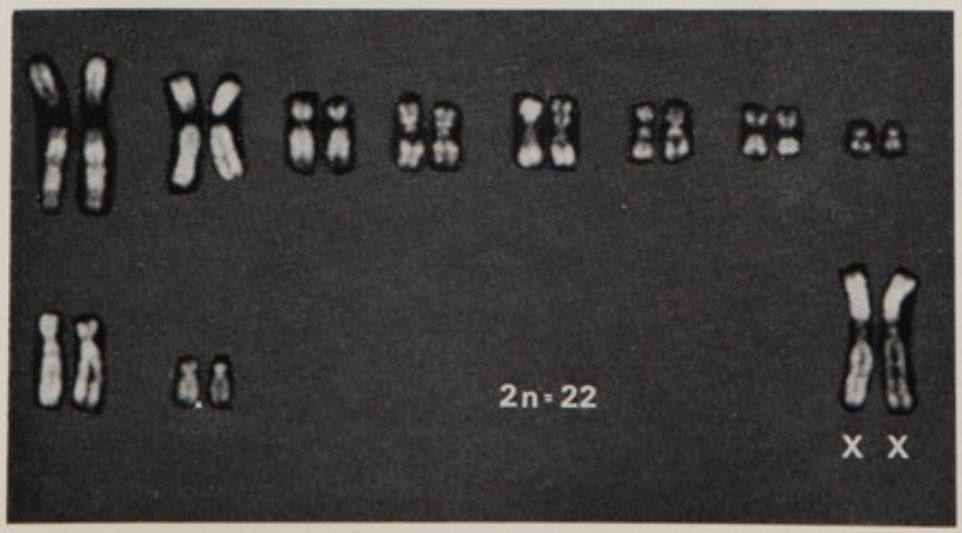
Insectivores

The European and the North African hedgehog species display large segments of distally located autosomal heterochromatin. Staining of the chromosomes of the North African hedgehog, *Aethechinus algirus* with the '33258' substance revealed weak or negative fluorescence of these segments [7] which at the same time are characterized by differential staining patterns with the Giemsa techniques for demonstration of *c*-heterochromatin [4], negative with the original Arrighi–Hsu-technique [1], positive with the technique according to Yunis et al. [20].

One female specimen of the common shrew, *Sorex araneus* Race A (according to Meylan [10]) or *Sorex gemellus* (according to Ott [12]), were obtained through the courtesy of A. Radbruch (Bonn) for a fluorescence study of the chromosomes. All chromosomes of the diploid set of 22 are devoid of bright fluorescence in the centromeric regions, except the larger subtelocentric element which shows a somewhat brighter fluorescence of that area (fig. 9). There is, however, a distinctive banding pattern which allows identification of each individual autosome, whereas both chromosomes are more diffusely stained or brightly fluorescing in the short arms. Knowledge of the distinguishable banding patterns in this species may be helpful in attempting recognition of species differences between *S. araneus* and *S. gemellus*, and of the identification of the chromosomes involved in the Robertsonian changes known to characterize *S. araneus*, viz. Meylan's Race B (see [3]).

Artiodactyl a

In cattle, the centromeric regions of the metaphase chromosomes are non-fluorescing with



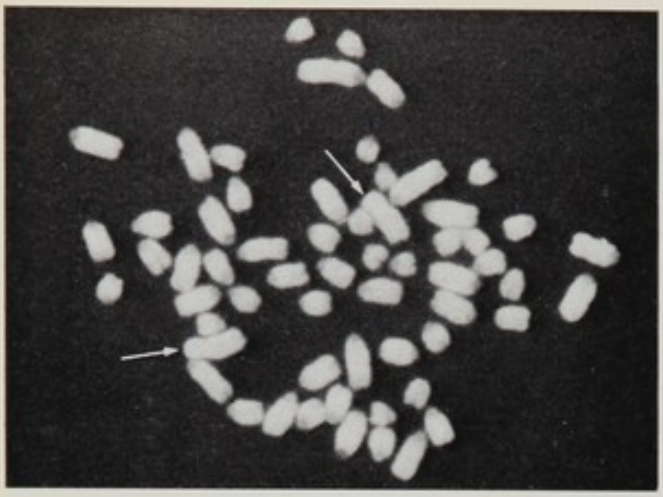


Fig. 10. Metaphase. Female cattle embryo. Benzimidazole staining. Centromeric regions display negative fluorescence. → X chromosomes.

fluorochrome 'Hoechst 33258' (fig. 10) [16]. They also display negative C-banding by the regular staining method of Arrighi & Hsu [1] and Sumner et al. [18]. These observations, though supporting the recent findings of Schnedl [15], have to be seen in the light of further results of a positive C-banding in the centromere region of cattle chromosomes [17] with the method described by Arrighi & Hsu [1] or with the technique of Yunis et al. [20]. Therefore the interpretation of Schnedl [15] that the highly condensed DNA localized at the centromeric regions of the autosomes is really removed during the incubation period in a buffer solution is contradictory. The foregoing observations suggest, however, the special chemical structure of the pericentric constitutive heterochromatin present in cattle. In this respect there is a marked similarity to the behaviour of the distally located heterochromatic segments of the hedgehog.

Binding properties of the benzimidazole compound

It can be assumed that the fluorochrome '33258 Hoechst' binds to DNA although the molecular mechanisms involved in the staining process remain to be elucidated. Some cytological experiments have been performed with spreads of mouse chromosomes and with mouse interphase cells, in order to shed some light on the binding properties of this substance. The results can be summarized as follows:

(*a*) Denaturation and reassociation procedures according to Arrighi & Hsu [1] and Yunis et al. [20] do not affect the bright fluorescence of the centromeric regions while the banding of the chromatids is replaced by a weak diffuse fluorescence.

(*b*) DNase pretreatment 5 to 15 min (0.2 mg/ml) leads first to an extinction of the stainability of the chromatids, and finally also to the loss of fluorescence of the pericentric regions of the chromosomes.

(*c*) DNase treatment of spread chromosomes after staining with the fluorochrome produces diffusion of the stain along the chromosome arms.

(*d*) DNase treatment of 5 up to 24 h (0.3 mg/ml) after 24 h exposure of the living cells or after staining of fixed interphase cells with the fluorochrome chases the stained material away from the nuclear structures to the nucleolus and cytoplasma, corresponding to areas rich in RNA (fig. 11*a*, *b*). Subsequent incubation with RNase leads then to a complete disappearance of the fluorescing material.

These experiments, though not conclusive as far as the exact mechanism of staining is concerned, nevertheless confirm at least the general assumption of a binding of the stain to DNA.

Conclusions

The observations described lead to the conclusion that, depending on the species and the species-specific molecular organization of the DNA complex, the fluorochrome '33258 Hoechst' visualizes heterochromatin either by a positive staining reaction as in the mouse and to a lesser and different extent in some of its allies among the Muridae, or by a specific failure of staining of the type present in the hedgehog and in cattle. The constitutive heterochromatin of the giant sex chromosomes in *M. agrestis* is brightly fluorescing. Besides that, the benzimidazole derivative has been shown to produce banding patterns similar to those observed with derivatives of the quinacrine series, though of different distinctness in different species. It is therefore evident that this fluorochrome reveals in certain species a prevailing pattern of C-banding, in other species preponderantly a Q-type banding, and sometimes also a mixed pattern of both. In view of the peculiarities of the staining patterns, the use of the benzimidazole derivative seems to be of interest as an additional means for the

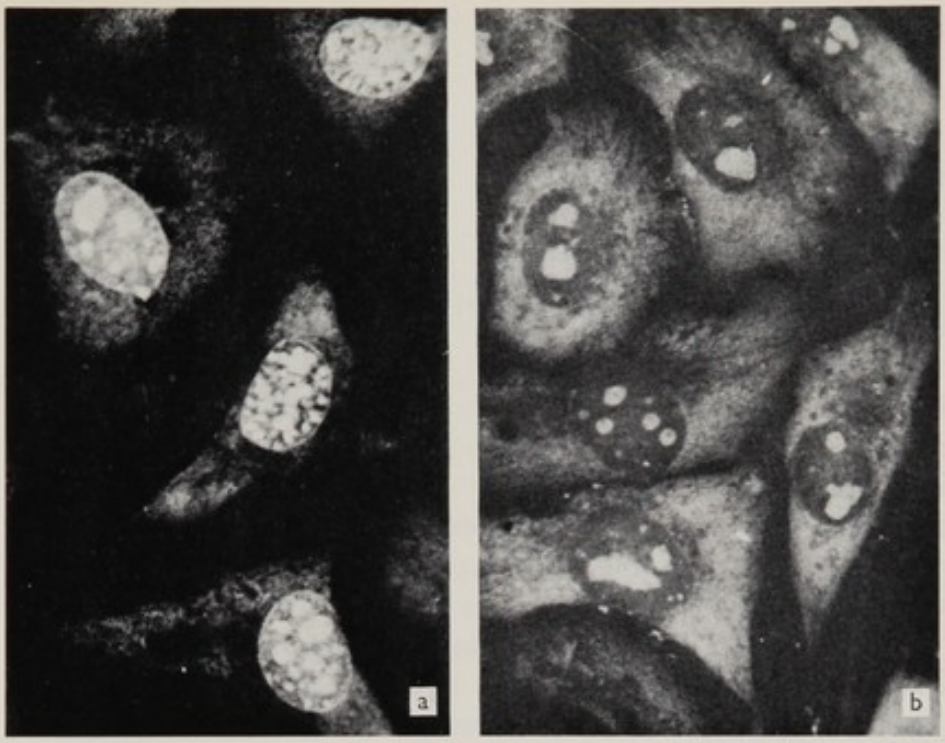


Fig. 11. Mouse interphase cells. Exposure of the living cell for 24 h to the benzimidazole derivative, fixation and treatment with DNase (0.3 mg/ml) (*a*) 6 h; (*b*) 24 h.

characterisation of the staining properties of constitutive heterochromatin and for the identification of individual chromosomes in comparative cytogenetics, in the analysis of karyotype rearrangements, and in the course of experimental procedures, e.g. cell hybridization.

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Discussion

Comings: Do you know the base composition of the satellite in the mouse in which the centromeric regions did not stain? Since this stain worked well with *Mus musculus* and AT-rich satellite, poorly in human and GC-rich satellites, the base composition may be important.

Gropp: Not yet. These other rodent species such as *M. pahari* and *A. agrarius* have not yet been studied in this respect.

de la Chapelle: We have now heard from Dr Hsu and Dr Gropp that the centromeric region of the mouse Y chromosome is different from that of

all the other chromosomes in that it does apparently not contain satellite DNA and does not stain brightly in Dr Gropp's system. This fact has been commented upon very little in the literature. I should like to mention an observation which may bear on this question.

Dr J Wennström in our laboratory has found that the two chromatids of the Y fall apart in ordinary bone marrow preparations far more often than those of any other chromosome. This may be interpreted in many ways, but one might hypothesize that the set DNA or repetitious DNA has something to do with the mechanical integrity of the centromere.

Gropp: It is true that the Y chromosome in the mouse as well as in *M. pahari* and in *Apodemus* does not show bright fluorescence of the centromere region. But it stains as a whole more brightly than the chromatids of the autosomes. Besides that, it is difficult to comment on the hypothesis concerning the importance of centromeric staining for the integrity of the chromosome.

Schnedl: (1) Which details of the technique were used for obtaining positive centromeric regions in cattle?

(2) We also used the procedure of Yunis et al. [20] and got holes at the centromeres of cattle chromosomes. Additionally, very good banding patterns could be obtained by this technique too in cattle.

Gropp: (1) We used the regular technique according to Arrighi & Hsu [1], but another brand of RNase (see text).

(2) Yunis & Yasmineh [19] published a photograph of a cattle metaphase prepared according to their own technique. In this figure all acrocentrics show dark centromeric Giemsa staining.

Pearson: General question: How important is RNase for C-banding?

Gropp: It is probably not very important.

Pearson: Does RNA has anything to do with C-banding?

Hsu: In in situ hybridization we remove RNA by both HCl and RNase treatments. Naturally, HCl also removes histones. In C-banding we have found that HCl and RNase treatments are not necessary.

Service of in Situ Nucleic Acid Hybridization to Biology

T. C. Hsu and Frances E. Arrighi

Department of Biology, The University of Texas at Houston
M. D. Anderson Hospital and Tumor Institute, Houston, Tex. 77025, USA

For more than a decade, the DNA/RNA and DNA/DNA hybridization techniques have contributed immensely to biological sciences. However, the earlier procedures employ molecules in solution, and an application of the hybridization principles to cellular components of higher order such as the nucleus, the chromosomes, and other structures was sorely needed. Many investigators in the field of cell biology (including ourselves) made half-hearted attempts to perform molecular hybridization in situ but abandoned it on account of various technical difficulties.

Without question, credit must go to the group at Yale University under the leadership of J. G. Gall for the elegant experiments which eventually led to a workable procedure [11, 12, 22]. The in situ hybridization procedure, in existence for only a few years, has already made significant contributions and it will become a powerful tool for geneticists as both molecular biology and cytology continue to improve. A human (or any species for that matter) chromosome map in respect to the distribution of DNA molecules is not an impossibility.

The Basic Principle

In the original form of molecular hybridization involving DNA/RNA or DNA/DNA, both component molecules are in solution and are, therefore, mobile. Thus hybridization results from the chance collision between molecules with complementary base sequences. In hybridization in situ, one component molecule, the DNA of the nuclei or the chromosomes, does not move about. It is presumably denatured but still embedded in the nucleus or the chromosomes. We may call these immobile DNAs in the nucleus or chromosomes "receptor DNAs." The other component is mobile.

The mobile component

The mobile component is the molecule being tested. Since in situ hybridization requires autoradiography, the molecules being tested must be radioactive, usually labeled with tritium for good resolution. This mobile molecule can be either DNA or RNA and can be either synthesized by the cells or by artificial transcription in cell-free systems.

(1) *Synthesis by the cells:* It is possible to extract the macromolecules for in situ hybridization by supplying the cells with radioactive precursors. Pardue et al. [22] used ^{3}H-rRNA synthesized by cultured kidney cells of *Xenopus laevis* for the identification of rDNA loci in *Drosophila*. Wimber & Stephensen [29] and Steffensen & Wimber [26] fed *Drosophila* larvae with large amounts of ^{3}H-uridine in order to extract 5S and tRNAs from the larvae. Jones [15] used ^{3}H-thymidine to feed mouse cells in culture to label the satellite DNA for his experiments.

The most obvious advantage for using this procedure is that the labeled molecules are directly extracted and purified from tissues or cells without going through an intermediate step of synthesizing complementary RNA. In studies involving tRNA or other RNA species, this is probably the most expedient way since isolating vertebrate tDNAs is not feasible at the present time. Nevertheless, this procedure has two major disadvantages:

(*A*) Obtaining large quantities of a specific ^{3}H-labeled DNA or RNA is impractical if the experiments require large quantities of these molecules. For example, Saunders et al. [25]

needed nearly 90 mg of DNA for fractionating repeated DNA sequences of the human genome in conjunction with thermal elution chromatography. It is not a difficult problem to isolate nonradioactive DNA from tissues in situ (liver, placenta, etc.), but it requires a prohibitive amount of labor and expense to obtain pre-labeled DNA because cells in most tissues are not in the S phase, thus requiring cells in culture as the initial material.

(*B*) The radioactivity of the pre-labeled molecules is usually not high enough for efficient autoradiography. This is particularly true for mammalian systems because the cells cease to grow when too much radioactive precursor is introduced. In our laboratory we failed to isolate ^{3}H-rRNA from a variety of cell types with a radioactivity exceeding 5×10^4 cpm/μg.

In *Xenopus* cell cultures, the situation appears to be not as critical since Pardue et al. [22] were able to obtain ^{3}H-rRNA with a radioactivity of 5×10^5 cpm/μg. The exposure time still required from 10 to 94 days for the in situ hybrids of the polytene chromosomes of *Drosophila*. In Wimber & Steffensen's report, the 5S RNA showed 4×10^6 dpm/μg. The mouse satellite DNA of Jones exhibited 2.2×10^6 dpm/μg. His autoradiographs exposed after 10 days showed a maximum of two grains on each chromosome whereas Pardue & Gall [21], using ^{3}H-labeled complementary RNA, could obtain a cluster of silver grains to completely cover the chromosome region in 7 days.

(2) *Synthesis of ^{3}H-complementary RNA (^{3}H-cRNA) in cell-free system:* This method takes advantage of the fact that the RNA polymerase is able to transcribe in cell-free system when a DNA template and all the nucleotides are present in a proper medium. Investigators first obtain the nonlabeled DNA fraction whose cytological locations are to be studied. This DNA is used as template to synthesize ^{3}H-labeled complementary RNA. Generally RNA polymerase from *Escherichia coli* is used, and 3 of the 4 nucleotides (ATP, CTP, UTP) are tritium-labeled while GTP is nonradioactive.

Although this method seems indirect, it has many advantages. The radioactivity of the cRNA can reach about 10^7 dpm/μg, thus drastically reducing the exposure time of the ensuing autoradiographs. Furthermore, many methods for isolating and purifying DNA fractions can be employed even though they require sufficient quantities of DNA initially.

The receptors

The receptors are the DNA molecules of the cell nuclei and chromosomes in the cytological preparations. They therefore assume fixed positions and will serve as landmarks. These molecules are presumably denatured prior to hybridization with the labeled mobile components.

When the cytological preparations containing the receptors are incubated with the isotopically mobile component(s) under proper conditions, molecular hybridization is expected to take place and the hybrid molecules do not move about on account of the fixed locations of the receptors. Thus the ensuing autoradiographs should show grain localization (or the lack of it) for cytologists to identify the cytological region(s) of a particular molecular species.

The Procedure

We shall describe only briefly the procedure for in situ hybridization since the readers can easily find the details in earlier papers. The procedure will be described in 3 parts; (1) the preparation of the mobile component; (2) the preparation of the receptors; (3) the hybridization procedure.

The preparation of the mobile component

As mentioned previously, the mobile component is the isotopically labeled nucleic acid molecules synthesized either in cell or cell-free system. The techniques for isolating and purifying the molecules of course vary, depending upon the kinds of the molecules (DNA or RNA), the biophysical characteristics of the molecules in question, and other properties. In other words, appropriate procedures used in molecular biology must be applied.

For experiments using the transcription process in a cell-free system, the important factor is the RNA polymerase. With a good polymerase preparation, a template of 5 μg DNA should yield sufficient ^{3}H-cRNA for a set of experiments.

The preparation of the receptors

As long as the cytological preparations are of good quality, it matters little which procedure is

used. The classic squash method works well, and in some instances, e.g., the polytene chromosomes, squashing is still the only effective way to flatten the long chromosomes. To prevent extensive cellular loss from squash preparations during the hybridization procedure, the slides should be coated (or 'subbed') before squashing with a mixture of chrome alum (0.01%) and gelatin (0.1%). Cleaned slides are dipped into this solution, drained and let dry. They can be kept in dust-free containers. Coverslips are removed by the conventional dry ice method.

It has been found that air-dried slides are also suitable for in situ hybridization experiments ([17], unpublished data of our laboratory). This method saves some time compared to squash preparation, particularly for mammalian cells in culture.

The slides are then treated with a succession of reagents, including HCl, RNase and NaOH. Presumably the last agent is to denature the DNA of the cell nuclei and chromosomes so that the DNA is available for hybridization. We have found that the HCl treatment is not necessary. Wimber & Steffensen [29] found that formamide is a better denaturation agent than NaOH for *Drosophila* polytene chromosomes because the morphology of the chromosomes is not as badly distorted. Gall et al. [10] avoided NaOH and used HCl for denaturation.

Hybridization

Hybridization is performed on cytological preparations by covering the slides with a solution (2 X SSC or 6 X SSC) containing the ^{3}H-labeled mobile component. Approx. 0.2 to 0.4 ml of the solution is placed on the cell surface of each slide which is then covered with a coverslip of appropriate size. The slides are incubated in moist chambers at 65°C for 16–20 h.

After the incubation period, if the mobile component is ^{3}H-RNA, the slides are treated with RNase to remove the unbound cRNA in order to reduce background radiation. Autoradiographic procedure is then applied, and after appropriate exposure time, the slides are developed and stained for analysis.

The Accomplishments

To present the record of accomplishments of the in situ hybridization technique it is necessary to first present its byproduct, i.e., the discovery of a method for revealing constitutive heterochromatin in mammalian chromosome complements. Pardue & Gall [21] found, in their in situ DNA/RNA hybrid preparations of mouse cells, that the centromeric regions, where the satellite DNA is located, stained deeply with Giemsa whereas the remaining chromosome regions stained faintly. These authors regarded the deeply stained areas as heterochromatin.

Arrighi & Hsu [1], Chen & Ruddle [7], and Yunis et al. [30] found the same phenomenon in human chromosomes. The differential staining is a result of the treatments, particularly NaOH and SSC incubation, and is not the result of in situ hybridization. In other words, Pardue & Gall accidentally found a simple and useful staining procedure to differentiate constitutive heterochromatin from euchromatin in the chromosome of higher animals which was not possible previously (for a detailed account of this area of research, cf [4]). These developments led to the numerous G-band techniques which enable laboratories without fluorescent optics to identify chromosomes and chromosome regions. The value of these techniques needs no emphasis in this paper, since many articles of this monograph delve into this subject.

This procedure turned out to be important not only for chromosome cytology in general but also for interpretations of results of in situ hybridization since there seems to be a correlation between highly repetitive DNA sequences and constitutive heterochromatin. The original work of Pardue & Gall showed that the satellite DNA is located in the centromeric heterochromatin of all the mouse chromosomes except the Y. Jones & Corneo [17] found that human satellite II DNA is primarily localized in the heterochromatin regions of human chromosomes 1 and 16 and less so in chromosome 9. On the contrary, Saunders et al. [24] found another human satellite DNA to locate primarily in the heterochromatin of chromosome 9. In *Drosophila* [10], in amphibia [18], and in birds [6], similar conclusions have been obtained, viz., satellite DNA fractions are primarily located in the chromocenter (or constitutive heterochromatin).

