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Fluorescence Banding Pattern of Human and Mouse Chromosomes with a Benzimidazol Derivative (Hoechst 33258)*

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Summary. The fluorescent banding patterns of human chromosomes and mouse chromosomes (from a tumour line MSWBS) following binding with a benzimidazol derivative (Hoechst 33258) were studied. Constitutive heterochromatic blocks in both materials fluoresced intensively with this stain. DNAase digestion or alkali denaturation and reassociation of the preparations increased the specifity of this fluorochrome to the constitutive heterochromatic blocks. The possible reasons for this differential binding of the stain to the heterochromatin are briefly discussed.

Zusammenfassung. Fluorescenz-Bandenmuster der Chromosomen von Mensch und Maus (letztere von dem Tumor-Zellstamm MSWBS) wurden nach Bindung mit einer Benzimidazol-Verbindung (Hoechst 33258) untersucht. Konstitutive Heterochromatin-Blöcke fluorescieren bei beiden Arten intensiv mit diesem Farbstoff. DNAase-Behandlung der Alkali-Denaturierung und Reassoziation der Präparationen erhöhen die Spezifität dieses Fluorochroms bezüglich der konstitutiven Heterochromatin-Blöcke. Die möglichen Ursachen für diese spezielle Bindung dieses Farbstoffs an das Heterochromatin werden kurz diskutiert.

Introduction

Since the introduction of the quinacrine fluorescence technique for the identification of chromosomes by Caspersson *et al.* (1969), several new techniques and many modifications have appeared in the literature. Of these, the C-banding technique is very useful for location of the constitutive heterochromatic regions in the chromosomes of several mammals, and also of plants and insects (Arrighi and Hsu, 1971; Klasterska *et al.*, 1973; Natarajan and Natarajan, 1972). In addition, Hilwig and Gropp (1972) found that another fluorochrome — a bis-benzimidazol derivative — was relatively specific to the constitutive heterochromatin in mammals. We have used this dye to identify individual chromosomes in widely different species of plants, insects and mammals (Sarma and Natarajan, 1973; Klasterska *et al.*, 1973; Natarajan *et al.*, 1973). In this communication we present our observations on the specificity of this fluorochrome to certain regions of human chromosomes and our results of experiments with this dye on human and mouse chromosomes, performed directly and also after different pretreatments

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to elucidate the mode of preferential binding of this dye to different regions of the chromosomes.

Material and Methods

Human chromosome preparations were obtained from phytohaemoglutinin-stimulated lymphocytes from normal male subjects by the routine air-drying technique. Mouse cells used in this study were from an ascites tumour line, MSWBS. This line has a stem-line chromosome number of 28—29 (Levan *et al.*, 1972) with the constitutive heterochromatin redistributed in the genome (Natarajan *et al.*, 1973). Host animals with tumours were given injections of colchicine (10 μg/g body weight) and after 2 hrs the ascitic fluid was drawn off by capillaries. The cells were washed with balanced salt solution and treated with hypotonic saline (medium + distilled water 1:4) for 20 min at 37°C. The slides were fixed in acetic methanol (1:3), dropped onto chilled slides and air-dried.

Staining

The slides were stored for not more then 10 days before staining with the fluorochrome. The slides were washed in 95% alcohol for 5 min and then incubated in benzimidazol dye solution (Hoechst 33258, kindly supplied by Dr. H. Loewe) at a concentration of 0.25 μg/ml for 20 min at 37°C (pH 5). The slides were rinsed twice in Soerensen's buffer (pH 5.5), mounted in buffer or glycerol and viewed under a Zeiss fluorescence microscope.

To allow some understanding of the mode of action of this fluorochrome the chromosome preparations were subjected to the following treatments before incubation in the dye solution.

1. Treatment with DNAase at a concentration of 100 μg/ml for 10 min at 37°C. The slides were then washed in the buffer and stained.

2. Treatment with 0.2 N HCl for 30 min at room temperature before incubation in the bis-benzimidazol derivative.

3. Processing according to the C-banding technique, i.e. HCl, RNase, 0.07 N NaOH for 90 sec and 6 × SSC overnight at 65°C before staining with Hoechst 33258.

