

DIFFERENT SENSITIVITY OF DNA *IN SITU* IN INTERPHASE AND METAPHASE CHROMATIN TO HEAT DENATURATION

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ABSTRACT

Heat denaturation of DNA *in situ*, in unbroken cells, was studied in relation to the cell cycle. DNA in metaphase cells denatured at lower temperatures (8°–10°C lower) than DNA in interphase cells. Among interphase cells, small differences between G₁, S, and G₂ cells were observed at temperatures above 90°C.

The difference between metaphase and interphase cells increased after short pretreatment with formaldehyde, decreased when cells were heated in the presence of 1 mM MgCl₂, and was abolished by cell pretreatment with 0.5 N HCl. The results suggest that acid-soluble constituents of chromatin confer local stability to DNA and that the degree of stabilization is lower in metaphase chromosomes than in interphase nuclei. These *in situ* results remain in contrast to the published data showing no difference in DNA denaturation in chromatin isolated from interphase and metaphase cells. It is likely that factors exist which influence the stability of DNA *in situ* are associated with the super-structural organization of chromatin in intact nuclei and which are lost during chromatin isolation and solubilization. Since DNA denaturation is assayed after cell cooling, there is also a possibility that the extent of denatured DNA may be influenced by some factors that control strand separation and DNA reassociation.

The different stainability of interphase vs. metaphase cells, based on the difference in stability of DNA, offers a method for determining mitotic indices by flow cytofluorometry, and a possible new parameter for sorting cells in metaphase.

The cell transition from interphase to metaphase involves a sequence of dramatic changes in the gross morphology of nuclear chromatin. The dispersed chromatin of the interphase nucleus undergoes condensation and packing into chromosome entities of a very characteristic morphology. Although the gross morphologic changes are well characterized (see references 9, 26, 30), the changes in molecular structure of chromatin that accompany this transition are poorly understood. This may be because most studies of chromatin structure have been carried out on isolated chro-

matin in solutions, i.e., under conditions where factors responsible for maintenance of the characteristic gross morphology are lost. Chromatin extraction which involves homogenization of nuclei, removal of divalent cations, shearing, and solubilization, destroys any chromatin superstructure that may exist *in situ* (4, 6, 15, 23) and that in turn may modulate the interactions between DNA and the macromolecules of the nuclear milieu. The gross morphology of chromatin, DNA-nuclear envelope interactions, and the presence of divalent cations appear to be of a special importance during transi-

tion from interphase to metaphase (9, 26, 30).

In previous papers (10–14, 35), we have presented a method for studying the thermal stability of DNA *in situ* in large populations of unbroken, individual cells. Since ionic interactions between DNA and neighboring macromolecules influence the stability of the double helix (3, 18–20, 32, 33, 36), the patterns of thermal denaturation of DNA *in situ* provide information regarding the molecular structure of nuclear chromatin. This method was used to investigate chromatin changes during the cell cycle.

MATERIALS AND METHODS

Cells

Most experiments were done on murine leukemic cells chronically infected with Friend leukemia virus (FL) which were kindly provided by Doctor Charlotte Friend of the Mt. Sinai School of Medicine, New York. The cells were grown exponentially in Eagle's Basal Medium containing Earle's balanced salt solution, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, 10% fetal calf serum, 200 U/1 ml of penicillin and 0.2 μ g/1 ml of streptomycin (14). Human leukemic cells (SK-L7) were maintained in McCoy's 5A medium supplemented with 20% fetal calf serum and antibiotics, as described (11). Before harvesting, the cells were treated with 0.1 μ g/1 ml of Colcemid (Grand Island Biological Co., Grand Island, N.Y.) or with 0.2 μ g/1 ml of vincristine (Eli Lilly and Co., Indianapolis, Ind.) for periods of time as specified in the figure legends.

When harvested, the cells were washed with a solution containing 0.25 M sucrose, 5 mM MgCl₂, and 20 mM Tris-HCl buffer, pH 7.4, (SMT), and were fixed in suspension in ethanol:acetone (1:1, vol/vol) for at least 16 h at 4°C (10–13). After fixation, cells were centrifuged, suspended in SMT solution, incubated with 1,000 U/1 ml of RNase (Worthington Biochemical Corp., Freehold, N.J., RASE) for 30 min at 35°C, then rinsed once with SMT, twice with the salt solution used for suspending the cells during heating, and finally suspended in that salt solution (see figure legends). In one experiment the incubations with RNase were carried out in the presence of inhibitors (see Table III). All inhibitors, except Na₂S₂O₄, were obtained from Sigma Chemical Co. (St. Louis, Mo.); Na₂S₂O₄ was purchased from Fisher Scientific Co. (Pittsburgh, Pa.)

In another experiment the cells were postfixed in formaldehyde. This was done by suspending already prefixed (in alcohol:acetone) cells in a solution containing 1% formaldehyde, 0.14 N NaCl, 2 mM MgCl₂, and 15 mM phosphate buffer (pH 7.0) for 30 min at 0°C. The cells were then rinsed in SMT and treated with RNase, as described above.