Satellite DNAs represent special classes of repetitive sequences within a genome, but satellite DNAs are not always resolvable, particularly

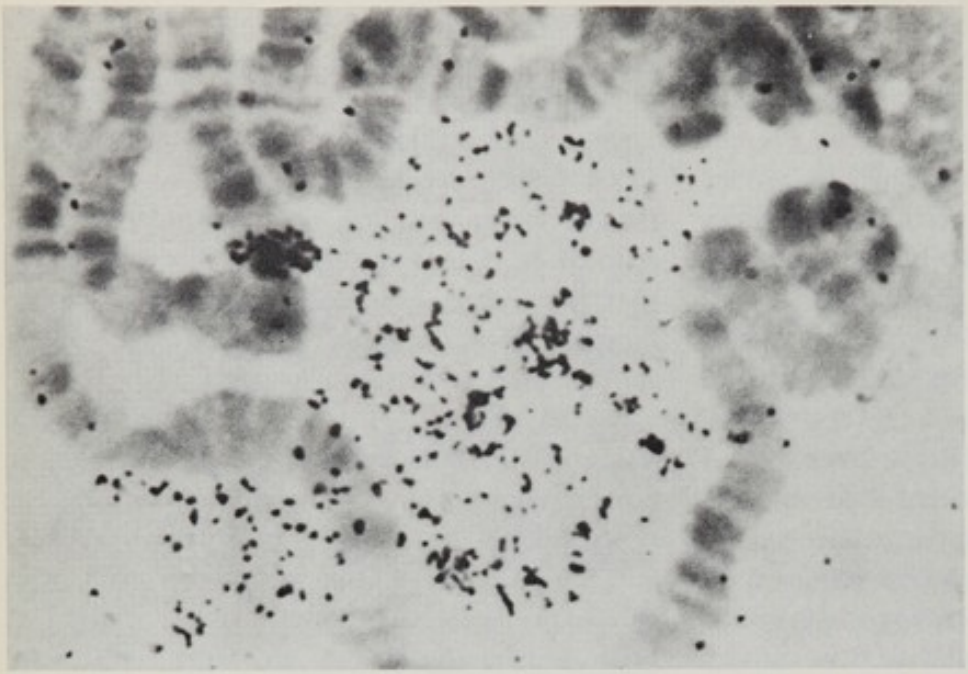

Fig. 1. Autoradiograph of an in situ hybrid from the salivary gland of *Drosophila melanogaster*, squashed lightly to preserve spatial relationships. Mobile component, ^{3}H-5S rRNA. Localization at one band on chromosome 2R, but the locus is seen to bend at the vicinity of the nucleolus. Courtesy of Dr Donald E. Wimber, University of Oregon.

those masked by the main-band. However, all eukaryote genomes contain repetitious sequences which can be isolated by the reassociation technique. Since satellite DNAs are found to be cytologically localized, it should not be a surprise to find a certain degree of localization of repeated sequences isolated by the reassociation technique. Indeed, in *Drosophila* [5, 23], in mammals [2, 3, 25], and in birds (Stefos. Unpublished data), sufficient demonstrations have been made to conclude that repetitious DNA sequences, especially the highly repetitious type (low C_0t fractions), are located preferentially in constitutive heterochromatin.

It should be borne in mind that isolation of nonsatellite repeated sequences depends on the reassociation rates of sheared DNA molecules. Two or more sequences having similar number of copies and similar complexities will have similar reassociation rates and will then be isolated in the same fraction. This is probably not a serious problem where highly repeated sequences are concerned; but in the so-called 'intermediate' and the 'slow' fractions, one should not expect purity. In their studies on the repetitious DNA of the human genome, Saunders et al. [25] isolated human DNA according to the melting temperatures into seven thermal elution fractions. From each fraction they obtained both highly repetitious and intermediate repetitious fractions. In situ hybrid analysis [14] showed that the highly repetitious fractions localize more at the centromeric regions (constitutive heterochromatin) and telomeric regions whereas the intermediate repetitious DNA sequences are more dispersed along all chromosomes.

The earlier works on in situ hybridization employed ^{3}H-RNAs synthesized in vivo. Gall's group used ^{3}H-rRNA as material for a number of reasons but the most important one was probably the knowledge about the cytological locations of the ribosomal cistrons. The in situ hybrids using rRNA would therefore serve to answer the question whether the procedure would give labeling at the right places. Applying this procedure, Wimber & Steffensen [29] identified the gene location for 5S-rRNA in the polytene chromosomes of *Drosophila melanogaster*. Of interest is the special relationships between the 5S rRNA gene and the nucleolus. Surprisingly, these two loci are quite separate, geographically, the rDNA being on the sex chromosomes and the 5S locus on 2R. In cytologial preparations of the salivary glands not heavily squashed, Wimber & Steffensen noted that the 5S locus actually bends to the proximity of the nucleolus. Fig. 1 shows such a cell. The grains over the nucleolus are believed to be contaminating 28S rRNA.

In situ hybridization also provides some infor-

mation in phylogenetic studies. Perhaps the best example to date was the work of Jones [16] regarding the location(s) of human satellite III. This satellite DNA is primarily located in the heterochromatin area of human chromosome No. 9. When its ^{3}H-cRNA was used to hybridize with DNA of chimpanzee chromosomes, the grain clusters were found to be located on several chromosome pairs. Since there is reasonably good paleontological evidence to estimate the time period of the emergence of man, Jones' data throw some light on the time regarding the emergence of this specific DNA fraction.

Jones' data are discrete enough to be qualitative, but such relatively clear-cut results probably cannot be obtained in too many cases. Nevertheless, a somewhat quantitative approach can be taken to estimate the degree of homology by using grain counts over interphase nuclei of the in situ hybrids. Arrighi et al. [2] found little homology between the repetitious DNA of *Microtus agrestis* and that of several other mammalian species. Saunders et al. [25] had similar findings with human repetitious DNA when in situ hybrids were compared.

Advantages and Limitations

Needless to say, the most important advantage of the in situ hybridization procedure is the cytological location of the DNA families under scrutiny which the conventional nucleic acid hybridization procedures cannot tell. In other words, in situ hybridization serves as one important link between molecular biology and cytology. However, this is not the only advantage. An experiment involving in situ hybridization requires relatively small amounts of DNA. We have used as little as 3 μg of DNA as template which yielded sufficient quantity of ^{3}H-cRNA for a set of preparations (ten or more slides, depending upon the areas to be covered).

An additional advantage of in situ hybridization may lie in the fact that the experiments must be carried out by autoradiographic techniques. In case of low resolution (low degree of homology, low annealing rates, etc.), which may be near the background level of radioactivity in scintillation counting, one can increase the resolution by prolonged exposures. Several such examples were found in our laboratories to reveal localization of grains after 6 to 12 months of exposure. In one case we received a sample of ^{3}H-rRNA from Dr J. G. Gall who isolated the fraction from *Xenopus* cell cultures. We used it to hybridize with human cells. There were no grains registered following a 5- to 8-week exposure time. This was confirmed by an experiment conducted by Dr Frank H. Ruddle (personal communication). However, after exposing the autoradiographs for 1 year, distinct label over the nucleoli was observed in every nucleus. This indicates that a low degree of homology between the ribosomal cistrons of *Xenopus* and those of man probably exists. If one considers a year or two too long a time to wait, all he needs to do is conduct some other experiments in the meantime.

It should be added that ribosomal cistrons still represent repeated sequences, even though the number of repeats may be relatively low. The in situ hybridization technique is too insensitive for single-copy sequences even after prolonged exposure times. Thus it may be nearly impossible to identify the chromosomal location or locations containing DNA homologous to a viral genome, even if such a homology exists. The report of McDougall et al. [19] on viral infection was for identification of viral genomes in the host cell, not the host genome.

The situation may be somewhat improved in Dipteran insects where polytene chromosomes can be used as receptors. Here each receptor gene is multiplied by endoreduplication even if it is a single-copy gene by definition. Indeed, Steffensen & Wimber [26] used pooled tRNA of *Drosophila melanogaster* to perform in situ hybridization experiments. This study was of course preliminary, but it pointed out the possibility of localizing genes in this species. In other materials (such as mammals) where no polytene chromosomes exist, the task will be extremely difficult.

Probably one should also keep in mind that grain registration in the autoradiographs does not necessarily mean strict homology between the receptor molecules and the mobile component. A stable duplex may be formed if a certain minimum number of base pairs match. Thus an RNA molecule and an entirely different DNA molecule sharing a segment of base sequence in common might show hybridization in that segment to give grain registration and hence a false positive reaction.

Technical Considerations

Some molecular biologists still question the validity of the term 'hybridization' because there is no conclusive proof to show what one obtains and observes from the autoradiographs are genuine DNA/DNA or DNA/RNA hybrids. It is difficult to explain without nucleic acid hybridization, however, the data from Gall's laboratory that rRNA labels only the nucleoli of *Xenopus* but not the chromatin, and that chromatin RNA only labels the chromatin but not the nucleoli. Similarly, it is hard to interpret the condensation of label on a specific segment of a chromosome when a specific fraction of DNA is considered. In fact, one would have to dream up some ingenious schemes to explain such specific and repeatable results without using hybridization as the mechanism. As far as cytologists are concerned, being able to localize a DNA or an RNA species on chromosomes is an exciting development; whether it is a real molecular hybrid or not is of secondary importance.

Perhaps it may be more worthwhile to consider some technical points in the in situ hybridization procedure since the procedure in use is still at its infancy. Steffensen & Wimber [27] discussed several pertinent points in their review on hybridization of nucleic acids to chromosomes, but they did not consider the possibility of DNA loss from the receptors (nuclei and chromosomes) following the series of treatments employed in the in situ hybridization system. We treated Chinese hamster and mouse LM cells in culture with ^{3}H-thymidine (0.5 μCi/ml, spec. act. 6.7 Ci/mM) and nonradioactive thymidine (5 μg/ml) for 18 h to give the cell population a low-level but continuous labeling. We then made squash preparations on subbed slides and subjected the preparations to a variety of treatments prior to the application of autoradiographic films. All autoradiographs were exposed for the same time period (4 days) and the grain counts over the nuclei (subtracting background) were used to estimate the effect on the DNA of the various procedures. Table 1 presents the average grain counts of each procedure converted into percentage of the grain counts of the untreated control samples. It can be seen that NaOH treatment followed by incubation in SSC drastically reduced the grain counts. We interpret this as the DNA loss. Comings et al. [8] arrived at the same conclusion using a different approach.

Table 1. *Grain counts (percentage of control) of nuclei prelabeled with ^{3}H-TdR and treated with a variety of chemicals*

Treatment	Chinese hamster cells	LM mouse cells
None (Control)	100.0 (58.5 Grains)	100.0 (155.2 Grains)
TCA	83.6	78.7
HCl	86.0	—
HCl-SSC	49.2	—
SSC	50.1	73.2
NaOH	—	75.3
HCl–RNase–NaOH	16.8	—
HCl–RNase–NaOH–SSC	15.0	—
NaOH–SSC	22.1	26.2

It is not known at this moment whether the loss of DNA following the treatments was random. Possibly repetitive DNA sequences situated close to one another renature quickly to minimize the loss whereas single-copy DNA will suffer more severely. If DNA loss following the treatments is nonrandom, this would certainly complicate the result of in situ hybridization.

Another serious consideration is false positive grain registration or artifact. Since investigations on in situ hybrids must depend on autoradiography, nonspecific or false grain registration could lead to erroneous conclusions. Several factors may contribute to false grain registration: (1) impure nucleic acid preparations; (2) nonspecific trapping of the mobile component in the chromosomes; (3) partial hybridization. Raising the incubation temperature close to the T_m of the nucleic acid fraction in question may be one way of reducing nonspecific silver grains. One might also utilize the repeated autoradiography technique [28] to increase resolution and to remove background. However, this method would not remove false-positive grains caused by nonspecific trapping or partial homology (different cistrons sharing segments of base sequence in common).

Finally, one does not know exactly how faithful is the polymerase of *E. coli* in transcribing mammalian DNA templates. It may be worth-

while to compare the transcripts produced by different types of RNA-polymerase, particularly those of mammalian origin.

Future Possibilities

It is anticipated that the in situ hybridization procedure will continue to make contributions to the field of biology, with the hope that eventually one may construct molecular maps of various species, at least in respect to repeated sequences. Indications are also available that one may use such techniques to trace molecular evolution regarding the geological time at which a particular repeated sequence came into existence or a particular sequence was eliminated. It is possible also to use this procedure to estimate the degree of homology between mitochondrial DNA and nuclear DNA of the same species. Cytologists must constantly watch the advances in the fields of molecular biology and biochemistry and apply these to their studies. For example, utilization of ^{125}I [9] as a label may offer advantages. Getz et al. [13] successfully used ^{125}I labeled RNA for molecular hybridization experiments. W. Prensky (personal communication) has iodinated *Drosophila* 5S RNA as the mobile component for its localization on the polytene chromosomes. His results were identical with those of Wimber & Steffensen who used ^{3}H-labeled 5S RNA. The beauty of ^{125}I label is that unlabeled RNA can be extracted and purified prior to labeling, thus avoiding treating cells, animals or plants with a large amount of ^{3}H-labeled precursors.

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Discussion

Gropp: Is there any evidence of reproducible and inherent differences in the amount of satellite DNA in individual mouse autosomes as visualized by different grain counts in the in situ preparation?

Hsu: The amount of constitutive heterochromatin is not the same from one chromosome to another in the mouse complement. Therefore it is conceivable that the amount of satellite DNA also differs to some extent from one chromosome to another. In the female mouse, centromeric areas of all chromosomes can be labelled heavily with satellite DNA hybridization, but in a particular metaphase one or more chromosomes may show little label. But this is not consistent. Since the label is so concentrated in the centromeric areas, quantitative analysis using grain counts is not expected to be accurate. In the metaphases of the male mouse, the Y chromosome consistently shows no label when satellite DNA is used for in situ hybridization, indicating that it does not contain that DNA fraction.

Rigler: In which way do chromosomal proteins affect DNA/RNA hybridization?

Hsu: The acetic acid in the fixative and the HCl-treatment probably remove most, if not all, histones. The NaOH-treatment may remove a considerable amount of acidic proteins. However, a certain amount of protein undoubtedly remains in the chromosomes because the chromosomes, after all the treatments, will disintegrate if they are treated with pronase. The extent of interference exerted by such proteins in DNA/RNA hybridization is not known.

Comparative Methodology and Mechanisms of Banding

H. A. Lubs, W. H. McKenzie and S. Merrick

Department of Pediatrics, University of Colorado Medical Center, Denver, Colo. 80226, USA

Summary

Optimal cytogenetic study currently requires the use of multiple technics. With an understanding of the factors which affect banding it is possible to obtain good banding routinely in all types of cytogenetic studies. Although the mechanisms of banding are not yet fully understood it is now clear that both variation in base ratios and overlying proteins are involved in the banding mechansisms. Denaturation or differential denaturation does not play a role in banding with the possible exception of R-banding.

Under the mantle of the word 'comparative' in the title we would like to make several types of comparisons and to apply the results of these comparisons to our understanding of the mechanisms of banding. Three types of studies, therefore, will be presented: (1) results of a comparative study of variables which affect the quality and frequency of banding; (2) a comparison of the details of G- and Q-, G- and G-, Q- and R-banding, utilizing successive banding technics on the same cell; (3) several studies which bear more directly on the mechanisms of banding. The special merits of the various technics will also be discussed.

Effect of Technical Variables

Slide preparation

The results of a survey of some of the preparative variables are summarized in table 1. Although these results are largely of practical importance they also have some theoretical implications. The method of slide preparation is quite critical to many of the banding technics. 'Heat drying' is our routine method of slide preparation. This involves dropping fixed cells on a wet slide, blowing abruptly at the drop and then heating the slide on a hot plate for 1½–2 min. The hot plate is set to maintain a beaker of water at 65°C. The surface temperature of the hot plate may vary from 70°C to 120°C, but the slide itself is under 100°C. As used here, the term 'air drying' is the same procedure but without heating. 'Flame drying' is predominantly ignition drying, not heating over a flame. Slide temperatures at the end of preparation are greater with heat drying than with flame drying, and both are greater than air drying. Two conclusions are apparent from the data in table 1: flame or ignition drying produces inconsistent results with all technics, in our laboratory and in most literature reports, and heat drying is necessary for the two abbreviated technics we use—the Giemsa 9- and HCl–SSC C-banding technic. The latter technic, namely, HCl treatment followed by a 24 h SSC incubation at pH 7.0 has proved the most workable C-technic in our laboratory, but only works well with heat-dried slides. With air-dried and flame-dried slides, brief NaOH treatment must be used in addition to HCl and SSC. With the exception of trypsin treatment, heat at some stage is necessary for consistent G-banding. Heat is applied during incubation in the ASG [1] and Schnedl [2] technics and during slide preparation in the Giemsa 9 technic [3]. The major advantage of the SSC treatment is that the temperature can be controlled more precisely than during the heat drying procedure. The major advantage of the heat drying technic is that if heat drying is accomplished successfully then the chances of getting good banding with other banding technics are also greatly enhanced and one step (SSC incubation) can be eliminated. Some banding can be obtained with orcein or conventional Giemsa staining after heat drying, but

Table 1. *Effects of slide preparation and other factors on banding*

		G-Banding			C-Banding	
	Q-Banding	ASG	G–9	Trypsin	HCl–NaOH–SSC	HCl–SSC
Slide preparation						
Air dry	+	+	−	+	+	−
Flame dry	V	V	V	V	V	−
Heat dry	+	+	+	+	+	+
Hypotonic R_x						
Dilute Hanks	V	V	V	V		
KCl & NaCitrate	+	+	+	+	+	+
Old slides						
(Air or flame dried)[a]	+	V	V	− orV	+	
Heat dried	+	+	+	+	+	+

+, Consistent banding; V, variable; −, inadequate banding.
[a] Data on air and flame dry from literature and unpublished data.

banding is sporadic and only rarely of adequate quality to permit construction of a reliable karyotype.

Hypotonic treatment

Poor banding results are obtained for every technic when Hanks is used as the hypotonic solution.

Age of slides

A number of laboratories have had difficulty obtaining G-banding in slides that are several months or more old. Comings et al. [4] observed that older air-dried slides showed a red, rather than the usual green fluorescence with acridine orange (AO) staining and suggested that slow denaturation was occurring with time. We have confirmed this observation and found that denaturation in our preparations usually begins between 9 months and one year; most slides are satisfactory at 6 months. Moreover, denaturation clearly affects the ease of banding, morphology, and even the type of banding obtained under various conditions. For example, in some 1-year-old slides kept at room temperature, C-banding was obtained under conditions that should have produced G-banding. G-banding with both ASG and trypsin treatment was either poor or not obtainable in cells with red AO fluorescence. Q-banding is somewhat more reliable in older slides but the quality tends to deteriorate with age. Thus good Q-banding may also be difficult to obtain in older slides. Differences in slide preparation, storage conditions and local atmospheric conditions may all influence the rate of change in stored slides.

Comparison of Q-, G-, R- and C-Banding

We have been particularly interested in comparing the details of banding when different technics are used sequentially on the same cell. All of our studies begin with heat dried slides and cells which have been G-banded either by the ASG or Giemsa 9 technic. Subsequently, slides are destained, then restained for Q-, R- or C-banding after appropriate treatment. This permits us to identify each chromosome prior to C-banding and to compare banding in the same cell with several technics.

The comparison of Q- and G-bands has been carried out using the automated analysis system described elsewhere in this symposium (p. 61). Curves of G- and Q-banding of the same cell determined by this technic are shown in fig. 1. All curves are from one cell; only one chromosome of each pair is shown. Except for the Y chromosome, the banding curves are remarkably similar and the only other differences are in 1qh and 16qh, where the Giemsa banding densities are greater.

Comparison of G- and C-banding can be made with a number of combinations of technics. In the course of evaluating the trypsin technic, we observed that slides first stained for G-banding by the ASG or Giemsa 9 technic, destained, then

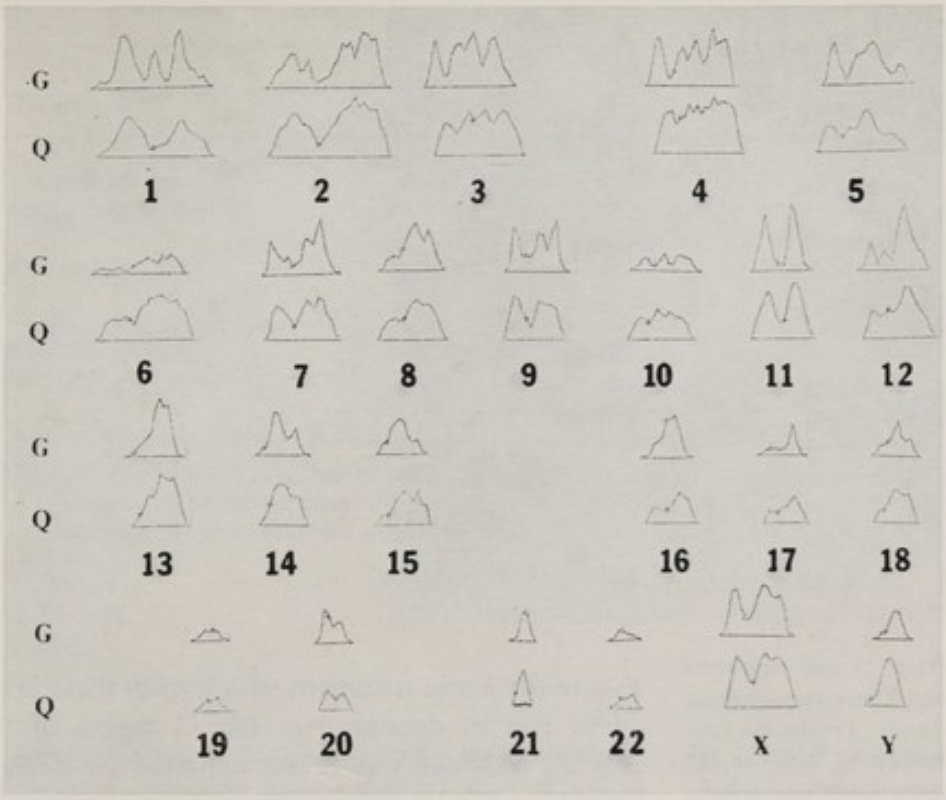
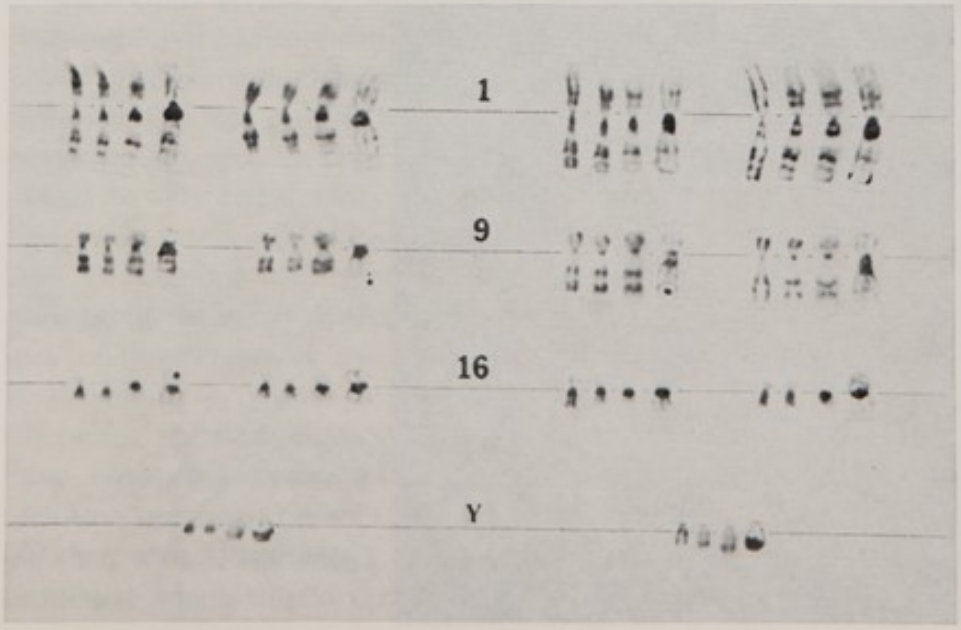


Fig. 1. Comparison of Q and G density curves in the same cell. Only one homologue is shown.

