STAINING PROPERTIES OF A BENZIMIDAZOL DERIVATIVE "33258 HOECHST" AND A SIM- PLIFIED STAINING METHOD FOR CHROMOSOME BANDING

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Summary To obtain good chromosome banding with "33258 Hoechst," various conditions were examined. For fluorescence microscopy, an UV filter for excitation and a BV filter for emission were selected from spectrofluorometric examination. "33258 Hoechst" was dissolved in distilled water and good chromosome banding was obtained when slides were stained at room temperature, for 10 to 20 minutes at the concentration between 4 $\mu\text{g/ml}$ to 0.2 $\mu\text{g/ml}$ of this dye. The aging of slides before and after staining was shown to be another important factor. Chromosome banding revealed with "33258 Hoechst" represents chromosome banding corresponding to both Q- and C-bandings in a very simple way. The mechanism of chromosome banding with "33258 Hoechst" is briefly discussed.

INTRODUCTION

Since the introduction of the quinacrine fluorescence technique for identification of human chromosomes (Caspersson *et al.*, 1970a), several new techniques and many modifications have appeared in the literature. A benzimidazol derivative designated as preparation "33258 Hoechst," belonging to a class of DNA binding fluorochrome, had been used to stain living cells *in vitro* (Lämmle and Schütze, 1969). Recently, Hilwig and Gropp (1972) found that this dye bound preferentially itself to chromosome segments composed of constitutive heterochromatin or sites of repetitive DNA's in mammals. Furthermore, a banding pattern similar to those of Q-banding was demonstrated under suitable conditions (Natarajan *et al.*, 1973; Latt, 1973), then this compound has gained increasing popularity among cytogenetists (Paris Conference (1971), Supplement (1975)).

The present paper is concerned with staining properties of this dye and a simplified staining procedure for demonstration of chromosome banding. And mechanism of chromosome banding with this dye is briefly discussed.

MATERIALS AND METHODS

Metaphase chromosomes were obtained from short term culture of human peripheral leukocytes and chromosome preparations were made by the conventional method with some modifications (Abe *et al.*, 1975a).

Spectrofluorometric examinations were performed by Nikon SPM-RFL system on preparations which were stained with 0.5 $\mu\text{g/ml}$ of "33258 Hoechst" for 10 minutes at room temperature. Following staining procedures were performed.

1) Slides were stained with "33258 Hoechst" dissolved in distilled water for 5 to 75 minutes at room temperature. The concentrations adopted were 40 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 0.4 $\mu\text{g/ml}$, 0.2 $\mu\text{g/ml}$ and 0.04 $\mu\text{g/ml}$. After staining, slides were rinsed and mounted in distilled water. Well spread metaphases were photographed by Nikon FM fluorescence microscope.

2) Sequential staining of C-staining following Q-staining. Using Q-staining method (Caspersson *et al.*, 1970a), several metaphases were photographed by fluorescence microscopy. Subsequently, these slides underwent C-staining procedure (Sumner, 1972) and the same metaphases were photographed by light microscopy (Abe *et al.*, 1975b).

3) Demonstration of DNA replicating regions (Latt, 1973; Dutrillaux, 1975; Eppelen *et al.*, 1975; Morita *et al.*, in prep.). Before harvesting cells 5-bromo-2'-deoxyuridine (BrdU) was added to medium, giving a final concentration of 100 $\mu\text{g/ml}$, corresponding to different phases of the cell cycle. Cells were stained with 0.5 $\mu\text{g/ml}$ "33258 Hoechst" for 10 minutes, 0.1 $\mu\text{g/ml}$ of acridine orange for 5 minutes or 50 $\mu\text{g/ml}$ of quinacrine mustard for 20 minutes.

RESULTS

The excitation and emission spectra of this dye were determined on interphase cells stained with "33258 Hoechst." The maximum excitation spectra were observed at 386 nm and 480 nm between 300 to 700 nm wavelength. The maximum emission spectrum was at 530 nm. Using 386 nm wavelength for excitation, cells were stained uniformly with banding structure along metaphase chromosomes. On the other hand, using 480 nm for excitation, interphase and metaphase cells were stained unevenly, that is, the circumference of cells fluoresced brighter and the center dull (Fig. 1). From these results, an UV excitation filter and a BV emission filter were selected for fluorescence microscopy and photography.