In yet another experiment to test the possibility that

cell fixation is responsible for the observed differences, the cells were subjected to heatings without the prior step of fixation in alcohol:acetone. In this case, the cells collected from cultures were washed in SMT and then were suspended and incubated in a solution containing 1 mM MgCl₂, 1 mM cacodylate buffer (pH 6.0), 40% ethanol, and 10³ U/1 ml of RNase for 30 min at 35°C. Control staining of cellular RNA with acridine orange (AO), as described in detail elsewhere (10–14), indicated that the RNase treatment of the nonfixed cells in the presence of 40% ethanol is as effective as it is in the case of the prefixed cells. The presence of ethanol in the incubation medium apparently makes the cells permeable to the exogenous RNase; the enzyme remains active during incubation under these conditions. After RNase treatment, the cells were washed in a solution containing 2 mM cacodylate buffer, 10⁻⁴ M EDTA, and 40% (vol/vol) ethanol (final pH 6.0), and then heated while suspended in this solution.

DNA Denaturation

A detailed description of the method is presented elsewhere (10–14). Briefly, the cells suspended in a solution containing 40% ethanol, 10⁻⁴ M EDTA-Na and 2 × 10⁻³ M cacodylate buffer (pH 6.0) (referred to hereafter as "standard" solution) were heated for 5 min at appropriate temperatures, as indicated in the figure legends. Ethanol was included in the solution in order to prevent the leakage of denatured DNA from the cells, at higher temperatures (10). The effect of the alcohol on DNA denaturation *in situ* is described elsewhere (12). After heating, the cells were stained with AO at a final concentration of 2.1 × 10⁻⁵ M in the presence of 5 mM MgCl₂ as described (10, 11). The excess of the cation (Mg²⁺) competing with AO for anionic groups increases the specificity of the interaction between nucleic acids and AO in whole cells, thus providing conditions similar in principle to those proposed by Rigler (27) who used an acetylation step and staining at pH 4.5 to prevent interactions between AO and anionic groups of proteins. Control experiments on DNase-treated cells performed as before (10–13) have shown that at least 85% of the measured fluorescence is due to AO interaction with DNA. The basis for differential staining of double- vs. single-stranded DNA with AO in cells depleted of RNA relies on the fact that AO binds to double-stranded DNA by intercalation and fluoresces in green (530 nm) (17), whereas AO stacking on single-stranded DNA involves dye-dye interactions and results in metachromatic red fluorescence (640 nm) (5). Thus, cell fluorescence intensities at 530 (F₅₃₀) and at 640 nm (F_{>600}) give relative measures of the extent of denatured DNA per cell (10–14, 16, 27–29).

The measurements of cell fluorescence were performed in a flow cytofluorometer (Bio/Physics Systems, Inc., Mahopac, New York) which is designed to measure the green (F₅₃₀) and red (F_{>600}) fluorescence of individual

cells as they pass through the focused beam of a 488 nm argon laser (21). Detailed descriptions of the instrument and the computer data handling system designed by us are given elsewhere (10, 11, 21). The data presented are mean values based on a total 5×10^8 cells measured in each sample at a given temperature. The extent of DNA denaturation within the cells is expressed as a value α_1 , representing the ratio of $F_{>600}$ to the total cell fluorescence $F_{>600} + F_{530}$ (10, 11). All experiments were repeated at least twice.

RESULTS

Nonheated, RNase-treated cells when stained with AO emit fluorescence mostly in the green (F_{530}); the red component ($F_{>600}$) is minimal. On the basis of the intensity of the green fluorescence (which is sensitive to DNase treatment), it is possible to estimate the relative content of stainable DNA per cell. Consequently, the histograms of this single parameter (F_{530}) indicate the proportions of cells in G_1 , S, and $G_2 + M$ phases of cell cycle (Fig. 1).

The G_1 , S, and $G_2 + M$ cells are represented on two-parameter ($F_{>600}$ vs. F_{530}) scattergrams by clusters occupying sites according to their respective $F_{>600}$ and F_{530} values (Fig. 2). As a result of cell heating, the $F_{>600}$ increases and the F_{530} decreases, reflecting denaturation of DNA *in situ* (10-14). On scattergrams, this is evident as a change in the position of the cells (or clusters) in respect to the $F_{>600}$ and F_{530} axes. As can be seen from Fig. 2, during cell heating there is a separation of cells within the original cluster representing $G_2 + M$ cells, i.e. having F_{530} value twice higher

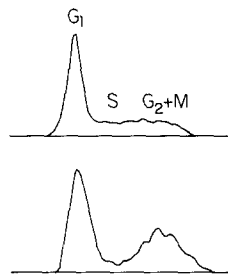


FIGURE 1 Computer-drawn frequency distribution histograms representing fluorescence at 530 nm of RNase-treated nonheated FL cells, stained with AO. The abscissa represents F_{530} in arbitrary units, and the ordinate, number of cells. Top: control cells from the nonheated culture. Bottom: cells from culture incubated with colcemid for 4 h. The intensity of staining of nuclear DNA with AO permits discrimination between cells in various phases of cell cycle.

than the G_1 cluster, into two subpopulations. One of them, namely the subpopulation with increased $F_{>600}$ and lowered F_{530} , was identified (see Discussion) as cells in mitosis. The respective positions of the clusters representing mitotic vs. G_2 cells indicate that, after heating at any given temperature, the DNA of mitotic cells is denatured more extensively (has increased α_1 value) than is the DNA of G_2 cells.

