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In situ Localization and Characterization of Different Classes of Chromosomal DNA: Acridine Orange and Quinacrine Mustard Fluorescence

Albert de la Chapelle, Jim Schröder and Ritva-Kajsa Selander

Folkhälsan Institute of Genetics, Helsingfors, Finland

Abstract. In situ denaturation of metaphase chromosomes with alkali results in a shift from green to yellow, orange, brown and red fluorescence with acridine orange, indicating increasing denaturation of chromosomal DNA. The kinetics and characteristics of denaturation are described. Mouse and *Microtus agrestis* chromosomes denature uniformly but human cells show sequential denaturation. With increasing concentrations of alkali, the secondary constrictions in chromosomes 1, 9 and 16 are the first, and the distal half of the Y chromosome the last, to become denatured. — Reassociation of chromosomal DNA occurs within seconds after the start of incubation in salt solution. Areas containing repetitious DNA, *e.g.* mouse centromeres, fluoresce much more strongly than other regions with acridine orange after prolonged reassociation. Since human and *Microtus* centromeric regions behave similarly, it is proposed that they, too, contain repetitious DNA. — Reassociation treatment leads to enhancement of bright quinacrine mustard fluorescence in regions already bright before treatment. Furthermore, regions containing repetitious DNA, *e.g.* the secondary constrictions in human chromosomes 1, 9 and 16, whose fluorescence is dull before treatment, turn bright after reassociation. — The methods of fluorescence analysis of mammalian chromosomes with acridine orange and quinacrine mustard permit the localization and study of different classes of chromosomal DNA.

I. Introduction

Attempts to determine the localization of different classes of DNA in different parts of the mammalian chromosomes are now under way in many laboratories. Major achievements in this field were obtained by the use of *in situ* nucleic acid hybridization techniques. Mouse satellite DNA occurs in most or all centromeres (Pardue and Gall, 1970; Jones, 1970). Repetitious DNA is found in the constitutive heterochromatin of the sex chromosomes and possibly elsewhere in *Microtus agrestis* (Arrighi *et al.*, 1970). Human satellite II DNA is found in the secondary constrictions of chromosomes 1, 9 and 16 (Jones and Corneo, 1971) and different classes of human repetitious DNA appear to be enriched in various chromosomal regions, notably some telomeric and centromeric areas (Saunders *et al.*, 1972).

Characteristic band patterns of fluorescence are obtained when chromosomes are stained with acridine derivatives (Caspersson *et al.*, 1970). Similar, but not identical, patterns result when staining with Giemsa is preceded by treatment such as with alkali, acids, enzymes and heat (Sumner *et al.*, 1971; Schnedl, 1971a; Finaz and de Grouchy, 1971; Drets and Shaw, 1971; Patil *et al.*, 1971; Dutrillaux *et al.*, 1972).

At present, the mechanisms leading to the emergence of darker and lighter regions (bands) after such treatment are poorly understood. We have previously used the fluorochrome acridine orange to determine under what experimental conditions chromosomal DNA becomes denatured and reassociated *in situ* (de la Chapelle *et al.*, 1971). In this paper, we report further studies with acridine orange and quinacrine mustard fluorescence of mammalian chromosomes. It will be shown that regions containing satellite DNA behave in a characteristic way enabling them to be distinguished from other parts of the chromosomes.

