Effects of Salt Concentration and H1 Histone Removal on the Differential Scanning Calorimetry of Nuclei†

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Received August 22, 1991; Revised Manuscript Received November 6, 1991

ABSTRACT: The effects of increasing NaCl concentrations on the melting profiles of chromatin in isolated nuclei contradicted published claims that structural transitions near 76 °C (Tn-7), near 89 °C (Tn-8), and near 105 °C (Tn-10) were respectively the melting of linker DNA, the melting of extended nucleosomal strands, and the collapse of nucleosomes in the 300-Å fiber. Contrary to expectations of such an interpretation, decreases in salt concentration stabilized Tn-7 and failed to eliminate Tn-10. Moreover, nuclei depleted of H1 histone, which is known to be essential for the formation of the 300-Å fiber, gave the same melting profile as intact nuclei with regard to the relative magnitudes of Tn-8 and Tn-10. The effect of salt concentration on the melting profiles and the insensitivity of Tn-8 and Tn-10 to H1 histone removal supports the notion that Tn-7 is the collapse of the nucleosome while Tn-8 and Tn-10 are respectively the unstacking of nucleotide bases in relaxed chromatin and supercoiled chromatin. The identification of Tn-8 as the unstacking of bases in relaxed DNA, and Tn-10 as unstacking in supercoiled DNA, shows that scanning calorimetry can be used to measure the state of repair of DNA in the nucleus. The gain in Tn-8 at the expense of Tn-10 that is seen as the mitotic index drops and differentiation occurs suggests that nicks accumulate in the DNA, perhaps because the gross aggregation of the inactive majority of the chromatin makes it inaccessible to repair enzymes.

Differential scanning calorimetry has been shown to be a useful technique to examine DNA structure in chromatin, intact nuclei, and whole cells (Touchette & Cole, 1985; Almagor & Cole, 1989a,b; Monaselidze et al., 1981; Nicolini et al., 1983; Balbi et al., 1989). Earlier we reported (Touchette & Cole, 1985) that nuclei from dividing cells melt in four thermal transitions.[1] The first transition (Tn-5) is complex, occurring at 55–60 °C, while the others are comparatively simple and occur at 76 °C (Tn-7), 89 °C (Tn-8), and 105 °C (Tn-10), respectively (Touchette & Cole, 1985). Tn-10 was the predominant endotherm in both dividing cells and other cells that retained the capacity to divide, while Tn-8 was predominant in cells with little or no proliferative potential (Almagor & Cole, 1987; Touchette et al., 1986). When cells were induced to stop dividing by serum deprivation or butyrate treatment, a progressive loss in enthalpy associated with Tn-10 was accompanied by a compensatory increase in Tn-8 (Touchette et al., 1986). Further studies indicated an association of Tn-8 with the G_0 phase of the cell cycle (Rice et al., 1988), whereas Tn-10 was associated with G_1 and to a lesser extent with S phase. It is thus of interest to identify the structures giving rise to the transitions observed in melting profiles.

Almagor and Cole recently found that although removal of proteins from isolated chromatin fragments by digestion with protease K resulted in a loss of Tn-7, Tn-8 was unaffected (Almagor & Cole, 1989a). Digestion of chromatin by DNase I eliminated Tn-8 and shifted Tn-7 to 58 °C. Since Tn-7 depended on the integrity of both protein and DNA while Tn-8 depended only on the integrity of DNA, these results indicated that Tn-7 represents the denaturation of the nucleosome while Tn-8 represents the unstacking of topologically unconstrained DNA. This interpretation is consistent with the melting temperature of the histones (and inner DNA) in core particles reported by Weischet et al. (1978).