Chromosome banding with "33258 Hoechst" is shown in Fig. 2. Bright fluorescent blobs at the centromeric regions and regions of secondary constrictions, especially on chromosomes nos. 1, 9 and 16 were demonstrated. Chromosome banding was revealed when slides were stained with 4 $\mu\text{g/ml}$, 0.4 $\mu\text{g/ml}$ and 0.2 $\mu\text{g/ml}$ of this dye for 10 to 20 minutes. When slides were stained 0.02 $\mu\text{g/ml}$ of this dye for 5 to

Fig. 1. a: An interphase cell 3 hours after staining, using an UV filter for excitation. b: An interphase cell immediately after staining, using a BV filter for excitation.

Fig. 2. Chromosome banding with "33258 Hoechst."

Fig. 3. A dual karyotype from a male. Top: Q-banding. Bottom: C-banding.

Fig. 4. Late replicating regions on chromosomes. BrdU was added 5 hours prior to harvest. Late replicating regions were stained dull with acridine orange.

75 minutes, metaphases were stained faintly fluorescent and no banding was observed. Staining with $40 \mu\text{g}/\text{ml}$, made it impossible to reveal chromosome banding, because chromosomes were stained diffusely without banding.

The aging of slides before and after staining was considerably important to demonstrate clear chromosome banding. Slides aged 2 to 10 days before staining offered good chromosome banding. Fluorescence microscopy immediately after staining demonstrated staining unevenness as Fig. 1b, but this staining unevenness disappeared and chromosome banding appeared with time. Good chromosome banding was observed from 2 hours to several days after staining when stained slides were stored in the dark or in a refrigerator.

Chromosome banding with "33258 Hoechst" was almost similar to those of Q-banding with some exceptions for the centromeric regions and regions of secondary constriction. These regions were bright fluorescent with "33258 Hoechst" but

negative with quinacrine mustard (Fig. 2,3). Chromosome banding corresponding both Q- and C-staining methods was demonstrated on the same time with this "33258-Hoechst" without pretreatment. Sequential staining of Q- and C-staining methods offered good chromosome banding on the same metaphases (Fig. 3).

BrdU substitution at early S phase revealed segments of early DNA replication fluorescently dull with "33258 Hoechst" or acridine orange and these segments roughly corresponded to R-bands and late S phase, to "33258 Hoechst"-bands or Q-bands (Fig. 4). Many intermediate bands were demonstrated according to periods of time by BrdU substitution. However, metaphase chromosomes substituted by BrdU, revealed Q-banding with quinacrine mustard.

DISCUSSION

Chromosome banding with "33258 Hoechst" under various conditions were reported by several authors (Hilwig and Gropp, 1972; Natarajan *et al.*, 1973; Seth and Gropp, 1973; Latt, 1973; Kim, 1974; Raposa and Natarajan, 1974; Holmquist, 1975; Jalal *et al.*, 1975). Natarajan *et al.* (1973) described that with the manipulation of the staining intensity with this dye, only bands corresponding to C-bands or those corresponding to both C- and Q-bands were obtained. In our experiments, chromosome banding corresponding to both Q- and C-bands were obtained when slides were stained for 10 to 20 minutes at room temperature with this dye at concentrations of 4 $\mu\text{g/ml}$, 0.4 $\mu\text{g/ml}$ or 0.2 $\mu\text{g/ml}$. The aging of slides before and after staining was relatively important to obtain good chromosome banding as described by some authors (Seth and Gropp, 1973; Raposa and Natarajan, 1974; Jalal *et al.*, 1975; Holmquist, 1975).

Another point for good chromosome banding is the selection of filters, especially filters for excitation as briefly mentioned by Seth and Gropp (1973) and Jalal *et al.* (1975), because this dye is shown to have two excitation peaks and either peak shows different staining properties.

Sequential staining of C-staining following Q-staining offered fine chromosome banding of C- and Q-bands respectively on the same metaphases (Fig. 3), but this procedures are troublesome and not always successful to obtain good dual karyotypes. On the other hand, "33258 Hoechst" reveals both Q- and C-bands in a very simple way.