The plots of mean α_1 values of G_1 , S, G_2 , and M subpopulations vs. temperature (Fig. 3) represent the curves of DNA denaturation ("melting profiles") *in situ* for cells in these phases of the cell cycle. The mean values of α_1 for cells in G_1 , S, G_2 , and M subpopulations have been obtained by computerized selection of cell clusters representing the above subpopulations (Figs. 1, 2, and 4) and by measurements of the mean F_{530} and $F_{>600}$ values of the cells within these clusters. Namely, G_1 cells were chosen as the main cluster (the highest peak on frequency distribution histograms) with fluorescence values representative of the 2c DNA content. G_2 cells were represented by the second peak, with fluorescence values twice as high as the G_1 peak (4c). Mitotic cells were indistinguishable from G_2 cells in nonheated samples, and therefore α_1 values at 24°C represent means of $G_2 + M$ cells. Upon heating, mitotic cells, which had clearly increased $F_{>600}$ and decreased F_{530} in comparison with G_2 cells, formed a separate cluster (Fig. 2 B-D); fluorescence of all cells within this cluster has been measured to obtain mean value of α_1 for the mitotic cells. At the same time, parallel control experiments were performed on synchronized cultures blocked with Colcemid to ensure that nearly all cells were in metaphase. The main cluster of cells in these cultures had fluorescence values similar to those of the M subpopulation from other nonsynchronized or partially synchronized cultures (Figs. 2 and 4). S cells were selected as those having fluorescence values between 2 and 4c; specifically to exclude any overlapping of G_1 and G_2 cells, only 20% of the total S cells from the middle regions, i.e., with $\sim 3c$ DNA content, were measured. Thus these cells are expected to be in the midterm of the S phase.

There are large differences between interphase and metaphase cells in the profiles of DNA denaturation *in situ*. When the cells are briefly pretreated with formaldehyde (1% at 0°C) and then heated in the standard medium, i.e., at low ionic strength in the presence of EDTA (Fig. 3 A), the

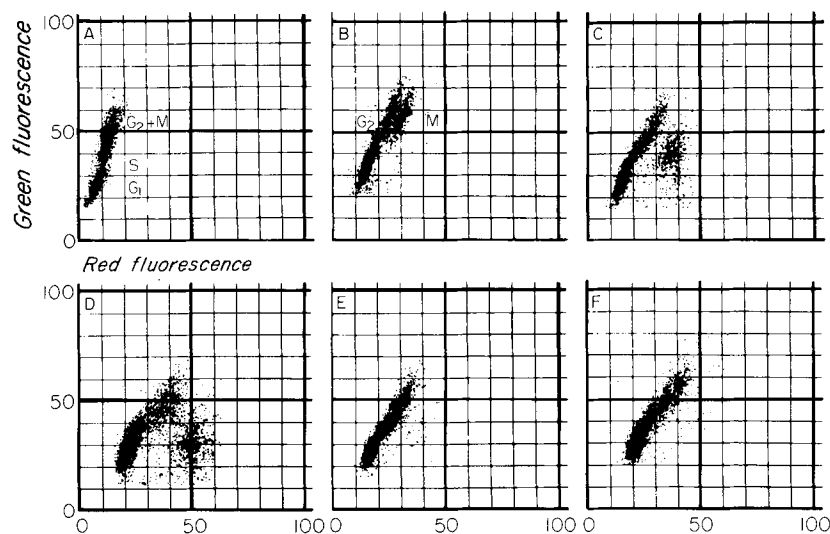


FIGURE 2 Computer-drawn scattergrams of the distribution of individual FL cells (3×10^3 cells in each scattergram) according to their green (F_{530}) and red (F_{600}) fluorescence intensities, after staining with AO. Each cell is represented by a single point; its distances from abscissa and ordinate are the measures of F_{530} and F_{600} , respectively. (A–D) The cultures were treated with vincristine for 6 h before harvesting; there were 15% metaphase cells in these cultures. (E and F) Control stationary cultures with $<0.2\%$ of cells in mitosis. (A) Nonheated cells. (B) Cells heated at 50°C . Note that two subpopulations become apparent within the cluster representing $G_2 + M$ cells. The cells with increased F_{600} were identified as the metaphase cells. (C) Cells heated at 85°C . Mitotic cells appear as a separate subpopulation (cluster) having higher F_{600} and lower F_{530} in comparison with the rest of the cells remaining within the upper (G_2) cluster. (D) Cells heated at 95°C . Further increase in F_{600} and decrease in F_{530} of the mitotic cells; the mean red fluorescence of M cells is about 50 U. (E and F) Cells from control cultures, heated at 85° and 95°C , respectively. There is a paucity of cells in the loci in which the clusters of mitotic cells were seen in vincristine-treated cultures. The conditions of cell treatment and heating were the same as those described in the legend for Fig. 3 A.

difference between the mitotic and interphase cells is the greatest. During the first phase of DNA denaturation (between 24° – 80°C , which corresponds to the α_1 increase of 0.1), the difference is within a range of 0.05–0.1 α_1 unit. During the second phase of DNA denaturation (over 85°C), the difference increases to 0.25 U.