Tn-10 appears to represent the melting of the topologically constrained DNA, since even slight nuclease digestion resulted in the loss of Tn-10 of chromatin and a compensatory gain in Tn-8 (Touchette & Cole, 1985). Vinograd et al. (1968) had previously shown that supercoiled polyoma virus DNA melted at 107 °C and nicked relaxed circular polyoma DNA melted at 89 °C. Since Vinograd's experiments were done at ionic strengths similar to ours, it seemed likely that the transition occurring in our experiments at 105 °C was due to the melting of topologically constrained, supercoiled DNA, while Tn-8, which occurred at 89 °C, represents the melting of relaxed DNA. It is not clear whether the DNA is free of histone while it undergoes Tn-8 and Tn-10 or is simply indifferent to the denatured histones if they are bound.

Balbi et al. (1989) have also attempted to assign structural meaning to the thermal transitions observed in the melting of nuclei. They attribute Tn-7 to the melting of linker DNA, Tn-8 to the denaturation of the core particle within an "unordered loop" of chromatin, and Tn-10 to the melting of a high-order solenoidal-type structure (Thoma et al., 1979). If such an interpretation were true, then Tn-10 would be dependent on both salt concentration and the presence of histone H1 (Thoma et al., 1979). The present study tested this interpretation by an examination of the effects of NaCl,

† This work was supported by National Institutes of Health Grants GM20338 and EHS-ES-01896 and by the Agricultural Experimental Station.

‡ Journal of NIH Research.

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[1] Previously we numbered the transitions I–IV, but Balbi et al. (1989) have since used Roman numerals, numbering the transitions differently. To avoid confusion, we are labeling the transitions according to their approximate melting temperatures at 100 mM NaCl: Tn-5 means melting between 51 and 60 °C, Tn-7 between 71 and 80 °C, etc. It must be kept in mind that any particular transition may occur at a somewhat different temperature under different salt concentrations.

0006-2960/92/0431-1842$03.00/0

multivalent ions, and histone H1 on the thermal denaturation of nuclei and showed that Tn-10 was essentially insensitive to all these factors and therefore could not be due to the melting of solenoidal chromatin.

Materials and Methods

Cell Culture. HeLa cells, strain S3, were maintained in suspension culture at (2–8 × 10^5 cells/mL in Joklik's modified spinner medium supplemented with 5% calf serum. Cells were harvested at (5–6) × 10^5 cells/mL.

Isolation of Nuclei. HeLa cells were harvested by centrifugation at 1500*g* and washed in swelling buffer (0.1 M hexylene glycol, 1 mM $CaCl_2$, and 0.06 mM Pipes, pH 6.8). After resuspension in swelling buffer for 10 min, cells were disrupted with 10 strokes in a loose-fitting Dounce homogenizer (pestle B). Nuclei were washed in primary buffer (5 mM Tris-HCl, 0.2 mM EDTA, and 250 mM sucrose, pH 7.5) plus NaCl, $MgCl_2$, $CaCl_2$, or polyamine, as indicated.

Nuclei from chicken erythrocytes were prepared according to the method of Olins et al. (1976). One hundred milliliters of chicken blood from freshly killed animals (a gift from Walter Olson) was collected into 30 mL of 2% citrate in phosphate-buffered saline (10 mM $NaHPO_4$/0.1 M NaCl, pH 7.4). Blood was filtered through eight layers of cheesecloth and diluted with 50 mL of STM buffer (10 mM NaCl, 10 mM Tris, and 2 mM $MgCl_2$, pH 7.4). Cells were centrifuged for 15 min at 4000*g*, and the pellet was suspended in 250 mL of STMN buffer (STM buffer plus 0.5% Nonidet P-40). Cells were lysed be stirring with a magnetic stirrer for 10 min at 4 °C, and nuclei were pelleted by centrifugation at 4000*g* and then washed 3 times in STMN buffer and 3 times in calorimetry buffers.

Histone Displacement and Reconstitution. HeLa nuclei were depleted of H1 according to the method of Lawson and Cole (1982), with the following modification. Nuclei were resuspended in depletion buffer (50 mM glycine, 250 mM sucrose, 25 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 2.8) and then gently stirred for 15 min at 4 °C. Depleted nuclei were pelleted and reextracted with depletion buffer. The supernatants (displaced H1 histones) were pooled and dialyzed against buffer C (5 mM Tris, 0.2 mM EDTA, 250 mM sucrose, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.5). Depleted nuclei were washed 3 times with buffer C.