4. For comparison, C banding of the chromosome preparations according to Arrighi and Hsu (1971).

Results

Human chromosomes show definitive fluorescent patterns following binding to this fluorochrome. The patterns are similar to those of Q banding in many respects (Fig. 1). However, heterochromatic regions of the autosomes show a differential response to these two dyes (Table 1). The Y chromosome is brightly fluorescent with both techniques. Centromeric regions fluoresce brightly, in contrast

Table 1. Comparison of fluorescence patterns between the Q banding and the bis-benzimidazol banding in human chromosomes

	Chromosomes No.	Quinacrine	Hoechst 33258
Secondary constriction regions in	1	negative	positive
	9	negative	positive
	16	negative	positive
	21	positive	positive
	22	dull	dull
In D group	short arm of acrocentrics	positive or negative	positive
Distal part of	Y	positive	positive

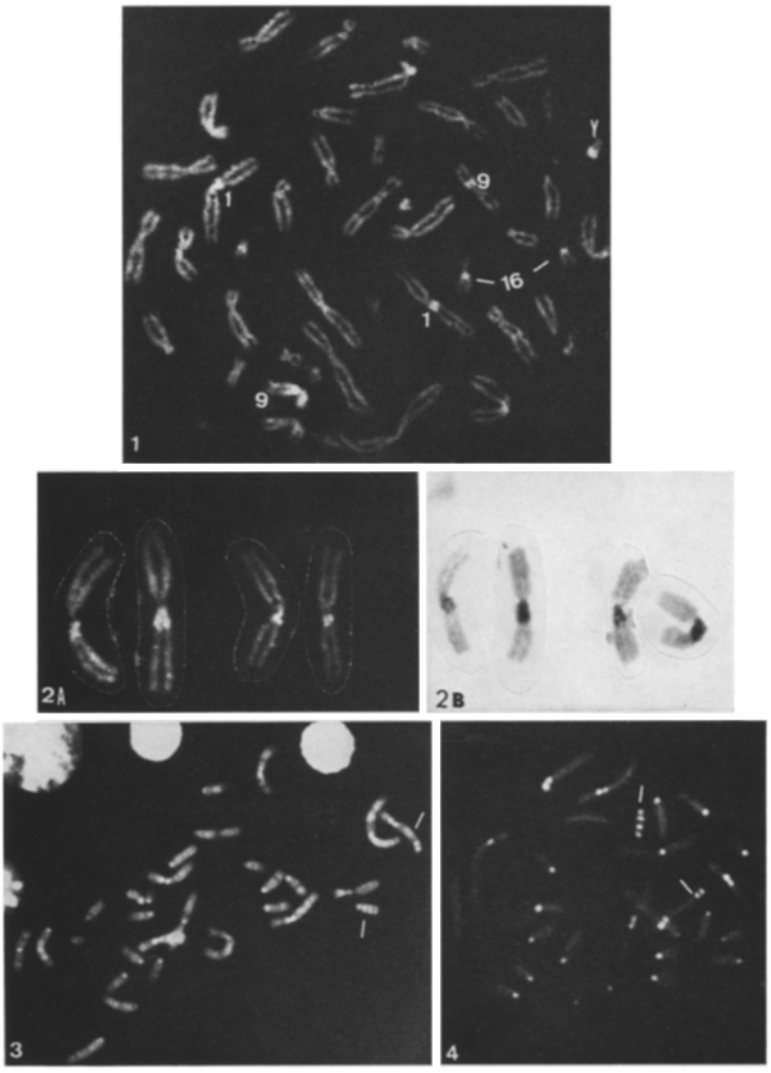

Fig. 1. Fluorescent pattern of normal human male metaphase chromosomes following binding to Hoechst 33258. Note the brightly fluorescent secondary constriction regions of chromosomes 1, 9, 16 and the distal part of Y. The chromosome arms also reveal patterns

Fig. 2A and B. A composite illustration for the polymorphism of the constitutive heterochromatic blocks in chromosome 1. A Hoechst 33258, B C banding

Fig. 3. MSWBS tumour metaphase stained with Hoechst 33258. Note the telocentric and biarmed chromosomes. In addition to brightly fluorescent centromeric heterochromatic regions, fluorescent bands on the chromosome arms are also seen. Arrows indicate the marker chromosomes

Fig. 4. MSWBS chromosomes after DNAase digestion. Only the constitutive heterochromatic regions show bright fluorescence. The chromosome arms fluoresce very dully with no banding patterns. Arrows indicate marker chromosomes

