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Microfluorometric Detection of Deoxyribonucleic Acid Replication in Human Metaphase Chromosomes

(33258 Hoechst/BrdU-dependent fluorescence/metaphase chromosome staining/autoradiography)

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ABSTRACT **Fluorescence of the dye 33258 Hoechst bound to chromosomes is partially quenched by the introduction of BrdU into chromosomal DNA. This finding has allowed microfluorometric detection of DNA synthesis in human metaphase chromosomes. Incorporation, shortly before cell harvest, of a pulse of thymidine into chromosomes otherwise substituted with BrdU results in sharply defined foci of bright 33258 Hoechst fluorescence, corresponding to regions of late DNA replication. The late-replicating X chromosome can thereby be clearly identified, and the time course of appearance of its fluorescent bands can be compared with that of its earlier replicating homologue. Growth of cells in medium containing BrdU for two generations allows fluorometric documentation of the semiconservative distribution of newly replicated DNA between sister chromatids, and regions of sister chromated exchange are demarcated. This approach should constitute, in many instances, a convenient, high-resolution fluorometric alternative to autoradiography.**

DNA replication in eukaryotic organisms is semiconservative (1), initiating at discontinuous foci (2) and terminating in a temporal sequence characteristic of the location of the DNA segment in metaphase chromosomes (3). Biochemical and cytological studies have correlated late DNA replication with both genetic inactivity and the persistence of chromosome condensation throughout most of the cell cycle (4, 5). Regions with these properties are termed heterochromatic and have been shown to correspond to regions of metaphase chromosomes staining intensely either with quinacrine or quinacrine mustard, or with Giemsa mixtures after any of a variety of pretreatments (6, 7). However, previous correlation of late DNA replication with banded regions of metaphase chromosomes has been limited by the resolution usually realized of the autoradiographic techniques used, and the chromosome-banding stains do not effectively differentiate the facultative heterochromatic X chromosome from its active homologue. More precise correlation of DNA replication with chromosome location in individual metaphases could be achieved were it possible to detect DNA synthesis at the level of resolution of optical techniques.

Direct light-microscopic visualization of DNA replication might use DNA base analogues with distinctive absorptive or fluorescent properties. However, available base analogues do not, at present, make such an approach practical. As an alternative method, one might use an indirect phenomenon, such as the environmental sensitivity of fluorescent chromosome stains, using them to report the incorporation of nucleotide analogues containing substituents, such as heavy atoms (8), perturbing dye luminescence.

Towards this purpose, we have examined several dyes, including the compound 33258 Hoechst [2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol·3 HCl], which has previously been used to demonstrate constitutive heterochromatin in rodent metaphase chromosomes (9). We observed that the fluorescence of a sample of 33258 Hoechst, kindly supplied by Dr. H. Loewe, Hoechst, is much less efficient when the dye is bound to poly(dA-BrdU) than when bound to poly(dA-dT). This paper introduces a method based on this observation. Reduction in the fluorescence intensity of 33258 Hoechst bound to chromatin, upon incorporation of BrdU, has been used to follow DNA synthesis in interphase nuclei and metaphase chromosomes.

MATERIALS AND METHODS

Cell Growth. Peripheral human leukocytes were cultured in Eagle's minimal essential medium (MBA) with 2 mM L-glutamine and 20% fetal-calf serum (GIBCO), to which crude phytohemagglutinin was added. BrdU and thymidine, added to cultures grown at 37°, as described in the figure legends, were obtained from Sigma and their tritiated derivatives from New England Nuclear Corp. Uridine was also obtained from Sigma, while FdU was purchased from Calbiochem.

Slide Preparation. Cells were harvested by centrifugation 2 hr after the addition of colcemid (Ciba) to a final concentration of 0.1 μg/ml, suspended in 75 mM KCl for 12 min, fixed in at least two changes of 3:1 methanol–acetic acid, and air dried on glass microscope slides.