Depleted nuclei were reconstituted with the displaced histones by direct mixing in buffer C. The reconstitution with displaced histones was achieved with an amount equivalent to that extracted.

Protein Analysis. Intact, depleted, or reconstituted nuclei were analyzed for protein content by extraction with 0.4 N H_2SO_4 and electrophoresis in 12.5% sodium dodecyl sulfate–polyacrylamide gels, as described by Laemmli (1970).

Electron Microscopy. Nuclei were washed 2 times in 0.1 cacodylate hydrochloride, pH 6.8, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, then fixed for 1 h at room temperature in 2% formaldehyde, 2% glutaraldehyde, and 0.1 M cacodylate hydrochloride, pH 6.8, and washed 2 times in 0.1 M cacodylate hydrochloride. After fixation in 1% osmium tetroxide/0.1 M cacodylate hydrochloride, pH 6.8, for 1 h at room temperature, samples were washed 2 times in distilled water. Samples were then stained in 2% uranyl acetate for 1 h at room temperature, washed 2 times in distilled water, and then embedded in 2% agar at 45 °C. Agar-embedded sample plugs were dehydrated in 100% ethanol, infiltrated with uncured resin, and then embedded in fresh resin and cured overnight at 70–75 °C.

Thin sections of resin-embedded nuclei were cut with a Sorvall Mp-2 microtome. The thin sections were poststained with 2% uranyl acetate for 45 min at room temperature, then examined, and photographed with a Philips EM 301 electron microscope.

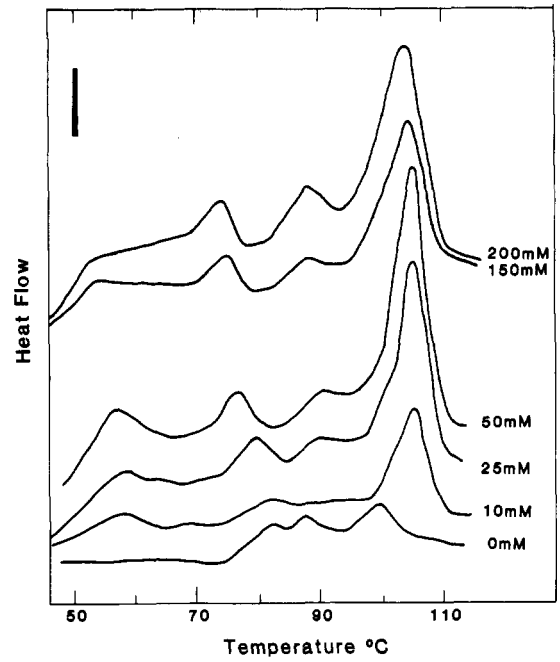

FIGURE 1: Effect of NaCl concentration in the differential scanning calorimetry of nuclei from proliferating HeLa cells. Heat absorption curves for intact nuclei in primary buffer and NaCl concentrations from 0 (bottom) to 200 mM (top) scanned at a rate of 5 °C/min. The amount of DNA in each sample of nuclei was as follows: 0 mM NaCl, 0.085 mg; 10 mM NaCl, 0.19 mg; 25 mM NaCl, 0.31 mg; 50 mM NaCl, 0.40 mg; 150 mM NaCl, 0.52 mg; 200 mM NaCl, 0.63 mg. The vertical bar indicates a heat flow of 0.05 mcal/s for the bottom three scans and 0.1 mcal/s for the top three.

Differential Scanning Calorimetry. All calorimetry experiments were done with a DuPont 99 thermal analyzer from 25 to 120 °C at a scanning rate of 5 °C/min. Output was plotted as a function of heat absorbed in millicalories per second per inch versus temperature. Prior to thermal analysis, nuclei were pelleted by centrifugation for 2 min in a microfuge, and pellets were transferred to aluminum pans and hermetically sealed. The sample sizes ranged from 10 to 20 mg of moist pellet. Total enthalpy was determined by cutting out peaks from tracings of the scans and weighing. After scanning, sample pans were opened, the contents were dissolved in 0.1 M NaOH, and absorbance was measured at 260 nm. DNA concentration was estimated, assuming A_{260} = 280 for a 1% solution.

