

Accessibility of DNA In Situ to Various Fluorochromes: Relationship to Chromatin Changes During Erythroid Differentiation of Friend Leukemia Cells¹

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Friend leukemia cells from exponentially growing or differentiated (DMSO-induced) cultures were permeabilized and their DNA was stained with 4'-6-diamidino-2-phenylindole (DAPI), Hoechst 33342, acridine orange, ethidium bromide, propidium iodide, quinacrine, 7-amino-actinomycin D, mithramycin, or chromomycin A₃. Accessibility of DNA to each of the above fluorochromes was compared in differentiated and nondifferentiated cells before and after nuclear proteins, mostly histones, were extracted with 0.1N HCl. A decrease in the accessibility of DNA to several dyes, especially pronounced in the case of some intercalators, was observed in differentiated cells. After extraction of nuclear proteins with HCl there was an increase in DNA accessibility, of varying degree depending on

the fluorochrome and the difference between differentiated and nondifferentiated cells was abolished for most of the intercalating dyes. The increase was the lowest for DAPI (45%), the highest for 7-amino-actinomycin D (13-fold), and in general was higher for the intercalating dyes that unwind DNA than for dyes binding externally to the double helix. The results are discussed in terms of the mode of interactions between DNA and the fluorochromes and factors associated with chromatin structure that may affect accessibility of DNA in situ in exponentially growing and differentiated cells.

Key terms: Flow cytometry, intercalation, DNA unwinding, external dye binding, histones

Cytochemical methods for nucleic acids have a long history of investigation (2,11), yet many facets of these reactions still remain unexplained. One such problem is accessibility of DNA in situ to the dye, and its relationship to chromatin structure. Thus, for instance, the binding of acridine orange or ethidium bromide to DNA in situ is restricted by chromosomal proteins; extraction of these proteins by acids or salt solutions of high ionic strength, or digestion with trypsin results in a marked increase in accessibility of DNA to these dyes (8,10,29). Furthermore, in certain cell systems, binding of these ligands correlates with changes in chromatin structure that accompany cell differentiation (see 5,6 for reviews). Specifically, a decrease in DNA accessibility to acridine orange (AO) was demonstrated during spermiogenesis (7,10,12) and erythropoiesis (8,18,29). Neither mechanisms responsible for the restriction are known, nor were the particular chromatin constituents of the differentiating cells causing the phenomenon identified.

In the present paper we analyze nine different DNA fluorochromes commonly used in flow cytometry (re-

view, see 19) or for chromosome analysis (3,4) with respect to: 1) the extent of restriction in their binding by nuclear constituents extractable with 0.1N HCl and 2) the difference in their binding as related to erythroid cell differentiation. Because the modes of binding of these ligands to free DNA are different (intercalation, various degree of unwinding, external binding, involvement of the double helix grooves) the extent of restriction for each of the ligands may shed some light on the possible mechanisms of interaction between these dyes and DNA in chromatin, and the nature of the restriction. A model of Friend erythroleukemia cells was chosen because it offers a possibility to study populations of relatively uniform differentiated cells when treated with dimethylsulfoxide (DMSO), which can be compared with cells growing exponentially (8,29). Chromatin changes

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associated with erythroid differentiation are quite well characterized at the molecular level (eg, 1,13,17,26).

MATERIALS AND METHODS

Cells

Friend leukemia (FL) cells, strain 745 (GM-86), were obtained from the Medical Research Institute, Camden, N.J. The cells were grown as a suspension culture in Dulbecco's modified Eagle's medium containing 25mM HEPES buffer (Grand Island Biological Co., GIBCO, Grand Island, N.Y.), supplemented with 10% fetal calf serum (GIBCO) and antibiotics as described (30). All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cultures were routinely passaged three times a week at a seeding density of 1–2 × 10⁵ cells per milliliter. To ensure exponential growth, the cultures were split 1:4 daily for 3 consecutive days prior to harvesting. To induce erythroid differentiation, the cells were grown in the presence of 2% (v/v) DMSO (Fisher Scientific Co., Fairlawn, NJ) for 6 consecutive days at densities not exceeding 1 × 10⁶ cells per ml as described (8,29). Growth in the presence of DMSO manifested as cell arrest in G₁ and the appearance of hemoglobin in the cytoplasm which could be detected cytochemically by the benzidine method (25).