Fig. 2. Chromosomes 1, 9, 16 and Y from 2 cells, showing loss of G- and appearance of C-banding. From left to right, for each homologue, banding after Giemsa 9 staining, 4, 12 and 24 min of trypsin treatment is shown. In 1 and 16, the secondary constriction (*h*) regions and adjacent centromeres are stained darkly and the area stained does not change during the progression from G- to C-banding. In 9, the h region becomes darkly stained and the C-band includes both the h region and the darkly stained centromere. In the Y, the distal 2/3 of the long arm becomes progressively darker with increased trypsin treatment.

treated with trypsin, restained etc. for a total of 20–30 min trypsin treatment (dilute Difco trypsin, final concentration 0.025%) showed loss of G-banding, apparent destruction of the chromosome and appearance of C-bands [5]. This sequence is shown for several chromosomes in fig. 2. In chromosomes 9 and Y, the C regions gradually became larger and more prominent, whereas in 1 and 16 the C regions were essentially the same size as in the original G-banded preparation. A more quantitative study carried out by the automated banding analysis system shows



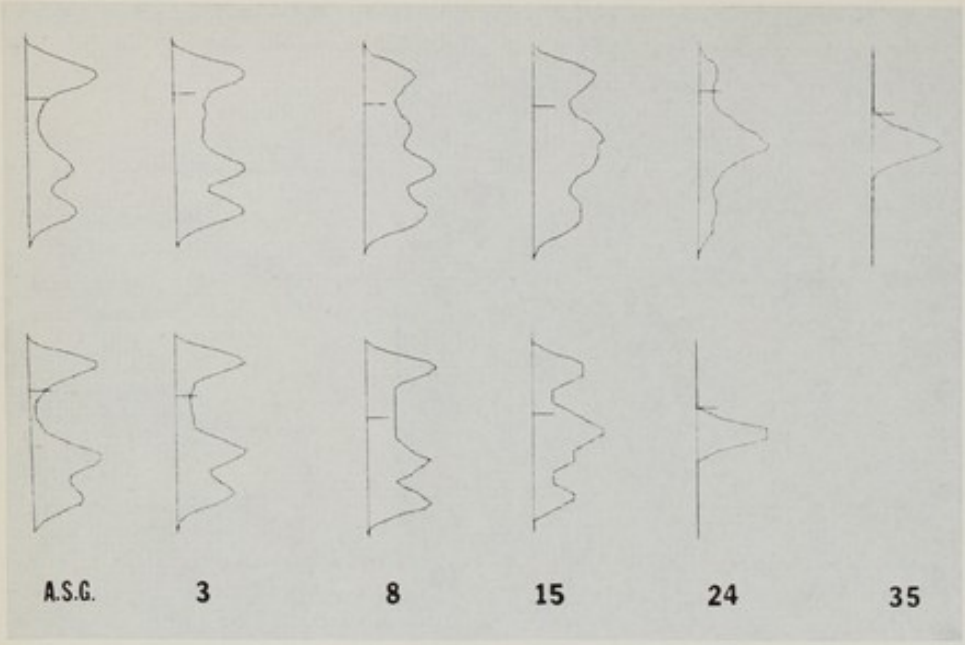


Fig. 3. Density curves obtained from 35 mm negatives for both 9s (same cell) are shown. These demonstrate loss of G- and appearance of C-bands. Treatment consisted of initial ASG-banding followed by 3, 8, 15, 24 and 35 min trypsin treatment. Even after 3 min trypsin treatment the secondary constriction (*h*) region is more intensely stained than after ASG treatment, as shown by the density curves.

Fig. 4. Dual karyotype of Q- and R-banding. A cell was first stained with quinacrine dihydrochloride, destained, incubated at 85°C for 6 min in phosphate buffer and then stained with acridine orange. The first two chromosomes in each set of four are Q-banded and the second set represents the same chromosomes with R-banding as shown by AO. In the latter pair, the bright regions showed a green fluorescence and the dim regions an orange-red fluorescence.

that in the 3 min treatment with trypsin there is a slight rise in density over the C region of 9 (fig. 3), although this is not apparent on direct inspection (fig. 2). Apparently, with even a short period of trypsin treatment there is a beginning of C-banding. This is not a major problem but those using the technic should be aware that a mixture of G- and C-banding may be obtained, and that the length of treatment should be kept to a minimum. Lastly, this unexpected result is of some interest in terms of the mechanism of the G- and C-bandings, since presumably the primary effect is on the protein of the chromosome.

It has been of interest to us to determine whether R-banding was in fact completely R

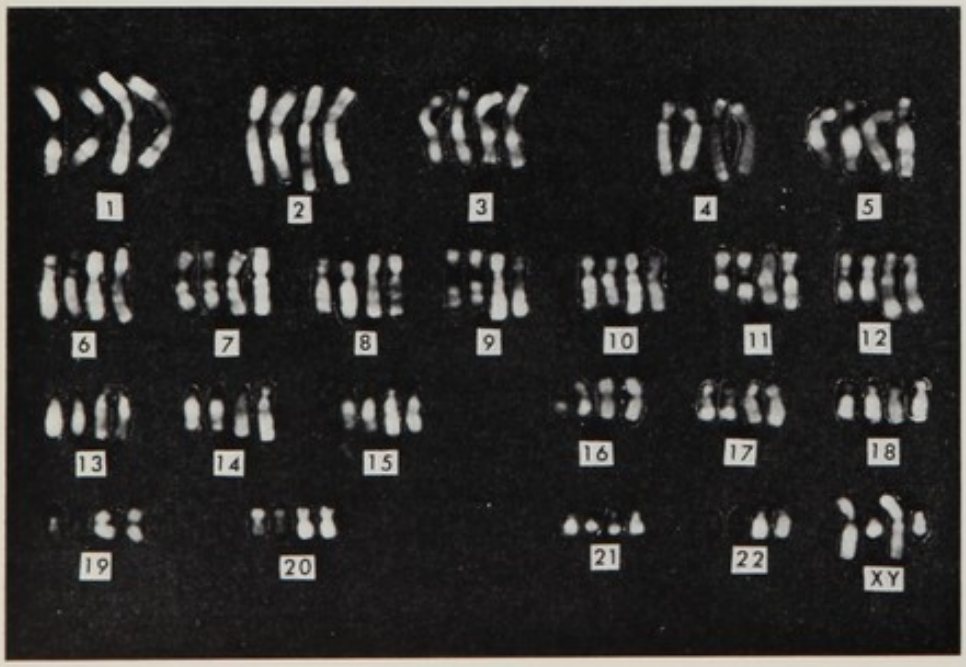


Table 2. *Acridine orange studies with and without formaldehyde—G-banding*

Treatment	Giemsa banding	AO color	Comment
Fix and air dry	−(G−9)	G	DNA denatures in methanol–acetic acid, but renatures on air drying (cooling) unless formaldehyde is added to fixative
$Fix_{(F)}$ and air dry	−	Y–O–R	
Fix and dry, 60°C	+(G–9)	G (Rare R)	DNA denatures in methanol–acetic acid, but renatures on heat drying and cooling unless formaldehyde is added to fixative
$Fix_{(F)}$ and heat dry	—	Y–O–R	
SSC 1 h at 60°C	+(ASG)	G	SSC and heat also denature DNA, but DNA renatures on cooling unless formaldehyde is added at end of incubation
$SSC_{(F)}$ 1 h at 60°C	—	O–R	

(F), Formaldehyde 4% added to indicated solution at end of incubation or time in fix.
+, Same cell Giemsa banding.
−, Not applicable or data not available.
G, Green.
Y, Yellow.
O, Orange.
R, Red.

or only largely R in relation to G- or Q-banding. We have obtained better definition of R-bands with AO staining than with Giemsa. There have been no difficulties in obtaining R-banding after Q- or G-banding. Of particular interest is the color differential along the length of the chromosome with acridine orange staining after R-treatment. The negative R regions (as defined by Giemsa staining) are red, the positive R regions are green. A dual karyotype of a Q-banded cell which was subsequently R-banded and stained with AO is shown in fig. 4. A dual karyotype which included R-banding has not been previously reported. Comparison with the Q-band banding patterns is complicated by the swelling which accompanies the R treatment. A preliminary review of cells in which either R- and Q- or R- and G-banding were employed has confirmed that R-banding is indeed reverse, with several minor exceptions, including the 9qh region. The weakly staining region in 9qh, seen by both Q- and G-banding, should be very bright with AO in R-banding if completely reverse; this is not the case, and the level of staining with R-banding is medium throughout the initial third of 9q. Similarly, the level of fluorescence of the distal Yq with AO in R-banding is greater than expected. As in C-banding, therefore, 9qh and Yq are exceptional. Does R-banding change arm ratios? If certain telomeres are stained more heavily with R-banding than with G-banding, a different arm ratio or relative length should result with R-banding. In certain chromosomes this is indeed the case; chromosomes with deeply staining telomeres generally show the greatest apparent increase in length. This is most marked in the short arms of 5, 7, 9, 13 and 17 and long arms of 7, 10 and 14. These are only preliminary impressions and we plan to do measurement studies to confirm these observations. In addition, a comparison of arm lengths between conventional Orcein- and R-banding needs to be carried out.

Observations on Mechanism of Banding

Our observations with AO on human lymphocytes with and without addition of formaldehyde at various treatment steps are summarized in table 2. Only by adding formaldehyde can it be shown that methyl-acetic acid fixation and SSC incubation are in fact denaturing, since both slide preparation and cooling ordinarily lead to renaturation. These results are similar to those of Comings et al. [4] in other species. In respect to Giemsa banding, we have confirmed the results of de la Chapelle et al. [6] in that the usual preparations for G-banding are double stranded, whether prepared by heat drying or air drying. Treatment with buffer at pH 9.0 for 6–10 min, similar to that done in the Giemsa 9 staining procedure, does not alter strandedness. Therefore, denaturation and differential renaturation is not the explanation for Giemsa banding. Whether the heat drying we employ effects the

Table 3. *Acridine orange studies with and without formaldehyde—C-banding*

Treatment	C-banding with Giemsa	C-banding with AO	AO color	Comment
HCl+(NaOH)*+24 HR. SSC	+	+	G	C-banding with renatured DNA
HCl+NaOH+24 HR. $SSC_{(F)}$	+	+	R	C-banding with denatured DNA

(F) Formaldehyde 4% added at end of SSC incubation.
+, Same cell C-banded.
[a] With or without NaOH.

denaturation or renaturation state cannot be determined, since renaturation occurs rapidly when slides are made with or without heat drying. In the ASG technic there are in fact 2 cycles of denaturation–renaturation: one during fixation and slide preparation and one with SSC incubation and cooling. In relation to C-banding, the critical point is that since chromosomes can be prepared which show C-banding and have either single-stranded or double-stranded DNA (table 3), neither denaturation nor differential renaturation can be the primary basis for the differential staining.

Discussion

What then are the mechanisms involved in the several types of banding? Denaturation-renaturation is not the primary mechanism by which C-banding is produced. Preferential loss of chromatin in the non-C regions, however, does provide an adequate explanation. Sodium hydroxide treatment, prolonged trypsin treatment, and prolonged SSC incubation all appear to produce significant chromosome destruction as visualized through the microscope. Moreover, the increase in time necessary to stain non-C regions after these treatments suggests loss of material. The data of Comings et al. [4], however, provide the best evidence of greater loss of both DNA and protein in the non-C regions than in the C regions. Their evidence for an additional component of DNA-protein interaction is also convincing.

In respect to G-banding, there is little evidence of loss of material [4]; differential renaturation is not the explanation. All of the various treatments devised for Giemsa-banding include heat at about 65°C, alkali or trypsin treatment. Both because of the negative results in studies with respect to renaturation of DNA and the nature of the treatments themselves an important role for proteins is likely. It should be recalled that Giemsa-banding involves a threshold or differential affinity for Giemsa [3]; by prolonging the staining time, an even Giemsa stain can be obtained, i.e. the threshold can easily be overcome. Heat, staining at pH 9.0 and trypsin, therefore, might alter this threshold by an effect on a DNA-protein interaction or on a certain class of proteins themselves.

At least 3 related factors may play a role in R-banding. (1) As discussed above, it is the only banding technic in which there is evidence that differential renaturation occurs. (2) Miller (p. 43] has shown that R-positive bands are GC-rich. GC-rich regions are predictably more likely to renature, hence these two observations are probably related. Whether either of these observations point to the primary mechanism of R-banding is not yet clear. (3) It is possible that differences exist in proteins overlying GC- or AT-rich regions and that these proteins respond to heating at 85°C in such a way as to alter the affinity or availability to Giemsa.

Q-banding is influenced least of all by the variables discussed above. The intensity of staining is directly related to AT-rich regions [7]. Miller's work (p. 43], has confirmed this finding. Finally, the findings reported by Bram [10] that there is a significant difference in secondary configuration of GC- and AT-rich DNA suggests that many of the effects discussed above may be closely interrelated, since base differences could affect the composition of overlying proteins.

Special Merits of the Several Technics

What then are the special merits and limitations of the various technics? To begin with, there remains a place for conventional staining (fig. 5).

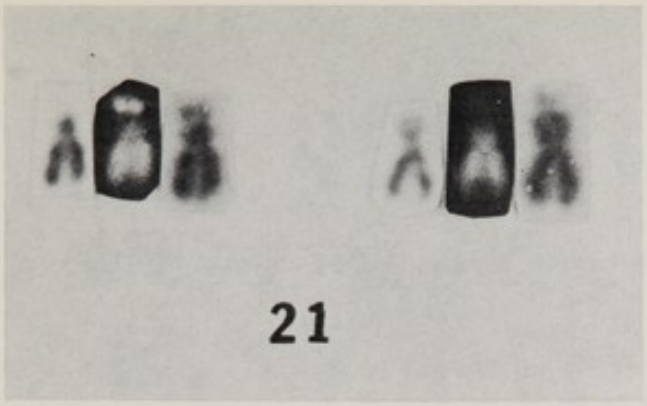


Fig. 5. Triple karyotype of both 21s from one cell stained first for G-banding, destained, stained for Q-banding, destained, and finally stained with orcein. In the chromosome to the left, the satellite can only be seen faintly with Giemsa banding. Its homologue to the right has longer short arms with orcein staining than with quinacrine and the satellite is not seen with quinacrine.

Most significantly, no single technic shows all features of the short arms and satellites. Optimal description of the karyotype is obtained only by use of multiple technics, preferably on the same cell. The choice of methods depend on the question being asked.

Currently, the best starting point for a cytogenetic study appears to be G-banding, although it may turn out, with more experience, that R-banding is even more useful. Many laboratories have obtained good G-banding with trypsin treatment. As discussed above, trypsin must be used with care or a combination of G- and C-banding will be obtained. The ASG technic has been widely used and the major strength of the technic, I believe, lies in the controlled method of heating the slides. Although this technic does not band all cells and all cultures satisfactorily, we have obtained two reasonably well banded cells in 291 of 300 newborns in a survey now in progress using ASG. The Giemsa 9 method has proven quite difficult to reproduce in many laboratories, probably because we did not initially realize or emphasize the importance of the heat drying step for successful Giemsa-banding with this technic. Its chief merit is simplicity. Moreover, if slides are prepared in the fashion described above, the chances of getting good banding with any technic are greatly enhanced. We have not had experience with the technic of Schnedl [2] since the simpler Giemsa-banding technics have proven effective in our laboratory. The major hazard would appear to be the chromosome destruction that occurs with NaOH treatment; if banding has proven difficult, however, this may be an asset. One of the major problems with the routine use of G-banding is the poor definition of details in the short arms of the D and G chromosomes and other weakly staining areas. In one of our current surveys of newborns we are finding only about half as many variants in D and G short arms with Giemsa-banding as we did in previous studies using orcein staining. After hearing the impressive reports on R-banding (pp. 16, 38, 205, 214) we will try a series with R-banding to see if it provides practical answer to this problem.

The C-banding technics are all relatively easy to reproduce, but it is critical to control pH precisely. We found that it was necessary to maintain pH close to 7.0 during the overnight SSC incubation. At pH 6.0 only Giemsa-banding was obtained; at pH 8.0 too much destruction of chromosomes occurred; only at pH 7.0 did optimal C-banding occur. The more slides that are included per coplin jar, the more alkaline the SSC solution becomes over a long incubation. It is often necessary to replace the solution with with fresh 2XSSC at pH 7.0 for the last 4–6 h of incubation. With more initial heat (i.e. slide preparation), less vigorous treatment can be used for C-banding. The technic of Arrighi & Hsu [9] is probably the most generally used C-banding technic, but NaOH exposure must be adjusted to the total preparative conditions. It is important to identify each chromosome before C-banding. Without dual (G–C) karyotypes, almost 3/4 of the chromosomes cannot be studied since C-banding does not permit identification of many similar sized chromosomes. In population studies and gene localization work, constructing dual karyotypes is particularly important; with this approach we have found an average of 4 C-band polymorphisms per infant. Moreover, these are distributed throughout the karyotype: polymorphisms were found in every chromosome. Examples of certain of these are shown in fig. 6.

Q-banding, of course, remains a tremendously useful technic. It is perhaps the best technic for use with old slides and offers the opportunity of identifying certain polymorphisms that cannot be identified by any other technic. We average

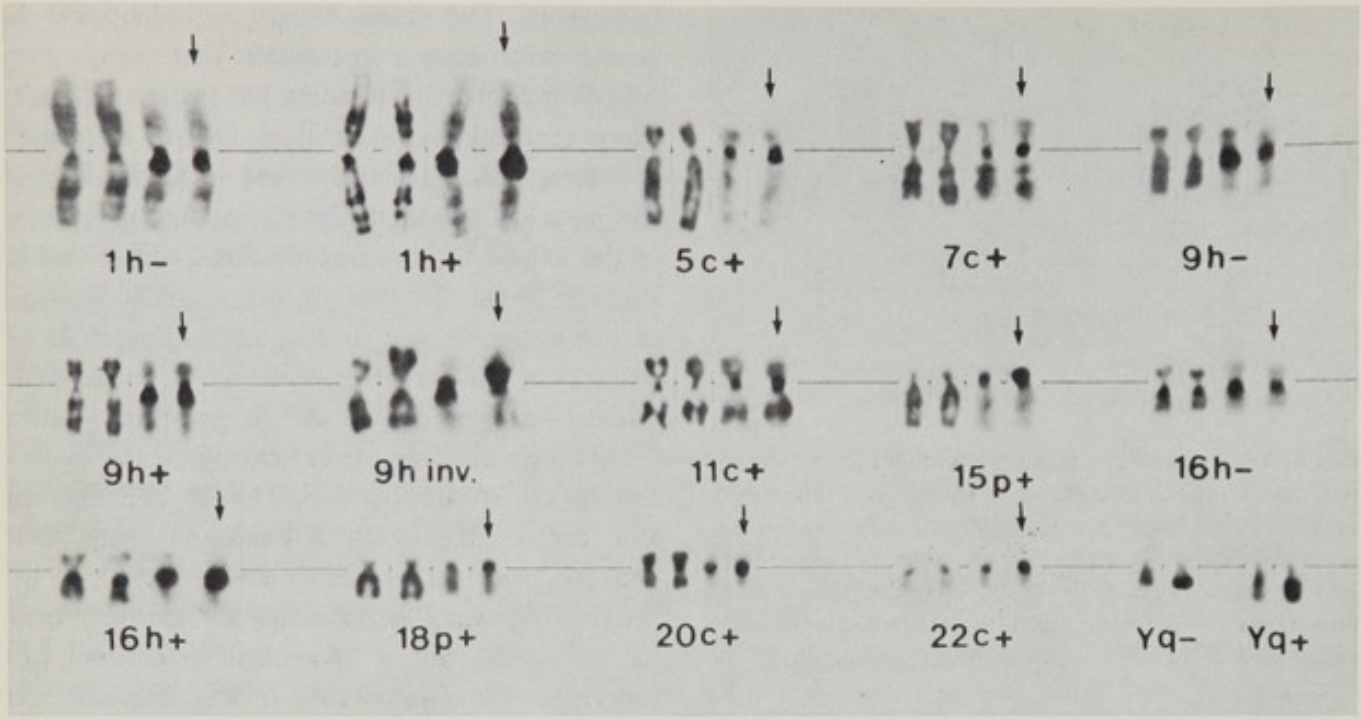


Fig. 6. Representative C polymorphisms found in a series of 50 newborns from a study carried out by W. McKenzie in Denver. Each pair of chromosomes is from a different person; dual karyotypes were prepared first by G-banding (first 2 chromosomes in each set of 4) and subsequent C-banding of the same cell (3rd and 4th chromosomes in each set of 4). C-regions in both G- and C-banded cells can be compared easily and each C-banded chromosome identified. The polymorphic C-bands are shown to the far right in each set of 4. A 50% difference in area from the norm was used to define a C-polymorphism.

3 per infant, or a total of 7 C- and Q-polymorphisms per infant. R-banding has several distinct advantages although it is still difficult to reproduce routinely. It is particularly helpful in defining the ends of arms and should increase precision in detecting and defining translocations, or possible translocations, that occur distally. Moreover, with R-banding it can be determined whether certain assumed reciprocal translocations are in fact reciprocal or whether there has been a transfer of material from only one chromosome to the other.

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Discussion

Lubs: (Response to Dr Hsu.) SSC treatment at 60–65°C is denaturing for metaphase chromosomes on slides, as shown by the red fluorescence when formaldehyde is added at the end of the incubation. Without the addition of formaldehyde, i.e. under usual conditions, renaturation occurs both at the time of slide preparation and on cooling after SSC incubation.

Comings: The denaturation temperature of fixed metaphase chromosomes may be different from that for DNA in vitro. For example, 2 × SSC at 60°C completely denatures DNA in situ. The time factor may be important.

Pearson: Do the fluorescent satellites on the No. 21 chromosomes you showed not stain up with the C-banding?

Lubs: The C-banding of intensely fluorescing QM material is unpredictable—it may be either positive or negative, although usually positive.

