DNA DENATURATION IN SITU

Effect of Divalent Cations and Alcohols

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ABSTRACT

Heat denaturation profiles of rat thymus DNA, in intact cells, reveal the presence of two main DNA fractions differing in sensitivities to heat. The thermosensitive DNA fraction shows certain properties similar to those of free DNA: its stability to heat is decreased by alcohols and is increased in the presence of the divalent cations Ca²⁺, Mn²⁺, or Mg²⁺ at concentrations of 0.1–1.0 mM. Unlike free DNA, however, this fraction denatures over a wide range of temperature, and is heterogeneous, consisting of at least two subfractions with different melting points.

The thermoresistant DNA fraction shows lowered stability to heat in the presence of Ca²⁺, Mn²⁺, or Mg²⁺ and increased stability in the presence of alcohols. It denatures within a relatively narrow range of temperature, consists of at least three subfractions, and, most likely, represents DNA masked by histones.

The effect of Ca²⁺, Mn²⁺, or Mg²⁺ in lowering the melting point of the thermoresistant DNA fraction is seen at cation concentrations comparable to those required to maintain gross chromatin structure in cell nuclei or to support superhelical DNA conformation in isolated chromatin (0.5–1.0 mM). It is probable that factors involved in the maintenance of gross chromatin organization in situ and/or related to DNA superhelicity also have a role in modulating DNA-histone interactions, and that DNA-protein interactions as revealed by conventional methods using isolated chromatins may be different from those revealed when gross chromatin morphology remains intact.

An assessment of the extent and the mode of interactions between DNA and nuclear proteins is necessary in elucidating the molecular structure of chromatin and in understanding the genome regulatory mechanisms. An important tool in the studies of these interactions is provided by analysis of thermal denaturation of DNA in chromatin (3, 30, 31, 42, 43, 45). Ionic interactions between DNA phosphates and positively charged neighboring macromolecules confer local stabilization of the double helix; the extent and strength of these interactions may be evaluated from the profiles of DNA denaturation (3, 30, 43, 45).

Biochemical methods used to study DNA denaturation of isolated chromatin involve breaking the nuclei, treatment with chelating agents (ethylenediaminetetraacetic acid, EDTA), and shearing of chromatin; DNA denaturation is assayed at low ionic strength and in the presence of EDTA (3, 30, 43, 45). It is obvious that any chromatin superstructure is irreversibly lost when the superstructure involves: interaction between
DNA and the nuclear envelope as proposed by Brasch and Setterfield (7); "suprachromosomal organization" as postulated by DuPraw (15); structures related to the specific morphology of chromatin as revealed by Berezney and Coffey (5). Indeed there is strong evidence from circular dichroism measurements (46) that breaking the nucleus changes chromatin conformation. In addition, chelation of divalent cations produces further changes, as evidenced by circular dichroism (47) and x-ray diffraction (17), which have been interpreted as the loss of the "superhelical" or "native" conformation of DNA. Furthermore, there is convincing evidence that divalent cations are an integral part of native chromatin and are involved in maintenance of the gross chromatin structure, modulating such features of the chromatin as its euchromatic or heterochromatic pattern, condensation, etc. (29, 35). These features, in turn, correlate with the functional states of the cell nucleus (RNA and DNA synthesis, template activity) and with cell differentiation (25, 29, 32).

Thus, the factors that influence DNA stability in isolated chromatin, in the presence of EDTA or citrate, may not be the same as those present in situ, i.e. in unbroken nuclei, in the presence of divalent cations.

We have recently developed a method to measure DNA denaturation in situ in large populations of unbroken, individual cells (12-14) which was used here to study the effect of divalent cations on DNA-protein interactions, at concentrations known to preserve and/or modulate gross chromatin structure. The method is based on staining of RNase-treated cells in equilibrium with acridine orange (AO) and measuring the fluorescence of individual cells with a flow-cytofluorometer (24).

AO binds to double-stranded DNA by intercalation; this type of interaction results in green fluorescence (530 nm) (26). AO stacking on single-stranded DNA involves dye-to-dye interactions and red metachromasia (640 nm) (6). Thus, cell fluorescence intensities at 530 nm ($F_{530}$) and at 640 nm ($F_{640}$) give relative measures of the extent of denatured DNA (20, 37-39). Although metachromatic staining with AO has been used to study thermal denaturation of DNA in cytological preparations on glass slides (1, 2, 34, 38-41, 48), the present method makes it possible to obtain more detailed DNA denaturation profiles from large cell populations (12-14).