II. Materials and Methods

1. Cell Cultures, Preparation of Slides

Human lymphocytes, mouse L cells and fibroblasts of primary explants of skin from *Microtus agrestis* were cultured *in vitro* using standard procedures. The cultures were terminated after 2–4 hours' treatment with Colcemid (Ciba). L cells and fibroblasts were detached from the surface of the culture flasks with 0.1% trypsin. After this, all the cell types were processed in an identical manner as follows: hypotonic treatment for 10 min in 0.075 M KCl, after which the cells were fixed in 3:1 methanol-acetic acid and stored at 4° C in the fixative for between 1 hour and 30 days. Before slides were made, the cells were washed twice with fresh fixative. Slides were prepared according to two alternative procedures: a) Slides washed in detergent and rinsed in water were stored in ethanol. Each slide was dried and dipped in tap water and the excess of water shaken off, after which 2–3 drops of cell suspension in fixative were allowed to fall on to the wet slide. After gentle blowing to distribute the suspension evenly, the slide was placed to dry on a hot-plate set at 40° C or in an incubator set at 40° C. b) Unwashed slides were wiped clean with Kleenex tissue and 2–3 drops of cells were pipetted onto the dry surface. Blowing and drying was as above. No consistent differences in denaturation or reassociation behaviour were observed between chromosomes on slides prepared in the two ways.

2. Further Treatments

Slides were stored for 1—100 days at 4° C before use. They were treated with alkali, acid and enzymes etc. at room temperature by placing the slides on a glass rack which was immersed in the solution indicated. After NaOH treatment the slides were transferred to 70% ethanol, 90% ethanol and then dried. After HCl treatment, they were washed with distilled water and dried. After enzyme treatment, they were washed in the corresponding buffer and dried. The slides were incubated in SSC solution by immersing them in a container with the solution in an incubator set at 67° C. After incubation, the slides were washed in 70% and 90% ethanol and then dried.

3. Staining, Microscopy, Photography

The staining method was as follows: The slides were processed through a series of decreasing concentrations of ethanol (absolute, 70%, 50%, distilled water). The staining time was 10 minutes with a 0.5% solution of acridine orange (AO) in McIlvaine's buffer, pH 7.0, or, alternatively, with a 0.005% solution of quinacrine mustard (QM) in the buffer just mentioned. Slides were sealed in the buffer.

The method and equipment used in the microscopy have been outlined previously (Schröder and de la Chapelle, 1972). A Leitz Ortholux microscope was fitted for fluorescence with an HBO 200 mercury lamp, a vertical Ploem Opaque illuminator and a 54 × achromatic lens. The filter combination was a 5 mm BG 12 excitor filter and a 490 nm barrier filter. The filter in the illuminator was set at 3. This combination gives the light a wavelength of approximately 510 nm.

4. Chemicals, Reagents

SSC is an abbreviation of 0.15 M NaCl, 0.015 M tri-sodium citrate, pH 7.0. Quinacrine mustard was obtained from the Sterling Winthrop Company. Acridine orange was purchased from G. T. Gurr, Ltd., trypsin from Difco Laboratories, pronase from Calbiochem Company, arginase, ribonuclease A and nuclease (Micrococcal, from strain SA-B) from the Sigma Company.

III. Results

1. Denaturation of DNA

The distribution of double-stranded and single-stranded DNA in the chromosomes *in situ* can be assessed from the colour of acridine orange (AO) fluorescence at 510 nm (Rigler, 1966; de la Chapelle *et al.*, 1971). While it is not possible to give a quantitative assessment of the extent of denaturation, the differences in colour of the fluorescence are clearcut and apparently reveal in qualitative terms the strandedness of the majority of DNA molecules present.

When untreated slides are stained with AO, the fluorescence of the chromosomes is green or yellow-green (Fig. 1). We have previously shown that this is certainly not always the case. If the slides are heated intensely during the drying procedure or if the fixative is ignited, the chromosomes in some or all of the cells may become red in colour (de la Chapelle *et al.*, 1971). We therefore stress that in order not to denature the DNA during preparation, procedures such as those described here (Materials and Methods) should be used. Interphase nuclei stain green but the nucleoli are often orange in colour. If abundant cytoplasm is present, scattered red fluorescence is seen throughout. According to our interpretation of these findings, undenatured DNA (double-stranded) stains green or yellow-green whereas nucleoli and cytoplasm stain orange or red because of their content of RNA (single-stranded).