Fluorescent Banding Agents

E. J. Modest

The Children's Cancer Research Foundation and Harvard Medical School, Boston, Mass. 02115, USA

Research on chromosome banding by fluorescence originated from the development by Torbjörn Caspersson and colleagues at the Karolinska Institute in Stockholm of high-resolution, rapid-scanning, ultramicrospectrophotometric techniques for the analysis of cellular components, including chromosomes and chromosomal regions [1, 2]. With this instrumentation the distribution of DNA along large plant and mammalian metaphase chromosomes can be measured with sufficient accuracy for preliminary identification purposes [3].

With the design of similar high-resolution ultramicrofluorometric instrumentation, whose increased sensitivity permits determination of DNA down to a theoretical level of 10^{-16} g of DNA or approx. 200 genes, Caspersson conceived the idea that the selective fluorescent staining of various chromosomal regions might be possible with an appropriate fluorescent agent that would bind selectively to bases in chromosomal DNA [4]. Through a collaboration with Caspersson started by Sidney Farber and George Foley of The Children's Cancer Research Foundation in Boston, a program was initiated to design or choose DNA-base specific fluorochromes that might produce selective fluorescence in chromosomal DNA.

The first proposal by Caspersson was to design and synthesize fluorescent analogs of actinomycin D, known to be DNA-guanine specificl [5]. While the actinomycin chemistry was being worked out, I selected as our first agent quinacrine mustard (fig. 1) [6] a nitrogen mustard analog of the antimalarial quinacrine, on the basis that this compound would satisfy our initial requirements: namely, a highly fluorescent aminoacridine nucleus that can intercalate into bihelical DNA; basic nitrogen atoms that can form ionic bonds with DNA-phosphate; and an alkylating group in the side chain capable of forming covalent bonds with DNA-guanine.

Exposure of a fixed metaphase plate of *Vicia faba* chromosomes to a solution of quinacrine mustard in buffer resulted in dramatic confirmation of the thesis: there are three intensely fluorescent regions near the centromere of the large *Vicia* M metaphase chromosome [4, 7, (cf p. 28, fig. 1). Even more interesting was the observation that these bands agreed in location with that of heterochromatin (cold treatment and alkylating agent breakage) and furthermore did not represent merely locally intense concentrations of DNA, since a DNA scan of the chromosome by ultramicrospectrophotometry did not reveal such a correlation, as indicated by the arrows in fig. 2 marking the fluorescent bands [7].

Other alkylating fluorochromes, including aminoacridine monofunctional nitrogen mustards and a bisbenzimidazole monofunctional nitrogen mustard (fig. 3), were found to produce similar fluorescent banding in *Vicia* chromosomes [7]. Non-alkylating fluorochromes (figs 3, 4), in particular quinacrine, proflavine, and acriflavine, also gave fluorescent banding in plant chromosomes [7, 8]. Quinacrine produces fluorescent banding in *Vicia* similar to that produced by quinacrine mustard, but the quinacrine banding is less intense and less stable to continued ultraviolet irradiation [7]. The superiority of quinacrine mustard as a fluorochrome has been reported [9]. Acridine orange was essentially negative for fluorescent banding in plant chromosomes, giving only occasional and variable banding in *Trillium erectum* [8]. Ethidium bromide, surprisingly, produced reverse banding in plant chromosomes, notably in *Scilla sibirica* [8].

The observation that each chromosome in a

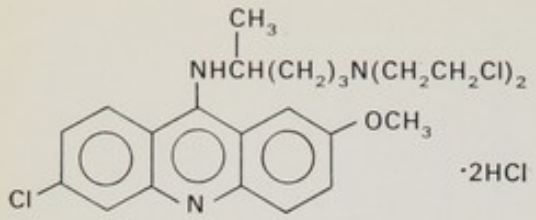


Fig. 1. Structure of quinacrine mustard dihydrochloride (QM, FLU 1)

quinacrine mustard-stained *Vicia* metaphase plate had an individual and characteristic pattern led Caspersson and Lore Zech first to the discovery that the distal end of the long arm of human chromosome Y is intensely fluorescent [10] and then to the observation that each human metaphase chromosome has a characteristic and reproducible quinacrine mustard-banding pattern, both fluorometrically and visually [11–14]. The quinacrine mustard method is now the primary international identification standard for human chromosomes (24 human chromosome types: 22 autosomes and 2 sex chromosomes) [15].

Except for minor variations caused by unfavorable (excessive or minimal) contraction, the fluorescence patterns in human chromosomes are entirely reproducible. The brilliant fluorescent regions first seen on the Y chromosome [10] and later identified on seven other chromosomes (3, 4, 13, 14, 15, 21, and 22) [14] are observed with varying degrees of frequency from individual to individual and are clearly different from the characteristic faint banding patterns seen on all human chromosomes after quinacrine mustard treatment. Thus, the quinacrine mustard fluorescence method provides *two* kinds of information when applied to human metaphase chromosomes [11–14]: (*A*) faint, reproducible *identification* banding (present in all normal metaphase chromosomes of all individuals); (*B*) intense, variable *polymorphic* staining (consistent in certain metaphase chromosomes of a given individual but variable from person to person).

The fluorescence technique with quinacrine mustard or quinacrine has already been extensively applied to a number of problems in human genetics, principally by Caspersson and collaborators and also by many other investigators [14]. These areas include detection and analysis of chromosome anomalies (including antenatal chromosome analysis by amniocentesis) [14], study of translocations [14], investigation of abnormal or marker chromosomes in neoplasia [14, 16], applications to analysis of somatic cell hybrids and human cell lines [14, 17], identification of the Y chromosome in interphase and in spermatozoa [14], and the analysis of human male meiotic chromosomes [14]. Technological advances have also been made in computer recognition and television analysis of quinacrine mustard-banded human metaphase chromosomes [14].

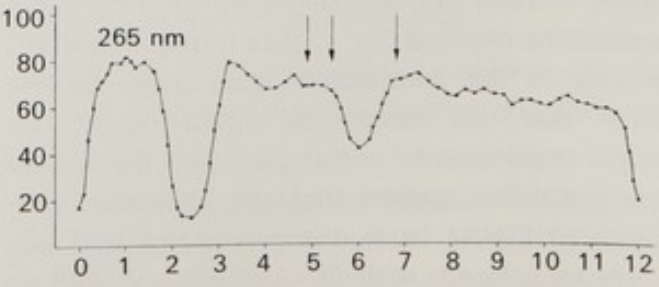
Research on newer fluorochromes for chromosome analysis continues. The original concept of the design of fluorescent actinomycin D analogs has led recently to our synthesis of 7-aminoactinomycin D (FLU 402), a fluorochrome which retains the DNA-binding properties of the parent antibiotic and which exhibits DNA-guanine binding specificity [18]. Other recent methods of promise involve the use of fluorescent antibodies to chromosomal protein [19] or nucleic acid components [20, 21] as differentially staining agents. Some of these newer methods may prove to be useful complements to the quinacrine mustard technique. Reports on other new fluorochromes and on newer techniques with existing fluorochromes are given elsewhere in this Symposium.

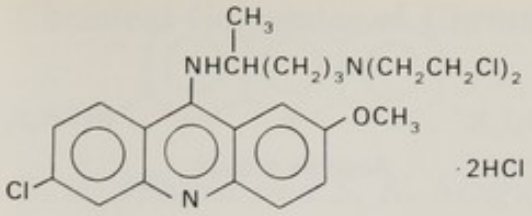
We are currently engaged in studies on mechanisms of binding and banding of fluorochromes in chromosomal DNA [18, 22, 23], particularly in light of the influence of DNA-base specific quenching and enhancement phenomena [24–26].

In conclusion, the quinacrine mustard methodology has in a very short time become the primary standard for human chromosome identi-

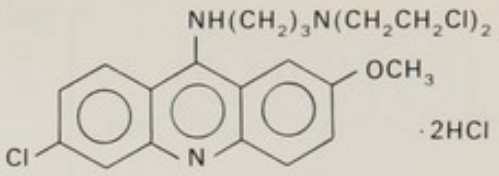
Fig. 2. *Abscissa:* relative length; *ordinate:* relative DNA absorption.

DNA distribution of *Vicia faba* M chromosome measured by ultraviolet scanning at 265 nm. Arrows indicate most intense QM bands and minima represent chromosome constrictions [7].

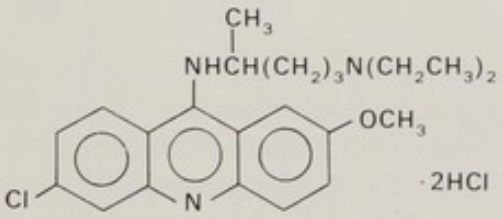




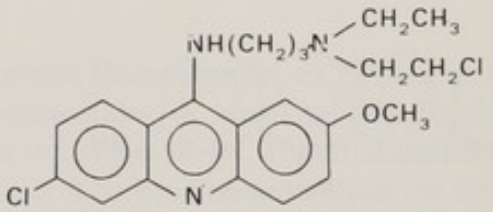
Quinacrine mustard
dihydrochloride (QM, FLU 1)



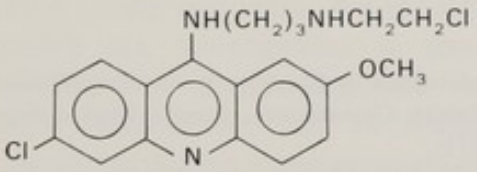
Propyl quinacrine mustard
dihydrochloride (PQM, FLU 2)



Quinacrine dihydrochloride (Q, FLU 6)



ICR 170 (FLU 21)



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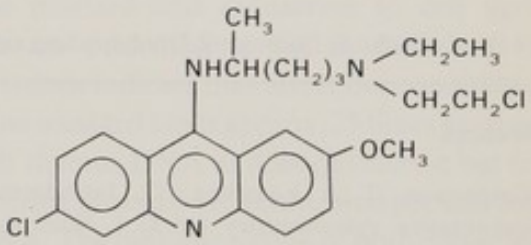

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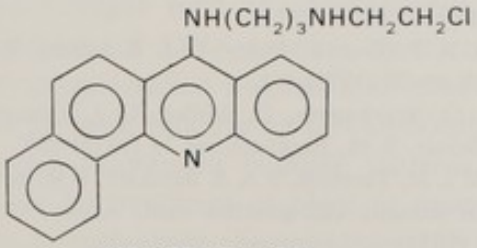
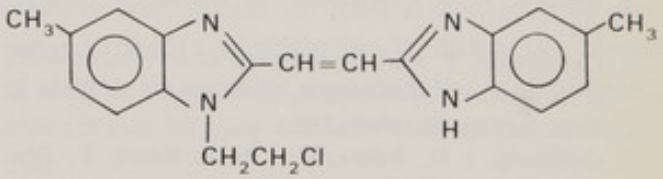


ICR 370 (FLU 24)



CIMDBE (FLU 25)

Fig. 3. Initially employed fluorescent DNA-binding agents [7].

fication, has already solved a number of problems in human genetics, and has stimulated the development of other techniques that provide complementary information in chromosome analysis. This approach will afford new information on the ultrastructure of the metaphase chromosome. The goal is chromosome mapping by fluorescence, through the development of a spectrum of selectively binding fluorochromes (both synthetic fluorochromes and immunofluorescent agents) for the visualization, analysis, and identification of a variety of chromosomal sites.

The work in Boston was supported by research grant C6516 and research career development award K3-CA-22,151 from NCI, NIH, USPHS, Bethesda, Md, USA. The work in Stockholm has been indicated by appropriate references. We acknowledge with appreciation our valuable collaboration with Dr Caspersson and his associates.

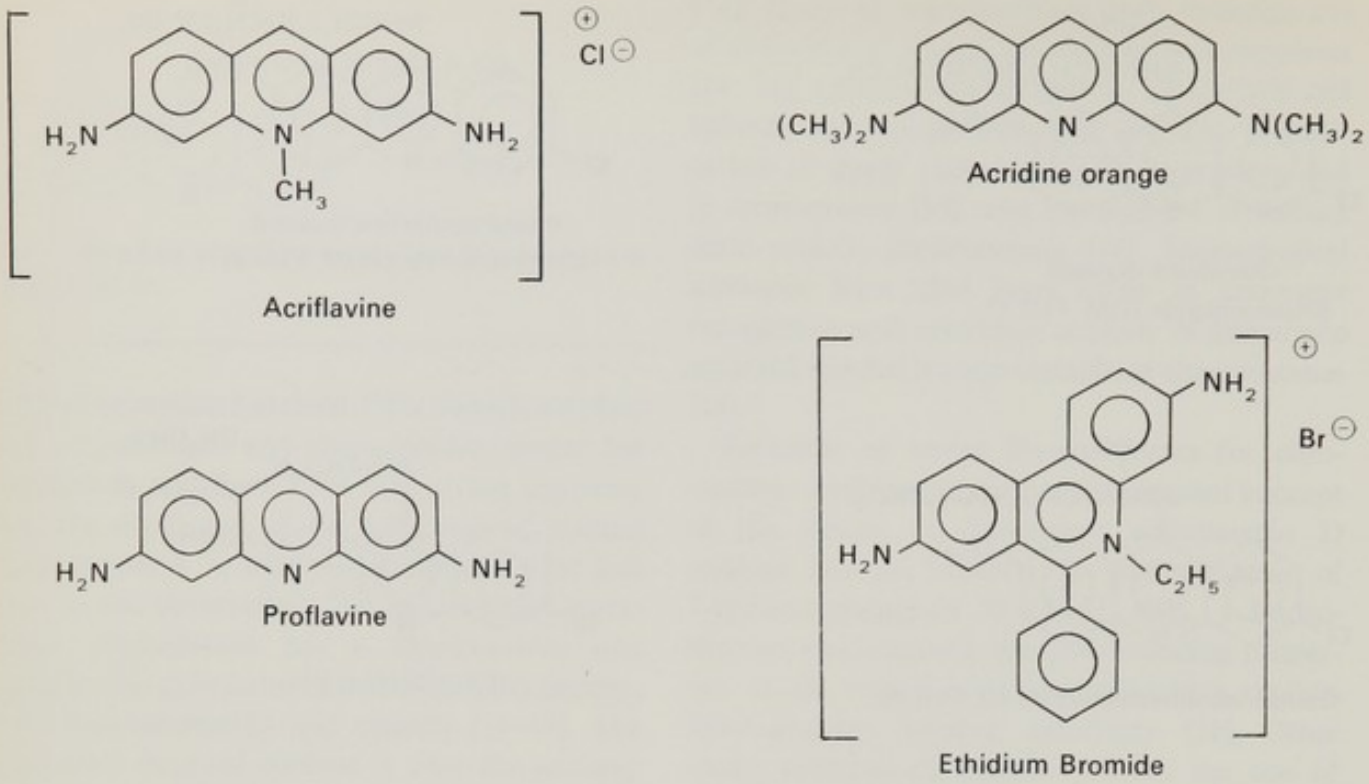


Fig. 4. Non-alkylating fluorescent DNA-binding agents.

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Chemical Correlates of Chromosome Banding

E. J. Modest and S. K. Sengupta

The Children's Cancer Research Foundation and Harvard Medical School, Boston, Mass. 02115, USA

Introduction of the technology of fluorescence labeling of chromosomes by quinacrine mustard and other DNA-binding fluorochromes through our collaboration with T. Caspersson and colleagues has become a powerful technique for the identification and analysis of chromosomes and chromosome regions [1–6]. Explanation of the mechanisms of fluorochrome banding and binding is clearly important. We should like now to outline our investigations in these areas, to correlate our observations with the results of other investigators, and to attempt to draw general correlative conclusions on the nature of banding in chromosomes.

Our first choice of a selective chromosome banding agent was quinacrine mustard (fig. 1*a*) [7] on the basis that this highly fluorescent compound might bind to chromosomal DNA by alkylation of DNA-guanine, by intercalation of the aminoacridine nucleus into bihelical DNA, and by ionic binding to DNA-phosphate [1, 2]. Unique fluorescent banding patterns were observed by treatment of plant or mammalian or human metaphase chromosomes with quinacrine mustard. The analogous antimalarial quinacrine (fig. 1*b*), which has the same fluorescence, intercalatory, and ionic binding capabilities but cannot form covalent bonds since it has no alkylating group, gave qualitatively similar although quantitatively less efficient fluorescent banding in both plant and human metaphase chromosomes [5, 8]. These empirical observations indicated even before mechanistic studies were undertaken that the primary mode of binding for both compounds is probably by intercalation of the aminoacridine nucleus into bihelical DNA, and that alkylation of DNA-guanine by quinacrine mustard must be a secondary binding mode.

We have measured the binding properties of several fluorochromes to DNA and to other polynucleotides in vitro by ultrafiltration and other techniques [8–10]. The ultrafiltration studies, equivalent to equilibrium dialysis for determination of apparent binding constants and apparent nucleotide:ligand ratios, were done with a Paulus ultrafiltration apparatus (fig. 2) [11].

Ultrafiltration data for the binding of quinacrine mustard and quinacrine to calf thymus DNA are presented in fig. 3. Scatchard plot calculations indicate that under our condition quinacrine mustard binds approx. 25 times as strongly to calf thymus DNA as does quinacrine but fewer quinacrine mustard molecules bind per unit length of DNA. The biphasic nature of both curves in fig. 3 suggests more than one mode of binding for each compound. The similarity of the two curves in fig. 3 indicates strongly that the primary binding mechanism of both compounds is the same, probably intercalation. If the binding mode of quinacrine mustard were exclusively alkylation, equilibrium binding curves would not be measurable.

In addition to these studies, we have observed that quinacrine shows no base specificity when bound to calf thymus DNA or poly d(AT), in contrast to quinacrine mustard, which, under our conditions, binds only to guanine-containing polynucleotides in bihelical configuration. No DNA–base binding specificity for quinacrine was found by Hahn [12], who used spectral shift techniques. Furthermore, the binding of quinacrine mustard, in contrast to that of quinacrine, is relatively irreversible: ethanol or dilute acid under mild conditions will extract quinacrine but not quinacrine mustard from the acridine–DNA complex. Hydrochloric acid under forcing conditions is required to disrupt the quinacrine mustard–DNA complex. Neither fluorochrome

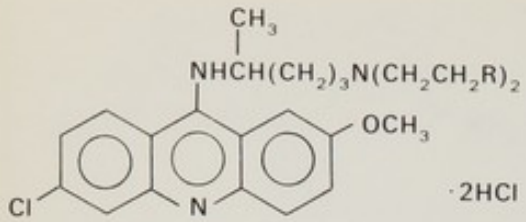


Fig. 1. Structure of (*a*) quinacrine mustard dihydrochloride (QM) R, Cl; (*b*) quinacrine dihydrochloride (Q) R, H.

binds significantly to calf thymus histone, but both bind to calf thymus nucleohistone.

Rigler [13, 14] and Weisblum [15] have recently described an apparent enhancement of fluorescence of certain aminoacridines (including quinacrine, quinacrine mustard, and proflavine) when bound to dAT base pairs and a quenching of fluorescence for these fluorochromes by guanine-containing polynucleotides, based on studies in solution. We have confirmed and extended these observations [10].

It was reported that the fluorescence of acriflavine when complexed with DNA is quenched [16] and then that proflavine or acriflavine fluorescence is selectively quenched at polynucleotide guanine sites but not at AT base pairs [17–21]. Furthermore, it was postulated that DNA-guanine quenches by a charge transfer complex with the aminoacridine [17, 21] and that the base pair sequence at the binding site has

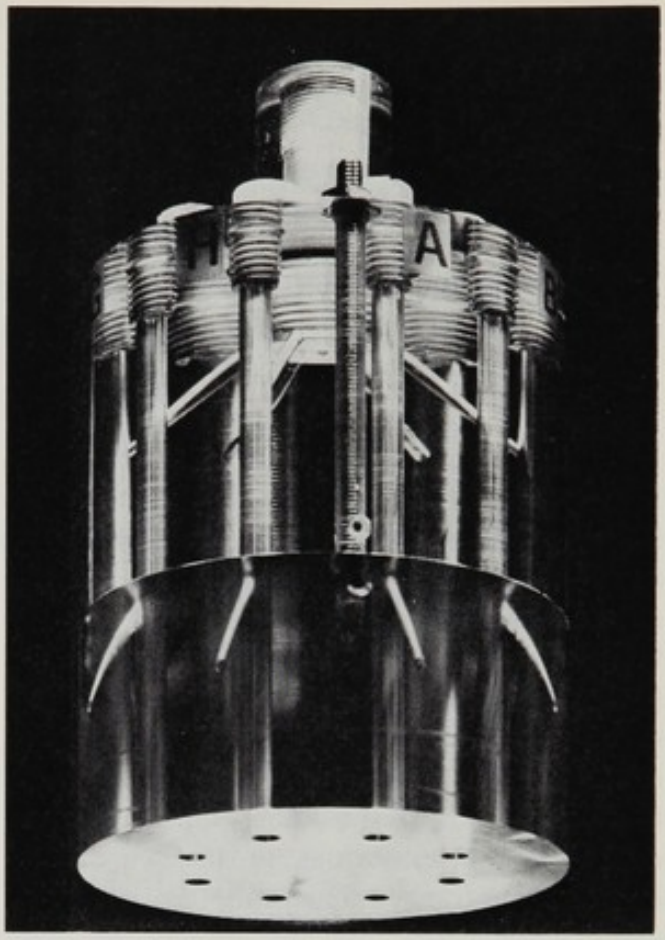
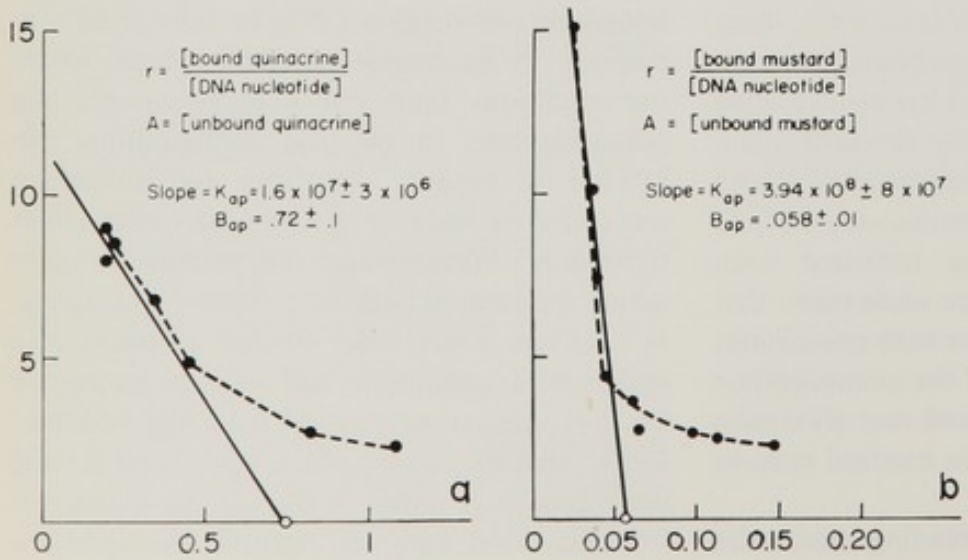


Fig. 2. Paulus ultrafiltration apparatus [11].

a strong influence on both binding and fluorescence efficiency [17, 21]. At least two adjacent AT base pairs seem to be necessary for aminoacridine fluorescence [21]. In recent work, these studies are being extended to quinacrine and other fluorochromes and to the mechanism of fluorescent banding in the chromosome [10, 13–15]. Rigler [14] has used fluorescence polarization to confirm binding of pro-

Fig. 3. *Abscissa:* r; *ordinate:* $r/A \times 10^6$.

