DETECTION OF DNA SYNTHESIS IN INTERPHASE NUCLEI BY FLUORESCENCE MICROSCOPY

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INTRODUCTION

It is often of interest to identify those cells in a mixed population which are synthesizing DNA. For example, division of thymus-dependent lymphocytes in response to phytohemagglutinin (1, 2) affords their differentiation from other peripheral blood cells. Previous cytological identification of replicating cells has employed autoradiography (3-6). These cells can now be identified by fluorescence microscopy (7, 8), due to the sensitivity of the fluorescence of the stain 33258 Hoechst (9) to...
5-bromodeoxyuridine (BrdU) incorporation into nuclear DNA.

Heavy polarizable atoms such as bromine have been known to quench the luminescence of organic dyes (10, 11). Since bromine can be incorporated into DNA in the form of the thymidine analogue, BrdU (12, 13), this effect can in principle be used for fluorometric detection of DNA synthesis. Therefore, an examination was made of the sensitivity of the fluorescence of a number of dyes to BrdU. At pH 7.0, the fluorescence of the bis-benzimidazole dyes 32020, 32021, 33258, and 33342 Hoechst was found to be significantly less when bound to poly(dA-BrdU) than when bound to poly (dA-dT) (7, 8). However, in experiments examining the mechanism of this quenching, it was found that the fluorescence efficiencies of the complexes of 33258 Hoechst (the most readily available compound of the group) with poly (dA-BrdU) and poly (dA-dT) did not differ significantly when the pH was reduced to 4.1 (S. A. Latt, unpublished experiments). These observations form the basis for the present communication.

MATERIALS AND METHODS

Human peripheral leukocytes were cultured in Eagle's minimal essential medium (Microbiological Associates, Inc., Bethesda, Md.) supplemented with 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 2 mM L-glutamine, 0.09 mM [3H]BrdU (1 µCi/ml) (New England Nuclear, Boston, Mass.), 6 µM uridine (Sigma Chemical Co., St. Louis, Mo.), 0.4 µM 5-fluorodeoxyuridine (Calbiochem, San Diego, Calif.), and a saline extract of red kidney beans containing phytohemagglutinin. After 48 h, at which time lymphocytes responding to phytohemagglutinin would be expected to have divided once (14), cells were harvested and slides were prepared by standard procedures (7). Slides were successively immersed in 0.01 M phosphate, pH 7, containing 0.15 M NaCl and 0.03 M KCl (5 min), in a 0.5 µg/ml solution of 33258 Hoechst in this buffer (10 min), and washed twice in buffer. (33258 Hoechst was a generous gift of Dr. H. Loewe, Hoechst AG, Frankfurt, Germany.)

FIGURE I Detection of DNA synthesis in interphase nuclei using fluorescence microscopy. Slides were prepared from peripheral leukocytes grown 48 h in medium containing BrdU as described in the text. Photographs in each row are of the same field but were taken under different conditions. (a) Cells were stained with 33258 Hoechst as described and mounted in pH 4.1 McIlvaine's buffer (0.08 M sodium phosphate, 0.12 M sodium citrate, 90% glycerol) for photography. (b) The slides were washed and mounted in pH 7 McIlvaine's buffer (0.16 M sodium phosphate, 0.04 M sodium citrate) and the same field was rerephotographed under the same optical conditions. Nuclei with relatively decreased fluorescence at pH 7 are indicated by arrows. (c) The slides were then dipped in Kodak NTB-2 emulsion for autoradiography, developed after 14-days' exposure, and stained with Giemsa. Nuclei which have incorporated [3H]BrdU and exhibit numerous grains correspond to those indicated by arrows in (b). Bar, 10 µm. x 825.
W. Germany.) Slides were mounted for observation as described herein. For autoradiography, slides were dipped in a 1:1 dilution of Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.) in water and exposed for 14 days before development. Photomicrography utilized the Orthoplan component of a Leitz MPV II microspectrophotometer (E. Leitz, Inc., Rockleigh, N. J.) (7). For fluorescence excitation, filters transmitting light between 360 and 400 nm were used, while fluorescence emission was viewed through a filter transmitting light above 460 nm.

RESULTS AND DISCUSSION

The fluorescence of metaphase chromosomes from lymphocytes grown one division in medium containing 0.09 mM BrdU and stained with 33258 Hoechst is about one third that of control metaphases when slides are mounted in pH 7 buffer (e.g. 0.16 M sodium phosphate, 0.04 M sodium citrate) (7). However, quenching of 33258 Hoechst fluorescence by BrdU is not observed if slides are mounted in a solution buffered at pH 4.1 and containing glycerol. (The pH 4.1 buffer contains 90% glycerol to decrease dye fading, presumably due to photodegradation. Glycerol is not used in the pH 7 buffer since it diminishes the quenching effect of BrdU.) Comparison of the fluorescence of nuclei stained with 33258 Hoechst and mounted first at pH 4.1 (with glycerol) and then at pH 7 (without glycerol) can be used to identify those nuclei which have incorporated BrdU.

Nuclei stained with 33258 Hoechst fluoresce brightly when mounted at pH 4.1 (Fig. 1 a). If the slides are then washed and mounted at pH 7, the fluorescence of certain nuclei is greatly diminished (Fig. 1 b, arrows). As expected from previous photometric measurements, correlating quenching of 33258 Hoechst fluorescence with BrdU incorporation (7), the nuclei with diminished fluorescence at pH 7 exhibit autoradiographic grains signalling incorporation of [3H]BrdU (Fig. 1 c). In contrast, those nuclei which fluoresce brightly at both pH 4.1 and pH 7 have not incorporated a significant amount of BrdU.

Those nuclei exhibiting reduced 33258 Hoechst fluorescence associated with [3H]BrdU incorpora-

**FIGURE 2** 33258 Hoechst fluorescence of nuclei from cells grown in the presence of thymidine but not BrdU. Cultures were duplicates of those of Fig. 1 except that the [5-3H]bromodeoxyuridine, 5-fluorodeoxy-
yridine, and uridine additions were omitted. Slide preparation and staining with 33258 Hoechst was identical to that of Fig. 1. Photographs of the same fields after mounting in pH 4.1 McIlvaine’s buffer containing glycerol (a) or pH 7 McIlvaine’s buffer (b) are shown. × 825.
The present method, employing fluorescence microscopy, can thus serve as a convenient, rapid alternative to autoradiography for detection of dividing cells in a mixed population. Visual interpretation of the photographs requires the presence of nondividing cells as an internal reference. Use of mounting media at pH 7 and pH 4.1 affords detection of fluorescence changes due specifically to BrdU incorporation. The sensitivity of the technique might be increased if the photographic negatives were analyzed densitometrically. Semi-quantitative measurement of different extents of DNA synthesis should thereby be possible.

Use of fluorescence quenching to detect DNA synthesis conveys a number of advantages in addition to rapidity and simplicity. Omission of autoradiography for this purpose would allow tritium autoradiography to be used for simultaneous analysis of other synthetic processes in the same nuclei. An analogous fluorometric method might also be applicable for detection of DNA synthesis in cell suspensions or in cell free systems. Potential uses include rapid quantitation of cell division by flow fluorometry and detection of DNA synthesis in living cells (18, 19).

SUMMARY

The incorporation of 5-bromodeoxyuridine into leukocyte nuclei markedly quenches the fluorescence of the stain 33258 Hoechst when cytologic preparations are mounted at pH 7, but not at pH 4.1. This effect can be employed for the detection by fluorescence microscopy of DNA synthesis in interphase nuclei.

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