Previous treatment with NaOH changes the colour of metaphase chromosomes stained with AO. There is a slight decrease in the intensity of fluorescence. With increasing concentrations of NaOH and longer

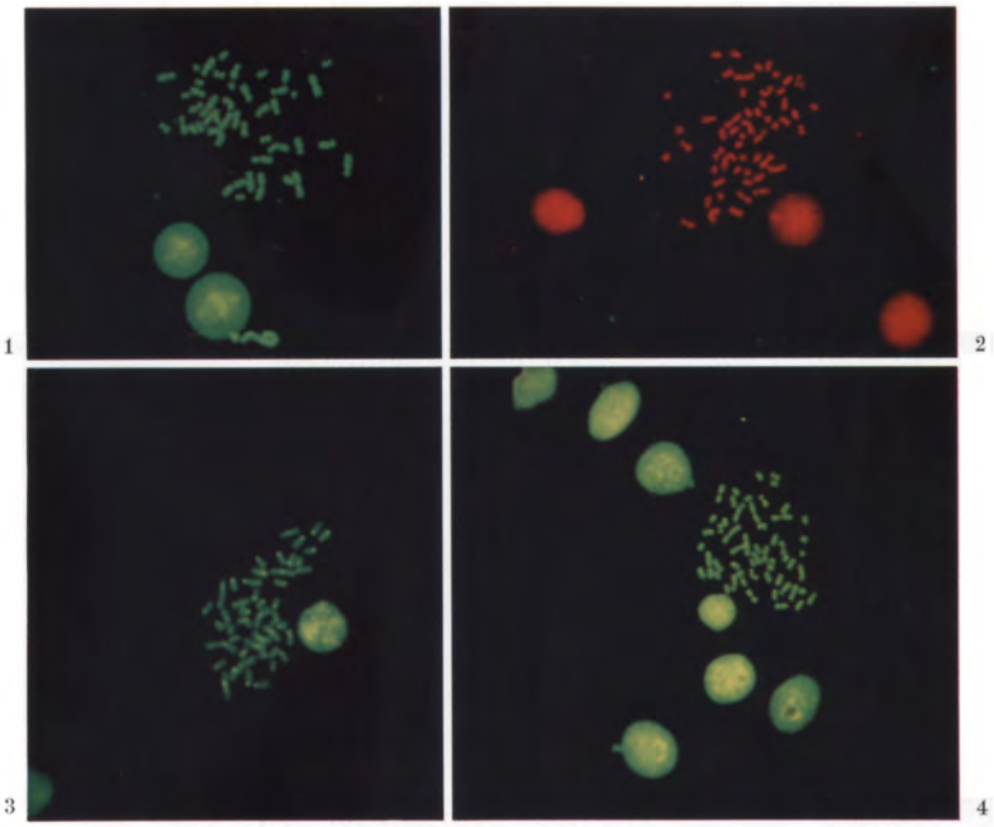

Fig. 1. Human metaphase from a lymphocyte culture stained with acridine orange. Uniform green fluorescence of the chromosomes

Fig. 2. Mouse L cell metaphase stained with acridine orange after prior treatment with 0.5 M NaOH for 2 minutes. Uniform red fluorescence of the chromosomes

Fig. 3. Mouse L cell metaphase and interphase stained with acridine orange after successive treatment with 0.5 M NaOH and incubation in 6 × SSC for 16 hours at 67° C. The fluorescence of the chromosomes is green. The centromeric regions have brighter fluorescence which is yellow or yellow-green in colour. In the interphase nucleus, areas with bright yellow-green fluorescence are seen

Fig. 4. Mouse L cell metaphase treated as in Fig. 4. In this cell, the fluorescence in the centromeric regions is red

periods of treatment, the fluorescence of the chromosomes (and interphase nuclei) becomes successively yellow, brown-red and bright red (Fig. 2). This is interpreted to signify an increase in the relative amounts of denatured (single-stranded) DNA.