No difference can be seen between G_1 , S, and G_2 cells at temperatures below 95°C . At the two highest temperatures (95° and 100°C), the extent of DNA denaturation appears to be different for representative cells of the G_1 , S, and G_2 clusters, increasing in the order G_1 -S- G_2 .

The difference between mitotic and interphase cells is markedly diminished and is seen only at temperatures over 60°C when cells are heated in the presence of Mg^{2+} (Fig. 3 B). No difference between G_1 , S, and G_2 cells is seen under these conditions.

DNA denaturation in cells not pretreated with

formaldehyde and heated in the standard solution is somewhat more extensive, and occurs at slightly lower temperatures than in formaldehyde-treated cells (Figure 3 C). The difference between mitotic and interphase cells is seen throughout the entire range of temperatures (above 45°C) and is the highest at temperatures over 75°C . The difference between G_1 , S, and G_2 is evident only at 95° and 100°C .

Pretreatment of cells at low pH changes the pattern of DNA denaturation *in situ* (Fig. 3 D–F and Table I). In general, in acid-treated cells DNA denatures at lower temperatures. After cell treatment at pH 2.2, the melting temperatures of DNA of mitotic and interphase cells are lowered by about 5° – 8°C , but the difference between these cells still remains. After extraction at pH 1.7, the melting pattern is entirely different than at pH 2.2. Namely, at the temperatures between 24° and 70°C , the DNA of the mitotic cells appears to be