Divalent cations, as counterions with a strong affinity for DNA, at low concentration, stabilize DNA against heat denaturation (16). If these cations were to be present in aqueous solutions at the desired concentrations, their stabilizing effect would be such that no DNA denaturation could be seen below 100°C (16). To lower the overall sensitivity of DNA to thermal denaturation, and thus to be able to observe DNA denaturation in the presence of divalent cations, we included alcohols in the solutions. Alcohols markedly decrease the melting point of free DNA (18, 28); consequently, they are expected to counterbalance the stabilization induced by divalent cations, thereby allowing the study of DNA denaturation at temperatures below 100°C. Interestingly, and as a by-product of our studies, DNA denaturation profiles in the presence of alcohols revealed certain novel, characteristic features of chromatin conformation in situ that will be described.

MATERIALS AND METHODS

Cell Suspensions

Thymus cells were obtained from 4-8 wk-old Sprague-Dawley rats as described elsewhere (12-14). The cells were rinsed in phosphate-buffered saline and fixed in ethanol-acetone (1:1 vol/vol) for at least 16 h (12-14). After fixation, the cells were centrifuged and suspended in a solution of 0.25 M sucrose, 5 mM MgCl₂, 20 mM Tris-HCl, pH 6.5 (SMT) containing 5 x 10⁸ U per 1 ml of RNase A (Worthington Biochemical Corp., Freehold, N. J., RASE) for 30 min at 37°C, then rinsed once with SMT, twice with the salt solution used for suspending the cells during beatings, and finally suspended in that salt solution (see legends).

DNA Denaturation

Tubes with 0.5-ml aliquots of cell suspensions (approximately 2 x 10⁶ cells) were heated for 5 min in a water bath at appropriate temperatures. The tubes were then transferred to an ice-cold bath, and 2 ml of ice-cold AO solution (National Aniline, division of Allied Chemical Corp., Morristown, N. J., dissolved in SMT, pH 7.4) were added to each tube to give a final AO concentration of 2.13 x 10⁻⁵ M. This concentration of AO, in the presence of 5 mM MgCl₂, has been found to give the highest resolution in differential staining of double- vs. single-stranded DNA in cells (13, 14). Taking into account AO concentration and quantity of cells (DNA)
per tube, there was several-fold excess of AO per DNA phosphate; under those conditions, variation in cell number per sample from $2.5 \times 10^4$ to $5 \times 10^5$, which corresponds to variation in AO per DNA phosphate molar ratio from 4:1 to 80:1, did not affect cell stainability (fluorescence at 530 nm and >600 nm) by more than 5%. A detailed description of the instrument (cytofluorograph, model 4801, Bio/Physics Systems Inc., Mahopac, N.Y.) is presented elsewhere (24). Fluorescence signals are generated by the individual cells as they pass through a focused beam of a 488-nm argon-ion laser. The red fluorescence emission ($F_{>600}$; measured in a band 600-650 nm) and green fluorescence emission ($F_{>500}$; measured in a band 515-575 nm) from each cell are separated optically, and quantitated by separate photomultipliers; 100-200 cells per s are measured. Background fluorescence is automatically subtracted. The measurements are filed by computer for further analysis. The data given are mean values for populations of diploid cells from a total of $5 \times 10^7$ cells measured in each sample. The variation coefficient of these means ranged from 6% to 12%, in most cases remaining close to 10%.

During measurements of DNA melting profiles, the photomultiplier sensitivities were adjusted to give the same numerical readings at 530 nm for the nonheated cells as at >600 nm for the cells that were heated at 100°C in solutions which facilitated full DNA denaturation (13, 14). This standardization was done to ensure that the recorded fluorescence intensities (yields) at 530 nm and at >600 nm remain in proportion to the extent of double- and single-stranded DNA in the cells, respectively. With such photomultiplier calibration, the gradual $F_{>600}$ decrease, representing disappearance of double-stranded DNA regions, was accompanied by a quantitatively comparable $F_{>500}$ increase, representing appearance of single-stranded DNA regions, throughout most of the temperature range at which DNA denatured. A decrease of $F_{>600}$ occurring at higher temperatures in aqueous solutions, as reported before (13) and attributed to leakage of denatured DNA from the cell, was not evident in the solutions containing methanol or ethanol.