a) Sequential Denaturation in Human Chromosomes

In human cells, different chromosomal regions show a conspicuous sequence of colour change (denaturation of the DNA). The first regions to become red are the paracentromeric secondary constrictions in chromosomes 1, 9 and 16. With low concentrations of alkali it is not uncommon to see the secondary constrictions bright red while the rest of the chromosomes still have unaltered green or yellow-green fluorescence (Fig. 5). When the concentration of NaOH is increased, all other regions of the human chromosomes turn yellow, brown and finally red, except the distal half of the long arm of the Y chromosome, which remains green or yellowish-green. Only after treatment with even stronger NaOH does this part of the Y turn red. It is therefore not uncommon to see mitoses in which the distal half of the Y chromosome has green, and the rest of the chromosomes brown or red fluorescence. These findings indicate that certain regions in human chromosomes contain DNA which is more easily denatured than the DNA in other regions.

Mitoses from mouse L cells and *Microtus agrestis* do not show any sequential denaturation. In these cells the changes in colour appear uniformly over all chromosomal segments.

b) Alkali Concentration

We have tested concentrations of NaOH ranging from 0.005 to 0.5 M. It is not possible to indicate any threshold value at which different colours appear in different regions. At 0.005 M NaOH no detectable change in colour occurs, and at 0.5 M NaOH, the fluorescence of all regions is red or brown-red in most instances. The secondary constrictions in human cells quite often turn red at a concentration around 0.01 M, while the Y chromosome is the only remaining green segment at 0.07 M NaOH. However, it should be emphasized that the concentrations of NaOH needed to achieve a certain degree of denaturation vary from experiment to experiment and even from cell to cell on the same slides. One factor of importance in this respect is the length of time for which the cells are stored before processing. The older the cells, the more resistant is the DNA to denaturation by NaOH. This is true whether the cells are stored in fixative or on ready-made slides. Furthermore, details in the various procedures preceding the NaOH treatment, notably the way in which the cells are dried, certainly have an influence

on the results of denaturation. We have found the methods difficult, if not impossible, to standardize so that the degree of denaturation could be predicted from the concentration of NaOH. However, in each experiment lower concentrations consistently lead to less, and higher concentrations consistently to more extensive denaturation.

The effect of the length of the NaOH treatment was tested in several experiments. It was shown that for each concentration used, maximum effect was obtained after 2 minutes. No additional denaturation occurred if the treatment was prolonged for up to 10 minutes. Hence 2 minutes was used throughout this study.

If the treatment with NaOH was preceded by treatment with 0.001—1 M HCl or RNase (20 μg/ml in 2 × SSC, pH 7.0), or both in succession, no effect was discernible on the parameters of AO staining. In this context it should be remembered that all the cells were in the fixative containing 25% acetic acid for at least 1 day.

The following sequences of acid and alkali treatment were tested without evidence of any effect on the denaturation parameters:

HCl—NaOH, NaOH—HCl and NaOH—HCl—NaOH.

2. *Reassociation of DNA*

a) Acridine Orange

In an attempt to achieve reassociation of denatured DNA, cells in which the DNA had been extensively denatured were incubated in 6 × SSC at 67° C, with the result that the colour of fluorescence with AO reverted from red to green (Fig. 3). We therefore conclude that reassociation of the DNA takes place. For convenience, the incubation procedure in 6 × SSC at 67 °C is henceforth referred to as "reassociation" or "reassociation procedure" in this paper.

An observation worth mentioning is that the colour of the chromosomes in cells subjected to denaturation-reassociation is different from that of untreated cells. While untreated cell nuclei and chromosomes mostly have yellow-green fluorescence (Fig. 1) denaturation-reassociation leads to green colour (Fig. 3).

