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Differential scanning calorimetry of nuclei reveals the loss of major structural features in chromatin by brief nuclease treatment

(nucleosome/chromatin melting)

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ABSTRACT **Differential scanning calorimetry revealed that chromatin melts in four distinct transitions in intact HeLa nuclei at 60°C, 76°C, 88°C, and 105°C. Calorimetry of whole cells was characterized by the same four transitions along with another at 65°C, which was probably RNA. Isolated chromatin, however, melted in only two transitions at 72°C and 85°C. Very brief digestion of HeLa nuclei with either micrococcal nuclease or DNase I resulted in the conversion of a structure that melted at 105°C to one that melted at 88°C. Further digestion with micrococcal nuclease to the level of the mononucleosome did not result in any further substantial changes in either enthalpy or melting temperatures. In contrast, further DNase I digestion that resulted in cleavage within the nucleosome produced a pronounced shift in melting temperatures to two broad transitions at 62°C and 78°C.**

Eukaryotic DNA exists as long continuous molecules but is condensed on the order of 1000-fold to fit within interphase nuclei (1). The lowest level of folding is that of the nucleosome, a repeating subunit consisting of two each of the core histones H2A, H2B, H3, and H4, along with about 200 base pairs of DNA and one molecule of histone H1. Histone H1 is believed to effect further condensation to a 300-Å fiber that has been postulated to be a solenoid (2).

Studies of chromatin structure above the level of the solenoid have been hampered due to the tendency of chromatin fibers to form aggregates and turbid suspensions at physiological salt conditions. The use of differential scanning microcalorimetry in studies of chromatin structure has the advantage that samples need not be optically clear as is required for spectral studies (3, 4). Moreover, aggregates that are too polydisperse for study by sedimentation can be studied calorimetrically. These advantages are significant, since most chromatin is aggregated in its natural state.

Despite its inherent advantages, only limited applications of differential scanning calorimetry have been made to the study of chromatin-related structures. Bina *et al.* examined the thermal denaturation of chromatin core particles (5). Nicolini *et al.* observed the effects of salt on calorimetric scans of chromatin and interpreted their results in terms of tertiary, quaternary, and quinternary structures of DNA in chromatin (6). Monaselidze *et al.* applied scanning calorimetry to cell suspensions, nuclei, and chromatin to conclude that secondary and tertiary structures are destroyed during the isolation of chromatin (7). These workers correlated the intensities of the endotherms observed with the relative amounts of DNA and RNA present in several samples.

The results of our study suggest that differential scanning calorimetry can be used to characterize the native chromatin structures in nuclei and that nuclei are to be preferred over isolated chromatin and whole cells. The thermodynamically significant chromatin structures of whole cells were shown to be retained in the nucleus, but a major structure was destroyed by brief micrococcal nuclease treatment.

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MATERIALS AND METHODS

Cell Culture. HeLa cells, strain S3, were maintained in suspension culture at cell densities of 2–8 × 10^5 per ml in Joklik's modified spinner medium supplemented with 5% calf serum. Cells were harvested at cell densities of 5–6 × 10^5 per ml.

Isolation of Nuclei. All steps were carried out at 0–4°C. HeLa cells were pelleted and washed in swelling buffer (0.1 M hexylene glycol/1 mM $CaCl_2$/0.06 mM Pipes, pH 6.8) and then resuspended in swelling buffer for 10 min. Cells were disrupted with 10 strokes in a loose-fitting Dounce homogenizer. For nuclease digestion experiments, nuclei were washed three times in buffer A (50 mM Tris/25 mM KCl/0.25 M sucrose/1 mM $MgCl_2$/1 mM $CaCl_2$, pH 7.5). For calorimetric scans of intact nuclei, nuclei were washed three times in buffer B (5 mM Tris/0.2 mM EDTA/150 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$, pH 7.5) or buffer C (5 mM Tris/0.2 mM EDTA/0.25 M sucrose/150 mM NaCl/1 mM MgCl/1 mM $CaCl_2$, pH 7.5). For calorimetric scans of whole cells, HeLa cells were harvested and washed three times in buffer C.