The extent of DNA denaturation within the cell is expressed as an index $a_t$, representing a ratio of $F_{>600}$ to the total cell fluorescence:

$$a_t = \frac{F_{>600}}{F_{>500} + F_{>600}}.$$  

Provided $F_{>600}$ represents AO interaction with denatured DNA and $F_{>500}$ with native DNA, and provided the $F_{>600}$ vs. $F_{>500}$ fluorescence intensities are in proportion to the quantity of denatured vs. native DNA, respectively, the $a_t$ index is expected to represent the proportion of denatured DNA within the cell, as discussed in detail elsewhere (13, 14). The temperature at which $a_t$ equals 0.5 ($T_m$) is thus somewhat comparable to the midpoint of the transition curves ($T_m$) obtained by conventional methods.

A derivative DNA denaturation profile (13) was obtained by using coordinates of the DNA thermal denaturation curve at 1°C intervals in the following equation:

$$\frac{da_t(T)}{dT} = \frac{a_t(T+1) - a_t(T-1)}{2},$$

where $a_t(T)$ is the value of $a_t$ at temperature $T$.

Control experiments provided evidence that at least 90% of the measured fluorescence of RNase-treated cells is due to AO interaction with DNase-sensitive macromolecules. The "conformationally nonspecific" components in DNA staining, namely $F_{>500}$ due to native DNA (i.e. some dye stacking, emission spectra overlap), and $F_{>600}$ as a result of AO binding to denatured DNA (i.e. due to spatial separation of bound AO molecules and absence of dye-dye interaction), cannot account for more than 5% of the $F_{>500}$ or $F_{>600}$, respectively, under the present conditions of cell staining and measurement (13, 14). Variation from the described conditions of cell staining (AO concentration, MgCl₂ concentration, ionic strength) decreases the specificity of the differential DNA staining with AO.

RESULTS

Effect of Methanol and Ethanol on DNA Denaturation

Fig. 1 illustrates DNA denaturation profiles of thymus cells heated in the absence of divalent ions, in aqueous solution and in a solution containing 50% methanol. In the case of cells heated without methanol, two main phases of DNA denaturation are distinctly evident. The first phase (I) during which $a_t$ rises to 0.4 is separated from the second phase (II) ($a_t$ increase from 0.4 to 0.9) by a transient plateau seen at 65-70°C. The rate of DNA denaturation ($a_t$ rise per °C increase) is slower during the first phase.

A considerably wider overall profile is seen for DNA denaturation of cells heated in 50% methanol. Here, also, two main phases of DNA denaturation may be distinguished. The first phase is very wide; an increase of $a_t$ up to 0.4 occurs during the 24-70°C temperature change. The width of the second phase (0.4-0.9 $a_t$ increase) is comparable to the width of the corresponding phase for cells heated in the absence of methanol. However, there is a shift by about 4°C toward higher temperatures for cells heated in alcohol as compared with cells heated in aqueous solutions. Thus, the main type of change for DNA denaturation in situ in 50% methanol appears to be related to that portion of DNA which denatures first. Methanol markedly destabilizes this fraction, lowering its melting point.
FIGURE 1 Profiles of thermal DNA denaturation in situ in rat thymus lymphocytes. Effect of methanol. The cells were heated in: solution containing $10^{-4}$ M Tris-HCl and $5 \times 10^{-4}$ M EDTA, pH 6.5 (O--O); and solution containing 50% (vol/vol) methanol, $10^{-4}$ M Tris-HCl and $5 \times 10^{-4}$ M EDTA, pH 6.5 (■—■).

and extending the range of temperature at which it denatures. In addition, in methanol solutions, this fraction appears to denature in a stepwise fashion since two distinct subphases become evident (at 40–55°C, $\alpha_1$ increases from 0.20 to 0.33; and at 64–72°C, $\alpha_1$ increases from 0.35 to 0.40).

