THERMALLY-INDUCED CHANGES IN CHROMATIN OF ISOLATED NUCLEI AND OF INTACT CELLS AS REVEALED BY ACRIDINE ORANGE STAINING

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SUMMARY: Thermal denaturation of DNA in situ is studied in individual cells (or isolated nuclei) by staining with acridine orange and measuring fluorescence in a rapid, automated flow-through cytofluorimeter. Acridine orange differentially stains native and denatured DNA and prevents DNA renaturation. Cells are studied in suspensions, in equilibrium with the dye. Denaturation of DNA in situ is sensitive to ionic strength and occurs at temperatures similar to those reported for preparations of soluble nucleo-histone. Histones appear to stabilize DNA in situ against denaturation. There are changes in chromatin at submelting temperatures as evidenced by increased binding of acridine orange to DNA and decreased light scattering. Present results differ from what was reported when formaldehyde is used to prevent DNA renaturation. It is suspected that formaldehyde produces severe artifacts when used in studies of DNA melting in situ.

Current methods of studying heat-induced changes in nuclear chromatin in situ are based on heating of pre-fixed, glass attached cells followed either by U.V. microspectrophotometry (1,2) or by cell staining with acridine orange (AO) and microfluorimetry (3-8). In the first case, hyperchromicity of cell nuclei at 260 nm is interpreted as a sign of thermal denaturation of DNA (1,2). In the case of AO staining, the strategy is based upon a specific reaction of AO with DNA; the dye interacts with double stranded DNA to fluoresce with maximum emission at 530 nm, while interaction with denatured DNA results in fluorescence with maximal emission at 640 nm. The ratio of fluorescence intensities of AO-DNA complexes at >600 nm to 530 nm (α) was proposed as a measure of the degree of DNA single-strandedness in DNA solutions (9) as well as in cell nuclei (3) and its increase during cell heating was accepted as evidence of thermal denaturation of DNA in nuclear chromatin (3-8). In all studies (2-8) but one (1)

Abbreviations: Acridine orange, AO; Fluorescence intensity within 600-650 nm band, $F_{>600}$; Fluorescence intensity within 515-575 nm band, F_{530}; Ratio of $F_{>600}$ to F_{530}, α.

formaldehyde was used during heating to prevent DNA renaturation.

We propose an automated method for studying chromatin of heated cells or isolated cell nuclei offering the following advantages: 1) Cells are heated, stained and measured in suspension. Thus, attachment of cells to glass, air drying and the phenomena related to variation in cell crowding, which affect staining with AO (10) are all eliminated; 2) The concentration of AO is constant during staining and measuring so that dye-DNA interaction is studied under equilibrium conditions; 3) Isolated, nonfixed cell nuclei can be studied; 4) Measurements are done rapidly (200 cells/sec) and automatically; 5) Subpopulations of cells from the sample can be chosen based on differences in staining or light scatter (11) and analyzed separately; 6) Formaldehyde is excluded from the procedure.

MATERIAL AND METHODS: Cells; cell nuclei: Thymic lymphocytes were obtained from 4 to 8 weeks old Sprague-Dawley rats, as described (12). Chick erythrocytes were collected from blood of 16-day old chick embryos. The cells were rinsed in a solution of 0.25M sucrose and 5mM $MgCl_2$ buffered with 20mM tris-HCl to pH 7.4 (SMT) and then fixed in ethanol acetone (1:1) for at least 16h. After fixation cells were suspended in SMT (pH 6.5) treated with 5000 units/ml of RNAse A (Worthington Biochemical Corp.) at 37°C for 30 minutes, rinsed twice in SMT (pH 7.4) and resuspended in media of various composition (see legends). In some cases to remove acid-soluble proteins, the cells after RNAse treatment were washed twice in media containing 0.25M sucrose and 5mM $MgCl_2$ at pH 1.8 or 1.0 (adjusted with HCl). Some cell preparations were treated with DNAse (RNAse-free DNAse, Worthington) as described (13).

Thymic cell nuclei were isolated as described before (12). Liver cell nuclei were prepared by a procedure described (12) from livers of 4 to 8 week old Sprague-Dawley rats. Chick erythrocyte nuclei were obtained by lysis of chick erythrocytes.