The intensity of the green fluorescence with AO after reassociation is much stronger in the following regions; in human cells: all centromeric regions, the paracentromeric secondary constrictions in chromosomes 1, 9 and 16, and the distal portion of the Y; in mouse cells: all centromeric regions (Fig. 3); and in *Microtus agrestis* cells: all centromeric regions and the constitutively heterochromatic portions of the X and Y chromosomes (shown in Fig. 1 b by de la Chapelle *et al.*, 1971). These strongly fluorescent regions are often yellow or yellow-green (Fig. 3). All these regions correspond to the so-called C bands after Giemsa staining. It is especially noteworthy that in human cells, the DNAs

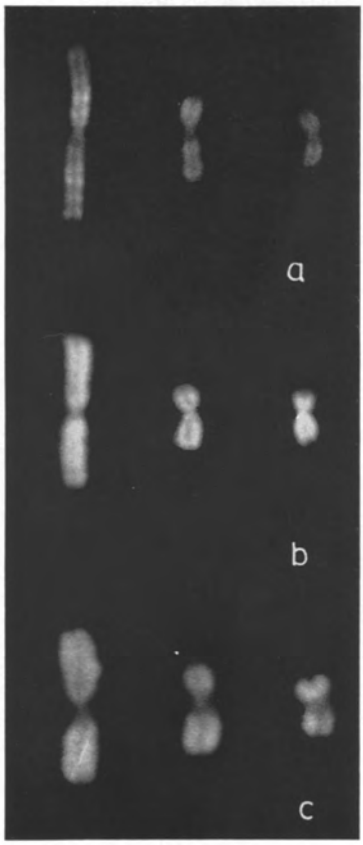

Fig. 5a—c. Human chromosomes Nos. 1, 9 and 16 from three different cells. a) Quinacrine mustard staining of an untreated cell. The fluorescence in the paracentromeric secondary constrictions is dull. b) Acridine orange staining of an untreated cell. The fluorescence is uniform. c) Acridine orange staining after denaturation in 0.01 M NaOH for 2 min. The colour of the chromosomes is green except for the secondary constrictions which are red. The red fluorescence appears dull on black and white photographs

in the secondary constrictions and the Y chromosome behave in an identical fashion after reassociation, whereas they are quite different in their susceptibility to denaturation by alkali.

In any case, most of the regions displaying strong AO fluorescence are known to contain repetitious DNA (cf. Introduction). However, there is no evidence as yet to show that all human centromeric regions or the distal end of Y contain repetitious DNA. Neither has it been shown that the autosomal centromeric regions in *Microtus agrestis* are enriched with repetitious DNA.

Another observation has to be reported in this connection. In occasional experiments involving denaturation-reassociation, the centromeric regions in mouse cells and the paracentromeric constrictions in human autosomes Nos. 1, 9 and 16 are red instead of yellow or green (Fig. 4). This exceptional behaviour after reassociation will be discussed below, but it should be borne in mind that we have shown above that the DNA in the constrictions in human chromosomes is very susceptible to denaturation.

b) Kinetics

The kinetics of the reassociation was studied using L cells. After only 30 seconds in the salt solution, yellowish-green fluorescence emerged. Hence, the reassociation is extremely rapid. Similar findings were recently reported after heat denaturation of mouse cells (Stockert and Lisanti, 1972). The appearance of stronger green fluorescence in particular regions described above (Fig. 3) is much slower; it begins to occur after 8 hours and is clearly discernible after 10 hours. When the treatment was prolonged for 16 hours, no additional changes occurred.