The phases of DNA denaturation are better visualized if the derivative of the melting profile is plotted as a function of temperature (Fig. 2). The phases are then represented by separate melting bands. The melting bands are wider and appear to be better separated in the case of cells heated in 50% methanol. Under these conditions, the first phase of DNA denaturation ($\alpha_1$ rises to 0.4) is characterized by a shoulder at 24–35°C, a wide band at 35–48°C and another wide band at 62–72°C. The second phase of DNA denaturation (0.4–0.9 $\alpha_1$ increase) consists of three high but relatively narrow bands with peaks at 75°C, 80°C, and 86°C.

The first phase of DNA denaturation of cells heated in aqueous solution consists of a shoulder and a very wide band with three peaks at 53°C, 59°C, and 63°C. There are four high bands with peaks at 74°C, 77°C, 81°C, and 86°C, representing the second main phase of DNA denaturation. It should be pointed out, however, that in repeated experiments on DNA denaturation in the absence of alcohol the reproducibility of the relative heights of these high bands was not always apparent. In addition, the second and third band were not always separated. In such cases, a single wide band at 75–83°C was present.

Experiments were performed also on cells heated in a solution of similar composition as described in Fig. 1, but containing ethanol instead of methanol. Profiles of DNA denaturation in these solutions were similar to the profiles seen in solutions containing methanol as presented above (Fig. 1), except that a minor shift (3–5°C) of the whole curve towards a lower temperature is observed (not shown). This would indicate that ethanol exerts a somewhat stronger effect in destabilizing DNA in situ than methanol.

**Effect of Divalent Cations**

Presence of divalent cations in the solution in which cells are suspended during heating dramatically changes the pattern of DNA denaturation (Fig. 3). The character of changes depends on the cation concentration. At $10^{-2}$ M concentration, MgCl$_2$ increases the stability of all cellular DNA. The stabilization, however, is uneven. Specifically, the portion of DNA denaturing at lower temperatures appears to be much more affected than the portion which is thermoresistant. Thus, while the

FIGURE 2 Derivative DNA denaturation profiles of cells heated in aqueous and in alcohol solutions. The cells were heated in: $10^{-4}$ M Tris-HCl, $5 \times 10^{-4}$ M EDTA, pH 6.5 (O--O); and 50% methanol (vol/vol), $10^{-4}$ M Tris-HCl, $5 \times 10^{-4}$ M EDTA, pH 6.5 (■—■).
heat resistance of DNA represented by the first phase is increased by as much as 20°C, only 1–3°C stabilization of the second phase of DNA is evident.

In contrast to the increased stability of DNA at 10^{-4} M MgCl₂, decreased resistance to heat of most of the nuclear DNA is observed at 5 × 10^{-4} and 10^{-3} M MgCl₂ concentration. MgCl₂ at these higher concentrations lowers the midpoint of the transition curves (T_{a1}) by about 15°C. At the same time, however, the small portion of DNA denaturing below 50°C in the absence of cations is stabilized in their presence. Consequently, the biphasic character of DNA denaturation disappears at concentrations of MgCl₂ above 5 × 10^{-4} M.

The melting profiles of cells heated in 10^{-3} M MgCl₂ or 10^{-3} M CaCl₂ are almost identical (Fig. 3). Monovalent cations at similar concentration exert only a minimal effect. Thus, NaCl included in the heating solutions at concentrations of 10^{-4} M to 5 × 10^{-3} M does not change the α₁ value of

The effect of divalent cations appears to be different depending on whether cells are directly heated in their presence following fixation (as in Fig. 3) or whether the endogenous ions are initially chelated with EDTA and then the chromatin ionic milieu is reconstituted by suspending cells in new solutions containing a single divalent cation (Fig. 4). In the latter case, as in the case of cells not treated with EDTA, the ions stabilize the heat-labile DNA portion (delaying the onset of DNA denaturation up to 45°C) and exert the opposite effect on the heat-resistant portion, i.e. lowering its melting point. Unlike the previous experiments, (Fig. 3) however, DNA denaturation profiles show differences depending on the specific ion. When one compares the effects of 10^{-3} M MgCl₂ in both these situations (see Fig. 3 and 4), it is evident that, although the general trend of Mg²⁺-induced
changes is similar, the extent of these changes is
greater if cells are not pretreated with EDTA.
Similarly, we observed nearly identical curves
when cells were heated with 10^{-2} M MgCl_2 and
10^{-3} M CaCl_2 without pretreatment with EDTA
(Fig. 3), while the effect of the same ions in
equimolar concentrations was different in cells
pretreated with EDTA (Fig. 4). Thus, reconstitution
of chromatin after EDTA treatment, either
with Mg^{2+} or with Ca^{2+} alone, cannot fully restore
the original chromatin structure, as revealed by its
thermosensitivity.