Cell Heating, AO Staining: Tubes with 1 ml aliquots of cell (nuclei) suspension (5×10^5 per ml) were heated in a water bath at appropriate temperatures. After 5 minutes the tubes were transferred to an ice-cold bath and 4 ml of ice-cold solution of AO (National Aniline Chem. Co.) in SMT medium was added to each tube to give a final AO concentration of 6.4×10^{-6}g/ml. The tubes were then equilibrated to room temperature for measurements.

Measurements: A Cytofluorograf 4801 (Bio/Physics Systems, Inc., Mahopac, N.Y.) interfaced to a Nova 1220 minicomputer (Data General) was used to obtain simultaneous two-color measurements of cellular fluorescence and light scatter. The Cytofluorograf system has been described elsewhere (11). Briefly, stained cells suspended in AO solution are rapidly transported through the instrument and fluorescence and light scatter are generated as the cells pass in single file fashion through an elliptically-focused 488 nm wavelength laser beam. The red fluorescence intensity (at >600 nm, $F_{>600}$) and green fluorescence intensity (at 515-575 nm, F_{530}) are separated optically and recorded by two photomultipliers. Forward light scatter at 1 to 19 degrees is recorded by the third sensor. The three measurements obtained on each cell are filed by the minicomputer for further analysis. A statistical program is used in which

the data, presented as scattergrams, serve to select the cell populations of interest and to obtain mean fluorescence and scatter values as well as the standard deviation, variance and skew. The data presented here are mean values or ratios of means for populations of diploid cells; 5-10 x 10^3 cells from each sample were measured.

Control experiments have shown that: 1) Heating for 5 minutes is adequate to induce near maximal changes in nuclear chromatin and cell cooling in the presence of AO and $MgCl_2$ stabilizes these changes. 2) RNAse treatment decreases $F_{>600}$ of thymus cells (nonheated) several-fold and has no effect on F_{530}. 3) DNAse treatment decreases F_{530} by over 80% with a negligible effect on $F_{>600}$. 4) Isolated nuclei have similar F_{530} and lower $F_{>600}$ in comparison with intact cells.

RESULTS: Fig. 1 shows the changes that occur when cells or cell nuclei are

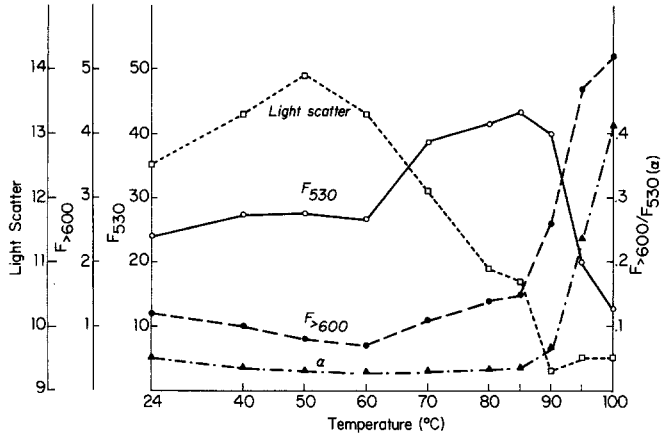

Fig. 1: Heat-induced changes in fluorescence and in light scatter of thymic cells stained with acridine orange following heating. Cells were heated while suspended in solution containing 0.15N NaCl, 0.01M phosphate buffer and 0.1mM EDTA, at pH 6.8.

heated in solutions at ionic strengths close to physiological saline. F_{530} remains nearly constant when cells are heated up to 60°C. Between 65-90°C, F_{530} increases nearly twofold, with a peak at 80-85°C. A rapid drop of F_{530} occurs at 95-100°C. $F_{>600}$ decreases slightly between 24-60°C then increases parallel to the rise of F_{530} between 65-90°C, and over 95°C increases rapidly. Light scattering properties of the cells increase up to 40°C then continuously decrease. The ratio of $F_{>600}/F_{530}$ (α) starts to increase at 85°C and at 90 or 100°C is several times the value at 24°C.