Results

If nuclei from rapidly dividing cells are scanned by calorimetry at 150 mM NaCl, four thermal transitions are observed at 55–60, 76, 89, and 105 °C (Touchette & Cole, 1985; Almagor & Cole, 1987, 1989a,b; Monaselidze et al., 1981; Nicolini et al., 1983; Balbi et al., 1989; Touchette et al., 1986; Rice et al., 1988). Balbi et al. (1989) contend that the transition occurring at 105 °C (Tn-10) is due to the melting of the solenoid. Thoma et al. (1979) showed that at low salt concentrations, chromatin assumes a beads-on-a-string conformation (100-Å fiber) which appears to be a solenoid. If Tn-10 is indeed due to the melting or denaturation of the solenoid, then one would expect its occurrence to be salt-dependent. Figure 1 shows the thermal denaturation profiles obtained by calorimetric scanning of HeLa nuclei in various concentrations of NaCl. It was difficult to obtain good scans in the complete absence of NaCl and divalent cations, because

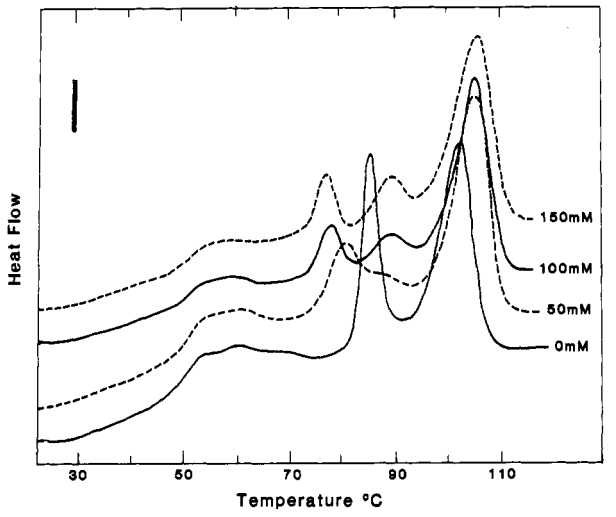

FIGURE 2: Effect of 1 mM $MgCl_2$ and 1 mM $CaCl_2$ on the NaCl concentration dependence of melting profiles of HeLa nuclei. Differential scanning calorimetry of nuclei was done as in Figure 1 except that 1 mM $MgCl_2$ and 1 mM $CaCl_2$ were added. The DNA in each sample was the following: 0 mM NaCl, 1.4 mg; 50 mM NaCl, 1.1 mg; 100 mM NaCl, 1.3 mg; 150 mM NaCl, 1.4 mg. The bar indicates 0.2 mcal/s.

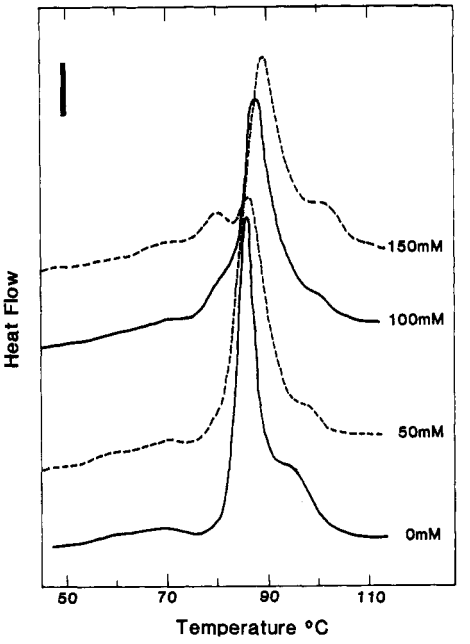

FIGURE 3: Effect of NaCl concentration in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$ on the melting profiles of nuclei from nonproliferative cells, mature chicken erythrocytes. Differential scanning calorimetry of nuclei was done as in Figure 2. The DNA in each sample was as follows: 0 mM NaCl, 1.7 mg; 50 mM, 1.9 mg; 100 mM, 1.8 mg; 150 mM, 1.8 mg. The bar indicates a heat flow of 0.5 mcal/s.