c) Quinacrine Mustard

In order to try to elucidate the mechanism of differential fluorescence along chromosomes after staining with QM, cells which had undergone the described denaturation-reassociation treatment were stained with QM. It is well known that in addition to the Y chromosome, certain autosomal regions in human cells have small areas with very bright fluorescence. Such regions are to be found near the centromeres in chromosomes 3, 4 and 13, and sometimes in 14, 15, 21 and 22 (Caspersson *et al.*, 1971). The bright fluorescence may occur in one or both homologues, or it may be of different strength in the homologues (Schnedl, 1971 b). There are indications that these traits are inherited in a Mendelian fashion (Uchida and Lin, 1971). Enhancement of the bright fluorescence after denaturation-reassociation has been reported (Gagné *et al.*, 1971; de la Chapelle *et al.*, 1971). We are now able to report that in 10 persons whose cells had quite different distributions of these brightly fluorescent areas with QM before treatment, extensive denaturation followed by reassociation treatment for 16 hours always resulted in an enhancement of the bright fluorescence (Fig. 6). Since the overall intensity of fluorescence decreases after the procedure described it is not possible to assess whether the apparent enhancement is an increase in actual intensity or a result of the decrease in intensity in surrounding areas. Nevertheless, wherever a region in at least one of the homologues of chromosomes 3, 4 and 13 was bright before treatment, it became bright in both homologues after the treatment. However, a difference in intensity between the two homologues was often noticeable. Further-

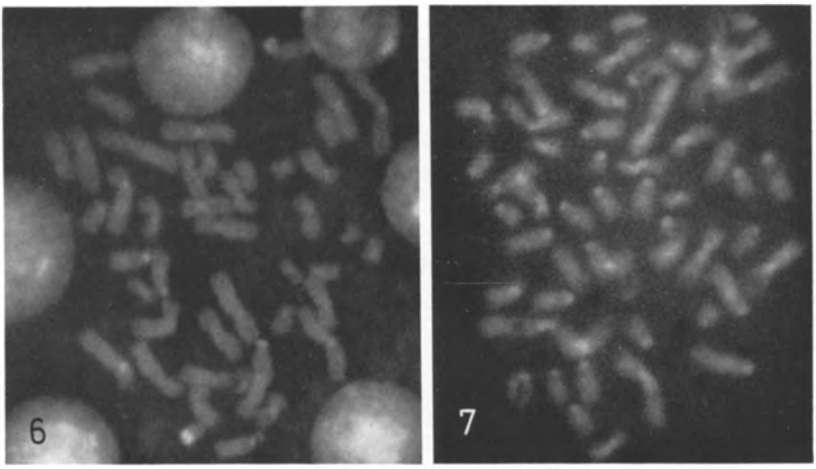

Fig. 6. A human metaphase and some interphase nuclei stained with quinacrine mustard after successive treatment with 0.5 M NaOH and incubation in 6 × SSC for 16 hours at 67° C. There is a marked decrease in the overall intensity of fluorescence and fuzziness of the chromosomes. The centromeric regions of most chromosomes and the distal part of the Y chromosome are more brightly fluorescent than other regions. Interphase nuclei contain brightly fluorescent spots

Fig. 7. Metaphase from a mouse L cell treated as in Fig. 6. Low intensity of fluorescence and fuzziness of chromosome structure are seen. There is bright fluorescence in all or most centromeric regions

more, strongly fluorescent regions in chromosomes 14, 15, 21, 22 and Y were exceptionally strong after treatment. Moreover, in most instances bright fluorescence can be seen in all human centromeric regions as well as in the paracentromeric constrictions in chromosomes 1, 9 and 16 (Fig. 6). Sometimes this is so conspicuous that the constriction, *e.g.* in chromosome 9, is as bright as the Y chromosome.

In mouse cells all centromeric regions behave in a similar way (Fig. 7), as was also shown in *Microtus agrestis* (Fig. 2b in de la Chapelle *et al.*, 1971). After denaturation-reassociation, the fluorescence with QM is practically indistinguishable from that with AO. This is in contrast to the very marked differences in untreated cells. The shift in intensity of QM fluorescence from very dull to very strong, *e.g.* in human secondary constrictions and mouse centromeres, is especially notable.