Staining and Photography. Slides were stained in 0.5 μg/ml of 33258 Hoechst in 0.15 M NaCl–0.03 M KCl–0.01 M phosphate (pH 7) and mounted in 0.16 M sodium phosphate–0.04 M sodium citrate (pH 7) for photography. Chromosome fluorescence was observed in a Leitz Orthoplan microscope equipped with incident illumination using a 200 W mercury light source, a UG-1 filter and TK 400 dichroic mirror for excitation, and K 400 and K 460 filters for emission. Photographs were taken through a 100 × achromat objective with an Orthomat II automatic camera on Tri-X film.

Quinacrine mustard and modified Giemsa staining and photomicrography followed established techniques (6, 7).

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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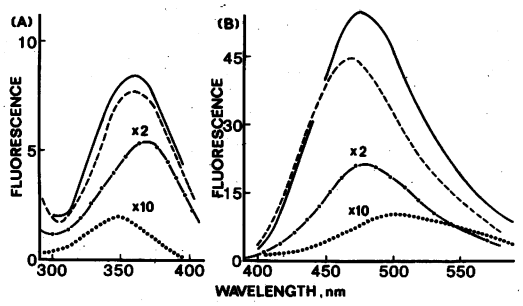

FIG. 1. The influence of binding to polynucleotides on the fluorescence of 33258 Hoechst. 33258 Hoechst (1 μM) was dissolved in 0.01 M NaCl–5 mM Hepes (pH 7.0). (*A*) Fluorescence excitation spectra (emission observed at 490 nm) and (*B*) emission spectra (excitation at 350 nm) were obtained on solutions of the free dye (· · ·, shown with a 10-fold scale expansion), or on solutions containing 0.05 mM poly(dA-BrdU) (·—·, shown with a 2-fold scale expansion), calf-thymus DNA (– – –), or poly-(dA-dT) (——).

Autoradiography. After fluorescent photomicrography, slides were dipped in Kodak NTB-2 emulsion and developed in Dektol. Chromosomes, lightly stained through the emulsion with Giemsa, were photographed in transmitted light through a 544-nm interference filter, using high-contrast copy film. Grains were counted on prints from this film.

Spectrophotometry. Absorptivity was measured either on a Cary 14 recording spectrophotometer or on a Gilford 2400 S spectrophotometer. Fluorescence spectra were obtained on a Hitachi MPF-3 spectrofluorometer operated in the ratio mode, and were corrected for instrument response by standard techniques (10). Microspectrophotometric measurements used a Leitz MPV II instrument.

Synthetic polynucleotides were obtained from Miles Chemical Co. or General Biochemical Co., and calf-thymus DNA from Worthington Biochemicals.

RESULTS

The fluorescence excitation spectrum of 33258 Hoechst in 0.01 M NaCl–5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.0 exhibits a maximum at about 345 nm, while the emission spectrum peaks at 505 nm (Fig. 1). Upon addition of polynucleotides, the fluorescence intensity greatly increases, the excitation maximum shifts to somewhat higher wavelengths, and the emission maximum shifts to lower wavelengths. Fluorescence efficiency of the dye is slightly greater when it is bound to poly(dA-dT) than when it is bound to calf-thymus DNA. The quantum yield of the former, after excitation at 350 nm, was estimated to be 0.75 relative to a value of 0.55 for quinine sulfate in 0.1 N H_2SO_4 (11). The fluorescence efficiency of 33258 Hoechst bound to poly(dA-BrdU) is less than one-fifth of that of the dye bound to poly(dA-dT), and the photostability of the former complex is very low*. These results suggested the use of 33258 Hoechst fluorescence as an indicator of BrdU incorporation into the DNA of metaphase chromosomes.

* In contrast to 33258 Hoechst, both quinacrine and ethidium bromide exhibit fluorescence intensities when bound to poly-(dA-BrdU) that are similar to those observed when these dyes bind to poly(dA-dT)

Human metaphase chromosomes exhibit a small unevenness in 33258 Hoechst fluorescence, corresponding to banding patterns found with modified Giemsa techniques, but of lower contrast (Fig. 2*A*). If BrdU is added to the leukocyte cultures and the cells are harvested after 2 days' growth, at which time most of the metaphases will correspond to cells that have divided once (12), the banding pattern is basically unchanged (Fig. 2*B*), although the fluorescence intensity of such metaphase chromosomes is reduced (Table 1).