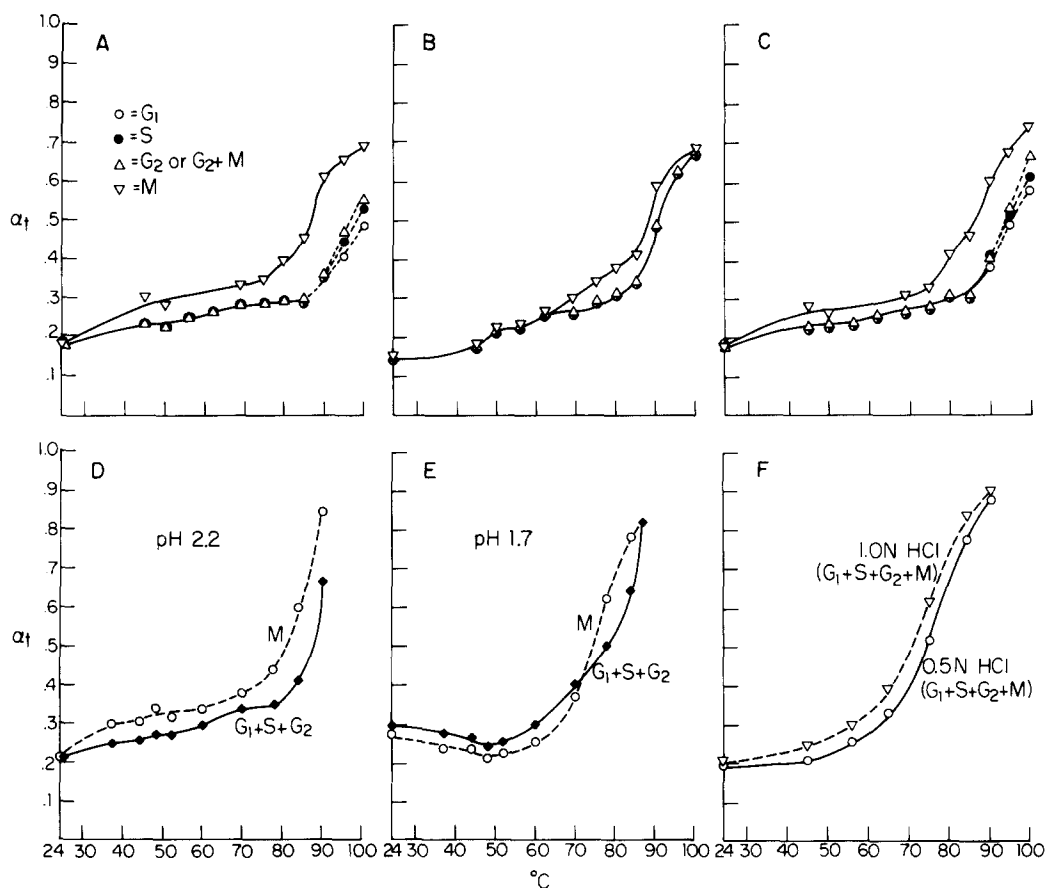


FIGURE 3 Profiles of thermal DNA denaturation *in situ* in FL cells heated at various conditions. DNA denaturation is represented as $\alpha_t = F_{>600} / (F_{>600} + F_{530})$ after heating at various temperatures. α_t of G_1 , S, and G_2 cells was obtained by interactive computer-programmed selection of the cells in the middle regions of the respective G_1 , S, and G_2 clusters and by measuring their $F_{>600}$ and F_{530} . (A-C) Separate symbols are used for cells in various phases of the cell cycle (as given in part A). (D and E) No difference was seen between G_1 , S, and G_2 cells; they are represented by one symbol. (F) All phases of the cell cycle are represented by a single symbol. (A) The cells were treated with 1% formaldehyde at 0°C for 30 min, then washed, and heated while suspended in a solution containing 2 mM cacodylate buffer, pH 6.0, 10^{-4} M EDTA, and 40% ethanol (standard solution). (B) The cells were heated in a solution containing 1 mM $MgCl_2$, 2 mM cacodylate buffer (pH 6.0), and 40% ethanol. No pretreatment with formaldehyde. (C) The cells were heated in a standard solution (see part A) without prior treatment with formaldehyde. (D and E) The cells were washed in 0.1 M acetate buffer, pH 2.2 (D) or 1.7 (E) and then heated in the standard solution. (F) The cells were washed with 0.5 or 1.0 N HCl and heated in the standard solution.

more stabilized than that of interphase cells, while above 70°C the DNA of metaphase cells denatures more extensively. This reversed pattern observed below 70°C correlates with an apparent lowering of α_t values at 24°–50°C. Analysis of $F_{>600}$ and F_{530} indicates that in cells pretreated at pH 1.7 and then heated at temperatures between 24° and 60°C, there is a marked increase in the F_{530} value, which is higher for mitotic cells than for

interphase cells, though there is no increase in $F_{>600}$. As a result, the α_t decreases and remains lower in the mitotic cells than in the interphase cells. This type of F_{530} and $F_{>600}$ change, as reflected by the lowering of α_t , is unique for cells treated at pH 1.7, since in all other cases there is an increase in $F_{>600}$ (and in α_t) during heating.

As a result of cell treatment with 0.5 or 1.0 N HCl, the DNA *in situ* denatures at temperatures

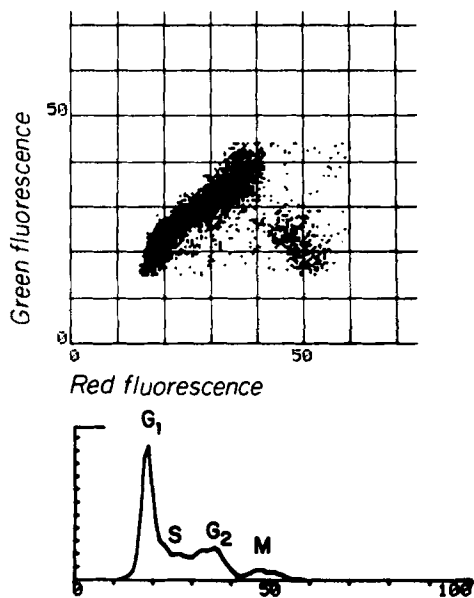


FIGURE 4 The cells from cultures treated for 2 h with colcemid were heated at 90°C in the standard solution. Clear separation of metaphase cells from other cells is seen in the upper scattergram. The frequency distribution histogram of $F_{>600}$ values of these cells, as given below, shows that it is possible to distinguish the subpopulations of G_1 , S, G_2 , and M cells on the basis of a single-parameter analysis.

TABLE I

Temperature of the Transition ($T_{0.5}$) of DNA *in situ* in FL Cells at Interphase and Metaphase after Cell Treatment at Various Conditions

| Cell pretreatment | Denaturation conditions | $T_{0.5}$ | | Difference |
|-------------------|-------------------------|------------|------------|------------|
| | | Interphase | Meta-phase | |
| | | °C | °C | °C |
| Formaldehyde | Standard | 97-100 | 87 | + 10 |
| None | With Mg^{2+} | 91 | 88 | + 3 |
| None | Standard | 94-96 | 86 | + 8 |
| pH 2.2 | " | 88 | 81 | + 7 |
| pH 1.7 | " | 78 | 74 | + 4 |
| 1.0 N HCl | " | 70 | 70 | 0 |
| Nonfixed | " | 88 | 78 | + 10 |

Temperature at which α equals 0.5 (10, 11) was determined from the slopes of the transition curves, as presented in Fig. 3. Cell pretreatment and the denaturation conditions are described in the legend to Fig. 3 and in Material and Methods.

20° or 24°C lower, respectively (Fig. 3 F). In both cases, no difference between mitotic and interphase cells is apparent.

Attempts were made to denature DNA in cells that were not fixed in alcohol-acetone. In these

cells the pattern (profile) of DNA denaturation was very similar to that of prefixed cells, except that DNA denatured at temperatures 4°-6°C lower. Moreover, the clusters of cells representing G_1 , S, and $G_2 + M$ subpopulations were less distinct due to markedly larger intercellular variation in $F_{>600}$ within the clusters (variation coefficient almost twice as high as that of fixed cells). However, the distinction between mitotic and interphase cells was clear at temperatures over 70°C. The transition temperature of the mitotic cells was 10°C lower than of the interphase cells (Table I).

The inhibitors of proteolysis (Table III), present during incubation with RNAase, are without any effect on the difference in stainability between interphase and metaphase cells.