3. Treatment with Enzymes

In cytological preparations, the chromosomal DNA is bound to proteins which have probably only partly become denatured or otherwise altered by fixation in alcohol and acetic acid. To evaluate the role of proteins in the fluorescence properties with AO and QM, cells were

stained after pretreatment for 1–30 minutes in 0.05 M Tris buffer, pH 7.5, with 0.01% trypsin, 20 μg/ml arginase or 30 μg/ml pronase. The longer the treatment, the more extensive was the destruction of normal chromosome structure. Therefore 2.5–5 minutes was the maximum period after which the fluorescence could be evaluated. No changes in the fluorescence with either fluorochrome was detected. We emphasize that enhancement of fluorescence, *e.g.* in human secondary constrictions, does not occur after enzyme treatment. This is in contrast to the strong Giemsa staining after similar procedures.

Conclusions from these experiments should be made with caution, because it is not possible to assess how completely the enzymes digest the proteins and to what extent certain proteins remain unaffected. Moreover, treatment with 20 μg/ml nuclease in the above-mentioned buffer did not destroy chromosome structure and the fluorescence pattern remained unchanged. This appears to indicate that digestion by enzymes of chromosomes *in situ* may be very incomplete. However, there is an indication that within the limits of the practicability of the method, proteolytic enzymes do *not* result in any change in fluorescence with AO and QM. Hence there is no present evidence to suggest that "unmasking" of the DNA *in situ* alters the fluorescence patterns.

IV. Discussion

1. Denaturation

The molecular explanation of the sequential denaturation of different regions in the human chromosomes should take into account not only the properties of the DNA, but also those of proteins (Kernell and Ringertz, 1972). Concerning the DNA itself, it is known that heat denaturation of DNAs rich in guanine (G) and cytosine (C) requires higher temperatures than of DNAs rich in adenine (A) and thymine (T) (Mandel and Marmur, 1968). Similarly, AT-rich DNAs presumably denature at lower concentrations of alkali than GC-rich DNAs (Gillespie, 1968). It is interesting, therefore, that the secondary constrictions in chromosomes 1, 9 and 16 denature first in our system, since they contain satellite II DNA (Jones and Corneo, 1971) which is rich in A + T (Corneo *et al.*, 1970). We failed to obtain any direct evidence to support the idea that the removal of chromosomal proteins affects the denaturation behaviour of the chromosomes. We suggest, then, that the human autosomal secondary constrictions denature before other regions because of the high AT-content of their DNA. Human satellite II DNA is highly repetitious as is shown by its behaviour at reassociation (Cornea *et al.*, 1970). However, the mouse satellite is also highly repetitious in nature (Waring and Britten, 1966) but not especially rich in A and T (Corneo, Ginelli, Soave and Bernardi, 1968). The mouse satellite is contained

mainly or only in the centromeric regions of the chromosomes which do not denature before other regions in our system. We therefore suggest that the susceptibility to denaturation is not due to the repetitious nature of human satellite II.

The molecular composition of the DNA at the distal end of the Y chromosome is not known. This region has very strong fluorescence with QM (Caspersson *et al.*, 1971). It has been stated that the strong fluorescence with QM may be due to a high G content (Caspersson *et al.*, 1970). If this were so, then the relative resistence to denaturation in our system could be explained by the low AT-content. However, there is as yet no direct evidence for a high G content in this region.

On the other hand, Weisblum and de Haseth (1972) showed that the higher the content of G + C in natural DNAs, the more they quenched fluorescence with quinacrine *in vitro*. Bihelical polynucleotides gave similar results. If these findings prove to be identical or comparative with QM, there is a discrepancy between the two sets of evidence. It should be remembered, of course, that fluorescence properties *in vitro* and *in situ* may be different. This could be due to different patterns of organization or of the secondary structure of DNA, the DNA-protein interactions or to a combination of these.

2. Reassociation

Reassociation of alkali-denatured DNA *in situ* is very rapid, as shown in this report. Stockert and Lisanti (1972) reported reassociation of heat-denatured mouse chromosomal DNA within seconds after incubation in a salt solution. In their system, the centromeric regions were the first to reassociate. The presumed reasons for the extremely fast reassociation were outlined by Stockert and Lisanti (1972). It should be added here that not only is repetitious DNA rapidly reassociated, but, in addition, all other chromosomal regions loose their red colour with AO within seconds after the start of incubation. This could be due to extremely high concentrations of DNA in the *in situ* situation, because reassociation of non-repetitious DNA *in vitro* requires very long incubation periods at standard concentrations (Britten and Davidson, 1969; Gelderman *et al.*, 1971).