the nuclei lysed to form a gel that did not pack into the sample pans in amounts sufficient to give strong signals. Nevertheless, as shown in Figure 1, Tn-10 was clearly the dominant endotherm even at 0–10 mM NaCl and therefore represents something other than the solenoid.

There was a modest increase in melting temperature with increasing salt for both Tn-8 (mainly complete by 25 mM) and Tn-10 (mainly complete by 10 mM), while Tn-7 showed a striking decrease in melting temperature as a function of NaCl concentration. Tn-5, which is a complex or multicomponent transition, also showed a substantial decrease in apparent melting temperature with increasing salt. Between 10 and 200 mM NaCl, there were no significant changes in enthalpy for any of the thermal transitions.

Figure 2 shows the effect of increasing NaCl concentration on the thermal melting profiles of HeLa nuclei in the presence of 1 mM $MgCl_2$. Although $MgCl_2$ had no obvious effect on the melting profiles at higher NaCl concentrations (>50 mM), in NaCl-free buffer, the presence of $MgCl_2$ resulted in a single structural transition at 85 °C in place of the transitions seen at 76 and 89 °C in the presence of NaCl. As increasing amounts of NaCl were added to $MgCl_2$-containing buffer, two transitions appeared to emerge from the single thermal transition that was seen at 85 °C in the absence of NaCl. Increasing the NaCl concentration to 50 mM resulted in a broadening of the 85 °C peak, more precisely described as a resolution into a major peak at 83 °C and a shoulder at approximately 88 °C. Further increases in the concentration of NaCl up to 200 mM NaCl resulted in the conversion of the shoulder to a peak at 89.5 °C and a clear decrease in the apparent melting temperature of the other transition from 83 to 76 °C. Increases in NaCl resulted in a slight shift on the melting temperature of the highest transition, from 103 to 105 °C.

The salt-induced stabilization of Tn-10 is clearly seen in the case of nuclei from rapidly dividing HeLa cells, but the stabilization of Tn-8 is equivocal because the peak is smaller and incompletely resolved from the peak of Tn-10. For nuclei from nondividing cells such as mature chicken erythrocytes, however, it is Tn-8 that is dominant, and as shown in Figure 3, the stabilization of Tn-8 by NaCl is obvious. Once again, it is evident that the structural feature giving rise to Tn-7 is destabilized by NaCl. (Tn-7 was not seen at 0 mM NaCl, but appeared as a very slight shoulder on Tn-8 at 50 mM, a more pronounced shoulder at 100 mM, and finally a fully resolved peak at 150 mM NaCl.) It has been suggested (Balbi et al., 1989) that our correlation of loss of mitotic potential with a shift from dominance of Tn-10 to dominance of Tn-8 was an artifact caused by degradation of chromatin to fragments too small to form solenoids. This suggestion was incorrect because we had shown (Touchette & Cole, 1985) that when nuclei were treated with nuclease, Tn-10 was almost completely replaced by Tn-8 when the fragments were still more than large enough (10–20 kbp) to form solenoids. Moreover, we had also reported (Touchette et al., 1986) that when whole cells or bits of whole tissue were submitted to scanning calorimetry the profiles looked like those for isolated nuclei except that they occurred on a high base line and showed an extra (large) peak at 65 °C, representing RNA. When bits of mature, differentiated tissues or mitotically impotent cells like mature chicken erythrocytes were observed by differential scanning calorimetry, the heat flow for Tn-8 was always an order of magnitude larger than that of Tn-10 (Touchette et al., 1986). We are confident, therefore, that the dominance of Tn-8 in Figure 3 is not an artifact.