DISCUSSION

Two grossly different phases of DNA denaturation
are seen on melting profiles of cells heated in
solutions lacking divalent ions. They reveal the
presence of two distinct fractions of DNA in situ.
The thermosensitive fraction, denaturing during
the first phase, consists of at least two subfrac-
tions, markedly differing in their melting point.
Since denaturation of the thermosensitive fraction
(in thymus cells) is completed when α_t reaches a
value of 0.4 (α_t increase by 0.3, from 0.1 to 0.4),
this fraction might be expected to represent about
30% of the DNA stainable with AO (13, 14).

The thermoresistant fraction, although it dena-
tures within a relatively narrow temperature range,
also appears to be nonhomogeneous. As judged
from the presence of separate melting bands, it
consists of at least three subfractions of different
heat sensitivity.

Divalent cations and alcohols influence these
two main DNA fractions in different ways.
Namely, in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+}
at concentrations over 5 \times 10^{-5} M, denaturation of the
thermosensitive fraction (at least of its major part)
is impeded while the denaturation of the
thermosensitive one is facilitated. Alcohols, on the
other hand, decrease the melting point of the
ermosensitive fraction and appear to further
stabilize the thermoresistant one.

Mg^{2+}, Ca^{2+}, and Mn^{2+}, as counterions for
DNA phosphates, stabilize free DNA in solutions
against thermal denaturation (16). The effect is
seen at a cation concentration of 10^{-6} to 10^{-9} M,
in solutions of low ionic strength, i.e. under
conditions similar to the present ones. MgCl_2 at
10^{-4} M concentration increases the T_m of calf
thymus DNA by 17°C (16). In the in situ situation,
we observed that the subfraction of the thermo-
sensitive DNA portion which denatures at 50–60°C
(corresponding to a rise of α_t to 0.28, see Fig. 3) is
delayed in denaturation by about 18°C in the
presence of 10^{-4} M MgCl_2. This subfraction,
therefore, exhibits properties similar to those of
free DNA in solution.

Alcohols lower the melting temperature of free
DNA, ethanol being more potent than methanol
(18, 28). We have observed that the thermosensi-
tive fraction was destabilized and that two subfrac-
tions now become apparent, with their melting
points separated by as much as 20°C (see Fig. 2,
the first and second melting bands) in the presence
of alcohols. Thus, both alcohols and divalent
cations influence the thermosensitive fraction of
DNA in situ (and particularly its first melting
subfraction) in the same way that they affect free
DNA in solution.

Despite these similarities, it is unlikely that a
thermosensitive fraction represents "free" DNA in situ,
because this fraction denatures over a very
wide range of temperature and is not homogene-
ous. Moreover, all attempts to denature any
significant portion of DNA in situ at temperatures
below 40°C in solutions of very low ionic strength
containing alcohols (5 \times 10^{-5} M EDTA with 50% ethanol), i.e. under conditions at which free DNA
denatures (18, 28), failed. Presumably, the ther-
osensitive DNA fraction represents DNA which
is only slightly stabilized, due to interactions either
with nonhistone proteins or with the nonbasic
histone regions. Regarding the lack of detectable
"free" DNA in situ, our results conform with
observations of Li et al. (31) who also failed to
detect any significant quantities of free DNA in
calf thymus nucleohistone.

The thermoresistant fraction most likely is the
fraction that remains in complexes with histones in situ.
The pattern of its denaturation is very similar
to denaturation of DNA bound to histones or to
histone basic-halves in solutions of native or
reconstituted nucleohistone (3, 30, 31, 42, 43, 45).
We have previously shown that histone extraction
from cells at low pH destabilizes DNA in situ;
while DNA denaturation after partial removal of
histones (at pH 1.8) remains biphasic, extraction of
all histones results in monophasic transition (12,
13).