If leukocytes are grown for 40 hr in medium containing BrdU and then for 6 hr in medium containing thymidine but not BrdU, late-replicating regions, which would be expected to incorporate thymidine rather than BrdU, exhibit very bright 33258 Hoechst fluorescence (Fig. 2*C*). The locations of bands observed under these conditions correspond in most cases with those of bands fluorescing brightly after standard quinacrine mustard treatment. A pattern consisting of dully fluorescing regions on a bright background, corresponding in many chromosomes to the reverse of Fig. 2*C*, is obtained if cells grown in medium containing thymidine are pulsed with BrdU before harvest.

If the leukocytes are grown in medium containing BrdU for 70 hr before harvest, two types of metaphase chromosome-staining patterns are observed. The first appears identical in intensity (Table 1) and distribution to that of Fig. 2*B* and probably corresponds to cells that have divided once. A second class of metaphase chromosomes, with somewhat further reduced total fluorescence intensity (Table 1), consists of chromosomes with one chromatid fluorescing distinctly

TABLE 1. *Autoradiographic and microfluorometric detection of BrdU incorporation into human metaphase chromosomes**

Growth conditions	Autoradiograph grain counts*		Relative metaphase fluorescence intensity†
Control (no BrdU)‡	1 (6)	1 (6)	1.00
46 hr in [^{3}H]BrdU§	192 (5)	178 (9)	0.40
70 hr in [^{3}H]BrdU			
Chromatid fluorescence equal§	174 (5)	194 (4)	0.43
Chromatid fluorescence unequal¶	265 (4)	241 (5)	0.32
Bright chromatids	95‖	86‖	
Dull chromatids	170‖	155‖	

* Peripheral leukocytes were obtained from a normal 46,XY human. [^{3}H]BrdU, when used, was at 0.09 mM, 1 μCi/ml. Slides were stained and photographed as described in *Methods*. Emulsion exposure for both slide sets was 11 days. Table entries represent the average grain number per metaphase for an individual slide. The number of metaphases per slide examined is given in *parentheses*.

† Determined microfluorometrically after staining with 33258 Hoechst. Each value represents an average of about 20 metaphases measured on each of three different slides.

‡ Fluorescence pattern like that of Fig. 2*A*.

§ Fluorescence pattern like that of Fig. 2*B*.

¶ Fluorescence pattern like that of Fig. 2*D*.

‖ These table entries each represent grain counts per set of chromatids, corresponding to one-half of the DNA of a metaphase spread.