Cell Staining

The experiments were performed both on isolated nuclei and in cells that were made permeable to the fluorochromes studied by pretreatment with the nonionic detergent Triton X-100. To isolate nuclei, the cells obtained from the cultures were rinsed once with Hanks' balanced salt solution (HBSS) and resuspended in a solution containing 0.2% (v/v) NP-40 (Calbiochem, La Jolla, CA), 3mM MgCl₂, 2mM CaCl₂, 0.32M sucrose, and 10mM PIPES buffer (Calbiochem) at pH 6.4. The cells were then homogenized in a glass pestle homogenizer, the nuclei were rinsed twice with the buffer described above but without NP-40 and incubated with 10³ units of RNase A (RASE, Worthington Biochem. Corp., Freehold, NJ) for 20 min at 24°C. All steps of nuclei isolation, excluding RNase treatment, were done at 0°C. Intact cells taken directly from culture were made permeable to the dyes by rinsing once with HBSS, suspending in HBSS (~10⁷ cells/1 ml), and treating with an equal volume of HBSS containing 0.2% (v/v) Triton X-100 (Sigma Chemical Corp., St. Louis, MO) at 0°C. The cells were then incubated with 10³ units of RNase A/1 ml for 20 min at 24°C. The suspensions of isolated nuclei or Triton X-100-treated cells were then stained with each of the fluorochromes either directly or after pretreatment with HCl. In the direct procedure, 0.2-ml aliquots of nuclei or cells (suspended in HBSS) were admixed with 2 ml of the respective dye solution in 0.1M PIPES buffer (pH 6.4), 2mM MgCl₂ and 0.1% Triton X-100 at 0°C. In the two-step procedure, 0.2-ml aliquots of nuclei or cell suspension were treated with 0.2 ml of a solution containing 0.2N HCl, 0.15N NaCl, and 0.1% Triton X-100 for 30 s at 0°C (the final HCl concentration

was thus 0.1M), and then with 2.0 ml of the respective dye solution in 0.1M PIPES buffer (pH 6.4) 2mM MgCl₂ and 0.1% Triton X-100, also at 0°C. The relatively small volume of HCl, as compared with the large volume and high concentration of the PIPES buffer (high buffering capacity) added during the second step resulted in a final pH of the staining solution close to 6.4, ie, similar to that of the direct staining reaction. Care was taken, by adjusting nuclei (cell) number and dye concentration, to ensure that at equilibrium there was an excess of free dye, and thus the staining was done at D/P > 2.0. Usually each dye was tested at different concentrations, ranging between 0.1 to 10.0 μM and the measurements were performed at various times after addition of the dyes (between 1 to 40 min). In most cases, the staining reaction was rapid and a stable staining pattern was achieved during the initial 10 min. In cases of Hoechst 33342, mithramycin, and chromomycin A₃, however, the staining reaction was slower and the equilibrium was observed after 30 min. While more detailed data, including that on isolated nuclei and the kinetics of the reaction, will be published at a later date, in the present communication we report results of the experiments on permeabilized cells (which are essentially similar to these on isolated nuclei) obtained at a single, equimolar dye concentration (5 μM), except in the case of DAPI which was studied at 1 μM, and Hoechst 33342 studied at 0.2 μM. The latter two fluorochromes produced unstable pattern of cell stainability, with broad G₁ distribution (high c.v. values) when tested at higher concentration. All reported measurements were done when equilibrium with the dyes was reached and the cell fluorescence was stable; the cells were equilibrated with the dyes at 0°C. The following fluorochromes were studied: 4'6-diamidino-2-phenyl-indole (DAPI; synthesized by Dr. Jan Kapuscinski—see ref 15), Hoechst 33342, acridine orange (AO; chromatographically purified), ethidium bromide (EB), and propidium iodide (PI) (all obtained from Polysciences, Inc., Warrington, PA), quinacrine hydrochloride (Sigma), chromomycin A₃ and 7-amino-actinomycin D (both from Calbiochem-Boehringer Corp., La Jolla, CA) and mithramycin (Pfizer, New York, NY).