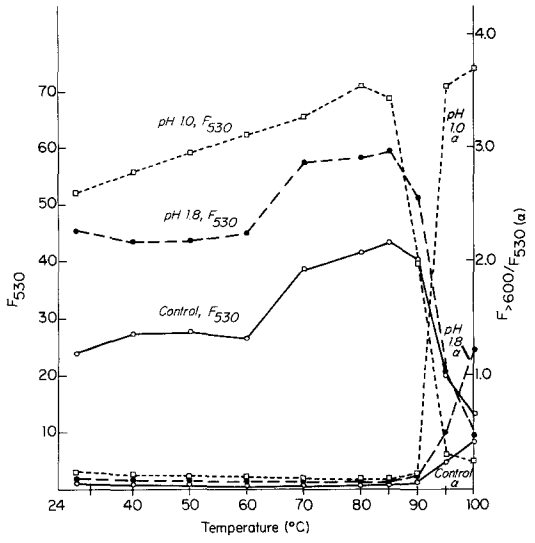

Fig. 2: Heat-induced changes in F_{530} and in $F_{>600}/F_{530}$ ratio (α) of normal thymic cells and cells treated with acids. Cells were heated in a solution containing 0.15N NaCl, 0.01M phosphate buffer and 0.1mM EDTA, at pH 6.8.

Thymus cells treated at pH 1.8 or pH 1.0 have increased F_{530} at 24°C (90% or 120% respectively) (Fig. 2). The heat-induced rise in F_{530} is comparable in absolute value to the rise seen in nontreated cells. However, this rise starts at lower temperatures in cells extracted at pH 1.0. The α values of both nontreated and acid-treated cells remain essentially the same up to 90°C. At 95°C and 100°C acid-treated cells have an α value several times that of nontreated cells. Heating of cells or cell nuclei in media of low ionic strength (<0.01N NaCl) gives different results. Namely, the rise of F_{530} seen at higher ionic strength between 65-90°C is not apparent and a drop of F_{530} correlated with a simultaneous rise of $F_{>600}$ (increase in α) occurs at lower temperatures. (Fig. 3). The cells extracted at pH 1.0 show maximal changes of F_{530} and $F_{>600}$ at 30-50°C; those extracted at pH 1.8 at 40-75°C; nontreated at 85-100°C. In the case of nontreated and pH 1.8 treated cells, the curve of α values indicate a two-step transition.

The results presented (Figs. 1-3) have been obtained from whole cells, prefixed in ethanol:acetone. Unfixed nuclei, isolated from thymus, liver or

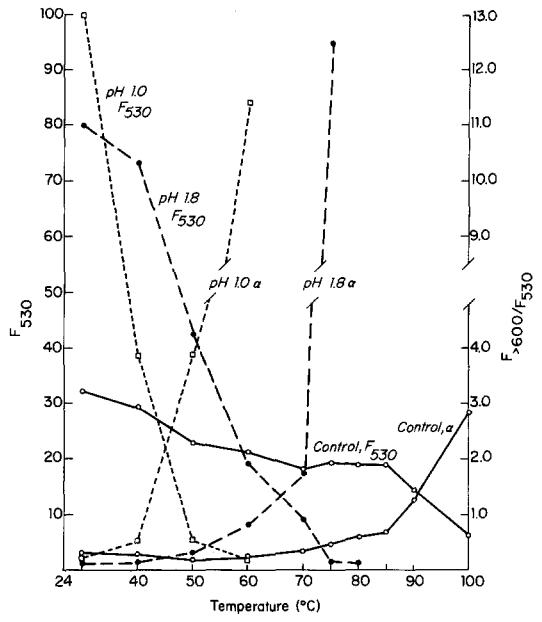

Fig. 3: Heat-induced changes in F_{530} and in $F_{>600}/F_{530}$ ratio (α) of normal chick erythrocytes and erythrocytes treated at pH 1.8 and 1.0. Cells were heated in a solution containing 0.1mM phosphate buffer and 0.1mM EDTA, at pH 6.8.

erythrocytes, heated under the same conditions exhibit similar changes in F_{530}, $F_{>600}$ and in light scatter.