Nuclease Digestion. Nuclei were resuspended in buffer A at an A_{260} of 30.0. Both micrococcal nuclease (Worthington) and DNase I (Worthington) digestions were carried out at an enzyme concentration of 25 units/ml at 37°C. Aliquots were removed at various times and quenched by the addition of 0.1 M Na_2EDTA to a final EDTA concentration of 5 mM. Nuclei were also digested with ribonuclease A (Worthington) for 30 min at 37°C at an enzyme concentration of 100 μg/ml. (RNase A was preincubated at 90°C for 15 min prior to use to destroy DNase activity.)

Preparation of Chromatin. HeLa nuclei were digested with micrococcal nuclease at 25 units/ml for 5 min at 37°C. The reaction was quenched with 5 mM EDTA. Nuclei were pelleted and resuspended in 0.2 mM EDTA and then lysed by intermittent shaking for 2 hr at 0–4°C. The suspension was centrifuged and the supernatant, which contained soluble chromatin, was dialyzed against buffer containing 5 mM Tris, 0.2 mM EDTA (pH 7.5). NaCl, $MgCl_2$, and $CaCl_2$ were added to final concentrations of 150 mM, 1 mM, and 1 mM, respectively, and the chromatin was incubated for 1 hr at room temperature. Insoluble chromatin was centrifuged for 10 min at 8000 × *g* and the pellet was used for calorimetric scanning.

Gel Electrophoresis of DNA Digestion Fragments. Following digestion by micrococcal nuclease or DNase I, nuclei were pelleted and dissolved in 5 ml of 1 M NaCl/0.6% $NaDodSO_4$ and then extracted two times with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase for 24 hr at −20°C by the addition of 2.5 vol of ethanol. Pellets were washed once in absolute

ethanol, dried under vacuum, and dissolved in gel electrophoresis buffer (0.25% bromophenol blue/40% sucrose) for agarose gels and 0.09 M Tris borate/2.5 mM EDTA, pH 8.3/90% formamide/0.2% bromophenol blue/0.02% xylene cyanol for DNA denaturing gels. DNA fragments were electrophoresed with either 1.6% agarose in Tris/phosphate buffer (0.036 M Tris/0.030 M NaH_2PO_4/1 mM EDTA) for 20 min at 100 V for DNA minigels (5 × 7 cm) or with 12% polyacrylamide/7 M urea in 0.09 M Tris borate/2.5 mM EDTA, pH 8.3, for 2 hr at 240 V for DNA denaturing gels (14 × 16 cm). Gels were stained for 5 min in ethidium bromide (1 μg/ml) and photographed. A *Hin*cII digest of III/φX-174 was run as a marker.

Differential Scanning Calorimetry. All calorimetry experiments were run on a Dupont 99 thermal analyzer, from 25°C to 120°C at a scanning rate of 5°C/min. Output was plotted as a function of the heat absorbed in mcal/sec/2.54 cm (1 cal = 4.184 J) vs. temperature. Prior to scanning, samples were centrifuged for 3 min in an Eppendorf Microfuge and the supernatant was removed. Pellets were transferred to aluminum pans and sealed. The sample size ranged from about 10 mg to 20 mg of moist pellet. Total enthalpy was measured by cutting out peaks from tracings of the scans and weighing them. After scanning, sample pans were opened and the contents were dissolved in 2% $NaDodSO_4$. The amount of DNA in each sample was determined by the absorbance at 260 nm, assuming an extinction coefficient of 200.0 for a 1% solution.

RESULTS

HeLa nuclei were compared to whole cells by differential scanning calorimetry. Fig. 1*A* shows the heat absorbed as a function of temperature for both samples in buffer C. Intact nuclei melted in four major thermal transitions, I, II, III, and IV, at 55–60°C, 76°C, 88°C, and 105°C, respectively. The melting profile was dominated by transition IV, with an enthalpy of 6.7 cal/g of DNA. Transitions II and III contribute 1.5 and 1.4 cal/g of DNA, respectively, whereas transition I showed a broad maximum (55–60°C) with an enthalpy of 1.1 cal/g. Whole cells gave a similar melting profile, but here an additional peak, at 65°C, was prominant. There was also a large, broad background enthalpy, which is presumably due to the melting of numerous cytoplasmic components.

The melting profiles of intact HeLa nuclei and isolated chromatin in buffer B were compared (Fig. 1*B*). The melting profile of the chromatin was quite different from that of the nuclei. The melting profile of the chromatin was characterized by two major endotherms at 72°C and 85°C. There was no thermal transition at either 60°C or 105°C, although a shoulder at ≈93°C was evident.