Fluorescence Measurements

The fluorescence of cells stained with DAPI and Hoechst 33342 was measured in an ICP 22 flow cytometer (Ortho Diagnostic Instruments, Westwood, MA) using the UG1 excitation filter and a combination of dichroic mirrors and secondary filters transmitting light between 460 and 500 nm. Cells stained with mithramycin and chromomycin A₃ were also measured in the ICP 22 instrument, using BG 12 excitation filter and a combination of dichroic mirrors and filters transmitting between 535 and 630 nm. Cells stained with AO were analyzed by both ICP 22 and FC 200 flow cytometers (Ortho Diagnostics) using excitation in blue light ("FITC special" dichroic filter for ICP) or the 488-nm excitation line of the FC 200 laser and measuring green fluorescence (515–560nm). Fluorescence of cells stained with

EB, PI, and quinacrine was measured in FC 200 flow cytometer, using the excitation wavelength of 488 nm and measuring emission above 590 nm for EB and PI or between 515 and 560 nm for quinacrine. Fluorescence of cells stained with 7-amino-AMD was also measured with the FC 200 flow cytometer using 514-nm excitation wavelength and measuring fluorescence above 590 nm. At least 10^4 cells were measured per sample and the peak values of the G_1 populations were compared. The experiments were repeated three times. Mean values \pm S.D. of these experiments are shown in Table 1. To avoid redundancy, the cell cycle distributions obtained with only five of the nine dyes are shown. These histograms were selected to illustrate the character of the raw data obtained after cell measurements using either the ICP 22 (following DAPI-Hoechst- or acridine orange-staining) or the FC 200 (following ethidium- or propidium-staining) flow cytometers. The distributions after staining with mithramycin or chromomycin A_3 were similar (with respect to the c.v. values) to those obtained after staining with the Hoechst dye, except that the difference between the exponentially growing and differentiated cells was minor. Quinacrine- and 7-amino-AMD-stained cells exhibited higher c.v. values, ranging between 10–14%.

RESULTS

Representative histograms illustrating exponentially growing and differentiated cells either untreated or ex-

tracted with 0.1N HCl and stained with some of the fluorochromes are shown in Figures 1–4. Quantitative data are summarized in Table 1. The staining characteristics for each of the fluorochromes is described below.

DAPI

The reaction with DAPI was rapid and the equilibrium was reached after 5 min. The resolution of the cell cycle phases was good, as reflected by low c.v. values of the G_1 population, varying between 2.0 and 3.0% in repeated measurements in different experiments. Differentiated cells exhibited 8% lower fluorescence than cells from exponentially growing cultures. This difference was significant and could be demonstrated in several experiments. Additionally, when the differentiated cells (or nuclei) were mixed with cells (or nuclei) from exponentially growing cultures and stained together, two G_1 -peaks were always apparent. Extraction with 0.1 N HCl resulted in 45% increase in DNA stainability, but the difference between differentiated and exponentially growing cells remained (Fig. 1).

Hoechst 33342

The staining reaction was slow and the equilibrium was reached only after 30 min. About a 4% lower stainability was observed for the differentiated cells in repeated experiments however, the presence of two distinct G_1 peaks in the mixed cell population could not be demonstrated prior to HCl treatment. Cell treatment with