DISCUSSION

A distinct cell subpopulation having an altered DNA stainability with AO (increased $F_{>600}$ and decreased F_{530} in comparison with other cells) appears as a result of heating of FL cells. The following evidence indicates that this subpopulation represents cells in mitosis, mostly in metaphase: a) it originates from the cluster representing $G_2 + M$ cells, i.e., the cells with a tetraploid DNA content (Fig. 2); b) the number of cells within this subpopulation closely parallels the number of cells at metaphase as counted by light microscopy in either synchronous or asynchronous cultures (Table IV); c) as a result of treatment of asynchronous cultures with colcemid or vinblastine, the number of these cells increases in proportion to the time of exposure to the mitotic inhibitor (Fig. 5); d) under UV microscopy the chromosomes of heated and AO-stained cells in metaphase exhibit more red and less green fluorescence than interphase nuclei of cells accompanying them, treated in the same way.

TABLE II

Effect of Washing of FL Cells at Various pH's on Binding of AO to DNA *in situ* by Intercalation (F_{530})

| Cell washing | F_{530} | Change |
|-----------------|-----------|--------|
| pH | U | % |
| 7.0 | 48 | 0 |
| 2.2 | 78 | + 63 |
| 1.7 | 90 | + 88 |
| 1.0 (0.5 N HCl) | 97 | + 102 |
| 0.9 (1.0 N HCl) | 98 | + 104 |

TABLE III
Effect of Various Inhibitors of Proteolysis on AO Stainability on Interphase and Metaphase Cells after Heating

| Cell treatment | Cells in mitosis | Metaphase-interphase difference ($d\alpha_1$) |
|---|------------------|---|
| | % | |
| None (control I) | 12.1 | 0.12 |
| Dimethylsulfoxide (control II) (Me_2SO , 0.1% vol/vol) | 11.9 | 0.13 |
| <i>N</i> - α -tosyl-L-phenylalanylchloromethane (Tos-PheCH ₂ Cl, 10^{-3} M) | 11.5 | 0.13 |
| <i>N</i> - α -tosyl-L-lysylchloromethane (Tos-LysCH ₂ Cl, 10^{-4} M) | 12.5 | 0.13 |
| Phenylmethyl sulfonyl fluoride (Bz-SO ₂ F, 10^{-4} M) | 11.8 | 0.13 |
| Sodium hydrosulfite (dithionite) ($\text{Na}_2\text{S}_2\text{O}_4$, 10^{-3} M) | 11.7 | 0.14 |

FL cells collected from cultures treated with Colcemid for 4 h, after fixation were incubated with RNase in the absence (control, Me_2SO) or presence of the inhibitors. Inhibitor solutions were prepared fresh before the experiment. Since Tos-PheCH₂Cl and Tos-LysCH₂Cl are poorly soluble in water, they were predissolved in a small quantity of Me_2SO and then in SMT solution (see Material and Methods); the final concentration of Me_2SO was 0.1% vol/vol. The possible effect of Me_2SO was tested in an additional control (II).

After heating at 85°C and staining with AO, the number of cells with increased α_1 (cells in mitosis) was counted and the α_1 values were calculated for all cells in interphase and in metaphase. Then, the value of α_1 for interphase cells was subtracted from the α_1 of metaphase cells to obtain $d\alpha_1$.

No effect was observed for any of the inhibitors specific for various classes of proteolytic enzymes, suggesting that the difference between interphase and metaphase chromatin is not a result of differential proteolysis of nuclear proteins (see Discussion).

TABLE IV
Correlation between Percent of Cells in Mitosis as Scored Visually under the Microscope and the Percent of Cells from the Same Cultures with High α_1 Values after Heating

| Time with Colcemid (h) | 0 | 1 | 2 | 3 | 7 | 16 |
|----------------------------------|-----|-----|------|------|------|------|
| Cells in mitosis (visual count) | 1.5 | 6.2 | 9.8 | 14.0 | 30.9 | 73.3 |
| Cells with high α_1 value | 2.3 | 4.9 | 10.1 | 15.2 | 32.7 | 74.0 |

Cultures of SK-L7 cells were treated with Colcemid for various periods of time. When harvested, a portion of each culture was used to make cell smears; the remaining cells were fixed in suspension and subjected to DNA denaturation as described in Material and Methods. The smears were fixed in alcohol:acetic acid (3:1), rinsed, stained with AO, and counted under the UV microscope. The percent of cells in mitosis was calculated on a basis of 200 cells counted from each smear. 5,000 cells from each sample were measured by flow-cytofluorometry after heating at 90°C and staining with AO.

We have shown previously (10-13) that the changes in α_1 of heated cells reflect DNA denaturation *in situ* and that the α_1 index represents the portion of AO-stainable DNA that is denatured at a given temperature. Increased α_1 values of the heated mitotic cells (Fig. 3) as compared with the

interphase nuclei indicate, therefore, that DNA *in situ* in metaphase chromosomes has markedly lower stability to heat. Since DNA in chromatin is stabilized against heat denaturation via interactions with local counterions, presumably histones (3, 18-20, 32-34, 36), our results suggest that the strength of these interactions under *in situ* conditions is lower in metaphase than in interphase chromatin. Indeed, when basic macromolecules are extracted with acids (Fig. 3F), DNA denatures at markedly lower temperatures and no difference between metaphase and interphase chromatin can be seen. We presume that 0.5 or 1.0 N HCl treatment, which abolishes the difference between metaphases and interphases and doubles the extent of unmasked, AO-stainable DNA (Table II), removes from the nucleus all histones and perhaps some other proteins soluble at that low pH.