Divalent cations decrease the melting point of the
thermosensitive DNA fraction. The effect is
seen at 0.5–1.0 \times 10^{-4} M cation concentrations
and appears to be cation specific (Fig. 4). Several
possible explanations might be advanced to explain
this phenomenon.
The cation effect may be linked to the unspecific action of the salts in raising ionic strength of the solution, which would facilitate dissociation of histones from DNA. This explanation is the least probable because concentrations of MgCl₂ 200-1,000 times higher (0.1-0.5 M) would be required to remove histones from chromatin (45). Furthermore, by increasing the ionic strength with NaCl (up to $5 \times 10^{-3}$ M) we observed only minimal effects. Also, histone dissociation from DNA results in a two- to threefold increase of AO binding to nuclear DNA (12-14), whereas addition of up to $10^{-3}$ M MgCl₂ produced a decrease in AO binding in these experiments and in prior studies (13).

A second possibility is that the destabilization of DNA by divalent cations might be a consequence of endogenous nuclease (or chromatin protease) action, activated by the cations and hastened by elevated temperature. We feel that this possibility may be ruled out because all cells were preincubated in the presence of 5 mM MgCl₂ (and RNase) at 37°C for 30 min, and then denatured in either the presence or absence of divalent cations. Thus, the conditions for activation of endogenous enzymes were equally optimal for all cells before they were divided into separate samples to be washed and heated with EDTA or with the cations. Yet, only the cells heated later for 5 min in the presence of the cations showed decreased DNA stability. In addition, since the effect is most evident at temperatures above 70°C, one would have to assume unusual heat stability of the enzymes in question.

At high concentration ($10^{-4}$ M), Mg²⁺ destabilizes free DNA (33, 36), perhaps by "charge reversal", i.e. by extensive binding to all phosphates. Such a Mg-DNA salt is expected to be a polycation having one positive charge per nucleotide residue (33). It seems unlikely that this phenomenon is responsible for the present effect of Mg²⁺ in lowering the stability of the thermoresistant DNA fraction because the effect we describe is seen at lower Mg²⁺ concentration. In addition, it is the thermosensitive DNA fraction that is expected to be destabilized first after the addition of the excess of Mg²⁺; the thermoresistant fraction, being involved in strong electrostatic interactions with histones, has DNA phosphates masked and unavailable for cations (9, 21).

The effect of divalent cations might be specifically related to their role in chromatin structure through the modulation of DNA-protein interactions. The following evidence may be advanced in favor of this notion.

The effect we observed occurs at cation concentrations comparable to those required to maintain gross chromatin structure in cell nuclei (29, 35) and to ensure "native" or superhelical DNA conformation in isolated chromatin (17, 46, 47). Furthermore, any direct effect of divalent cations on DNA, not involving rearrangement of DNA-protein interactions, is expected to stabilize rather than destabilize all DNA and to produce a parallel shift of the melting profile towards higher temperature. Yet, we observed a dramatic change in the shape of the profile; the total width of the transition was decreased and most DNA denatured at lower temperature. Only at $10^{-4}$ M MgCl₂ was there a change that could be explained by a direct effect on DNA since all DNA was stabilized, although not equally. The monophasic transition and decreased stability of most of the nuclear DNA seen in the presence of divalent cations suggest that under these conditions (as opposed to the presence of EDTA) the histone basic charges are more uniformly redistributed along the entire length of nuclear DNA and the strength of histone-DNA ionic interactions is lowered when compared with the fraction of DNA which is stabilized in the absence of divalent cations (the thermoresistant DNA fraction).

Is it possible that the profiles of DNA denaturation as observed here in the presence of divalent cations reflect melting of superhelical DNA, while the profiles seen after chelation with EDTA characterize DNA denaturation when superhelicity is already destroyed before heating? At present, direct evidence to support this notion is lacking. However, the data of Wagner and Vandergrift (47) and of Garrett (17) indicating that divalent cations are essential in maintaining DNA superhelicity in chromatin clearly suggest this possibility. The results of Barclay and Eason (4) who observed that superhelical SV40 DNA binds twice as few histones as the circular form also favor this possibility. On the other hand, it is possible that factors which play a role in the organization of gross chromatin structure (chromatin packing into the nucleus, DNA-nuclear envelope interactions, chromatin condensation, DNA-structural matrix interactions, etc.) and which also require divalent cations may be responsible for this effect of divalent cations on DNA denaturation in situ.