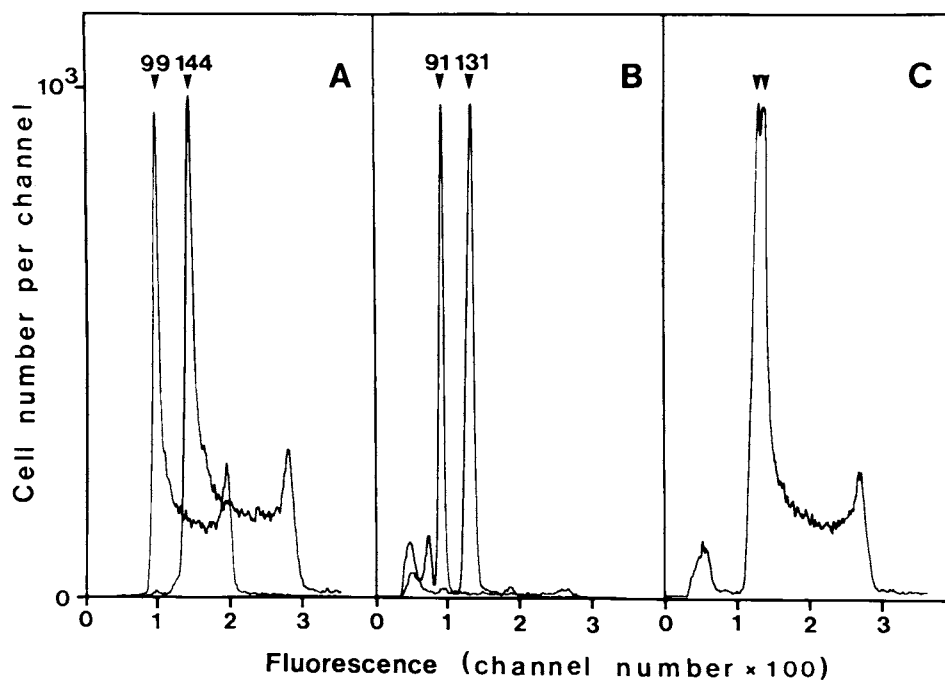


FIG. 1. Frequency distribution histograms representing fluorescence of FL cells stained with 4'6-diamidino-2-phenylindole (DAPI). A) Exponentially growing cells before (peak value = 99) and after treatment with 0.1N HCl (peak value = 144). B) Differentiated cells before (peak = 91) and after extraction with 0.1N HCl (peak = 131). C) Differentiated cells were mixed with exponentially growing ones in 1:2 proportion, treated with 0.1N HCl, and stained. In these mixed cell populations, cells in G_1 exhibit different stainability, as manifested by the divided G_1 peak (arrow). One-step staining (without HCl-treatment) of mixed populations resulted also in a bimodal cell distribution within the G_1 peak in repeated experiments (not shown).

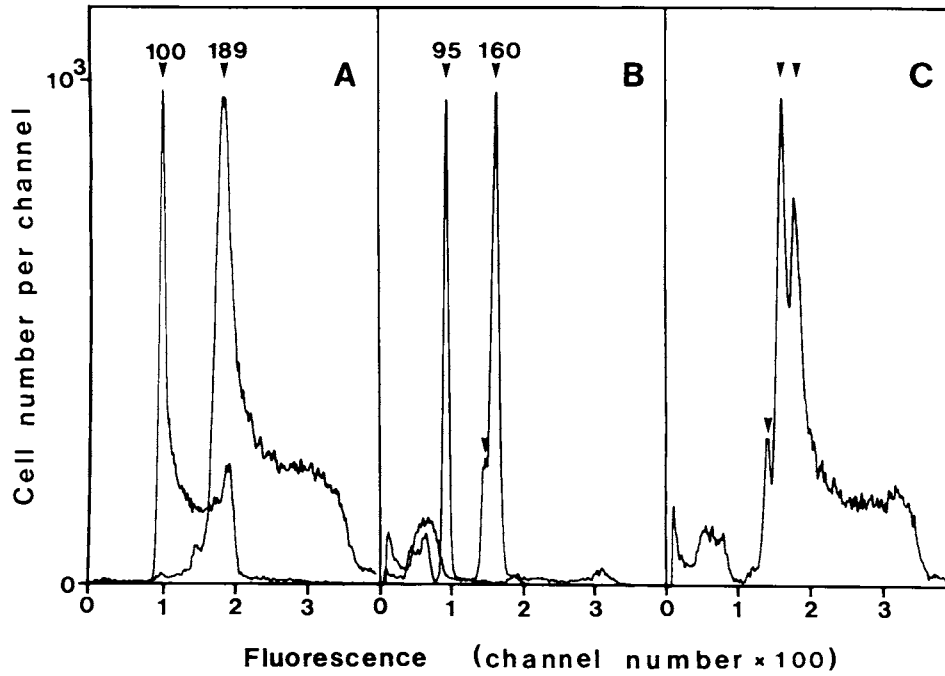


FIG. 2. Frequency histograms of cells stained with Hoechst 33342. A) Exponentially growing cells before (peak = 100) and after extraction with 0.1N HCl (peak = 189). B) Differentiated cells before (peak = 95) and after extraction with HCl (peak = 160). After acid extraction, a