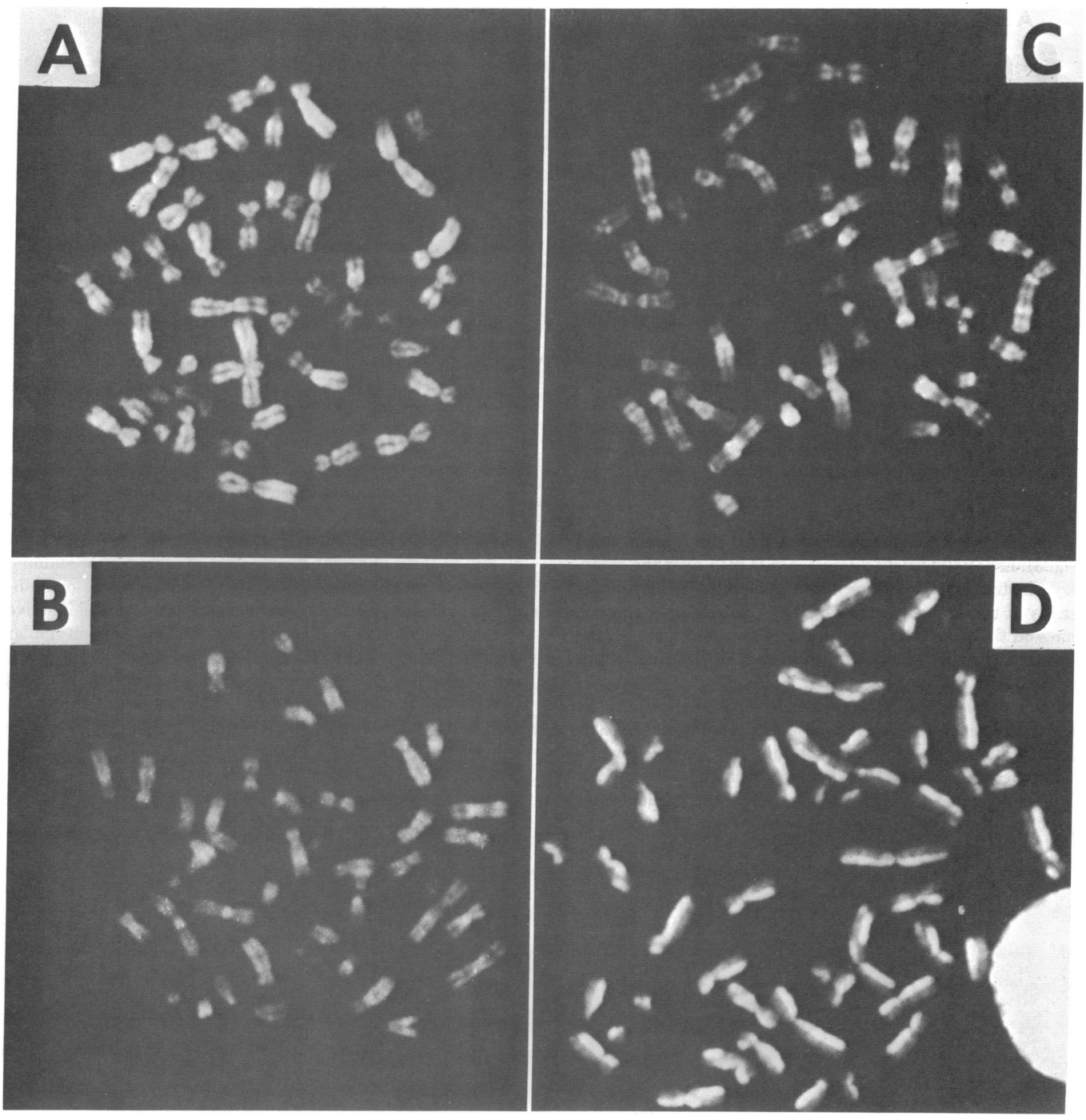

FIG. 2. Fluorescence of human 46,XY metaphase chromosomes stained with 33258 Hoechst. (*A*) Control. (*B*) Cells grown 46 hr (one generation) in medium containing 0.09 mM [^{3}H]BrdU (1 μCi/ml), 0.4 μM FdU, and 6 μM U. (*C*) Cells grown 40 hr in 0.09 mM BrdU, 0.4 μM FdU, and 6 μM U, followed by growth in Ham's F-10 medium supplemented with 20% fetal-calf serum and 3 μM [^{3}H]dT (2 μCi/ml) for 6 hr before harvest. Bright regions of chromosomes 13 and Y, as well as the secondary constrictions of chromosomes 1, 9, and 16, are especially prominent. (*D*) Cells grown 70 hr (two generations) in 0.09 mM BrdU, 0.4 μM FdU, and 6 μM U.

brighter than the other (Fig. 2*D*). Autoradiographic estimation of the incorporation of tritiated BrdU into these chromosomes (Table 1) shows that the dull chromatid regions have incorporated nearly twice as much BrdU as the brightly fluorescing regions, while the total incorporation over this type of metaphase is nearly 40% greater than that of the first type of metaphase. The simplest interpretation of these results, consistent with a semiconservative model for the distribution of newly replicated DNA, is that these cells have undergone two divisions and that the brighter chromatids contain DNA with only one base-sugar-phosphate chain substituted with BrdU, while the duller chromatids contain DNA with both chains substituted with BrdU. A striking feature of these chromosomes is the sharp demarcation of sister chromatid exchanges.