The absence of transitions I and IV in isolated chromatin might suggest that these endotherms are due to the melting of nonchromatin components. Alternatively, these transitions may represent the melting of higher order chromatin structures destroyed during micrococcal nuclease digestion and chromatin isolation. To test the last possibility, we performed a series of nuclease digestion experiments on nuclei.

Intact HeLa nuclei were digested for varying amounts of time with micrococcal nuclease, which cleaves chromatin between nucleosomes (8). Melting profiles (Fig. 2) of undigested nuclei are dominated by transition IV (105°C). After digestion for only 1 min, however, there was a marked change in the melting pattern and the predominant endotherm shifted to 88°C. Quantitative comparisons of these changes are represented in Fig. 3. There was a large change in the melting enthalpies of transitions III and IV after digestion with micrococcal nuclease for 1 min but no further changes

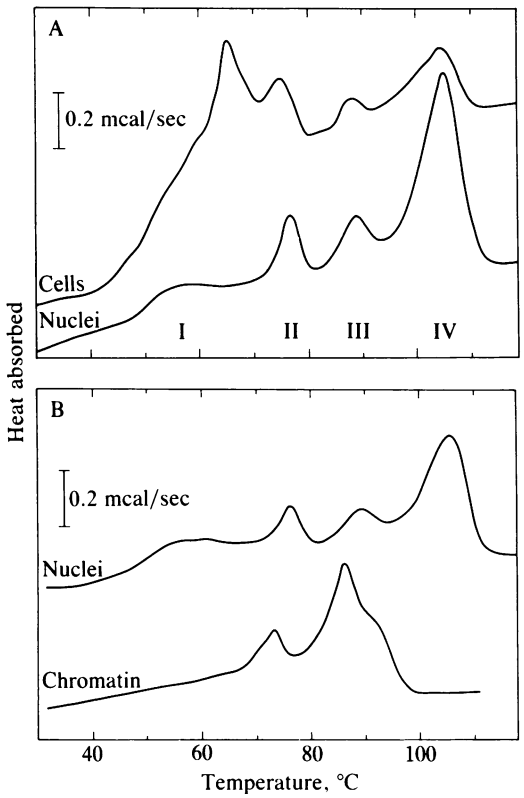

FIG. 1. Differential scanning calorimetry of HeLa cells, nuclei, and chromatin. Heat absorption curves as a function of temperature for whole cells and intact nuclei from HeLa in buffer C (*A*) and nuclei and chromatin from HeLa in buffer B (*B*). Bar indicates 0.2 mcal/sec absorbed at a scanning rate of 5°C/min. The scans are translated arbitrarily along the ordinate to simplify comparisons. The amount of DNA in each sample was 1.1 mg (cells) and 1.4 mg (nuclei) in *A* and 0.9 mg (nuclei) and 0.4 mg (chromatin) in *B*.

as digestion proceeded. After 1 min, transition IV, with an enthalpy of 6.8 cal/g of DNA, completely disappeared while the enthalpy associated with III increased from 1.0 to 6.2 cal/g of DNA. The enthalpies of transitions I and II appeared to be relatively insensitive to increasing digestion with micrococcal nuclease. The dramatic change from a dominance of transition IV to a dominance of transition III was effected with very slight digestion. After 1 min of digestion, the size of the chromatin fragments ranged from 1 to 200 nucleosomes, with a majority of the material existing as large fragments (Fig. 2 *Inset*). Further digestion to a majority of monomers and core particles had only the modest effect of shifting transition temperatures; after 15 min, the digestion decreased transition III from 88°C to 86°C and transition II from 77°C to 74°C. The low melting endotherm I at 55–60°C appeared as a broad transition, probably a doublet at early digestion times, but as digestion progressed, the peak appeared to sharpen at ≈60°C.