There are evidences that at least the regions carrying large constitutive heterochromatin blocks contain specific type of DNA (sattelite DNA) characterized by short reiterated sequences as relatively high adenine-thymidine base content (Corneo *et al.*, 1970; Jones and Corneo, 1971; Gosden *et al.*, 1975). These regions show bright fluorescence with "33258 Hoechst," but dull with quinacrine mustard.

This fluorochrome "33258 Hoechst" was reported to bind to DNA bases by hydrophobic interaction (Comings, 1975b). The fluorescence intensity of this dye was enhanced in relation to DNA concentration and A-T base composition (Weis-

blum and Haenssler, 1974; Comings, 1975b). Quinacrine fluorescence was also enhanced by A-T bases, but guanine bases and the degree of its interspersion could cause the lack of quinacrine fluorescence (Ellison and Barr, 1972; Weisblum and de Haseth, 1972; Selander and de la Chapelle, 1973). But, fluorescence efficiency of "33258 Hoechst" is rather unaffected by G-C pairs (Weisblum and Haenssler, 1974; Comings, 1975b).

A fluorochrome, acridine orange, reveals R-bands according to single- and double-stranded DNA along metaphase chromosomes (Bobrow and Madan, 1973). Raposa and Natarajan (1974) described that "33258 Hoechst" may preferentially binds to DNA with a greater affinity to double stranded DNA. Staining procedure with this dye, however, is performed without pretreatment of denaturation and re-association. Furthermore, acridine orange stains metaphase chromosomes uniformly without banding, if denaturation and reassociation were excluded.

Another factors having effects on chromosome banding have been reported. The differential chromosome condensation was reported to have an important effect responsible for quinacrine banding (Golomb and Barr, 1974) and G- and C-bandings (McKay, 1973) or few effect to quinacrine banding (Caspersson *et al.*, 1970b; Comings *et al.*, 1975a) or no effect to G- or C-bandings (Comings, 1973). The fluorescence intensity with "33258 Hoechst" was reported not to be so dependent on chromosome condensation (Comings, 1975b).

An interaction of DNA-nonhistone protein complex was reported to play an important role on chromosome banding (Comings *et al.*, 1973; Vogel *et al.*, 1974), but "33258 Hoechst" binds to DNA bases and almost not to be affected (Comings, 1975b).

Fluorescence efficiency of "33258 Hoechst" bound to poly (dA-BrdU) is less than one fifth of the dye bound to poly (dA-dT) (Latt, 1973; Latt, 1974). Substitution of chromosomal DNA by BrdU provided a degree of resolution for the replication of chromosomal DNA by staining with "33258 Hoechst" (Latt, 1973; Latt, 1974; Morita *et al.*, in prep.), acridine orange (Dutrillaux, 1975) or "33258 Hoechst"/Giemsa technique (Epplen *et al.*, 1975; Grzesechik *et al.*, 1975). This procedure demonstrates early, intermediate or late replicating regions correspond to the periods of time substituted by BrdU. These authors describe that early replicating regions correspond to R-bands and late replicating regions to Q-, G- or "33258 Hoechst"-bands.

Fluorescence efficiency of the complex of quinacrine with poly(dA-BrdU) is little different from that of the corresponding complex with poly(dA-dT) (Latt, 1974) and metaphase chromosomes substituted by BrdU represent Q-bands with quinacrine mustard (Latt, 1974; Bühler *et al.*, 1976; Abe *et al.*, in prep.) From these experiments, the banding of chromosomes and replication patterns are thought to be independent phenomena.

These results proved that the precise mechanism of the chromosome banding with "33258 Hoechst" is still to be elucidated. Holmquist (1975) suggested that nonhistone protein-DNA interaction, "B"-form to "C"-form transitions, late replica-

tion, quinacrine brightness, G-banding, chromatid mass discontinuities and differential chromatin condensation may be different aspects of the same phenomenon. DNA is coated with protein, folded and condensed into the final package we call chromosome, and these factors may possibly altering its fluorochrome brightness, much or less.

Staining with "33258 Hoechst" eventually contribute to elucidating the hitherto unknown molecular mechanism involved in the relationship between DNA sequence and chromosome banding.

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