Because the effects of changing the NaCl concentration on the melting profile of HeLa nuclei were more pronounced in the presence of a constant concentration (1 mM) of $MgCl_2$ ion, we also examined the effects of changes in the concentration of the divalent cation Mg^{2+} and the polyamine spermidine in the presence and absence of 200 mM NaCl, as shown in Figure 4. Along with Tn-10, we observed (Figure 1) two distinct thermal transitions in the absence of NaCl, $MgCl_2$, and spermidine. In the absence of NaCl, there was a convergence of those two peaks to a single endotherm at 85 °C even upon increasing the spermidine concentration to only 0.05 mM or the $MgCl_2$ concentration to only 0.5 mM. In the presence of 200 mM NaCl, however, we observed two distinct

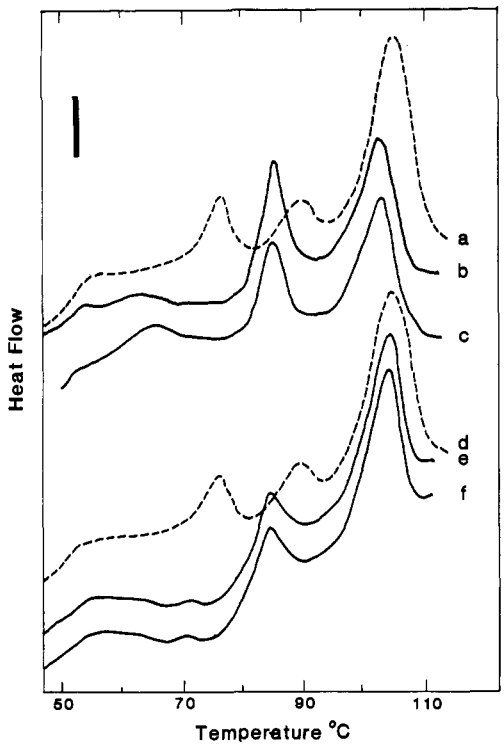

FIGURE 4: Comparison of the effects of $MgCl_2$ and spermidine concentrations in the melting profiles of HeLa nuclei. The upper set of profiles shows the effect of $MgCl_2$ concentration while the lower set shows the effect of spermidine concentration. The conditions for the profiles were as follows: (a) 1 mM $MgCl_2$ + 200 mM NaCl, 0.64 mg of DNA; (b) 2 mM $MgCl_2$, 0.51 mg of DNA; (c) 0.5 mM $MgCl_2$, 0.43 mg of DNA; (d) 0.2 mM spermidine + 200 mM NaCl, 0.54 mg of DNA; (e) 0.2 mM spermidine, 0.64 mg of DNA; (f) 0.05 mM spermidine, 0.28 mg of DNA. The bar indicates a heat flow of 0.1 mcal/s for (a–e) and 0.05 mcal/s for (f).

thermal transitions at 76 and 90 °C, whether or not $MgCl_2$ or spermidine was present.

The observed decrease in melting temperature of Tn-7 with increasing salt concentration seemed inconsistent with the suggestion of Balbi et al. (1989) that this transition represents the melting of linker DNA since salt concentrations in this range are known to stabilize DNA. As a further test, we examined the effect of histone H1 depletion of nuclei. In addition, the effect of histone H1 removal on the melting profile of nuclei provided a further test of the claim than Tn-10 represents melting of the solenoid. If Tn-10 is due to the denaturation of the solenoid or 300-Å fiber, then the removal of histone H1 should eliminate Tn-10, because solenoid formation is dependent upon the presence of histone H1 (Thoma et al., 1979).

HeLa nuclei were depleted of histone H1 without loss of core histones by exposure to buffer at pH 2.8 (Lawson & Cole, 1982), where more than 90% of histone H1 was extracted from the HeLa nuclei as measured in stained electrophoretic gels. Some of the depleted nuclei were washed with buffer at pH 7.5 and reconstituted with their extracted histone H1. The histone content of reconstituted nuclei was also examined electrophoretically to confirm that the linker histone had reentered the nucleus when the pH was raised to 7.5.