Correspondence between autoradiographic and 33258

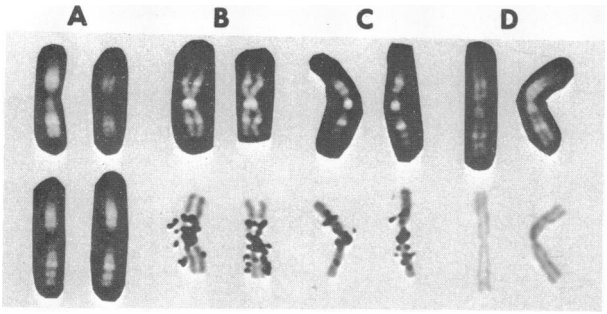

FIG. 3. Comparison of fluorometric and autoradiographic detection of the regions of late DNA replication of human chromosome no. 1. Cells from a normal 46,XY individual. (*A*) Control, stained with 33258 Hoechst (*upper pair*) or quinacrine mustard (*lower pair*). (*B*, *C*, and *D*) Cells grown in medium containing BrdU and then pulsed with [^{3}H]dT as described in Fig. 2*C*. Chromosomes of (*B*), (*C*), and (*D*) are all from metaphase cells on the same slide. *Upper pairs*, stained with 33258 Hoechst; *lower pairs*, autoradiographs of same chromosomes, stained with Giemsa, after exposure of emulsion for 11 days.

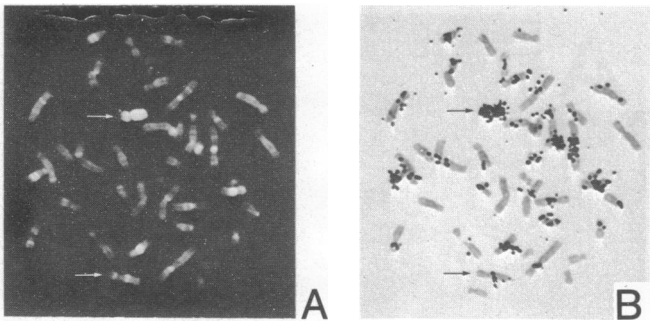

FIG. 4. Fluorometric detection of the late-replicating human X chromosome. Human 46,XX cells grown 45 hr in medium containing BrdU, FdU, and U before a 6-hr terminal pulse of dT as in Fig. 2*C*. (*A*) Staining with 33258 Hoechst. (*B*) Autoradiograph of the same metaphase, after 11 days' exposure, stained with Giemsa. The *upper* and *lower arrows* indicate the late and early replicating X chromosomes, respectively.

Hoechst fluorometric patterns is shown for chromosome no. 1 (Fig. 3). Especially bright fluorescence is seen over the secondary constriction, correlating with the high grain-distribution density of this late-replicating region. A pair of no. 1 chromosomes on the same slide, but lacking regions of bright fluorescence, exhibits no thymidine incorporation by autoradiography (Fig. 3*D*).

The present approach can be used for fluorometric detection of the late-replicating, inactive (13) X chromosome. 33258 Hoechst fluorescence of chromosomes from cells grown in medium containing BrdU and given a terminal pulse of [^{3}H]-dT highlights one X chromosome (Fig. 4*A*), which corresponds to the late-replicating X chromosome in the companion autoradiograph (Fig. 4*B*). The other X chromosome can be identified by its characteristic fluorescent banding pattern.

Regions of bright 33258 Hoechst fluorescence in the earlier replicating X chromosome (Fig. 5*B*, bottom row) correspond closely to those of bright fluorescence observed after staining of control chromosomes with either 33258 Hoechst or quinacrine mustard (Fig. 5*A*). Bright fluorescence in the inactive X chromosome is more extensive, although it occurs in similar regions (Fig. 5*B*). The proximal region of the long arm of the late-replicating X chromosome from this individual, in all cases, exhibits a very bright band. This chromosome appears somewhat more contracted than its homologue, and the fluorescent band at the distal end of the long arm appears less distinct. Extension of bright fluorescence in the inactive X chromosome involves the long arm distal to the proximal bright band and the middle of the short arm.