We can propose no clear-cut explanation for the strong fluorescence with AO after denaturation-reassociation. The phenomenon occurs in regions known to contain repetitious DNA: in human chromosomes the secondary constrictions in chromosomes 1, 9 and 16, in mouse chromosomes all centromeric regions, and in *Microtus agrestis* the constitutive heterochromatin in the X and Y. In addition, strong fluorescence with AO occurs in all centromeric regions in human and *Microtus* cells. These regions have not been shown to contain repetitious DNA. In view of their analogous behaviour, *e.g.* with mouse centromeres in our system,

we tentatively propose that they contain repetitious DNA. Other explanations may be possible.

In molecular terms, explanations should be sought of this behaviour with AO, as well as of the exceptionally bright fluorescence with QM after the procedure. Possible explanations are: (i) there is more DNA in these areas than in other regions after the treatment. This could be a consequence of the loss of DNA from regions not containing repetitious or highly repetitious sequences. (ii) There could be a different organization of the DNA in the brightly fluorescent areas as demonstrated *in vitro* by Corneo *et al.* (1968), who showed that reassociated mouse satellite DNA has a density which is significantly higher than that of native satellite DNA. Furthermore, the thermal denaturation characteristics of native and reassociated DNA are different (Waring and Britten, 1966; Corneo *et al.*, 1971). On the other hand, base mispairing is common in the experimental hybridization procedures (McConaughy and McCarthy, 1970) and could account for a decrease in intensity in chromosome segments containing mainly non-repetitious DNA. (iii) The availability of DNA in brightly fluorescent areas to AO and QM could be improved after denaturation-reassociation. The failure to observe any change in AO or QM fluorescence after treatment with various proteolytic enzymes is an indication that the removal of proteins may not significantly affect fluorescence. However, it is likely that the removal of proteins by enzymes is only partial. The failure of nuclease to alter the fluorescence further indicates that the effects of enzyme treatment *in situ* should be interpreted with caution.

The shift in the quality of the green colour after denaturation-reassociation is worth noting and could well be due to the differences in the chemical properties of the native and reassociated DNA mentioned above. This remains to be experimentally verified by *in vitro* tests. Another, less intriguing possibility is that the colour becomes a clearer green after treatment because *e.g.* ribosomal and nuclear RNA (red in fluorescence) have been washed away during the treatment. The yellowish-green colour of untreated chromosomes would then be due in part to interference with the redness of RNA.

Mouse centromeric regions occasionally turn red instead of yellow-green after the denaturation-reassociation procedure (Fig. 4). This exceptional behaviour could be due to (i) spontaneous denaturation after the reassociation procedure or (ii) irreversible denaturation.

A feature of the fluorescence with QM is that the brightly fluorescent areas in certain chromosomes become even brighter after denaturation-reassociation. The treatment further brings out bright fluorescence in homologues which did not show the phenomenon in untreated cells. This phenomenon, also recorded by Gagné *et al.* (1971), should be better characterized using larger samples of test subjects.

3. Use and Significance of the Methods

There is little doubt that much can be learned about the composition and organization of the chromosomal macromolecules by the use of *in situ* fluorescence analysis. Conclusive evidence as to the chemical nature of dye-chromosome interactions will probably come from experiments *in vitro*. The great value of *in situ* techniques such as those described here is that they allow precise chromosomal or cellular localization of various classes of DNA. We therefore feel that more can be learned through these techniques.

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Dr. A. de la Chapelle
The Folkhälsan Institute of Genetics
P. O. Box 819
00101 Helsingfors 10
Finland