Scatchard plots (by ultrafiltration) of binding of (*a*) quinacrine (Q) and (*b*) quinacrine mustard (QM) to calf thymus DNA in 0.01 M phosphate buffer at pH 7. K_{ap}, apparent binding constant in moles^{-1}; B_{ap}, apparent number of binding sites/nucleotide.



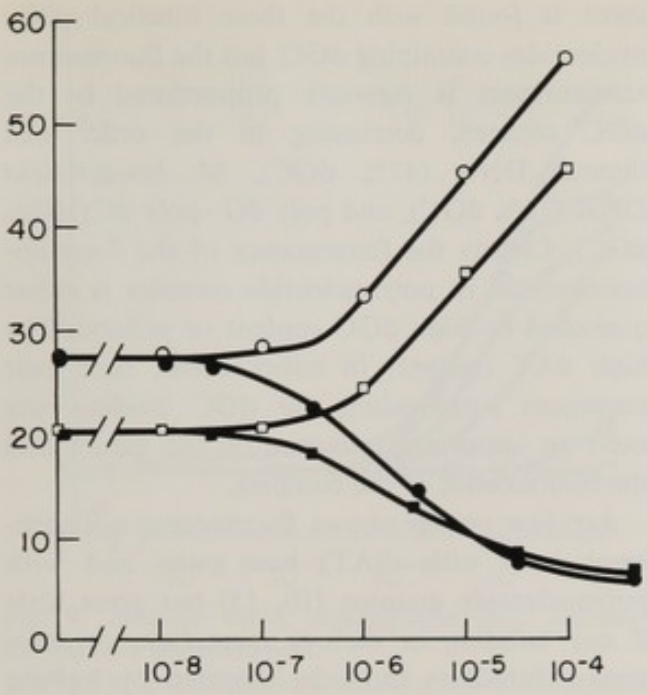


Fig. 4. *Abscissa:* DNA concentration (M); *ordinate:* fluorescence units (500 nm).

Effect of polynucleotides on quinacrine mustard (QM) and quinacrine (Q) fluorescence in 0.1 M phosphate buffer at pH 6.8 (QM and Q, 2×10^{-7} M). Excitation wavelengths 415 nm for QM and 430 nm for Q. ■—■, QM–calf thymus DNA; □—□, QM–poly d(AT); ●—●, Q–calf thymus DNA; ○—○, Q–poly d(AT).

flavine or quinacrine to polynucleotides in solution fluorescence measurements.

Fig. 4 indicates graphically changes in relative fluorescence intensity for quinacrine mustard and quinacrine at a fixed concentration in the presence of increasing concentrations of calf thymus DNA or poly d(AT). In agreement with the published data, quenching of fluorescence is observed with calf thymus DNA and enhancement with poly d(AT). The reduced quenching for quinacrine mustard/calf thymus DNA may reflect some alkylation of DNA-guanine with consequent reduction in the efficiency of charge transfer complex formation.

Although quinacrine mustard was the first fluorochrome chosen for this program, our first discussions centered on designing a fluorescent analog of actinomycin D. In order to preserve the DNA-binding capacity and DNA–guanine specificity of the molecule in the process of introducing a fluorescence, enhancing group, we deduced on the basis of published information [22, 23] that substitution could be introduced at position 7 (and perhaps at position 8) of the

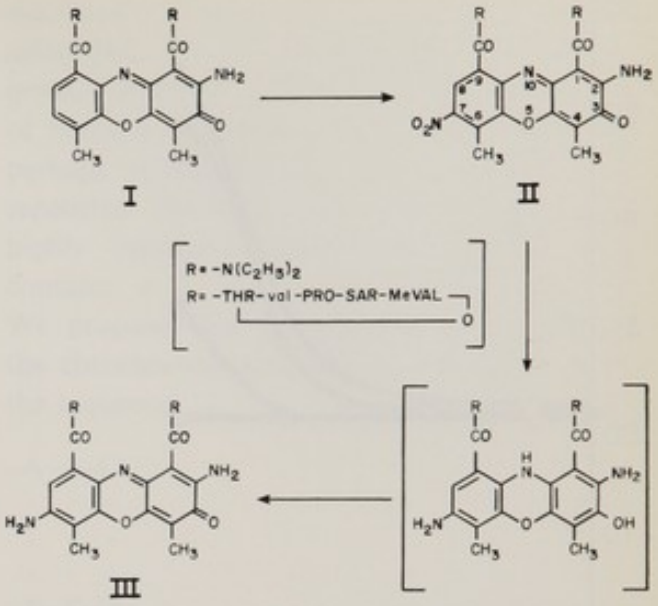
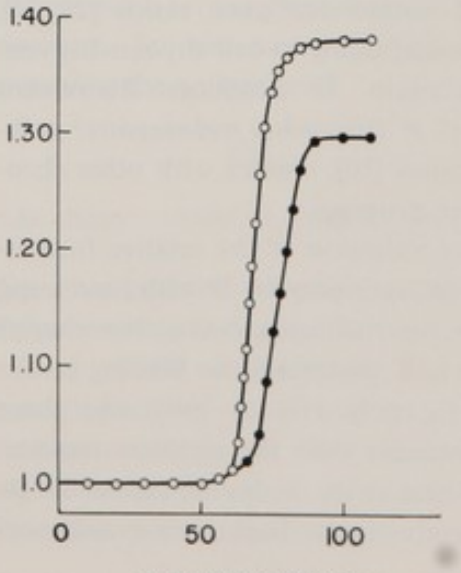


Fig. 5. Synthesis of the model compound 7-aminoactinocyl bis(diethylamide) (7-amino ACT–DEA) III : R, $N(C_2H_5)_2$ and 7-aminoactinomycin D (FLU 402), III : R, pentapeptide lactone.

phenoxazinone chromophore without endangering the selective binding properties of the molecule. We decided to introduce an amino group at position 7 [24], since such an electron-donating group can enhance both the fluorescence and absorption properties of a conjugated aromatic ring system [25]. After appropriate chemical studies with model compounds, 7-aminoactinomycin D was synthesized via direct nitration of

Fig. 6. *Abscissa:* temperature (°C); *ordinate:* hyperchromicity (260 nm).

Thermal denaturation profile of calf thymus DNA in the absence (○—○) and in the presence (●—●) of actinomycin D (AMD) or 7-aminoactinomycin D (FLU 402) (coincident curves). Measurements were done in 0.01 M phosphate buffer at pH 7.0.



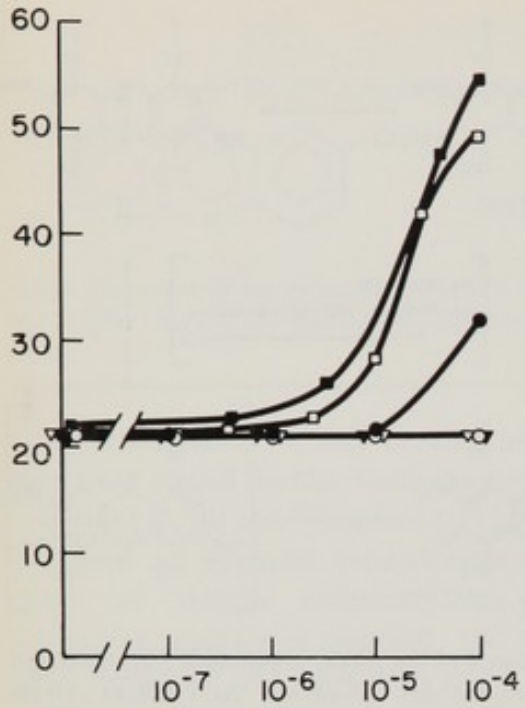


Fig. 7. *Abscissa:* DNA concentration (M); *ordinate:* fluorescence units (595 nm).

Effect of polynucleotides on 7-aminoactinomycin D (FLU 402, 5×10^{-6} M) fluorescence in 0.1 M phosphate buffer at pH 6.8. Excitation wavelength 540 nm. ■—■, calf thymus DNA (42% dGC); □—□, *Micrococcus lysodeikticus* DNA (73% dGC); ●—●, poly dG·poly dC (100% dGC); ○—○, poly d(AT); ▼—▼, poly rG; ▽—▽, poly rA.

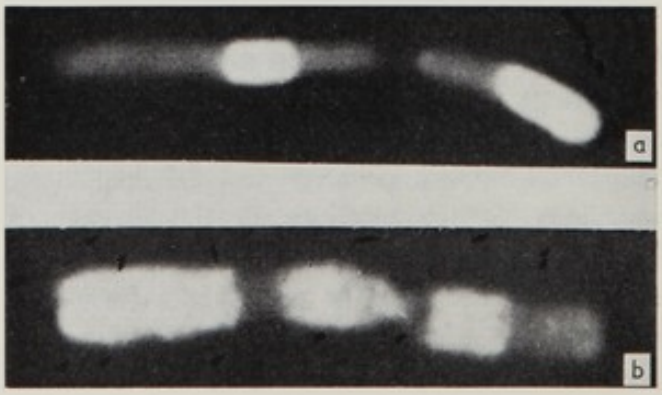
actinomycin D, followed by reduction (fig. 5). This compound binds to bihelical DNA with guanine specificity in the same way as actinomycin D and exhibits strong fluorescence in the red. Melting temperature curves for 7-aminoactinomycin D and actinomycin D bound to calf thymus DNA (fig. 6) show that both compounds produce the same increase in melting temperature. Ultrafiltration experiments indicate that the two compounds have comparable binding constants and nucleotide:ligand ratios [23, 24]. Neither compound binds to calf thymus histone. 7-Aminoactinomycin D produces fluorescent DNA segments in *Drosophila melanogaster* polytene chromosomes [10]; studies with other chromosomes are in progress.

Fig. 7 shows variation of the relative fluorescence of 7-aminoactinomycin D with increasing polynucleotide:fluorochrome ratios. No change in fluorescence and presumably no binding is seen for poly d(AT), poly rG, or poly rA; these results are consistent with the absolute requirement of dGC base pairs in double helical DNA for binding. Interestingly, fluorescence enhancement is found with the three bihelical polynucleotides containing dGC, but the fluorescence enhancement is *inversely* proportional to the dGC content, decreasing in the order calf thymus DNA (42% dGC), *M. lysodeikticus* DNA (72% dGC), and poly dG · poly dC (100% dGC). Clearly the fluorescence of the 7-aminoactinomycin D:polynucleotide complex is either quenched by high dGC content or enhanced by high dAT content; in either event, base pair sequences surrounding the dGC binding site exert an important influence on the nature and the fluorescence of the complex.

Acridine orange shows fluorescence enhancement both with d(AT) base pairs and with polynucleotide guanine [10, 15] but gives little if any banding in various metaphase chromosomes. Ethidium bromide, reported by LePecq to exhibit fluorescence enhancement in the presence of polynucleotide guanine [26], gives 'reverse' banding in metaphase chromosomes in a limited number of species: *Scilla sibirica* [27], *Vicia faba* [27], *Trillium erectum* [3, 27], Chinese hamster [28], and the Australian wallaby [29]. In these species, the ethidium bromide fluorescent bands are precisely the reverse, mirror image of the quinacrine mustard bands: those regions that are fluorescent with quinacrine mustard are less fluorescent with ethidium bromide and vice versa. Fig. 8 shows the fluorescent banding of the large *Scilla* metaphase chromosome with quinacrine mustard and with ethidium bromide [3].

In fig. 9 ethidium bromide shows enhanced fluorescence when bound to several polynucleotides. The degree of enhancement is inversely

Fig. 8. *Scilla sibirica* metaphase chromosome treated (*a*) with ethidium bromide (*EB*); (*b*) with quinacrine mustard (QM). ×2 000. Note reversal of fluorescent banding pattern [3].



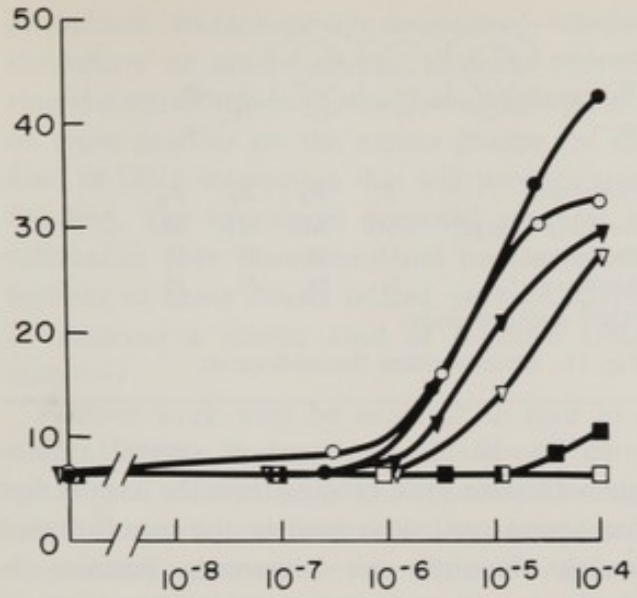


Fig. 9. *Abscissa:* DNA concentration (M); *ordinate:* fluorescence units (575 nm).

Effect of polynucleotides on ethidium bromide (EB 4×10^{-7} M) fluorescence in 0.1 M phosphate buffer at pH 6.8. Excitation wavelength 465 nm. ●—●, poly d(AT) (0% GC); ○—○, calf thymus DNA (42% GC); ▼—▼, *M. lysodeikticus* DNA (72% GC); ▽—▽, poly dG·poly dC (100% GC); ■—■, poly rG; □—□, poly rA.

proportional to the guanine content, decreasing in the order poly d(AT) (0% GC) through calf thymus DNA (42% GC) to poly dG · poly dC (100% GC). Since ethidium bromide effects fluorescent banding in chromosomes (even though reverse banding), the differing degrees of fluorescence enhancement of ethidium bromide–polynucleotide complexes seem insufficient to explain banding. We propose that base dependent enhancement/quenching phenomena play a role in fluorescent chromosome banding but we argue against the simplistic postulate that fluorescent banding in general is entirely a function of this mechanism.

There are two distinct kinds of fluorescent banding in human metaphase chromosomes: (*A*) faint, reproducible identification bands (present in all normal metaphase chromosomes of all individuals) [4, 5]; (*B*) intense, variable polymorphic regions (consistent in certain metaphase chromosomes of a given individual but variable from individual to individual) [4, 5, 30]. An understanding of the nature of these fluorescent bands will provide valuable information on the composition and ultrastructure of the metaphase chromosome. The identification banding (type *A*) correlates to some extent with DNA distribution along the chromosome, and is influenced by the distribution of DNA and protein along the chromosome. The second kind of banding represents a special kind of DNA, perhaps supercoiled or d(AT) rich or highly repetitive. Southern has found recently that highly repetitive guinea pig satellite DNA contains a 6 base sequence—CCCTAA [31]. We propose that fluorescence enhancement in the chromosome depends on the occurrence of the sequence

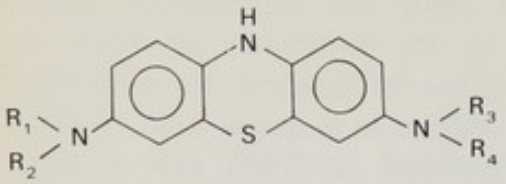
–A–A–C–
. . .
. . .
. . .
–T–T–G–

in a modification of the concept of Chan & McCarter [21], with the fluorochrome intercalating between the adjacent AT base pairs. In order to explain the increased efficiency of fluorescence and the relative persistence to fading of quinacrine mustard compared with quinacrine, we suggest that the antimalarial side chain with its terminal nitrogen mustard function alkylates a neighboring guanine residue, either in the same double helix or in an adjacent double helix; model studies should indicate the feasibility of this mechanism of binding. A final comment on the advantages of quinacrine mustard or quinacrine as fluorescent banding agents in human metaphase chromosomes is based on the observations of Caspersson and Zech that aminoacridines such as acriflavine and proflavine exhibit identification banding (type *A*) but not intensely fluorescent polymorphic staining (type *B*), at least in the Y chromosome. It might be necessary, for reasons related to tertiary DNA structure, for the aminoacridine fluorochrome to possess the antimalarial

side chain $-NHCH_3CH(CH_2)_3N(CH_2CH_2R)_2$ (CH_3 on the CH_3CH group)

(fig. 1) at position 9 for the visualization of type *B* fluorescence in human chromosomes.

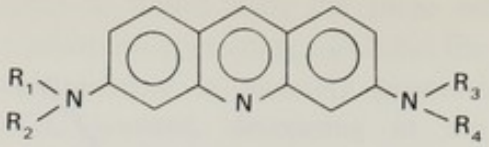
Very useful information will result from measurement of plant and human metaphase chromosomes banded with a differentially staining fluorochrome, with the object of correlating the absorption, fluorescence, quantum yield,



Compound	R_1	R_2	R_3	R_4
Methylene blue	Me	Me	Me	Me
Azur B	Me	Me	Me	H
Thionine	H	H	H	H
Toluidine blue*	Me	Me	H	H

*(2-methyl substituted)

Fig. 10. Aminophenothiazine chromophores (shown in reduced form).



Compound	R_1	R_2	R_3	R_4
Acridine orange	Me	Me	Me	Me
Proflavine	H	H	H	H
Acriflavine*	H	H	H	H

*(N^{10} methochloride)

Fig. 11. Aminoacridine fluorochromes.

and fluorescence polarization properties of the bound fluorochrome with observed banding and with DNA distribution along the chromosome. Caspersson and his colleagues are now carrying out such studies with proflavine- and acriflavine-banded plant and human metaphase chromosomes. In proflavine-banded *Vicia* chromosomes, increased quantum yield has been detected in the banded regions [14].

Demonstration of the feasibility of fluorescent banding of human metaphase chromosomes has stimulated the development of nonfluorescent banding techniques for human chromosomes involving primarily the use of Giemsa stain [32–40]. Giemsa banding of human metaphase chromosomes accords reasonably well with the fluorescent identification banding (type *A*) produced by quinacrine mustard, but requires some technical manipulation after preparation of the air-dried metaphase slide, such as treatment with alkali [35, 36], trypsin [39, 40] or a special buffer [33, 37]. The polymorphic variant regions intensely stained by quinacrine mustard (type *B*) are *not* visualized by Giemsa techniques.

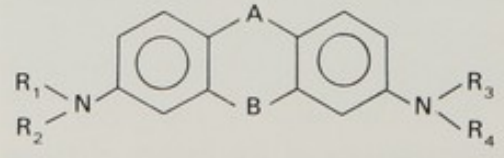
Giemsa stain is a mysterious mixture of methylene blue and eosin Y [41]. The mechanism of staining by Giemsa is unclear. Neither pure eosin Y nor pure methylene blue gives Giemsa banding, but certain impure samples of methylene blue do give such banding [42]. Since impure samples of methylene blue might well contain one or more of the demethylated analogs of methylene blue, namely, the azurs, we have investigated with Caspersson and Zech the banding potential of the azurs in human metaphase chromosomes. In preliminary experiments, azur B and also thionine and toluidine blue give banding, although not the same as Giemsa banding.

In fig. 10 the structures of several aminophenothiazine dyes, ranging from the tetramethyl compound methylene blue to the unsubstituted analog thionine, are presented. Banding is obtained only with those aminophenothiazines in which one or more of the protons on the exocyclic amino groups are present.

It has already been mentioned that proflavine and acriflavine, aminoacridines having no substitution at position 9, give fluorescent chromosome banding (type *A*) qualitatively similar to that produced by quinacrine mustard and quinacrine. On the other hand, acridine orange, the tetramethyl analog of proflavine (fig. 11), gives weak or variable banding, if any. We conclude that one or more of the protons on the exocyclic amino groups at positions 3 and 6 of the acridine ring system must be present for fluorescent banding to occur. This conclusion is to some extent consistent with the observations of Löber & Achtert [18] that alkylation of the amino groups of proflavine or acriflavine decreases the binding strength to DNA and decreases the DNA-quenching of fluorescence.

It may be of more than academic interest to note that the aminoacridine and aminophenothiazine structures are nearly isosteric (fig. 12), and to speculate that in order to give effective banding

Fig. 12. Structural relationship of isosteric aminoacridines and aminophenothiazines (reduced form).



Agent	*A*	*B*
Aminoacridine	CH	N
Aminophenothiazine	NH	S

in human metaphase chromosomes, whether fluorescent or nonfluorescent, diamino tricyclic ring systems of this kind must have available one or more protons on the amino groups for the kind of DNA-interaction that will produce such banding. The hypothesis proposed attempts to rationalize both fluorescent and nonfluorescent banding as being closely related, perhaps having in common a similar kind of selective DNA reactivity.

Further work may be expected to lead to a more definitive explanation and analysis of the nature and mechanism of chromosome banding, which is clearly conditioned by the properties of the binding agent and by distribution and composition of DNA, histone, and nonhistone protein along the metaphase chromosome.

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Discussion

Gropp: If the assumption of a specificity of a side chain of the Q-molecule for the staining of Q-band were valid then it is difficult to understand how the benzimidazole derivative '33258 Hoechst' is capable of producing a banding which in several species (mouse, *Microtus agrestis* and others) is similar to Q- or QM-banding.

Rigler: Regarding the interaction of acridines with polynucleotides of different base composition, one has to be careful to conclude from an unchanged fluorescence intensity during titration that no interaction takes place, unless one has other independent proof. One could very well imagine interactions which do not affect the quantum yield of the acridine. A simple way to check a situation of this kind is to measure the polarized components of fluorescence.

Modest: I agree that binding must be proved by fluorescence polarization or other methods, and I hope you will discuss that in your paper.

Rigler: When investigating the interaction between quinacrine mustard and polynucleotides in aqueous solution you may be dealing with a mixture of hydrolyzed and non-hydrolyzed mustard, the latter leading to an irreversible reaction with nucleic acids. Would you care to comment on your thermodynamic data in view of this situation?

Modest: There are two possibilities for explaining our Scatchard binding curve for QM. One (extremely unlikely) is that there is partial hydrolysis of QM during the experiment. The other (most probable) is that the primary binding of QM is by intercalation, with alkylation secondary. The nonlinear Scatchard plot reflects two binding modes—presumably intercalation and ionic binding.

Rigler: Is the secondary ionic binding on the outside of the DNA helix?

Modest: Probably.

Comings: Then how does quinacrine (Q) bind?