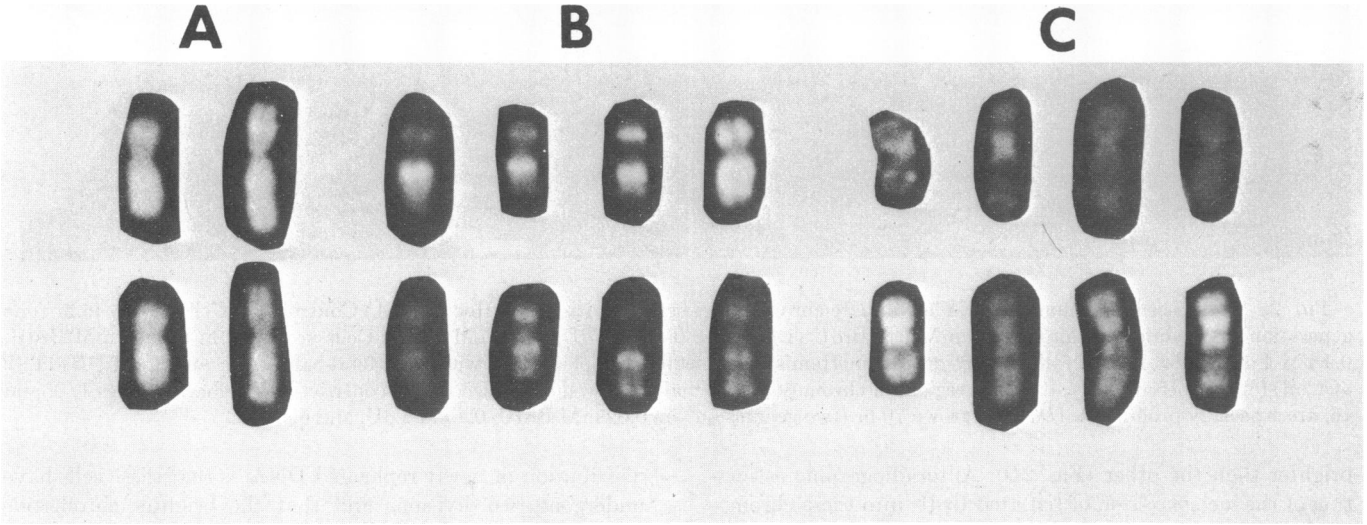

FIG. 5. Fluorescence patterns of the late- and early-replicating human X chromosomes. Cells were from a normal 46,XX woman. (*A*) Control cells, stained with 33258 Hoechst (pair at the *left*) or quinacrine mustard (pair at the *right*). (*B*) Cells grown as described in Fig. 4, stained with 33258 Hoechst. *Upper chromosomes* of each pair: late-replicating X, confirmed by autoradiography with [^{3}H]dT; *lower chromosomes* of each pair: early-replicating X. (*C*) Cells were grown in medium containing thymidine to which BrdU, FdU, and U (as in Fig. 2*B*) were added 6 hr before harvest. Chromosomes were stained with 33258 Hoechst. Arrangement is homologous to that of *B*, confirmed by [^{3}H]BrdU autoradiography.

Chromosomes from cells given a pulse of BrdU before harvest exhibit a nearly complementary X-chromosome fluorescence pattern. Regions last to complete DNA synthesis incorporate BrdU, which quenches 33258 Hoechst fluorescence (Fig. 5*C*). The persistently bright regions are located on both arms near the centromere, while more extensive fluorescence also involves the tip of the short arm and a region below the middle of the long arm. Foci of dull fluorescence in the earlier replicating X chromosome (Fig. 5*C*) are much less extensive.