minor population in G₁ with somewhat lower stainability is apparent (arrow). C) Exponentially growing and differentiated cells were mixed, treated with HCl and stained. Two main peaks and the minor one in G₁ were apparent.

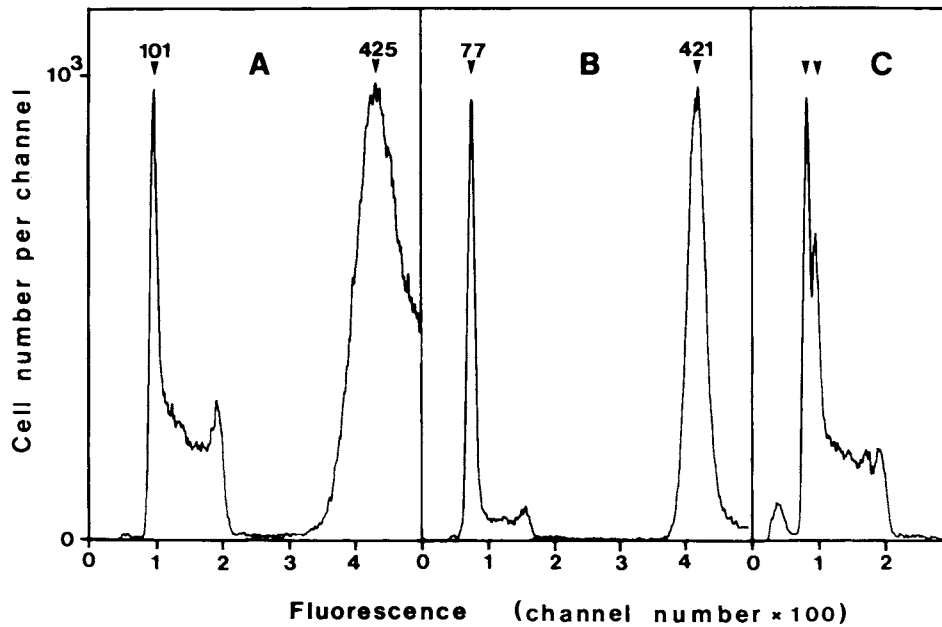


FIG. 3. Frequency histograms representing green fluorescence of cells stained with acridine orange. A) Exponentially growing cells before (peak = 101) and after (peak = 425) extraction with HCl. B) Differentiated cells before (peak = 77) and after (peak = 421) extrac-

tion with HCl. C) Differentiated and exponentially growing cells were mixed and stained together with AO (without HCl treatment). A bimodal cell distribution in G₁ is evident (arrows). Mixed cells stained after HCl treatment exhibited a single G₁ peak (not shown).