Irrespective of the mechanism by which divalent cations influence DNA stability in situ, our results
indicate that the cations markedly change molecular structure of nuclear chromatin. This implies that DNA-protein interactions as revealed either in conventional studies of DNA denaturation in nucleohistone, or by other methods using isolated chromatin in the absence of divalent cations, might be quite different from the situation in situ when gross chromatin morphology remains intact.

The question may be asked how relevant to the in vivo state are our results obtained on fixed cells. Unfortunately, cell fixation is a necessary prerequisite for this kind of study. Fixation stabilizes macromolecular cell constituents in situ, allows controlled equilibration of the cell interior with solvents and dye, and prevents cell destruction by media of low ionic strength and/or heat. The cold alcohol-acetone fixation was chosen since it offers a fast and partially reversible means of precipitating various macromolecules in situ. The major advantage of alcohol-acetone fixation is that it leaves the reactive groups of many macromolecules, including enzymes, in their original state. Thus, DNA polymerase remains active (10), as does RNA polymerase, which also retains the restrictions in activity related to the extent of genome activation (8). This indicates that the DNA template and the proteins which restrict its transcription or replication remain in their native state. The restrictions in the availability of DNA to various intercalating probes, such as AO or actinomycin D which correlated with the genome transcriptional activity, also remain preserved after fixation (11, 19, 37). Furthermore, our results on EDTA-treated cells, indicating biphasic DNA denaturation, are similar to that data on DNA denaturation in isolated nucleohistone (3, 30, 31, 43-45). This suggests that the fixation step does not induce large changes in the interactions between DNA and proteins and confirms recent observations (22, 23) that a number of organic solvents, including alcohols, which previously had been thought to denature DNA, are in fact supporting the double-stranded and helical secondary DNA structures. By contrast, aldehyde fixatives such as formaldehyde chemically modify the reactive groups of various macromolecules and markedly change the stability of DNA in situ (44).

The technique used by us to study DNA denaturation in situ differs in many respects from the methods based on measurements of UV-absorption. The specifics of this technique are discussed in detail elsewhere (13, 14). One point should be stressed here, however, namely that the $\alpha_i$ index cannot be regarded as an absolute measure of the extent of denaturation of the total nuclear DNA; $\alpha_i$ represents rather the relative proportion of the AO-stainable, denatured DNA to the total DNA (denatured plus native) that is stainable with AO at a given temperature. The portion of stainable DNA depends on the extent of unmasked DNA; the latter presumably varies with temperature since DNA denaturation might coincide or be preceded by the dissociation of histone. The concentrations of AO and Mg$^{2+}$ during cell staining were chosen to provide optimal differential stainability of denatured vs. native DNA (13). At an excess of AO per DNA phosphate, the method is not sensitive to uncontrolled variations in cell number per sample or to small changes in AO concentration, i.e. due to the dye binding to glassware, tubing, etc. MgCl$_2$ at a concentration of 5 mM was included in order to suppress ionic binding of AO (nonintercalation) to double helical DNA; in the presence of counterions the electrostatic interactions between charged dye molecules and DNA phosphates are hindered while the intercalation is affected to a lesser degree (27). These conditions are similar in principle to those described by Ichimura et al. (20) for quantitative measurements of single-vs. doubled-stranded regions of DNA.

Observations of heated and AO-stained cells by UV-microscopy confirm the fact that DNA denaturation in situ does not progress evenly in all chromatin. At any given temperature, with partial DNA denaturation, some areas of nuclear chromatin fluoresce red, while other areas remain green. Mapping of the interphase nucleus with respect to the distribution of heat-sensitive and heat-resistant DNA fractions, and correlation of that distribution with chromatin morphology (condensed vs. dispersed chromatin in various cell types), will be the subject of another report. It should be stressed here that there appears to be a high degree of tissue-specificity in respect to the pattern of DNA denaturation in situ, in both morphological (specific pattern of red-green stainability of interphase nucleus under conditions of partial DNA denaturation) and quantitative terms (melting profiles). In our observations to date on various cell types, including normal vs. 

\[^4\text{Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M. R. Melamed. Manuscript in preparation.}\]
malignant cells, we have observed that DNA denaturation profiles differ so markedly that the extent of DNA denaturation (α, value) at certain temperatures and divalent cation concentrations may provide a useful parameter in diagnostic cytology (see footnote one).

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