To learn whether any of the transitions depended on the integrity of the nucleosome or core particle, HeLa nuclei were digested with DNase I, which cleaves DNA at 10-base-pair intervals within the nucleosome (9). DNA isolated from the nuclei was run on DNA denaturing gels. According to the DNA gel in Fig. 4*B*, digestion for 1 min generated a distribution of chromatin fragments in the high molecular weight range, which could not be resolved by gel electrophoresis. With increasing digestion, the DNA was cleaved into smaller fragments (10–100 bp), beyond the nucleosomal level. As shown in Fig. 4*A*, digestion for 1 min resulted in an increase in transition III (87°C), apparently at the expense of transition IV (105°C). The calorimetric scans show a striking contrast to

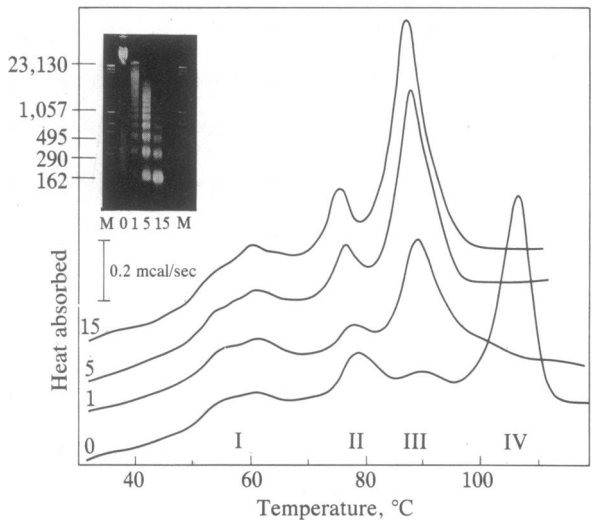

FIG. 2. Calorimetry of HeLa nuclei digested with micrococcal nuclease. HeLa nuclei were digested with micrococcal nuclease at 25 units/ml at 37°C in buffer A and quenched with 5 mM EDTA. Heat absorption curves as a function of temperature are shown for nuclei digested for 0, 1, 5, and 15 min. Bar indicates 0.2 mcal/sec absorbed at a scanning rate of 5°C/min. The amount of DNA in each sample was 0.98 mg (0 min), 0.80 mg (1 min), 0.93 mg (5 min), and 1.1 mg (15 min). (*Inset*) Agarose gel electrophoresis of DNA fragments isolated from nuclei digested for 0, 1, 5, and 15 min; M = molecular weight markers calibrated in base pairs.

those observed for micrococcal nuclease digestion in which the nucleosomal structure was preserved. Increasing digestion with DNase I resulted in a greater shift in melting temperatures from 87°C for transition III at 1 min to 78°C at 15 min. Transition II shifted from 76°C at 0 min to 62°C at 15 min, where it appeared to merge with transition I. It is difficult to precisely measure changes in enthalpy with DNase I digestion since all endotherms were destabilized and seemed to merge at lower melting temperatures as digestion progressed.

To determine if any of the thermal transitions observed in the melting of intact nuclei were due to the melting of RNA, intact nuclei were digested with ribonuclease A. No significant changes were observed after digestion for 30 min (data not shown).

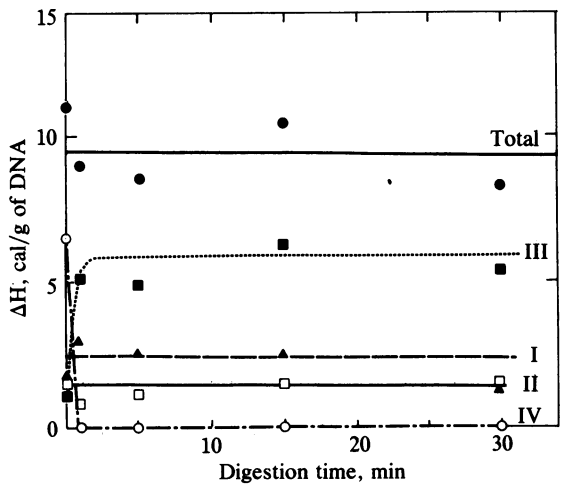

FIG. 3. Dependence of the melting enthalpy ΔH (cal/g of DNA), for the thermal transitions observed in the melting of HeLa nuclei, as a function of time of digestion by micrococcal nuclease (as shown in Fig. 2). ▲, Transition I (55–60°C); □, transition II (76°C); ■, transition III (89°C); ○, transition IV (105°C); and ●, total enthalpy. The peaks in Fig. 2 were integrated by dividing at minima, and the areas were corrected for the amount of chromatin used for analysis.