Modest: In the same way—primarily by intercalation but without alkylation, of course. The similarity of the Scatchard plots for both QM and Q points to similar binding mechanisms for the two compounds.

Lejeune: What do you call intercalation? Is it the insertion of the whole tricyclic molecule inside the double helix of DNA?

Modest: By intercalation I mean insertion of the acridine nucleus between base pairs—probably (but not necessarily) adjacent AT base pairs. There is room inside the double helix for intercalation. During intercalation the helix unwinds and is distorted somewhat. This effect is measurable.

Pearson: Have you an explanation for the fading of QM and Q in the chromosome preparations?

Modest: The fading must be a photodynamic process under ultraviolet irradiation. We find that QM fades more slowly than Q, and we attribute this difference to covalent binding of the nitrogen mustard side-chain of QM to DNA-guanine.

Pearson: We have conducted fading experiments in chromosome preparations with QM and Q. One sample of QM gives the longest lasting fluorescent bands. Other samples of QM are no better and some are worse than various samples of Q. These results indicate the chemical variability of samples of QM and of Q, and also perhaps the variability of the chromosome preparations used.

Ford: Dr Pearson's observations are terribly important. We must standardize our preparations.

Zech: The studies with the azures, thionine and toluidine blue which Dr Modest, mentioned are of very preliminary character. We got bands on the chromosomes with all these substances. How far the patterns obtained resemble the Q and G patterns is too early to decide.

Interactions between Acridines and DNA

A key to the understanding of banding patterns in chromosomes

R. Rigler

Institute for Medical Cell Research and Genetics, Medical Nobel Institute,
Karolinska Institutet, S-104 01 Stockholm 60, Sweden

In this symposium exciting examples have been given for the use of fluorescent dyes to identify individual chromosomes ([4, 5], and this volume, pp. 28, 50, 290, 293).

The basis of identification is the specific banding pattern characteristic of each chromosome. In all these cases we are dealing with dye compounds which show a pronounced specificity for nucleic acids either because they react with DNA by alkylation of certain purine bases (quinacrine mustard) or because they bear positive charges (acridine orange, proflavine, acriflavine) which allow them to interact with the DNA phosphate backbone. These interactions are in addition stabilized by the intercalation of the flat acridine rings between nucleic acid base pairs.

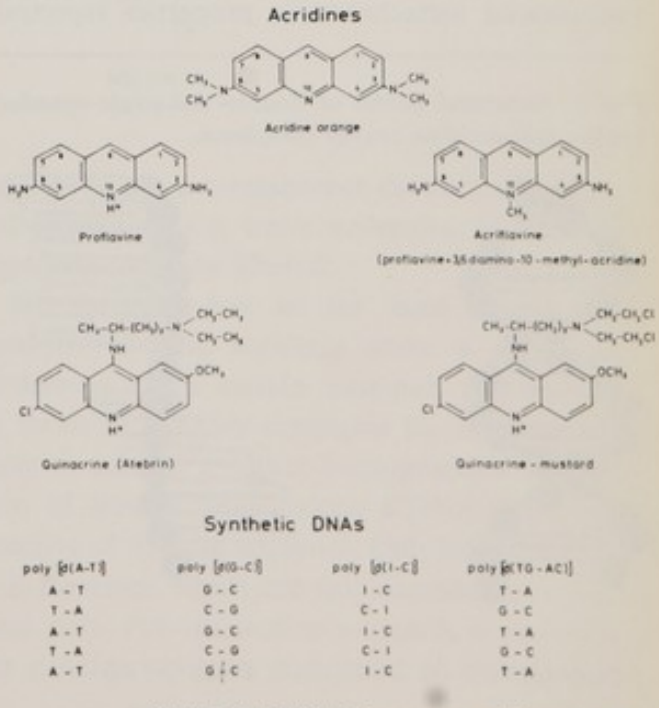
Out of a variety of factors influencing the dye–DNA interactions at least three are known: (*a*) the secondary structure of DNA (strandedness); (*b*) the base composition and distribution along the DNA chain; (*c*) the interaction of proteins with DNA. There exists an overwhelming literature on the physical chemistry of acridine DNA interactions [1, 14]. I want to restrict myself only to those points which seem to be of immediate interest to the observed banding patterns and their explanation.

The Structure of DNA

Among the fluorescent dyes, acridine orange (AO, fig. 1) exhibits large changes in its emission properties when interacting with nucleic acids with different degree of strandedness [20, 21]. A pronounced red shift of the emission spectrum of AO (paralleled by a blue shift in the absorption spectrum) is observed when AO is bound to single-stranded nucleic acids such as heat-denatured DNA or poly U. Interaction between AO and double-stranded nucleic acids such as DNA, however, results in a small blue shift of the emission spectrum (red shift of the absorption spectrum, fig. 2). The physical basis of these spectral shifts can be explained by the formation of associated dye molecules. Their formation is facilitated by the rather flexible single-stranded nucleic acid chain, but is restricted by the highly ordered structure of a DNA–helix. Under conditions usually applied in staining reactions AO molecules are dispersed within the helix of a double-stranded DNA without intermolecular interactions and fluoresce green, while stacks of AO molecules are formed on single-stranded DNA which fluoresce red (fig. 3).

This remarkable property of AO provides a powerful tool to probe the strandedness of nucleic

Fig. 1. Structure of acridines and synthetic DNAs.



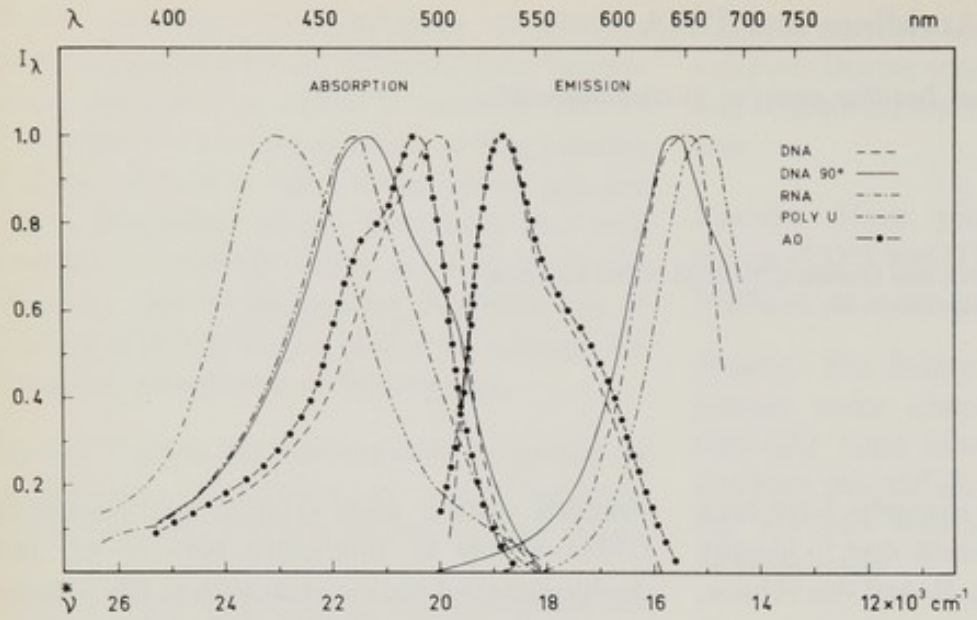


Fig. 2. Absorption and emission spectra of acridine orange-nucleic-acid complexes [21].

acids, particularly that of DNA in chromatin and chromosomes. Several examples have been given in the literature [21, 22, 24].

The Base Composition of DNA

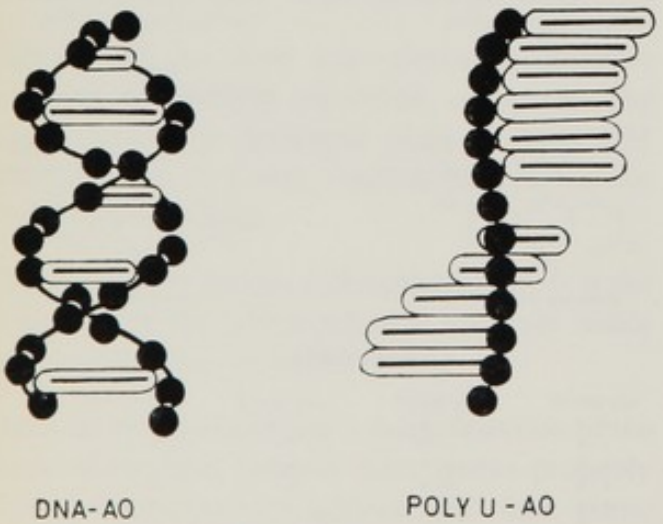
Certain acridines, particularly those which have been used for chromosome identification (quinacrine (QAC), proflavin (PF), acriflavin (AF), fig. 1) exhibit sensible changes in their quantum yield of fluorescence when complexed with natural [26] or synthetic DNA [19, 22, 27] of different base sequence. Their relative absorption and emission spectra remain almost unchanged (fig. 4*a*, *b*). In contrast to AO these dyes do not show pronounced metachromatic properties (spectral shifts) on aggregation. The quantum yield of both QAC and PF is (table 1) strongly reduced when interacting only with G–C-containing DNA (poly [d(G – C)]), while it is enhanced, particularly with QAC, when interacting with A–T-containing DNA (poly [d(A–T)]). The quantum yield of natural DNA with an intermediate A–T/G–C content as calf thymus DNA can be explained from the data obtained from the poly [d(A–T)] and poly [d(G–C)]-acridine complexes. In this case it is assumed that the fluorescence of acridines is almost completely quenched in GC–GC and GC–AT base pair combinations while their fluorescence is strongly (QAC) or slightly enhanced (PF) in AT–AT combinations. No distinction between the sequence of purines and pyrimidines is made

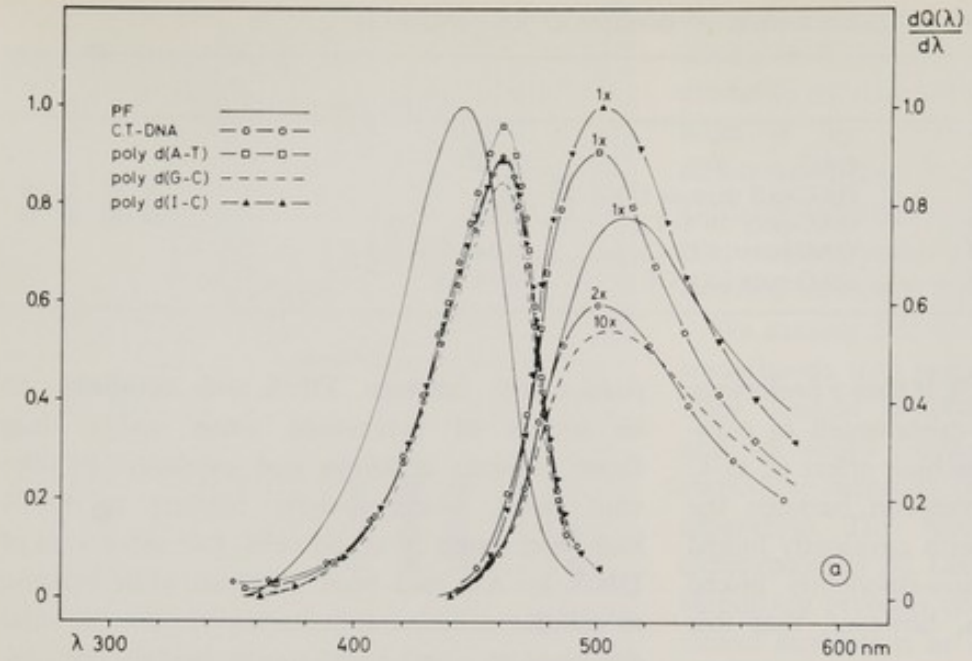
$$\left(\text{e.g.}\quad \begin{matrix}\text{A–T}\\ \text{T–A}\end{matrix}\ \text{or}\ \begin{matrix}\text{A–T}\\ \text{A–T}\end{matrix}\right)$$

Studies on the interaction of PF with DNA of alternating T–A/G–C sequence (poly [d(TG–AC)] which quenches almost as effectively as poly [d(G–C)], indicate that an A–T base pair must apparently be adjacent to either side of an intercalated acridine to retain or increase its quantum yield.

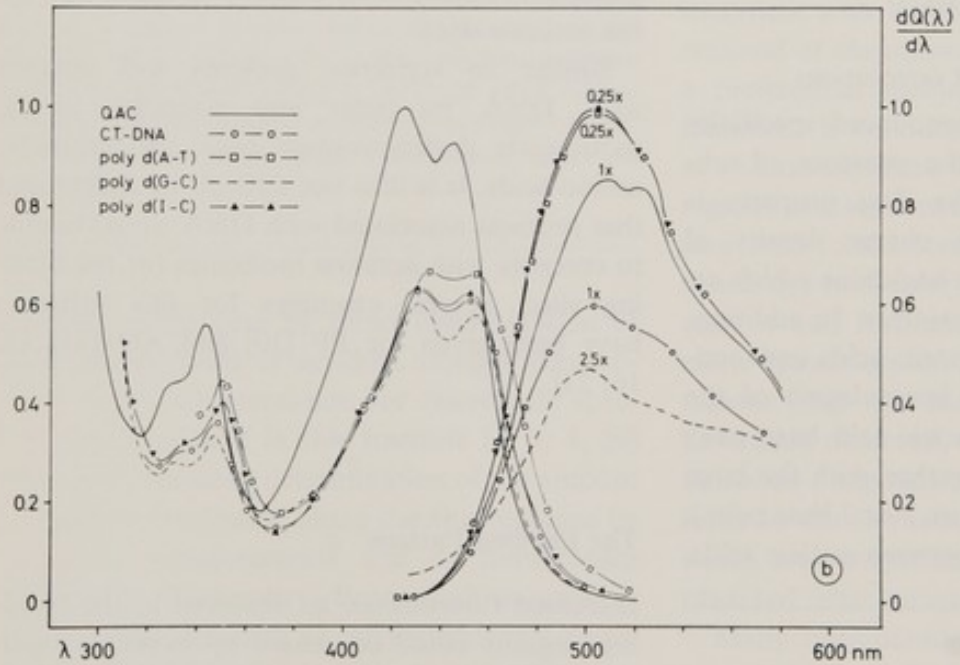
The physical basis of these quantum yield changes is not fully understood as yet. A reasonable, however unproven explanation seems to be an electron exchange between guanine and acridine. That guanine might be involved is supported by the observation that replacing guanosine by inosine which lacks an amino group

Fig. 3. Structural model of double- and single-stranded nucleic acid-acridine orange complexes.





a



b

Fig. 4. Absorption and quantum spectra of (*a*) proflavin and proflavin–DNA complexes; (*b*) quinacrine and quinacrine–DNA complexes [19].

in position 2 of the purine ring (fig. 5) abolishes the quenching of QAC and PF (table 1). Besides the altered electronic structure of inosine which might affect the quantum yield of these acridines in a different way than does guanosine, one has to consider that the I–C base pair, like the A–T pair has only two H-bonds. Thus the stability of base-pairing is different from that of the G–C pair. This in turn could influence the stereochemical position of acridine in relation to the base pairs. As a consequence the access of external quenchers, e.g. water molecules, to the acridine ring could be altered.

No evidence has so far been found that reversibly bound acridines show a significant preference for a certain base-pair combination in terms of stability constants [6, 19]. We thus have to assume a rather homogeneous distribution of these acridines along a DNA chain irrespective of its base sequence. Only those acridines will fluoresce which are not exposed to a G–C base pair. For quinacrine mustards a specificity for guanine has been postulated on experimental

Table 1. *Quantum yield (φ) of fluorescence of acridine DNA complexes*

	φ		φ
PF	0.7	QAC	0.17
PF-calf thymus DNA	0.25	QAC-calf thymus DNA	0.12
PF-poly [d(A–T)]	0.74	QAC-poly [d(A–T)]	0.74
PF-poly [d(G–C)]	0.05	QAC-poly [d(G–C)]	0.04
PF-poly [d(I–C)]	0.83	QAC-poly [d(I–C)]	0.74

and theoretical grounds [4, 5]. If thus a preference exists it has to be rather pronounced to compensate for the strong quenching effect of G–C base pairs. The good correlation between the banding pattern obtained with covalently linked quinacrine mustard and non-covalently linked quinacrine (QAC) suggests, however, that this is not the case.

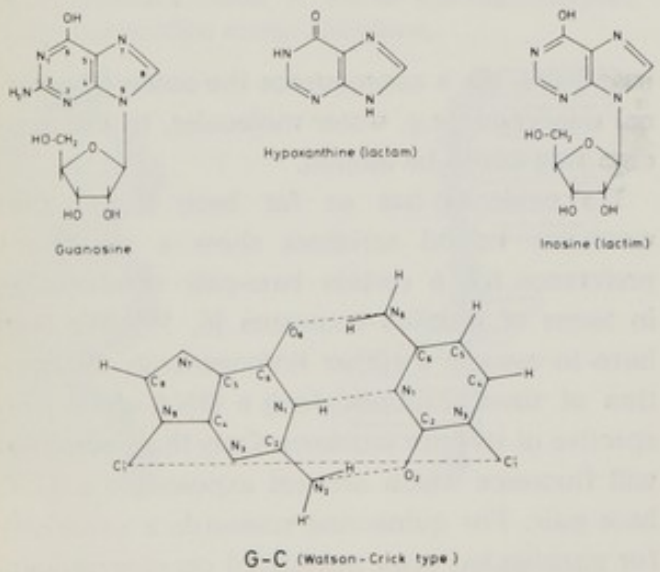
Specificity of Acridines with DNA

Interference by protein–DNA interactions

Acridine dyes exhibit a pronounced specificity to nucleic acids even in the presence of substantial amounts of protein. This property is due to the high negative charge density of the nucleic acid phosphate backbone which attracts positively charged acridines. In addition, the interaction between nucleic acids and acridines is stabilized by the intercalation of the planar acridine between nucleic acid base pairs [13]. These conditions, together with the large number of binding sites (1 per 2 or 3 base pairs), causes complex formation between nucleic acids, particularly between DNA and acridines, to be orders of magnitude more stable than those between acridines and proteins. In relevant cases, where specific binding to DNA had been tested in single cells, full saturation of DNA by AO had been obtained while binding to cellular proteins constituting almost the total dry mass of a cell could hardly be detected [20]. There may be exceptions. Certain proteins, e.g. phosphoproteins, may exhibit increased affinity for acridine dyes.

Similar to acridines, proteins will interact with DNA by polar and nonpolar forces, particularly if they contain basic and aromatic amino acids. It is thus not difficult to understand that proteins associated with DNA would be able to compete with acridine molecules for the binding sites. Several examples for this situation have been given for PF [10] and AO [11, 15, 16, 23].

Fig. 5.



The Banding Pattern

Increased fluorescence as observed in the banding regions could be caused by increased binding of acridines to parts of DNA where interaction with DNA and surrounding proteins is less strong. A good example is the observation that DNA in nuclear chromatin of cell cultures activated to cell growth is able to bind AO in higher amounts [15, 16]. Although PF and QAC have not been tested in this respect in vivo, it is likely that these compounds would behave in in vivo systems similar to AO.

The studies of the PF and QAC binding to DNA of different base composition [19, 22, 27], however, suggest that the banding pattern could equally well be accounted for by regions in DNA predominantly consisting of A–T base pairs. A decision between these two possibilities is in

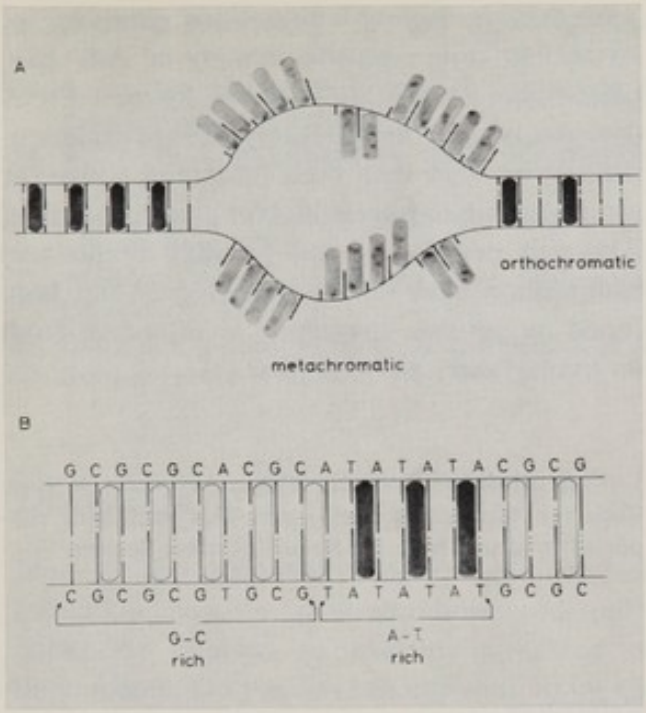


Fig. 6. Schematic model (*A*) of a structure-sensitive acridine (acridine orange) within double- and single-stranded DNA regions: (*B*) of base-sensitive acridines (proflavin, quinacrine) within an A–T-rich DNA region. Symbols for fluorescence colour of acridines: blank, no fluorescence; filled, green fluorescence; grey, red fluorescence.

principle rather easy by direct determination of the quantum yield of acridine along a chromosome. The instrumentation for microscale spectroscopy developed in this Institute [2, 3, 4, 20] allows simultaneous determination of the amount of acridine distributed along the chromosome by absorption measurements and its fluorescence intensity by fluorimetry. From both parameters the quantum yield can be calculated. Preliminary measurements of this kind (Caspersson. Personal communication) on PF-stained *Vicia faba* chromosomes indicate that the distribution of PF parallels the DNA distribution along the chromosome indicating a constant stoichiometry between DNA and PF. It thus follows that the increased fluorescence intensity in the banding regions must be due to an increased fluorescence quantum yield of PF. Thus, at least in this case, the fluorescent bands apparently represent A–T-rich regions. This interpretation is further supported by the observation that the banding pattern obtained by the use of fluorescence-labelled antiadenosine antibodies (p. 43) is generally equivalent to the pattern obtained by quinacrine staining. Discrepancies related to the Y-chromosome are rather interesting and can probably provide further insight in the molecular basis of the banding pattern. An essential difference in the two methods is the fact that the antibody-banding technique requires formation of single-stranded DNA while the acridine banding technique does not.

We assume that we are dealing in the chromosome bands with regions rich in A–T base pairs. They contain an H-bond less than G–C pairs and we would predict in these regions a lower stability to thermally or chemically induced strand separation. This behaviour is well established experimentally [9]. Likewise removal of structure-stabilizing proteins could also cause preferential strand separation in A–T-rich regions. Turning back now to AO staining this would mean that procedures inducing strand separation by heat, treatment with NaOH or enzymatic or chemical removal of chromosomal proteins would lead to a preferential melting of A–T regions. If the treatment is conducted properly one can arrive at a situation where only the strands of A–T-rich regions are separated. Staining with AO would now lead to the formation of red bands (associated dye molecules) in the single-stranded A–T regions and formation of green bands (monomeric dye molecules) in the intact double-stranded regions (fig. 6). Indeed AO-banding patterns have been observed, where AO-staining of chromosomes exposed to denaturing conditions leads to the formation of red fluorescing bands which are compatible with the banding patterns observed after quinacrine staining (pp. 77, 293).