When cells are pretreated with formaldehyde, the difference between metaphase and interphase increases (cf. Fig. 3A and C). This appears to be due to the fact that the effect of formaldehyde in stabilizing DNA against heat is more pronounced in the interphase than in the metaphase cell. Since the stabilization of DNA in chromatin by formaldehyde is a result of cross-linking and the appearance of covalent DNA-histone interactions (7, 20, 35), the different sensitivity of metaphase vs. interphase cells to this agent indicates that the molecular structure of chromatin during these two

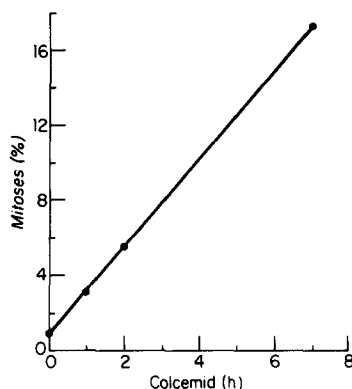


FIGURE 5 Increase in the percentage of metaphase cells in logarithmically growing FL cultures in relation to the time of exposure of the culture to Colcemid. The percent of mitoses was calculated on a basis of analysis of 5×10^3 cells per sample. The cells were heated at 90°C; the mitoses were scored automatically by computer-programmed selection based on their α_1 values.

phases is different. The structure in the interphase nucleus, as opposed to metaphase chromosomes, seems to favor the cross-linking which again suggests a tighter interaction between DNA and histones during interphase.

We have previously shown that denaturation of DNA *in situ*, in thymus cells, is markedly affected by divalent cations. The fraction of DNA resistant to heat was found to be destabilized by Mn^{2+} , Ca^{2+} , or Mg^{2+} at millimolar concentrations (12), suggesting that divalent cations significantly modulate the DNA-histone interactions. It appears that the FL interphase cells respond to Mg^{2+} (although not so dramatically) as do thymus cells, i.e., their DNA is destabilized (Fig. 3 B). In contrast, the effect of Mg^{2+} on metaphase cells is minimal. As a result, the difference in DNA denaturation between interphase and metaphase chromatin is decreased in the presence of Mg^{2+} . This difference in the response of metaphase and interphase chromatin to Mg^{2+} provides additional evidence that the molecular structure of chromatin in these phases of the cell cycle is different.

Extraction of basic proteins at low pH reduces the difference between metaphase and interphase chromatin. Yet, after extraction at pH 1.7, which is associated with an increase in the extent of the unmasked (AO binding) DNA by 88%, DNA denaturation in metaphase and interphase chromatin still proceeds differently (Fig. 3 E). In the stepwise extraction of histones that results from lowering the pH, the H1 histone is removed first. It is likely, thus, that H1 is removed at pH 1.7; the difference between metaphase and interphase chromatin seems to remain after its removal.

While the present investigations performed on chromatin *in situ* in unbroken cells reveal a difference between the metaphase and interphase chromatin, the detailed biochemical studies by Shih and Lake (33) failed to detect any differences. In their studies both the thermal stability and the circular dichroism of chromatin isolated from metaphase and interphase cells were found to be identical (33). The authors caution, however, that since the superstructure characteristics of chromosome morphological appearance are not preserved in the isolation procedure, its possible influence on the molecular structure of nucleohistone may escape detection (32). The present results appear to confirm this notion; they suggest that some factors influencing conformation of nuclear chromatin *in situ* are indeed lost during extraction of chromatin.

Perhaps the gross chromatin structure, and associated with it, the superstructure organization of chromatin (4, 6, 15, 23) modulate interactions between DNA and other macromolecules of the nucleus. As the gross chromatin structure and the molecular superstructure are destroyed during isolation, the difference between interphase and metaphase chromatin is lost.

Additional evidence suggesting that chromatin extraction may markedly change the interactions between DNA and other macromolecules as reflected by the thermal stability of DNA is provided by numerous other studies on DNA denaturation *in situ*. In all these reports a large difference between various cell types is stressed (1, 2, 13, 28, 29, 31). As an example, the melting profiles of DNA in FL cells as presented here are markedly different from the profiles in thymus cells reported by us before (12). The difference is evident in the proportions of the thermosensitive vs. thermoresistant DNA fractions as well as in the melting temperature of the thermoresistant fraction (12). In contrast, the differences in melting profiles of chromatin isolated from various cell types and assayed biochemically are of a lower magnitude (3, 18–20, 32–34, 36).