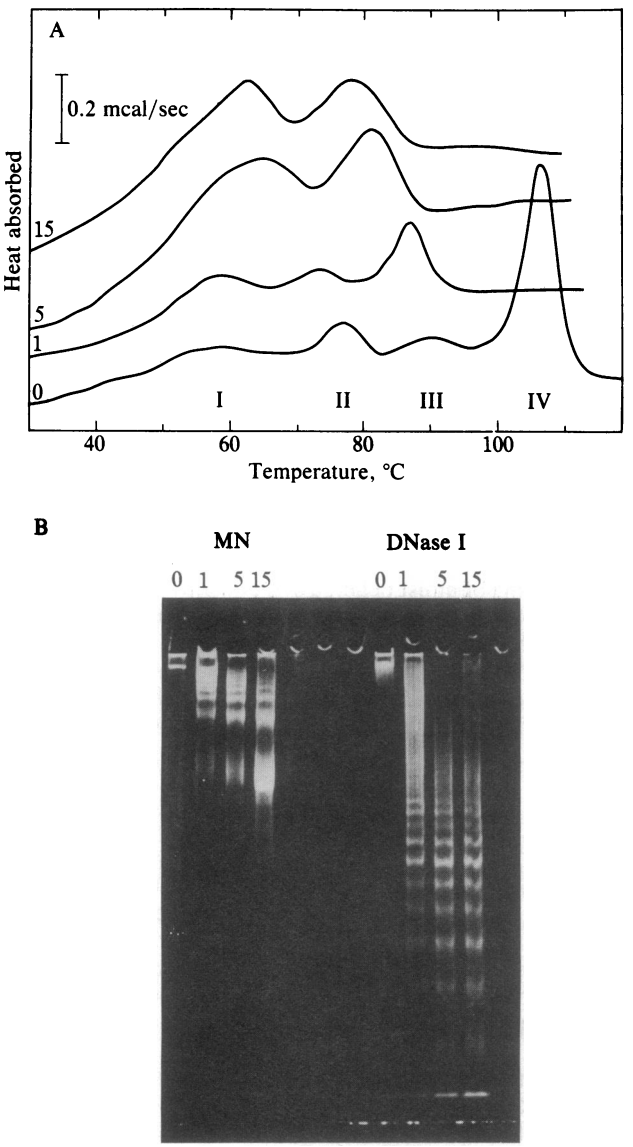

FIG. 4. Calorimetry of HeLa nuclei digested with DNase I. HeLa nuclei were digested with DNase I at 25 units/ml at 37°C in buffer A and quenched with 5 mM EDTA. (*A*) Heat absorption curves as a function of temperature are shown for nuclei digested for 0, 1, 5, and 15 min. Bar indicates 0.2 mcal/sec absorbed at a scanning rate of 5°C/min. The amount of DNA in each sample was 0.74 mg (0 min), 0.52 mg (1 min), 0.60 mg (5 min), and 0.97 mg (15 min). (*B*) DNA denaturing gels of DNA fragments isolated from nuclei digested for 0, 1, 5, and 15 min with either micrococcal nuclease (MN) or DNase I.

DISCUSSION

We used differential scanning calorimetry to probe the structure of chromatin. Most conventional methods of chromatin analysis depend upon the optical clarity of chromatin solutions, and so studies on chromatin structure have been carried out usually at low ionic strength on chromatin that has been rendered soluble by nuclease digestion (10). Such studies may not represent the true structure of native chromatin. Since calorimetry does not require prior solubilization of the chromatin its use allows us to test whole cells and nuclei in order to ask which form of chromatin best reveals the native structure. Whole cells would guarantee the structure as native, but calorimetric scans might be complicated

by the superposition of nonchromatin components. Isolated chromatin would be free of nonchromatin elements but would have the greatest risk of structural damage. Our results suggest that isolated nuclei have their native chromatin structures preserved and are also free of components whose transitions might interfere with those of chromatin.