DISCUSSION: We assume that under the conditions described we are measuring the interaction of AO with nuclear DNA and not with other cell components because: 1) RNAse treated cells were studied in which $F_{>600}$ at 24°C was minimal. Thus, the contribution of RNA to cell staining is negligible. 2) DNAse treatment decreased F_{530} by over 80%. 3) Isolated nuclei and intact cells have similar F_{530}. Since AO stains native and denatured DNA differentially, (3-9), it seems reasonable to assume the changes we observe reflect heat-induced alterations in secondary structure of DNA in nuclear chromatin. The drop in F_{530} coupled with a simultaneous rise in $F_{>600}$ (represented by a rise in α) indicates thermal denaturation of DNA ("DNA melting") in situ. A change in nuclear structure as reflected by decreased light scatter, accompanies this transition. At ionic

strengths close to physiological saline, DNA melting starts at 90°C for all cell types studied, including acid-treated cells. However, the degree of denaturation (slope of α value) at 95 and 100°C is higher for cells treated at low pH.

At low salt concentration, denaturation occurs at lower temperatures. In addition, the width and the biphasic nature of curves representing α values suggest that various portions of nuclear DNA melt at different temperatures.

Cell treatment with acid at pH 1.8 has been shown to remove all histone I and also histone V specific for avian erythrocytes (14-16). Thermal denaturation of DNA in such cells seems to occur in at least two phases and at temperatures approximately 20°C lower than in nontreated cells. Treatment of cells at pH 1.0 removes all tightly bound histones IIb1, IIb2, III, and IV (15-17). In cells so treated, DNA melting appears to be monophasic and is seen at temperatures 50°C lower. These results conform with biochemical observations on DNA melting in nucleohistone which show that histones stabilize DNA against denaturation (18-21) and explain multiphasic, wide DNA melting in nucleohistone as representing different melting points of "naked" DNA and DNA in complexes with histone (20,21). Since the melting we observed occurs at similar temperatures as in the case of soluble nucleohistone (studied at comparable ionic strength) (18-21) the factors affecting thermal stability of DNA in intact cells seems to be no different than those present in nucleohistone preparations.

An increase of F_{530} at submelting temperatures, correlated with a decrease of light scatter by cells or cell nuclei, reflects alterations of chromatin structure that result in increased AO binding to DNA. Increased AO binding to nuclear DNA in a variety of systems associated with "genome activation" has been explained as an unscreening of AO reactive DNA sites due to dissociation of DNA-histone complexes (22,23). Indeed, in the present studies, we observed that removal of histones markedly increases AO binding at 24°C both in thymus cells and in chick erythrocytes. However, since the F_{530} peak seen at submelting temperatures is of the same magnitude in cells extracted at pH 1.0 as in nontreated cells, some other mechanism must be considered.

"Melting" of the DNA superstructure maintained by DNA association with the nuclear membrane (16) loosening of the double helix, or other conformational changes of DNA (24) might be responsible for this phenomenon.

Our results are in contrast with most of the data on DNA melting in situ (2-8). Namely, other studies indicate that DNA in situ is more sensitive to thermal denaturation than pure DNA in solution. In cells heated in 0.15N NaCl, melting was seen in the 30-80°C range (2-8). At that ionic strength, isolated DNA of the same species melts above 85°C (T_m=90°C) (25). Moreover, chromatin changes at submelting temperatures shown here were not evident in studies by conventional methods (3-8). These discrepancies could be due to the use of formaldehyde. We have studied the effect of formaldehyde and we provide evidence (manuscript in preparation) that this agent produces artifacts when used to study DNA denaturation. Its reactivity with amino groups of DNA bases and crosslinking of chromatin components affect DNA stability in situ. AO prevents DNA renaturation in solutions (26) and we have found that it also stabilizes heat-induced changes of DNA in situ. This enabled us to avoid the use of formaldehyde.

We believe that the method described in this report, yielding results comparable with biochemical data on nucleohistone, provides a better insight into DNA stability in situ than has been heretofore available.

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