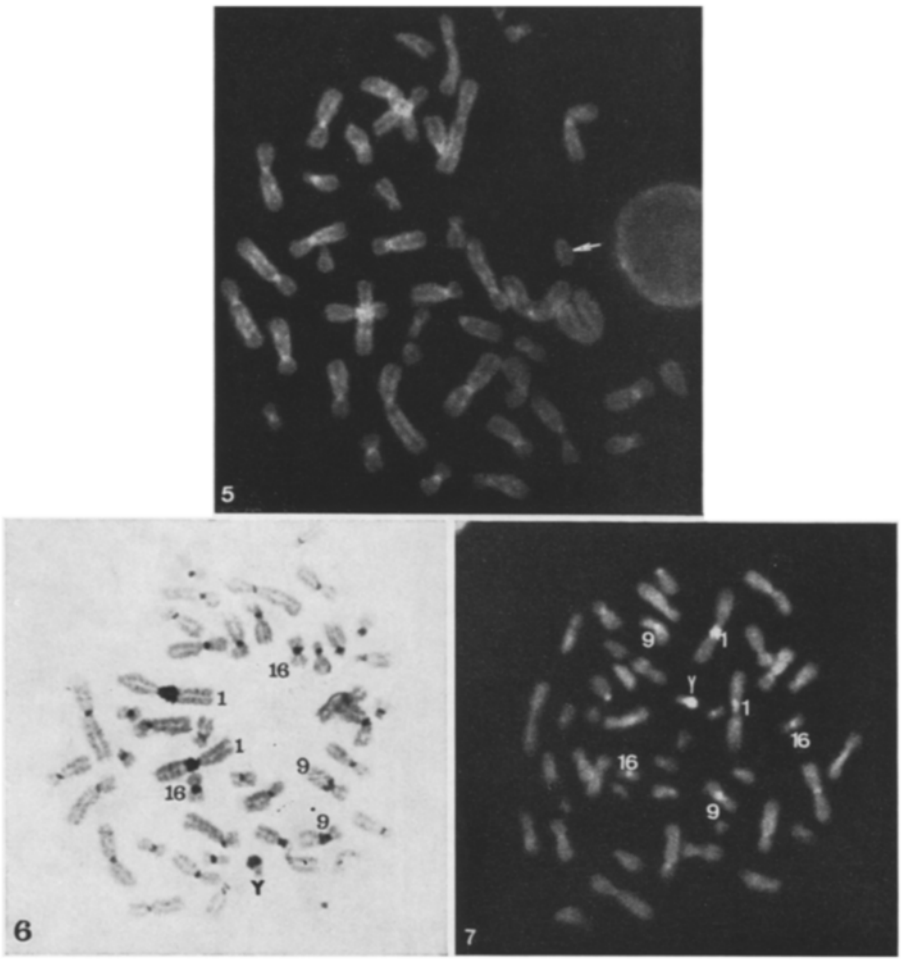

Fig. 5. Human male metaphase treated with 0.07 N NaOH for 30 sec following immediate staining with Hoechst 33258. Only the centromeric regions are brightly fluorescent, while Y shows dull fluorescence. Arrow indicates the Y

Fig. 6. C banding of a normal human male metaphase. Chromosomes 1, 9, 16 and Y are pointed out

Fig. 7. Normal human male metaphase treated according to the C-banding procedure but stained with Hoechst 33258. Note the bright fluorescent regions of chromosomes 1, 9, 16 and Y and of the short arms of acrocentrics

to what is seen in Q banding. The heterochromatic regions of chromosomes 1, 9, and 16 are very distinctly fluorescent, and polymorphisms existing between the homologous chromosomes can easily be identified, as illustrated for chromosome 1 in Fig. 2.

As is known, the constitutive heterochromatin in mouse chromosomes is restricted to the centromeric regions. The ascites tumour line, MSWBS, was shown to have a redistribution of the heterochromatin (Natarajan *et al.*, 1973). This

redistribution pattern is very evident after staining with this fluorochrome (Fig. 3).

Comparable results were obtained for both human and mouse chromosomes when different pretreatments were applied before staining.

When the preparations were subjected to mild treatment with DNAase before staining, the heterochromatic regions fluoresced intensely (Fig. 4), which may indicate that the DNA in the heterochromatic regions of the chromosomes in fixed preparations is more difficult to digest than that in the euchromatic regions and that this dye is specific to DNA. When the preparations were treated with alkali and stained immediately, the chromosome arms did not fluoresce, except for the centromeric regions, which were also relatively dull (Fig. 5). Mild acid treatment before staining did not alter the fluorescent pattern.

When the slides were processed according to the C-banding procedures (i.e. reassociated after alkali denaturation) and stained with Hoechst 33258 instead of Giemsa, precisely the same picture as the C-banding pattern was obtained, i.e. all the centromeres, short arm of acrocentrics, the heterochromatic blocks of 1, 9, 16 and the long arm of the Y chromosome fluoresced very brightly (Figs. 6 and 7).

Discussion

The precise mechanism by which the different chromosome-banding techniques work is not clear. The extensive literature on this topic indicates that the production of these patterns with Q or C banding is influenced by factors such as (a) base composition of the DNA in these regions (Chapelle *et al.*, 1973, and references quoted), (b) concentration of repetitive DNA of different classes (Chapelle *et al.*, 1973), (c) double-stranded *vs* single-stranded DNA in different regions (Bobrow and Madan, 1973), (d) selective loss of DNA in various regions (Ahnström and Natarajan, 1973) and (e) organization of nucleoprotein at different stages of the cell cycle (Kernell and Ringertz, 1972; Natarajan and Gropp, 1972). It is difficult to specify which of these factors are important in the staining reactions. The experiments designed to elucidate this problem indicate that each of these plays a smaller or greater role in the differential staining achieved.