DISCUSSION

The approach described in this paper should provide, in many instances, a convenient fluorometric alternative to autoradiography. The fluorescence of chromosomes stained with 33258 Hoechst and observed under the conditions described appears to differentiate regions according to the number of chains of the DNA into which an appreciable amount of BrdU has been substituted. Dye bound to chromosomes containing DNA without BrdU substitution fluoresces with the highest efficiency, while that bound to chromosomal regions containing DNA with both chains substituted fluoresces the most weakly (Table 1).

While there is variation in 33258 Hoechst fluorescence along a chromatid of uniform BrdU content (Figs. 2*B* and *D*), the staining patterns obtained on chromosomes grown one generation in BrdU (Fig. 2*B*) are of lower contrast than those of similarly grown cells pulsed with thymidine (Fig. 2*C*). Moreover, reversal of the sequence of thymidine and BrdU incorporation into DNA of the late-replicating chromosome regions (e.g., Fig. 5*C*) to obtain complementary patterns of fluorescence suggests that differences in dye fluorescence due to variations in BrdU incorporation predominate. Effects due to chromosome uncoiling by BrdU (14) or the influence of proteins on the accessibility of chromosomal DNA to 33258 Hoechst may exist, but appear to be of secondary importance in interpreting the present results.

The results described are consistent with the correspondence between regions staining intensely with quinacrine or Giemsa and those of late DNA replication, although, within these regions, there appears to be a variation in the order of DNA replication. The chemical nature of the remarkably sharply demarcated, brightly fluorescent regions remains to be established. Regions of the secondary constrictions of chromosomes 1, 9, and 16 are exceptions to this association (Fig. 2*C*), in that they complete replication late in the DNA synthesis period but do not fluoresce brightly after quinacrine mustard staining. By use of 33258 Hoechst fluorescence, growth of cells for nearly one division in BrdU followed by a terminal pulse of thyrmidine should constitute a convenient method of identifying regions of late DNA replication, while growth in thymidine followed by a pulse of BrdU should allow identification of regions that finish DNA replication early. A particularly informative potential application of this approach is in the examination of the order of DNA replication in the late-replicating X chromosome. Detailed examination of the sequence of late- and early-replicating regions in the two X chromosomes in normal individuals and individuals possessing X-chromosomal abnormalities may provide insight into the scope and spread of X-chromosomal inactivation.

This photographic technique appears to be able to detect bright regions ranging in size from 0.1 to 1% of the length of the total chromosome complement. This might ultimately allow fluorometric detection of DNA hydridized *in situ* to chromosomes containing BrdU.

The results with chromosomes composed of chromatids containing DNA with either one or two chains substituted with BrdU (Fig. 2*D*) document the semiconservative chromatid replication previously established by autoradiography (15), and clearly show sister chromatid exchanges. Use of [^{3}H]BrdU(11 Ci/mol) does not appear to significantly increase the number of exchanges compared with that found with unlabeled BrdU. An average of about 15 such exchanges after two divisions were observed per metaphase both in the presence and in the absence of a radioactive label. However, the present results do not rule out the possibility that the nonradioactive BrdU, which was incorporated into the chromosomes, influenced the chromatid exchange frequency. It will be of interest to see if the chromosomal breakpoints, which can now be precisely localized, correspond to particular chromosomal regions, as defined, e.g., by the quinacrine banding patterns. In addition, it may prove useful to quantitate these breakpoints in chromosomes from individuals with diseases such as Fanconi's anemia or Bloom's syndrome (16) in which an increase in chromosome breakage is observed.

Determination of the mechanism by which BrdU substitution into DNA reduces the fluorescence of bound 33258 Hoechst will be useful for further applications of this dye. The quenching mechanism will be examined by appropriate studies of the fluorescence and phosphorescence intensities and lifetimes of 33258 Hoechst bound to DNA substituted to different extents with BrdU. Were the interaction between BrdU and the dye of short range (e.g., a heavy-atom or charge-transfer effect), dye binding would be localized near the bromine atom, which is in the large groove of the DNA, making 33258 Hoechst a useful probe of interactions in this groove. Other dyes exhibiting luminescence responsive to similarly located heavy-atom substitution into DNA could also be used for this purpose.

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