Many considerations relevant to the fluorescent dyes are valid also for non-fluorescent dyes used in chromosome staining. One of the main ingredients of the frequently used Giemsa stain is methylene blue. This dye is one of the classical examples of the metachromatic behaviour as elucidated by Michaelis [17]. Like AO it aggregates on nucleic acids with a blueshift in the absorption spectrum. The aggregates will appear to the eye with a deep purple blue colour. It is thus not surprising that a correlation between Giemsa-, quinacrine- and AO-bands can be found.

Still, the interpretation of the banding pattern given is rather tentative and probably represents an oversimplification of the actual situtation. It does not take into account specific DNA–protein

interactions which might influence the banding pattern in addition to the base sequence and structure of DNA. Evidently there is still much to learn about these interactions. There is good hope that refined biophysical analysis of the banding pattern by fluorescence spectroscopy [25] in combination with biochemical studies will give important information.

A–T-rich Regions, What do They Mean?

The size of chromosome bands is in the range between 0.1 and 1 μm. We may conclude that such a band consists of DNA with a chain length of at least 300 to 3 000 base pairs. This would be enough to code between 1 to 10 proteins of average size. This estimate, however, does not take into account the complicated three-dimensional folding of DNA in a chromosome. Thus, the size of DNA in these regions is probably much larger. Faced with this fact and the tentative explanation given to these chromosome bands, we have to ask about the biological meaning of large A–T-rich stretches of DNA. A decisive answer cannot be given at present. However, I want to close my contribution by presenting some suggestions, some of which can be tested rather easily.

(*a*) We are dealing with regions with a structure differing from the rest of the DNA. From the physico-chemical properties of DNA containing only A–T base pairs, single-strandedness, chain branching, and increased breathing of the DNA helix are expected to occur. These structures meet many criteria for the 'globular control DNA' postulated by Francis Crick in his functional model for chromosomes of higher organisms [7].

(*b*) We are dealing with recognition sites for proteins controlling gene activity. This feature might be closely connected with the previous suggestion. A likely candidate for specific interactions with A–T regions are lysine-rich proteins, e.g. lysine-rich histone fractions. This assumption is supported by the observation that polylysine exhibits a significant specificity for A–T-rich regions in DNA [12, 18]. It could be tested easily by producing fluorescent antibodies against several histone fractions [28] and by comparing banding patterns obtained by antibody labelling with classical acridine bands.

(*c*) We are dealing with regions consisting of an ancient code consisting simply of A–T base pairs. The amino acids coded by this DNA (lys, asn, tyr, phe, leu, ileu) are able to provide peptides or proteins with both polar and non-polar groups known to be essential for protein function. Although proteins of this peculiar amino acid composition have—to my knowledge—not been found as yet this suggestion is attractive from an evolutionary point of view [8].

I thank Professor Torbjörn Caspersson and Dr Lore Zech for stimulating discussions. This study was supported by grants from the Swedish Cancer Society.

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Discussion

Modest: You suggested charge-transfer complex formation between aminoacridines and polynucleotide guanine to explain quenching of fluorescence. Do you have an explanation for the enhancement of fluorescence with A–T?

Rigler: A likely explanation, though not tested as yet, is a change in perturbation of the acridine ring by solvent molecules when the acridine ring is buried in the hydrophobic region of DNA.

Lejeune: The effect of enzymes demonstrate that proteins play a role in the banding. Hence if we are dealing as proposed with AT and GC sequences of DNA, then a different kind of protein should be related to each segment. Why could not the dye be selectively sensitive to one of these two types of protein?

Rigler: Professor Lejeune's suggestion that certain banding patterns might be caused by binding of dyes to protein instead of nucleic acid is very interesting. Howewer, the stability between basic dyes used in staining procedures and nucleic acid is usually much higher than between these dyes and cell proteins under comparable conditions. Therefore I think the view that one deals with a competitive binding of dye and chromosomal proteins to DNA is more relevant (cf also [20, 21]). The banding pattern could thus be modified in numerous ways in addition to the banding pattern based on differences in the DNA structure alone (cf Rigler, p. 338).

Observations on the Mechanisms of Giemsa Staining Methods

W. Schnedl

Histologisch-Embryologisches Institut der Universität Wien, 1090 Wien, Austria

Several methods have been described for demonstrating banding patterns in chromosomes which can be made visible by Giemsa staining (for instance [2, 3, 4, 5, 6]). It is not easy to find a common interpretation of all these differing methods. This study is therefore confined to some of the earlier methods [5, 6]. Originally it was believed that DNA denaturation and renaturation is involved in these techniques. This interpretation is still in question. The present report shows that, at least in the method analysed here, DNA is partially removed by the treatment.

Material and Methods

Blood cultures were established from normal human males and females and chromosome preparations were made by the air-drying procedure. The cultures were labelled 9 h prior to harvesting of the cells, with ^{3}H-thymidine (0.3 μCi/ml medium) or with ^{3}H-deoxycytidine (1.0 μCi/ml medium) respectively, for 1 h. In this way the metaphase cells in the preparations had received their label in the middle phase of their DNA-replication period.

All chromosome preparations (including the control preparations) were treated with RNase (100 μg/ml in 2 × SSC) at 37°C for 1 h.

The control preparations and the preparations which had been treated with various agents (NaOH, Sörensen's buffer) were analysed autoradiographically by the stripping film method (Kodak AR 10) and developed exactly 48 h after coating the slides with Kodak D19b at 14°C for 2 min. The mean grain count in the control preparations was about 150 grains/metaphase cell. About 50 metaphase cells were analysed in every case.

Results and Discussion

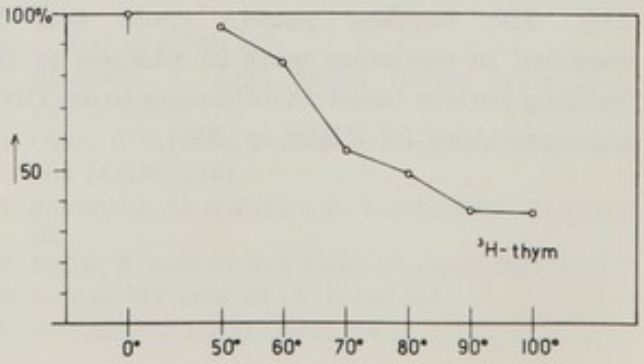
The rather laborious method of grain counting is necessary for analysing the DNA of the metaphase cells. It is possible that DNA in interphase nuclei which make up the majority of the cells in chromosome preparations is affected in a different way than DNA in metaphase chromosomes.

In one experiment the effects of sodium hydroxide on DNA in the chromosomes were tested (table 1). It can be seen that a low concentration of NaOH has no or only very little effect on the grain count. However, there is some evidence that DNA can be lost if higher concentrations and longer treatment times are used.

In the method of Yunis et al. [7] the preparations are treated first with phosphate buffer at high temperatures (80–100°C) and subsequently incubated at 65°C in the same buffer. Fig. 1 shows the effects of incubation in phosphate buffer (Sörensen's buffer, M/15 KH_2PO_4/Na_2HPO_4) at pH 6.8 for 8 min and at various temperatures. It can be seen in fig. 1 that at elevated temperatures more DNA is removed than at lower temperatures.

Incubation in Sörensen's buffer at 60°C for 8 min reduced the grain count only to about 85%. Several Giemsa banding methods use a treatment

Fig. 1. Effects of incubation in M/15 phosphate buffer (pH 6.8) (*abscissa*) at various temperatures for 8 min on the grain count; (*ordinate*) of ^{3}H-thymidine in metaphase cells.



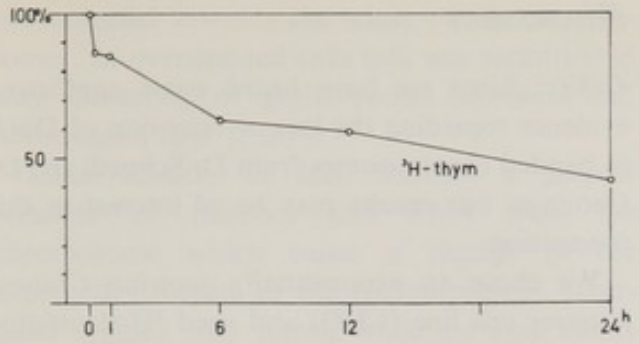


Fig. 2. Reduction of the grain count (*ordinate*) of ^{3}H-thymidine in metaphase cells by a prolonged treatment in M/15 phosphate buffer (pH 6.8) at 60°C (*abscissa*).

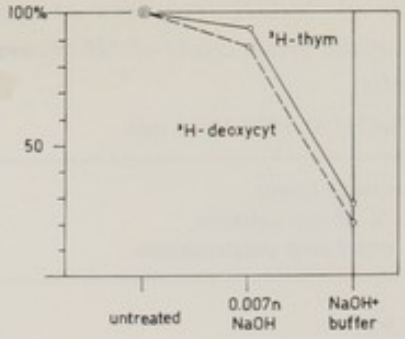


Fig. 3. Effects of a NaOH treatment and a subsequent incubation in M/15 phosphate buffer (pH 6.8) at 60°C (*abscissa*) for 12 h. Large amounts of ^{3}H-thymidine and ^{3}H-deoxycytidine are removed by the buffer incubation if a NaHO-pretreatment is used (*ordinate*) grain mean count).

in neutral buffer solutions at about 60°C for longer incubation times. In fig. 2 the effect of prolonged incubation with phosphate buffer at 60°C is shown. The grain count drops very rapidly at first, but later the curve becomes more horizontal.

The banding patterns in chromosomes can be enhanced if a treatment with low concentrations of NaOH is used prior to the incubation in buffer at pH 6.8 and 60°C. Incubation with buffer for 12 h reduced the grain count of ^{3}H-thymidine to about 60%. If a treatment with NaOH is used prior to the incubation in buffer, much lower values are reached (fig. 3). NaOH alone has almost no effect on the grain count (table 1). If, however, an NaOH treatment and a subsequent buffer incubation is used, the grain count drops to about 20–30%. We have not yet been able to find a statistically significant difference between the grain count reduction for ^{3}H-thymidine and for ^{3}H-deoxycytidine, respectively.

In another series of experiments some effort was made to analyse the mechanisms of the Giemsa staining method used in this laboratory from a morphological point of view. The Nomarsky interference microscope (Reichert, Vienna) seemed to be especially suited for analysing the morphological changes occurring during the Giemsa staining procedure. Fig. 4 shows 3 steps in the formation of the banding patterns. Fig. 4*a* shows an untreated cell. The chromosomes appear thick and no striking substructures can be detected. After a treatment with NaOH

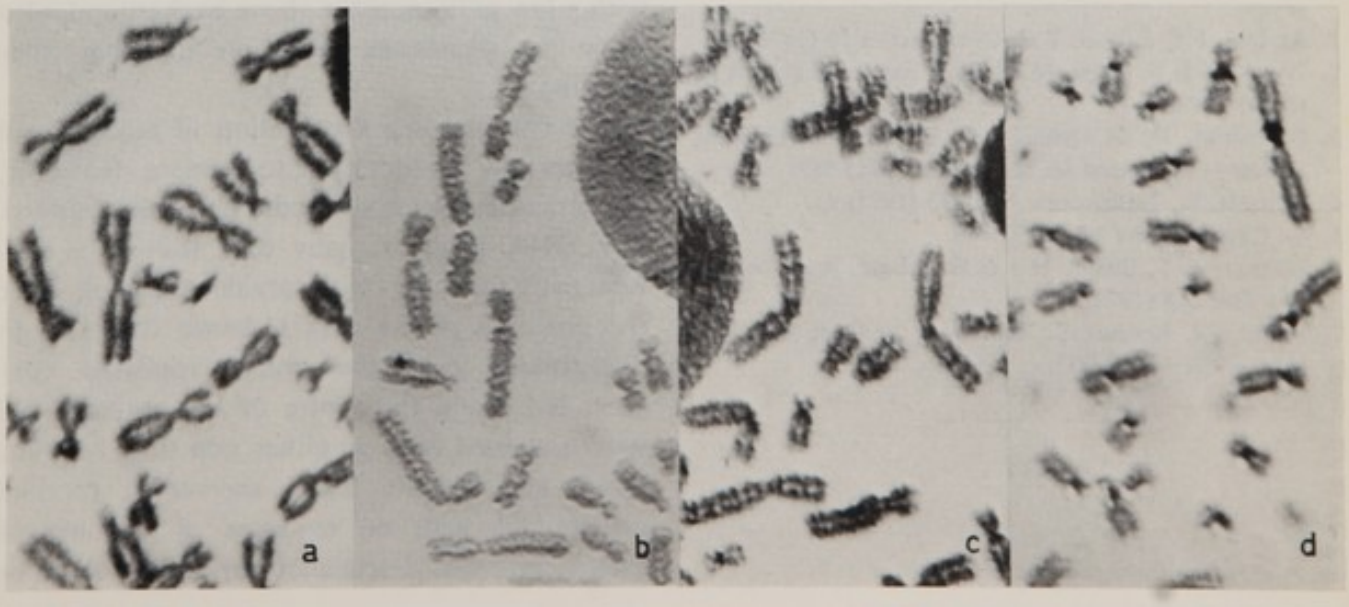


Fig. 4. Details of metaphase cells (human) analysed by the Nomarsky interference microscope. (*a*) Untreated chromosomes; (*b*) chromosomes after a treatment with 0.007 N NaOH for 2 min; (*c*) chromosomes after NaOH treatment and incubation in phosphate buffer; (*d*) a cell showing the effect of the Arrighi & Hsu method produced by a prolonged NaOH-pretreatment and buffer incubation.

Table 1. *Effects of NaOH treatment at various concentrations on the grain count of ^{3}H-thymidine in metaphase cells*

Treatment with NaOH at 10°C for 1 min

NaOH concentration (N)	Grain count % of the value in untreated preparations
0.007	95
0.035	98
0.07	98

(fig. 4*b*) the chromosomes show a somewhat fuzzy appearance and fine, equally spaced substructures, the chromosome coils, can be visualized. After a subsequent buffer incubation (M/15 phosphate buffer, pH 6.8, 59°C) for 12 h sharply defined raisings can be seen in the chromosomes (fig. 4*c*). These regions correspond to the bands visualized by the Giemsa stain method. In interference contrast, the bands are visible also in unstained preparations which have been treated accordingly; however, staining enhances the raisings in the chromosomes.

If the chromosome preparations are treated with higher concentrations of NaOH (0.07 N) prior to the buffer incubation a result similar to the Arrighi & Hsu method [1] is obtained. In the interference microscope it can be seen that most of the material in the chromosomes is removed. Only at the regions occupied by the so-called "C-bands" are larger amounts of remaining material found.

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Discussion

Ockey: Since we have heard some conflicting evidence regarding the loss or retention of DNA in banded chromosomes from Dr Schnedl and Dr Comings, our results may be of interest in this connection.

We chose an exponentially growing Chinese hamster cell line (CHO) and used ^{3}H-thymidine as the label for DNA, ^{3}H-uridine for RNA, ^{3}H-lysine and ^{3}H-arginine for the histone fractions and ^{3}H-glutamic and aspartic acids for the non-histone proteins.

The cells were treated for 8 h with ^{3}H-thymidine and 2.5 h with each of the other precursors. Colcemid was used to accumulate metaphases. G-banding was produced by 0.0001% trypsin for 4 sec, followed by 70% methyl alcohol, air drying and a 1 h treatment in Hanks solution pH 7.5 at 60°C. The chromosomes were banded in about 80% of the cells, the remainder were over-treated.

Grain counts were carried out over one of the large metacentric pair from each of 50 early metaphase cells from both pre- and post-banded slides. This pair shows a similar banding pattern (fig. 1*a*). The mean control pre-banded counts were normalized at 100 for each isotope.

The effect of the trypsin treatment was to remove 20–30% of the labelled RNA and histones from the chromosomes. Each histone fraction is presumably equally extracted. The DNA shows no reduction but a slight increase due to the exposure of the now expanded chromosome to a greater film area. The glutamic and aspartic acid labelled chromosomes, although with a low grain number above background, also show no significant reduction in label after banding.

The chromosome distribution of label for all isotopes was random prior to banding. However, after treatment each showed a particular pattern. The DNA label in many cells showed a characteristic banding with grains clustered over the positions of the dark G-bands (fig. 1*b*). In over-treated cells the grains appeared concentrated along the centre of the chromosome with a stained area on either side (fig. 1*c*). The RNA and histone labels showed a random distribution with no evidence of banding of grains but with a tendency for the label to be

concentrated towards the edges of the chromosomes. In over-treated cells this was accentuated with clustering of grains round telomeres and centromeres (fig. 1, *d–f*).

These results, we feel, indicate a general removal of histones and RNA from the chromosome which cause a change in the supercoil structure of the nucleo-protein. Interband regions may uncoil while the dark bands remain condensed. This could result in a structure analagous to the prophase structure described by Dr Bahr.

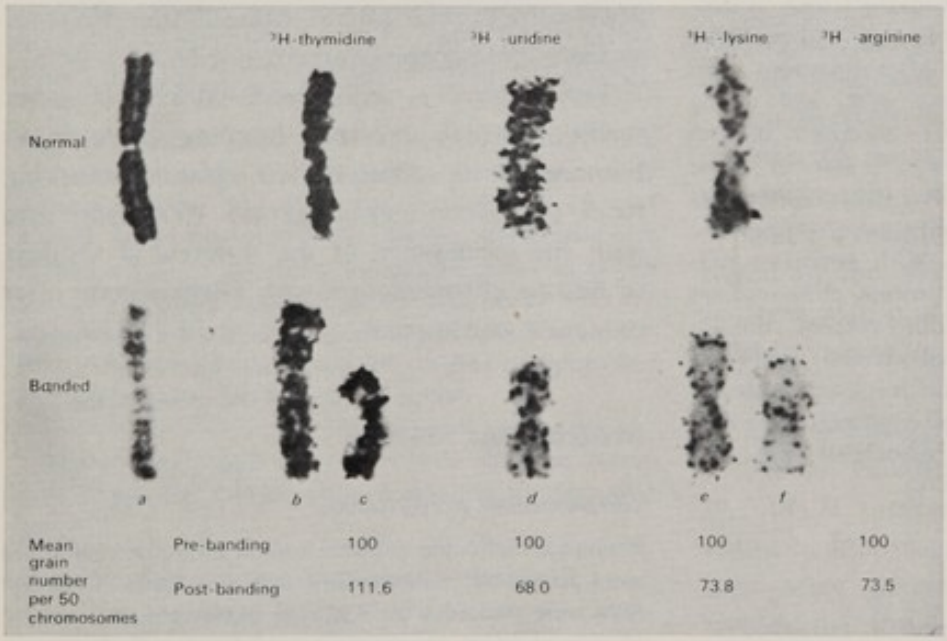


Fig. 1.

Differential Staining of Human Chromosomes after Oxidation Treatment

T. Utakoji

Pathology Department, Cancer Institute, Toshima-ku, Tokyo 170, Japan

Summary

Differential staining patterns of human chromosomes were studied with Giemsa stain after treatment with potassium permanganate, peracetic acid, and cupric sulfite reagent. These treatments produced distinct banding patterns in chromosomes similar to those produced by proteolytic enzymes. No distinct differences could be observed among the banding patterns produced by these three different reagents. With potassium permanganate and peracetic acid treatments, chromosomes were swollen, but with cupric sulfite reagent chromosomes did not swell and remained slender. Characteristics of these reagents in chemical reactions suggested that the differential stainings of chromosomes after oxidation treatments are closely associated with sulfhydryls/disulfides in the chromosome.

Chemical differentiation along metaphase chromosomes of higher organisms has been demonstrated by Caspersson et al. by the fluorescent technique [1]. Differential affinity of chromosome segments to certain chemicals was shown as bright and dark fluorescent patterns which were interpreted as reflecting the differences in chemical composition in different chromosome segments. Since 1970 numerous methods of demonstrating differential affinity to Giemsa stain have been reported [2] in chromosome regions after various treatments. Both fluorescent and Giemsa stain banding techniques greatly facilitated the chromosome identification in cytogenetics and in cell biology. Yet the mechanism of chromosome banding is still a subject of controversy.

It was reported in the previous study that the permanganate treatment of human chromosome preparations under very mild conditions produced a banded appearance after Giemsa staining [3]. Apparently the oxidation of some chromosome constituents by the treatment with potassium permanganate caused alterations of stainability in chromosome regions.

This report is concerned with two other methods which produce banding patterns in human chromosomes similar to those resulting from potassium permanganate treatment, and with the mechanism of the differential staining of human chromosomes with Giemsa stain after oxidation treatments.

Materials and Methods

Chromosome preparations

Human lymphocyte cultures with a phytohemagglutinin were harvested without Colcemid treatment. Collected cells were treated with 0.075 M potassium chloride for 15 min at room temperature, and fixed with 3 changes of a mixture of 3 parts methanol and 1 part glacial acetic acid. Chromosome spreads were made on clean dry slides by air-drying in the ordinary way without heat treatment.

Staining with dilute Giemsa stain

Giemsa stock solution was prepared by dissolving 1 part "Azur-eosin methylene blue according to Giemsa" (E. Merck, Darmstadt) in 66 parts glycerol at 60°C, and after cooling, diluted with 66 parts methanol. The stock solution was diluted 100 times with 1/75 M phosphate buffer according to Sörensen at pH 7.0. Slides were stained with freshly diluted Giemsa stain for 10 min at room temperature.

Oxidation of chromosome preparations with peracetic acid

Peracetic acid was prepared according to Greenspan [4]. A mixture of 50 ml glacial acetic acid, 150 ml 30% hydrogen peroxide, and 2 ml concentrated sulfuric acid was kept in an ordinary refrigerator for 3 days. Chromosome preparations were treated with peracetic acid in an ice-bath for 10 min, washed quickly with running tap water for about 30 sec, then with absolute ethanol for 5 min, and air-dried.