Is it possible that the difference between interphase and metaphase chromatin, as presented here, may be the result of some undetected artifact related to the *in situ* technique, i.e., due to cell fixation, and that the biochemical studies which show no such difference (33) truly reflect the situation as it exists *in vivo*? We think this is unlikely for the following reasons: *a*) The results of our *in situ* studies are highly reproducible; the difference can be seen after cell heating in solutions of various chemical composition and also after heating of nonfixed cells. The difference disappears only after extraction of basic proteins; *b*) We also observe the difference between metaphase and interphase in cell types other than FL and SK-L7 cells (manuscript in preparation). These include Chinese hamster ovary cells, i.e., the cells studied biochemically by Shih and Lake (33). *c*) The correlation of differences in chromatin *in situ* with cell cycle phase has been shown by other methods. Thus, actinomycin D binding to DNA of fixed cells or of isolated nuclei varied during the cell cycle (24). The template activity of metaphase chromosomes was different from that of interphase nuclei (8). *d*) Contrary to the original observations of Shih and Lake, there are re-

cent studies that do indicate a difference in conformation of chromatin isolated from cells at various phases of cell cycle. Thus, Nicollini et al. (22) observed differences in circular dichroism and in binding of the intercalating dye ethidium bromide, while Pederson (25) noted a variation in sensitivity to nuclease digestion.

Another possibility of artifact would be a differential (interphase vs. metaphase) proteolysis of histones by endogenous proteases after cell fixation. We exclude this possibility for the following reasons: *a*) Fixation immobilizes all macromolecules in the cells; the cytoplasmic (lysosomal) enzymes cannot migrate into the nucleus. Thus, the protease(s) in question, even if remaining active, cannot extensively hydrolyse histones while both the enzyme and the substrate are fixed and spatially immobilized; *b*) If the effect we observe was due to differential proteolysis, then the difference is expected to be higher in nonfixed cells. Yet, the difference was highest in the formaldehyde-post-fixed cells. *c*) Proteolysis and removal of histones increase binding of intercalating dyes to DNA, and thus the stainability of chromatin (10, 11). Since at 24°C the stainability of G₂ and M cells is identical, this again indicates that histones remain intact; *d*) The highest difference is seen at 90°–100°C, i.e., at temperatures at which all known proteases are inactive; *e*) Cell treatment with a variety of inhibitors of proteolysis is without any effect (Table III).

The results presented herein suggest that interactions between DNA and proteins which stabilize DNA are stronger in interphase nuclei than in metaphase chromosomes. This is somewhat surprising, considering that chromatin in metaphase, as opposed to interphase, is more condensed (9, 26, 30), binds less actinomycin D (24) and is a poor template for RNA polymerase (25). DNA of metaphase chromosomes is thus expected to have rather more extensive interaction with histones. At present, we cannot provide an explanation for this apparent discrepancy. In other studies (13), condensed chromatin of dormant lymphocytes has been found to denature *in situ* at lower temperatures than the active chromatin of leukemic cells. These *in situ* data agree also with the biochemical observations of Tsai et al. (36) and of Subirana (34) who reported the presence of poorly stabilized DNA regions in chromatin preparations that are transcriptionally inactive. For instance, 20% of the DNA of sea urchin sperm chromatin denatured at lower temperatures than the least stabi-

lized regions of transcriptionally active liver chromatin (36). Chicken erythrocyte chromatin also exhibited a surprisingly high (higher than the liver chromatin) extent of poorly stabilized DNA (36). Thus, the final strength of DNA stabilization and the extent of DNA masking (suppression of template activity, chromatin condensation) might not necessarily be positively correlated in chromatin of various template activities as was previously postulated on the basis of DNA denaturation *in situ* in the presence of formaldehyde (27–29).

It should be pointed out, however, that since the measurements of DNA denaturation are performed after cell cooling, the α_t index represents a fraction of the denatured, stainable DNA which does not renature after cooling. The extent of DNA renaturation which cannot be measured by the present method might vary in different chromatins. Consequently, the difference between metaphase and interphase chromatin might be related to some structural properties which influence DNA renaturation rather than denaturation. It is possible, for instance, that strand separation might occur more easily in metaphase than in interphase chromatin. Although there is no direct evidence on the extent of DNA renaturation in this system, there are certain data which suggest that renaturation may not be the factor responsible for the differences described. Thus, DNA renaturation in chromatin is only partial (34) and cell cooling is done in the presence of AO, an agent which additionally impedes DNA renaturation (10, 11, 16). Furthermore, strand separation in chromatin is a monophasic phenomenon (34) as compared with DNA denaturation, which is biphasic (32–34, 36). The transition curves presented here are biphasic and similar in shape to DNA denaturation rather than strand-separation curves (34). Nonetheless, additional studies aimed at assaying the extent of DNA renaturation in interphase and metaphase chromatin are required to prove that the observed difference does indeed reflect the different melting points of DNA in interphase vs. metaphase chromosomes.

The possibility of differential staining of metaphase vs. interphase cells based on a difference in sensitivity to heat (Figs. 2 and 4) offers a new tool for rapid, automatic quantitation of mitotic cells. Since several thousand cells may be measured within seconds by flow cytophotometry, the method allows accurate calculation of mitotic indices even in cases where the number of cells in mitosis is small (manuscript in preparation). This

approach might be useful in cancer research to investigate the effectiveness of drugs that influence the cell cycle, particularly the mitotic blockers.

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