Our experiments with the benzimidazol derivative indicate that this dye is preferentially bound to DNA, with a greater affinity to double-stranded DNA. The DNAase-digestion studies indicate that the DNA in the heterochromatic regions is organized in such a way that it is less accessible to the enzyme. This could also be due to the quantitative difference in the amount of DNA per unit area between constitutive heterochromatin and euchromatin, but there is no evidence for this so far. The sequential disappearance of the bands following alkali treatment and the appearance of strongly fluorescent bands following reassociation in salt solution in the present study is similar to the results obtained in such studies with acridine orange (Chapelle *et al.*, 1973; Bobrow and Madan, 1973). If we can use the same reasoning for the differential fluorescence with the acridine orange as for the binding of Hoechst 33258, we can conclude that this dye has a higher affinity to double-stranded DNA. The regions which fluoresce brightly with this dye following denaturation and reassociation (i.e. secondary constriction regions of chromosomes 1, 9, 16, the distal part of Y, etc.) are the same which bind strongly to Giemsa stain

following processing by the C-banding technique. This parallelism suggests that Giemsa stain is also specific to double-stranded DNA. However, *in-vitro* studies based on the equilibrium-dialysis technique have shown that the Giemsa stain has a higher affinity to single-stranded DNA than to double-stranded or repetitive DNA (Ahnström and Natarajan, 1973). Thus it is very difficult to draw any definitive conclusions on the mode of specifity of these dyes to the chromosome regions in cytological preparations at present, as we know too little about the molecular organization of the eukaryotic chromosomes.

It should be pointed out that the benzimidazol derivative is a very useful fluorochrome, as it combines the advantages of Q banding and C banding. The preparations stained with this dye are more stable than quinacrine-stained ones. In addition, it has affinity to regions which show green fluorescence with acridine orange following denaturation and reassociation. Thus, this dye may be as useful as acridine orange in the study of the various regions of chromosomes with differential rate of reassociation following denaturation.

References

Ahnström, G., Natarajan, A. T.: Localization of repetitive DNA in mouse cells by *in-situ* hybridization and dye binding technique. In preparation

Arrighi, F. E., Hsu, T. C.: Localization of heterochromatin in human chromosomes. Cytogenetics **10**, 81—86 (1971)

Bobrow, M., Madan, K.: The effects of various banding procedures on human chromosomes, studied with acridine orange. Cytogenet. Cell Genet. **12**, 145—156 (1973)

Caspersson, T., Zech, L., Modest, E. J., Foley, G. E., Wagh, U., Simmonsson, E.: Chemical differentiation with fluorescent alkylating agents in *Vicia faba* metaphase chromosomes. Exp. Cell Res. **58**, 128—140 (1969)

Chapelle, A. de la, Schröder, J., Selander, R. K.: *In-situ* localization and characterization of different classes of chromosomal DNA: Acridine orange and quinacrine mustard fluorescence. Chromosoma (Berl.) **40**, 347—360 (1973)

Hilwig, I., Gropp, A.: Staining of constitutive heterochromatin in mammalian chromosomes with a new fluorochrome. Exp. Cell Res. **75**, 122—126 (1972)

Kernell, A. M., Ringertz, N. R.: Cytochemical characterization of deoxyribonucleoprotein by UV-microspectrophotometry on heat denatured cell nuclei. Exp. Cell Res. **72**, 240—251 (1972)

Klasterska, I., Natarajan, A. T., Ramel, C.: Heterochromatin distribution and chiasma localization in the grasshopper *Bryodema* tuberculata (Fabr.), Acrididae. Chromosoma (Berl.) (in press)

Levan, A., Bregula, U., Klein, G.: The stemline idiogram of the MSWBS tumour of the mouse and the problem of centric fusion. Hereditas (Lund) **70**, 283—294 (1972)

Natarajan, A. T., Ahnström, G., Raposa, T.: Distribution of constitutive heterochromatin in the chromosomes of MSWBS ascites tumor cells. J. nat. Cancer Inst. **50**, 1721—1726 (1973)

Natarajan, A. T., Gropp, A.: A fluorescent study of heterochromatin and nucleolar organization in the laboratory and tobacco mouse. Exp. Cell Res. **74**, 245—250 (1972)

Natarajan, A. T., Natarajan, S.: The heterochromatin of *Rhoeo discolor*. Hereditas (Lund) **72**, 323—330 (1972)

Sarma, N. P., Natarajan, A. T.: Identification of heterochromatic regions in the chromosomes of rye *(Secale cereale L.)*. Hereditas (Lund) (in press)

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