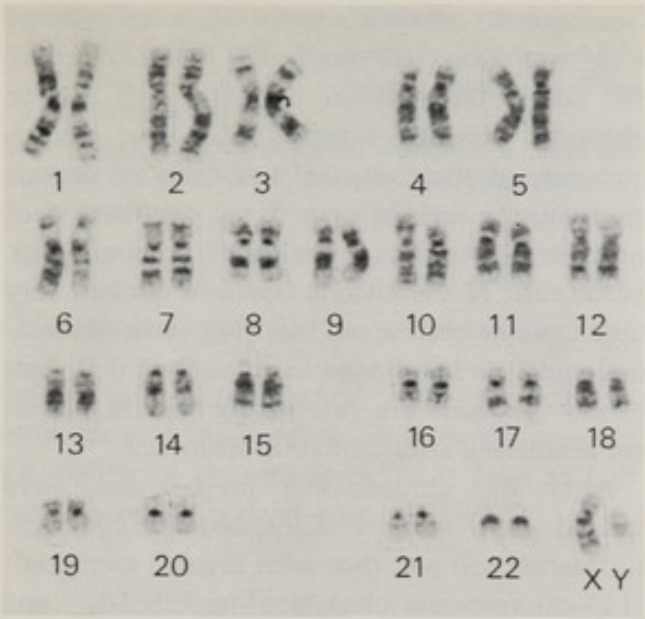


Fig. 1. Normal human male karyotype. Chromosome spreads were made by the standard air-drying method without heating. The slide was treated with 10 mM $KMnO_4$ at pH 7.0 and at 0°C for 30 min, and stained with Giemsa stain [3]. Arrangement of chromosomes within certain groupings may not be in accordance with the agreement on the nomenclature at the Paris Conference, 1971. Distinct and conspicuous banding patterns and swelling of chromosomes may be noted.

The peracetic acid did not contain divalent cations such as calcium and magnesium and pH was less than one.

Treatment of chromosome preparations with cupric sulfite reagent

To convert sulfhydryls and disulfides to thiosulfates, cupric sulfite reagent [5] was applied to chromosome slides. To 200 ml 0.025 M copper-(II) sulfate solution about 1 ml ethylenediamine was added drop by drop to bring the pH of the solution to approx. 9.0. Fifty ml 0.25 M sodium sulfite solution was added to above solution. All the solutions were prepared carefully with oxygen-expelled water to avoid air oxidation. Chromosome slides were placed in a Coplin jar full of freshly prepared cupric sulfite reagent and the cover of the jar was sealed with plastic adhesive tape. Slides treated with the reagent overnight at room temperature were washed with running tap water for about 30 sec, with absolute ethanol for 5 min, and air-dried.

According to Swan [5], the reactions are specific to convert sulfhydryls and disulfides to thiosulfates:

$$R-SH+2Cu^{2+}+SO_3^{2-}\rightarrow R-SSO_3^-+2Cu^+ +H^+$$

$$R-SS-R+2Cu^{2+}+2SO_3^{2-}\rightarrow 2R-SSO_3^-+2Cu^+$$

Results and Discussion

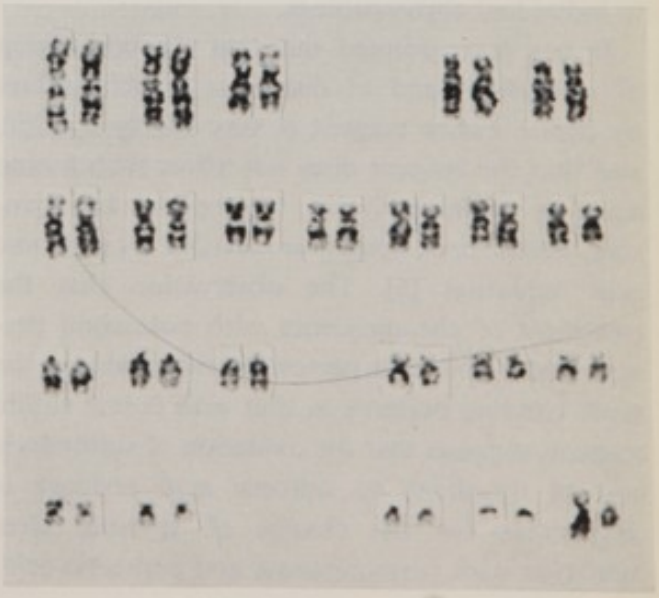
Staining of chromosome preparations with dilute Giemsa stain after peracetic acid treatment produced distinct banding patterns similar to the patterns produced by permanganate treatment (figs 1, 2). In the majority of metaphase plates observed in a single slide, chromosomes became swollen by the treatment with peracetic acid.

With respect to the banding patterns in each homologous pair which were identified by their sizes and their arm ratios, the arrangement of light- and dark-stained segments appeared to be identical within a single diploid complement and among metaphase plates.

The swelling and the patterns of differential staining of human chromosomes produced by permanganate and by peracetic acid treatments appeared generally to be similar to those illustrated in the studies on chromosome banding patterns with proteolytic enzymes [6, 7, 8].

Both potassium permanganate and peracetic acid have been known to oxidize sulfhydryls ($-SH$) and disulfides ($-SS-$) to sulfonic acid ($-SO_3H$) residues in peptides and proteins [9, 10]. If these changes in chromosome constituents were responsible for chromosome bandings after permanganate and peracetic acid treatments, the conversion of sulfhydryls and disulfides to thiosulfates ($-SSO_3H$) might also

Fig. 2. Banding patterns after oxidation of chromosomes with peracetic acid [4] at 0°C for 10 min. Stained with Giemsa stain. Banding patterns and swelling of chromosomes are generally similar to those in fig. 1.



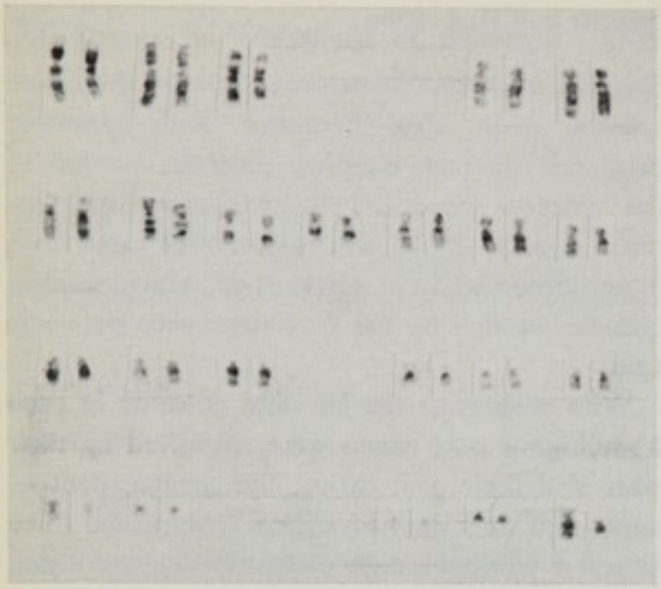


Fig. 3. Differential stainings of chromosomes by the treatment with cupric sulfite reagent [5]. Chromosomes did not swell with this reagent. Similarities in the banding patterns of certain homologous chromosomes treated with different reagents may be observed in figs 1, 2, 3.

produce similar banding patterns after Giemsa staining. This hypothesis was tested by treating chromosome preparations with cupric sulfite reagent. Fig. 3 shows the staining patterns of chromosomes after the treatment with cupric sulfite reagent. As is evident, chromosomes failed to swell by the treatment with this reagent. The banding patterns were generally the same as those treated with potassium permanganate and with peracetic acid. In particular, careful observation of the banding patterns in "A" group chromosomes differently treated, and which were positively identified by their sizes and arm ratios, revealed the close similarities in the arrangements of light- and dark-stained segments in individual chromosomes.

It has been pointed out that the conversion of sulfhydryls and of disulfides to thiosulfates by cupric sulfite reagent is very highly specific, and that the reagent does not affect such amino acids as arginine, lysine, tryptophan, and tyrosine, which are destroyed or modified by peracetic acid oxidation [5]. The observation that the treatment of chromosomes with potassium permanganate and with peracetic acid produced the same banding patterns as that with cupric sulfite reagent, suggests that the oxidation of sulfhydryls and of disulfides to sulfonic acid residues is responsible for the change of staining after oxidation with permanganate and peracetic acid. Consequently the differential staining patterns after oxidation treatment may indicate either the uneven distribution of sulfhydryls and/or disulfides along the longitudinal axis of a chromosome, or their unequal reactivity in various chromosome regions even if the distribution of sulfhydryls/disulfides was relatively homogeneous. Since most of the histone fractions contain very little cysteine/cystine residues [11], these observations may be interpreted as to suggest that non-histone proteins are responsible for the differential staining after oxidation treatment.

After the chromosome preparations were treated first with a disulfide-reducing agent (dithiothreitol) and then with organic mercurials [1-(4-chloromercuriphenylazo)-naphthol-2, and 3,6-bis-(acetatomercurimethyl)-dioxane], the permanganate oxidation and Giemsa staining did not produce the banding patterns. Chromosomes were stained uniformly dark blue [12]. These findings on the blocking of chromosome bandings by mercurials also tend to confirm that the differential staining after oxidation treatment is closely associated with sulfhydryls/disulfides in chromosomes.

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Discussion

Lejeune: If I understood right Dr Utakoji supposes that SH-bonding could be the cause of the coiling of chromosomes, as it does to hair keratin and gives curly hair.

Concluding Remarks

Trying to summarize a meeting like this is very difficult, since so many of the individual presentations were themselves summaries of a great deal of work. But in another sense it is rather easy, because Jerome Lejeune has done such a thorough job in his introductory talk on the scientific impact of Torbjörn Caspersson's discovery of chromosome banding. Professor Caspersson very modestly understated the importance of this discovery by implying it was simply an outgrowth of the basic science of cytochemistry. But such an outgrowth could only take place after he had the insight to ask the critical question and make the decision that a specific chemical marker of one purine or pyrimidine base in DNA would be useful, and should be sought. In any case, Torbjörn Caspersson cannot hide his own role so easily from us, because we know that the field of quantitative cytochemistry owes so much to his work.

Banding Techniques

Lore Zech described the quinacrine or quinacrine mustard fluorescent Q-banding technique, which remains the standard against which other methods are compared. The other major banding techniques were described briefly: C-banding by T. C. Hsu, Giemsa G-banding by Wolfgang Schnedl, and reverse Giemsa, or R-banding by Bernard Dutrillaux. I described an immunofluorescent technique for producing chromosome banding with antinucleoside antibodies. Antiadenosine produced a banding pattern closely resembling that of Q- or G-banding while anticytidine produced the reverse pattern clearly resembling R-banding. John Hamerton presented the main features of the report of the Paris Conference on Standardization in Human Cytogenetics.

Automated Banding Pattern Analysis

Caspersson has been successful in devising techniques for quantitative recording and analysis of the chromosome banding patterns and in stimulating other people to work towards automated and computerized analysis of banded human chromosomes. Aage R. Møller described a method of chromosome identification based on Fourier analysis of microdensitometric tracings of fluorescence intensity. Gösta Granlund presented a different method which, unlike the Fourier harmonic analysis, did not imply a relationship between fluorescent intensities of different regions along the chromosomes. Charles Ford pointed out the potential usefulness of such a sensitive method of analysis in recognizing genetic recombination between parents and progeny or between sibs.

Herbert Lubs and Kenneth R. Castleman discussed the potential of automated analysis in more general terms. Goals of machine methods for chromosome identification are becoming more well-defined, and perhaps less ambitious as the inherent superiority of man over the machine becomes more obvious. The object is not to replace the cytologist who is an expert at pattern recognition, but to relieve him of those parts of the task which can be done better or faster by machine so that he will have more time to do his own thing, interacting with the machine for maximum benefit.

Banding Techniques in Relation to Chromosome Structure and Composition

The chemistry of eukaryotic chromosomes was discussed by C. Ockey and that of polytene chromosomes by J. Edstrom. G. Bahr and D. Comings described their fine structural studies of chromosomes and the effect of banding procedures. However, the major part of the time devoted to techniques and mechanisms of chromosome banding

was taken up by short descriptions of an impressively large number of methods for producing banding visible with the ordinary light or fluorescence microscope. Chromosome banding has obviously attracted the attention of many people, and the field has grown rapidly in many directions. This is an exciting field right now, and the excitement was apparent throughout this meeting as more and more new techniques, new approaches, fresh ideas and new findings were described. A new experimental spirit clearly pervades almost every chromosome laboratory, and both naive and scientific attempts have been made to produce chromosome banding, in some cases using known fluorochromes or other specifically designed chemicals, in others taking reagents almost at random from the nearest shelf; almost every substance has been tried except beer and ketchup, and I would not be surprised if one of those worked quite well!

Fluorescent Banding

A growing number of techniques for producing chromosome banding have been discovered as a result of this new interest. Edward Modest described some of the fluorescent dyes that have been tested. Quinacrine mustard (QM) has been generally the most useful, and the fluorescent banding it produces, Q-banding, is the accepted standard in human cytogenetics. In addition, certain specific differences in reactivity have been noted, e.g. proflavine and acriflavine produce fluorescent bands in the same regions as QM on plant chromosomes but fail to produce bright fluorescence of the human Y. Similar differences involving other fluorochromes were described by various speakers, and the advantage of using several methods made clear. E. Modest discussed the relation of molecular structure to the chromosome banding effect of fluorochromes and other dyes: this effect is only shown by chemicals in which hydrogen atoms have replaced one or more of the four methyl groups attached to the two nitrogen atoms at opposite ends of the tricyclic compounds, acridine orange and methylene blue. In addition, 7-amino-actinomycin D binds specifically to guanine in double-stranded DNA, but shows the same quenching of fluorescence as quinacrine as the GC ratio in the DNA rises. Rudolf Rigler presented evidence that the quenching of fluorescence of GC-rich regions was due to the presence of the amine group on the guanine moiety.

Acridine orange is turning out to be an increasingly useful fluorochrome, primarily because it produces green fluorescence when complexed to native DNA and red fluorescence when complexed with single-stranded DNA or RNA. Its use in conjunction with the various banding techniques promises to clarify the role of denaturation and reassociation of chromosomal DNA in chromosome banding. In addition, several speakers reported the development of methods by which distinctive chromosome banding patterns could be produced with acridine orange, and the use of acridine orange must be included as a key method because of its importance in analysing the molecular basis of chromosome banding. Alfred Gropp described a new fluorochrome, a benzimidazole derivative, which produces either very bright or almost no fluorescence of heterochromatin (or C-banding regions), and in addition produces a very clear banding pattern, comparable to Q-banding, in some species but not in others. From such findings, the potential usefulness of still other fluorescent dyes for understanding the chemical structure of chromosomes is increasingly obvious, and the search for more agents goes on.

Giemsa Banding

One of the most exciting, and confusing, developments of the past three years has been the discovery that various types of pretreatment, or mistreatment, as Lejeune calls it, of the chromosomes prior to staining with Giemsa or certain other basic dyes, also leads to chromosome banding, and that these bands correspond, by and large, to those revealed by the fluorescent dyes. I think it is of more than symbolic significance that these techniques were developed as a by-product of research in molecular hybridization, that is, they had a strong scientific underpinning despite the accidental nature of the discovery by Gall & Pardue that Giemsa staining is restricted to a very small portion of the chromosomes, in the C-banding region, after the pretreatment schedule required for DNA-RNA hybridization in situ. As T. C. Hsu pointed out, molecular hybridization itself, in conjunction

with other techniques of molecular biology, such as those which make it possible to fractionate DNA into various classes, is now one of the most promising approaches to chemical mapping of chromosomes, if not gene mapping itself.

I won't attempt a detailed summary of all the papers dealing with Giemsa or G-banding techniques or those dealing with the mechanism of chromosome banding. More people wanted to speak on these topics than the time would allow. Nevertheless, it became clear as speaker followed speaker, that G-banding can be obtained in a number of ways, using salt solutions varying in their pH, temperature, ionic strength, divalent cation content and proteolytic enzyme content. The effect of various treatments on the DNA or protein content of chromosomes as well as on chromosome banding was studied by several people. Although all the data do not yet appear to be fully consistent, they may lead to some revision in our thinking, although it is not yet clear what the mechanism of G-banding is, or how many underlying mechanisms are involved.

Mechanisms of Chromosome Banding

Rudolf Rigler reviewed the biophysical studies of fluorochrome–DNA interactions. These provide strong support for the idea that quinacrine banding reflects differences in the base composition of the DNA along the chromosomes, with brighter fluorescence occurring in AT-rich regions. This view is supported by some of the results with acridine orange binding as well as by the findings I described with anti-nucleoside antibodies, anti-A producing a banding pattern generally closely similar to that of quinacrine, and anti-C producing the reverse banding pattern which resembles the R-banding with Giemsa described by B. Dutrillaux. However, the view that DNA is the important chromosome constituent in producing banding does not fully account for all the available information. Giemsa and other basic dye staining is largely dependent upon the availability of binding sites in the DNA, and this is determined largely by whether the sites are blocked by protein. The importance of chromosomal proteins for banding is supported by the effectiveness of proteolytic enzymes in producing banding. Fine structural studies suggest that G-banding procedures produce a distortion of the normal distribution of chromosome fibers, and this fits with other evidence that divalent cations also play a role in banding. A syncretic view, holding that DNA-protein interactions are involved, seems necessary, and seems to account in a general way for almost all the available evidence, since lysine-rich histones appear to bind preferentially to AT-rich DNA. It is still difficult, if not impossible, to fit all the evidence together, perhaps because not all the reports are correct! One last bit of evidence which may be of some importance is the finding reported by T. Utakoji that permanganate, peracetic acid or a cupric sulfite reagent, each of which may act primarily on the cystine-cystein residues of the non-histone proteins, can produce G-banding.

With such overwhelming amounts of new and undigested information, the time is ripe for a synthesis. This certainly did not occur at this meeting—we hardly agreed even on the fact—but the interaction of all these points of view may provide the stimulus for the development of a new model of the chromosome which could account for the important facts recounted at this meeting. I'm sure everyone would have liked to have more time to devote to the sessions on banding techniques and mechanisms if only to have seen David Comings' slides again, and for somewhat longer than the brief instant allowed each one. But the presentations dealing with various applications of the banding technique were equally crowded with new information, and perhaps even more interesting.

Applications to Medicine

There has been a veritable explosion of knowledge as a result of the application of the new banding techniques to the study of clinical disease. Speaker after speaker presented vast accumulations of new data, showing the existence of almost every kind of chromosome change imaginable. Insertions and other complex rearrangements requiring three or more chromosome breaks have been identified in profusion, evidence presented of the correctness of McClintock's interpretation of the mechanism of formation of double-sized rings, and new mechanisms proposed to account for some of the abnormal

chromosomes which can now be characterized so much more adequately.

The greater precision and accuracy of chromosome identification promises to lead to the delineation of many new clinical syndromes, and enhance our understanding of the role of chromosomal causes of maldevelopment, mental retardation, abortions, behavior disorders, and also of cancer. Caspersson and his associates first showed the specific association of a 22q – chromosome in the bone marrow with chronic myelogenous leukemia. That the Ph^1 chromosome is a deleted 22 has now been confirmed by O'Riordan et al. and many others, and other specific associations, e.g. Yq – and erythemic leukemia, are beginning to appear. Levan presented an optimistic summary of the progress his group has made in recognizing specific chromosome changes in the evolution of specific types of tumors. I can be forgiven for my personal view that gross chromosome changes may not play a role in the origin of most tumors, although an evolution of the karyotype may very well be found in the majority of more advanced tumors. On the other hand, the banding techniques will certainly make it possible to recognize the existence and type of altered karyotype in a much larger proportion of the positive cases than was possible before, as well as permitting new experimental approaches, using heteroploid and somatic hybrid cell lines.

Applications to Biology

Chromosome mapping

This brings us to the subject of the application of the new banding techniques to biology. The most impressive progress has been made in chromosome mapping. An almost complete mapping of mouse chromosomes has been achieved, as I described earlier, building on the great amount of genetic information available in this widely used species. The new banding techniques have also permitted rapid progress in assigning genes to human chromosomes, using rodent-mouse somatic cell hybrids from which human chromosomes tend to be lost. Chromosome banding techniques have greatly increased the power of this method, and reduced its margin of error, so that one can confidently expect that genes will have been assigned to virtually every human chromosome within the next year or two by the use of this technique. Already, as D. Bootsma described, definite or tentative assignments have been made to more than half the chromosomes in the human complement.

John Hamerton described stable man–Chinese hamster hybrids in which human chromosomes were not progressively lost. I described a simple graphical method for estimating the stability of hybrid or heteroploid lines. Nils Ringertz showed how a series of species-specific antibodies to proteins of the nucleus or chromosomes can be used to analyse the mechanism of gene activation in interspecific hybrid cells. Peter Pearson gave a very helpful summary of the various problems which must be dealt with in using interspecific hybrids to assign genes to specific chromosomes or chromosome segments. He presented more data, derived from rodent–man hybrids in which the human parent has an X-autosome translocation, in an attempt to resolve the problem of the location of the human hypoxanthine-guanine phosphoribosyl transferase, glucose-6-phosphate dehydrogenase and phosphoglycerate kinase loci. These data tend to support the idea that all three are on the long arm, but it is clear that the interpretation of data obtained in such hybrids is not yet routine. Nevertheless, this method is the most powerful tool we have right now for linkage analysis and for assigning genes to chromosomes in man.

The construction of genetic maps in man and the mouse is only the prelude to far more extensive understanding of linkage relationships in many species. This kind of information is essential for understanding many problems in evolution and genetic regulatory mechanisms. It would not be out of place here to mention that linked genes provide banding patterns of even higher resolution than quinacrine or Giemsa banding. In this regard, it seems only natural that Q- and G-banding are being used successfully to further the development of standard linkage maps.

Evolution

J. de Grouchy and P. L. Pearson showed how useful banding techniques can be in studying primate evolution. I described evolutionary changes limited to the satellite DNA of two

species of mice. D. Comings attempted to show the presence of homologies within the human karyotype, reflecting profound stability of banding patterns since the postulated occurrence of tetraploidization some 200 million years ago. Although some remained unconvinced, it is easy to see that the increased resolving power made possible by the banding techniques will play a role in providing tests for important hypotheses such as this one.

Studies on many different plant and animal species were presented by Karl Fredga, Alfred Gropp, Canio Vosa, Gunnar Östergren, and others, and many important principles delineated in this way, particularly by the combined use of several techniques. Biological variation has produced a vast array of life forms, some much better adapted than others to provide the answer to specific scientific questions. I would like to make one last comment, or perhaps it should be called a personal reaction. One of the underlying themes that became apparent during this symposium is that any field of applied science advances more rapidly when it builds on more basic sciences. Paper after paper dealt with the utilization of knowledge, concepts or techniques derived from biophysics, chemistry, molecular biology and immunology. This entire meeting attests to the rejuvenating effects of this approach.

Finally, I would like to take this opportunity, speaking on behalf of all the participants in this symposium, to thank our hosts for making this delightful and exciting meeting possible. To Professor Caspersson and the other members of the organizing committee; to the sponsors: the Nobel Foundation and its Nobel Symposium Committee, and the Tri-Centennial Fund of the Bank of Sweden; and to the members of the Congress Bureau who have been of so much help to us during the past few days, I would like to extend our warmest thanks and sincere appreciation of their unflagging interest and support.

Orlando J. Miller

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