The chromatin in intact nuclei seems to represent the *in situ* structure of chromatin. This was established when whole HeLa cells and intact nuclei were melted under similar conditions and found to exhibit similar melting profiles (Fig. 1*A*). Evidently, the structural integrity of chromatin was preserved in the isolated nuclei, at least in thermodynamic terms. There were two major differences between the scans of nuclei and whole cells—the absence of a thermal transition at 65°C in nuclei that was present in whole cells and the much higher background enthalpy in the low-melting region of whole cells. The latter was not surprising, since the whole cell contains an abundance of organelles, membranes, proteins, etc., which are not found in nuclei and which would be expected to melt as a mixture over a broad range of temperatures (3). We believe the transition at 65°C represents RNA. The melting of base-paired RNA would be expected to give a sharp transition, which would be prominent in whole cells and absent in nuclei. Indeed, Doty *et al.* observed ribosomal RNA to melt at 65°C under similar conditions (11). Moreover, in a study of cells of varying RNA/DNA ratios, the data of Monaselidze *et al.* (7) show a correlation between RNA content and the 65°C transition. [Monaselidze *et al.* chose to attribute a 75°C transition to the melting of RNA, but our data rule out this conclusion by showing that this transition had the same enthalpy in nuclei and whole cells (Fig. 1*A*) and that it was insensitive to digestion with RNase A and micrococcal nuclease.] With the exception of the high general background and the 65°C transition, all of the features observed in calorimetric scans of whole cells were found in isolated nuclei, and since all of the major transitions observed in nuclei proved sensitive to DNase I treatment, we conclude that all of the native forms of chromatin are retained in isolated nuclei and that the calorimetry of nuclei represents chromatin without substantial interference by extraneous components.

While native chromatin structure was retained in nuclei, the isolation of chromatin resulted in a loss of structure. In intact nuclei, the major thermal transition (IV) melted at 105°C, but in chromatin there was no endotherm at this temperature. Also, the broad thermal transition (I) observed at low temperature in nuclei (55–60°C) was not present in isolated chromatin. Instead, transition III was the predominant peak in chromatin. The damage was done by micrococcal nuclease digestion before the chromatin was isolated from the nuclei. Even the mildest digestion brought about the complete conversion of transition IV to III. It was also observed that as digestion with micrococcal nuclease proceeded to the level of mononucleosomes and core particles, transition III persisted as the major thermal transition. This suggests that transition III corresponds to the melting of chromatin at the level of the nucleosome and not at some higher level. Transition III and transition II as well are clearly dependent on the integrity of the DNA within the nucleosome or core particle because DNase I, which cleaves only DNA, affected the melting temperatures of those transitions along with transition IV and possibly transition I. This shows that all of these transitions are due to the melting of duplex DNA as it exists in discrete conformational states in chromatin. It may be presumed that proteins also play significant roles in the determination of the conformations represented by the transitions. Weischet *et al.* (12) demonstrated clearly that in core particles, the majority of the DNA melted concurrently with the collapse of the core histone complex. This analysis used a combination of circular dichroism and calorimetry, and the samples were analyzed at low salt concentrations to maintain optical clarity. It is unfortunate that these and many other studies (13–21) of nucleosome and chromatin melting cannot be strictly compared with our results because of the substantial differences in salt concentrations. Weischet *et al.* (12) found the melting of core particles to be quite sensitive to salts in the range 0.1–10 mM. In any case, it seems reasonable to suppose that transitions II, III, and IV all represent the melting of deoxyribonucleoprotein (none of them was sensitive to RNase). We conclude, then, that isolated chromatin retains nucleosomes and perhaps other forms of deoxyribonucleoprotein in states represented by transitions II and III but that, compared to nuclei, it has lost some higher order structure, on which transition IV depends.

The most intriguing feature in the calorimetry of nuclei was transition IV (105°C), which was completely shifted to a 88°C transition by brief exposure to either micrococcal nuclease or DNase I. In differential scanning calorimetry of calf thymus chromatin, Nicolini *et al.* (6) assigned a similar high-melting endotherm (107°C) to the helix-to-coil transition in duplex DNA. Although it is likely that the melting of double-stranded DNA is a source of enthalpy observed in transition IV, it is clear from our studies that it is not simply a helix-to-coil transition. The endotherm we observed at 105°C was due to the melting of chromatin under a higher order structural constraint. These constraints might be related to those expressed in the work of Benyajati and Worcel (22), who described interphase *Drosophila* chromatin prepared without nuclease digestion as large supercoiled loops that could be relaxed by limited nuclease digestion. The nature of this higher level architecture is a matter of conjecture, but it would certainly be consistent with chromatin stabilized by the nuclear matrix or scaffolding structure described by Laemmli (23, 24). If this high temperature endotherm represents large loops of chromatin DNA stabilized by attachments to scaffolding proteins or any such matrix, it is reasonable that the few cleavages produced by a mild DNA digestion could completely relax this structure, resulting in a lower melting temperature. Although this profound alteration of the endogenous structure of chromatin opens studies of solubilized chromatin to certain questions, it demonstrates strikingly the power of differential scanning calorimetry and the suitability of whole nuclei, rather than chromatin, for such studies.

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