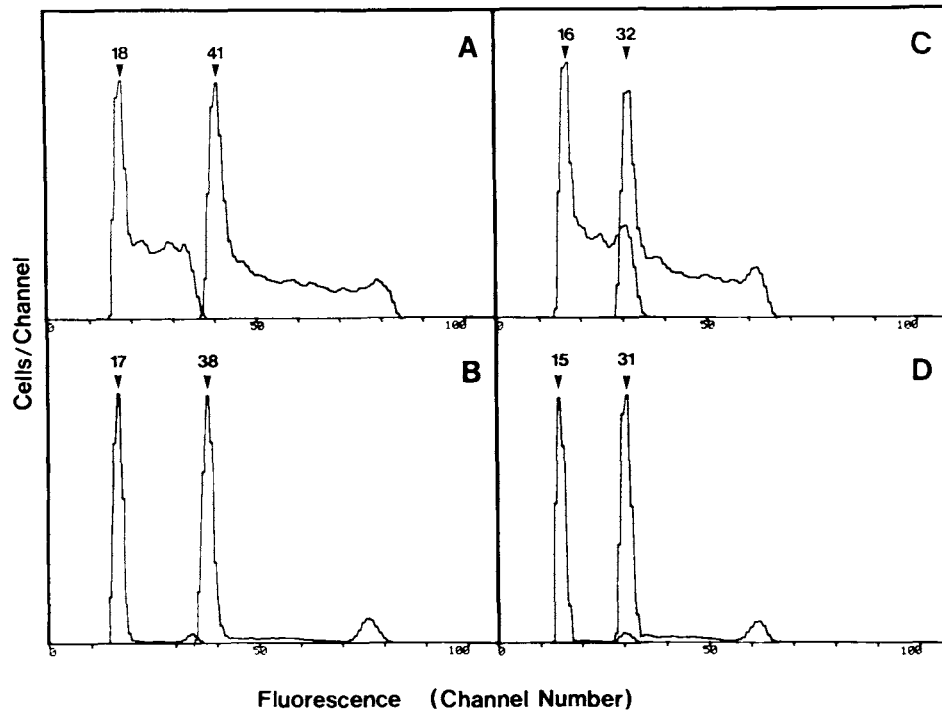


FIG. 4. Frequency distribution histograms of cells stained with ethidium bromide (A and B) and propidium iodide (C and D). A and C) Exponentially growing cells stained before (peaks = 18 and 16) and after extraction with 0.1N HCl (peaks = 41 and 32). B and D) Differ-

entiated cells stained prior (peaks = 17 and 15) and after extraction with HCl (peaks = 38 and 31). Mixed cell populations of exponentially growing and differentiated cells did not show a bimodal distribution within the G_1 population, either before or after HCl treatment.

HCl raised fluorescence of exponentially growing and differentiated cells by 88 and 69%, respectively. Thus, following the acid treatment, the difference between exponentially growing and differentiated cells was actually increased (14%) and in the mixed cell population two peaks became evident (Fig. 2).

Acridine Orange

The equilibrium was reached rapidly (5 min) and the differentiated cells showed 24% lower stainability. Acid pretreatment increased fluorescence of nondifferentiated and differentiated cells by 328 and 451%, respectively. Following HCl treatment, therefore, both exponentially growing and differentiated cells exhibited the same stainability and when mixed populations of these cells were stained, only a single G_1 peak could be seen.

Ethidium Bromide

A stable staining pattern was attained after 10 min. The fluorescence of differentiated cells was 6% lower than that of cells from exponentially growing cultures and two G_1 peaks could not be seen in mixed cell populations. Acid treatment of nondifferentiated and differentiated cells increased cell fluorescence by 125 and 132%, respectively.

Propidium Iodide

Stainability of cells with PI was similar to EB (Fig. 4). The difference between nondifferentiated and differentiated cells was 6% and the result of the treatment was an 103 and 105% increase in cell fluorescence to similar levels for both exponentially growing and differentiated cells.

Quinacrine

The equilibrium was obtained after 10 min. The difference between exponentially growing and differentiated cells was 6%. An increase in cell fluorescence by 315 and 323% was observed following HCl extraction of exponentially growing and differentiated cells, respectively. A single G_1 peak was observed in mixed cell populations both before and after acid treatment.

7-amino-actinomycin D

A stable pattern was observed after 10 minutes incubation. The difference between exponentially growing and differentiated cells was 33%, the highest of all the fluorochromes tested. Although the resolution was rather poor (c.v. values 10–14%), two G_1 peaks could be observed in mixed cell populations. A dramatic 13-fold increase in fluorescence was observed in cells extracted with HCl; following HCl treatment, differentiated and nondifferentiated cells had similar fluorescence.

Table 1
Results of the Experiments in Which Exponentially Growing and Differentiated Cells Were Stained With Various Fluorochromes Before and After Extraction of Nuclear Proteins With HCl^a

Dye	Treatment	Cell Fluorescence	
		Exponentially growing cells	Differentiated cells
DAPI	None	1.00	0.92 ± 0.04 ^b
DAPI	0.1N HCl	1.45 ± 0.04	1.32 ± 0.05 (-9) ^b
Hoechst 33342 ^c	None	1.00	0.96 ± 0.03
Hoechst 33342 ^c	0.1N HCl	1.88 ± 0.05	1.62 ± 0.06 (-14) ^b
Acridine orange	None	1.00	0.76 ± 0.04 ^b
Acridine orange	0.1N HCl	4.28 ± 0.16	4.19 ± 0.19 (-1)
Ethidium bromide	None	1.00	0.94 ± 0.05
Ethidium bromide	0.1N HCl	2.25 ± 0.09	2.18 ± 0.10 (-4)
Propidium iodide	None	1.00	0.94 ± 0.05
Propidium iodide	0.1N HCl	2.03 ± 0.06	1.94 ± 0.11 (-3)
Quinacrine	None	1.00	0.94 ± 0.04
Quinacrine	0.1N HCl	4.15 ± 0.18	3.98 ± 0.19 (-5)
7-amino-actinomycin D	None	1.00	0.67 ± 0.03 ^b
7-amino-actinomycin D	0.1N HCl	13.06 ± 0.51	13.24 ± 0.60 (+1)
Mithramycin ^c	None	1.00	0.96 ± 0.05
Mithramycin ^c	0.1N HCl	1.96 ± 0.08	1.90 ± 0.10 (-2)
Chromomycin A ₃ ^c	None	1.00	0.97 ± 0.04
Chromomycin A ₃ ^c	0.1N HCl	2.04 ± 0.10	1.97 ± 0.11 (-2)

^aThe data are expressed as normalized peak values (exponentially growing, not treated with HCl cells = 1.00) when all measurements for each fluorochrome were done at the identical photomultiplier settings. Figures in brackets show the change (percent) of fluorescence of differentiated cells treated with HCl with respect to exponentially growing, HCl-treated cells.

^bThe difference between exponentially growing vs differentiated cells could be additionally demonstrated in mixed populations of these cells, stained and measured together, when a bimodal G₁-peak distribution was evident (Figs. 1-3).

^cThe staining reaction was slow, the equilibrium was established after 30 minutes.

Mithramycin and Chromomycin A₃

Both dyes showed similar characteristics. The staining reaction was slow; the equilibrium was established after 30 min. No significant difference between differentiated vs exponentially growing cells was evident. Pretreatment with HCl doubled cell fluorescence.

DISCUSSION

The aim of these experiments was to estimate for each of several different DNA dyes whether a difference in DNA stainability in situ exists between exponentially growing vs differentiated FL cells, and also the extent of the restriction in DNA stainability by nuclear proteins extractable with 0.1N HCl. Before discussing the results in terms of differences between various fluorochromes, their mode of binding and chromatin structure, certain technical points require comment. First of all, we assume that under the present conditions the staining was specifically restricted to DNA. This assumption is based on the following observations: 1) all fluorochromes exhibit rather high specificity toward nucleic acids and were tested at low concentrations; 2) the results were similar regardless of whether whole cells or isolated nuclei were measured; 3) both cells and iso-

lated nuclei were preincubated with RNase prior to staining; 4) although the staining conditions (ionic strength, pH, dye concentration) were not tailored for optimal stainability for each individual dye, high resolution (low c.v. values of the G₁ population) characterized nearly all measurements, especially prior to cell treatment with HCl; 5) in control experiments, nearly all fluorescence measured was sensitive to DNase (not shown).

The second important consideration is that the results were not affected by changes in permeability of the cell membrane. The cells were fully permeable to the dyes, and thus the measurements reflect chromatin stainability at equilibrium. Both detergent-treated cells and isolated nuclei exhibited similar staining characteristics. Triton X-100-treated cells were also permeable to RNase inasmuch as RNase-treatment diminished their red luminescence (after differential staining of DNA vs RNA with AO, see ref. 9) which was as low as that of isolated nuclei treated with RNase (not shown).

The cells were treated with 0.1N HCl to extract basic nuclear proteins, including histones. Extensive biochemical literature indicates that most histones are soluble in 0.1N HCl (review, 13). It is thus presumed that the acid treatment removes histone H1 and in all prob-