Intact, H1-depleted, and reconstituted nuclei were examined by electron microscopy. As shown in Figure 5, the chromatin in histone H1 depleted nuclei took on a disaggregated appearance, relative to either intact or reconstituted nuclei. Thus, at least in the sense that it aggregates chromatin, histone H1 was bound to chromatin in a physiological relevant manner, upon reconstitution.

Intact, H1-depleted, and reconstituted nuclei were scanned calorimetrically in buffers containing 150 mM NaCl (in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$). As shown in Figure 6, H1 depletion did not affect Tn-7, Tn-8, or Tn-10. We did observe that histone depletion appeared to remove the 60 °C component of the low-temperature complex transition. Reconstitution with histone H1 failed to reconstitute this portion of the calorimetric profile. The indifference of Tn-8 and Tn-10 to the presence of linker histones is inconsistent with the notion that Tn-10 is the denaturation of the 300-Å fiber and Tn-8 the denaturation of nucleosomes in the 100-Å fiber.

Discussion

Differential scanning calorimetry has been used to study chromatin structure under physiological conditions in whole cells and intact nuclei (Touchette & Cole, 1985; Almagor & Cole, 1987, 1989a,b; Monaselidze et al., 1981; Nicolini et al., 1983; Balbi et al., 1989; Touchette et al., 1986; Rice et al., 1988). These studies indicated that chromatin in nuclei from a number of sources melts in four thermal transitions. We designate these as a complex transition (Tn-5), occurring at 55–60 °C, and transitions Tn-7, Tn-8, and Tn-10, occurring at 76, 89, and 105 °C, respectively. Balbi et al. (1989) considered the first complex transition to be two distinct endotherms at 58 and 68 °C, which they suggested were due to the melting of scaffolding structures and RNA-associated proteins. Their transition at 75 °C was thought to be due to the melting of DNA in the linker domain, while the transitions at 92 and 107 °C were believed to be due respectively to the melting of the core particle placed within an "expanded loop" of chromatin and within the 300-Å fiber. Our interpretation of the melting profile of nuclei in physiological salt conditions differs substantially from that of Balbi et al. We also demonstrated that most of the first transition is due to the melting of non-chromatin components, but we attributed Tn-7 to the denaturation of the nucleosome, Tn-8 to the unstacking of bases in unconstrained chromosomal DNA, and Tn-10 to the unstacking of bases in constrained chromatin.

The interpretation of Balbi et al. included the extrapolation of previously published thermal denaturation data obtained from core particles and digested chromatin in low ionic strengths (<5 mM monovalent cations) to their own conditions (133 mM). Since electrostatic and hydrophobic interactions for DNA and nucleosomal proteins respond differently to salt and heat, there is a risk in making such extended extrapolations, especially since differences in the length of DNA (comparing 145 bp to intact chromosomal length) could introduce further complications. The sensitivity of chromatin to subtle changes in conditions at low salt concentrations can be seen in the reports of Reczek et al. (1982) and Riehm and Harrington (1987), who studied the thermal stability of long chromatin fragments. These workers found that at low ionic strength chromatin melts in three endotherms. The first thermal transition, at 60 °C, was attributed to the melting of both linker DNA and loosely bound core DNA. At 0.2 mM EDTA, Reczek et al. found that 48% of the DNA in chromatin melted at this temperature, while in 5 mM NaCl, only 22% of the chromatin was in this state (Reczek et al., 1982). Riehm and Harrington reported that 9–10% of the DNA in chromatin melted at 60 °C in both 0.2 mM EDTA and 5 mM sodium phosphate (Riehm & Harrington, 1987). Despite their quantitative differences, both groups concluded that the 60 °C transition was due to the melting of DNA not stabilized by protein or salt. Both Reczek et al. and Riehm and Harrington concluded that in low salt concentrations, their second transition (66 or 73 °C, respectively) was due to the melting

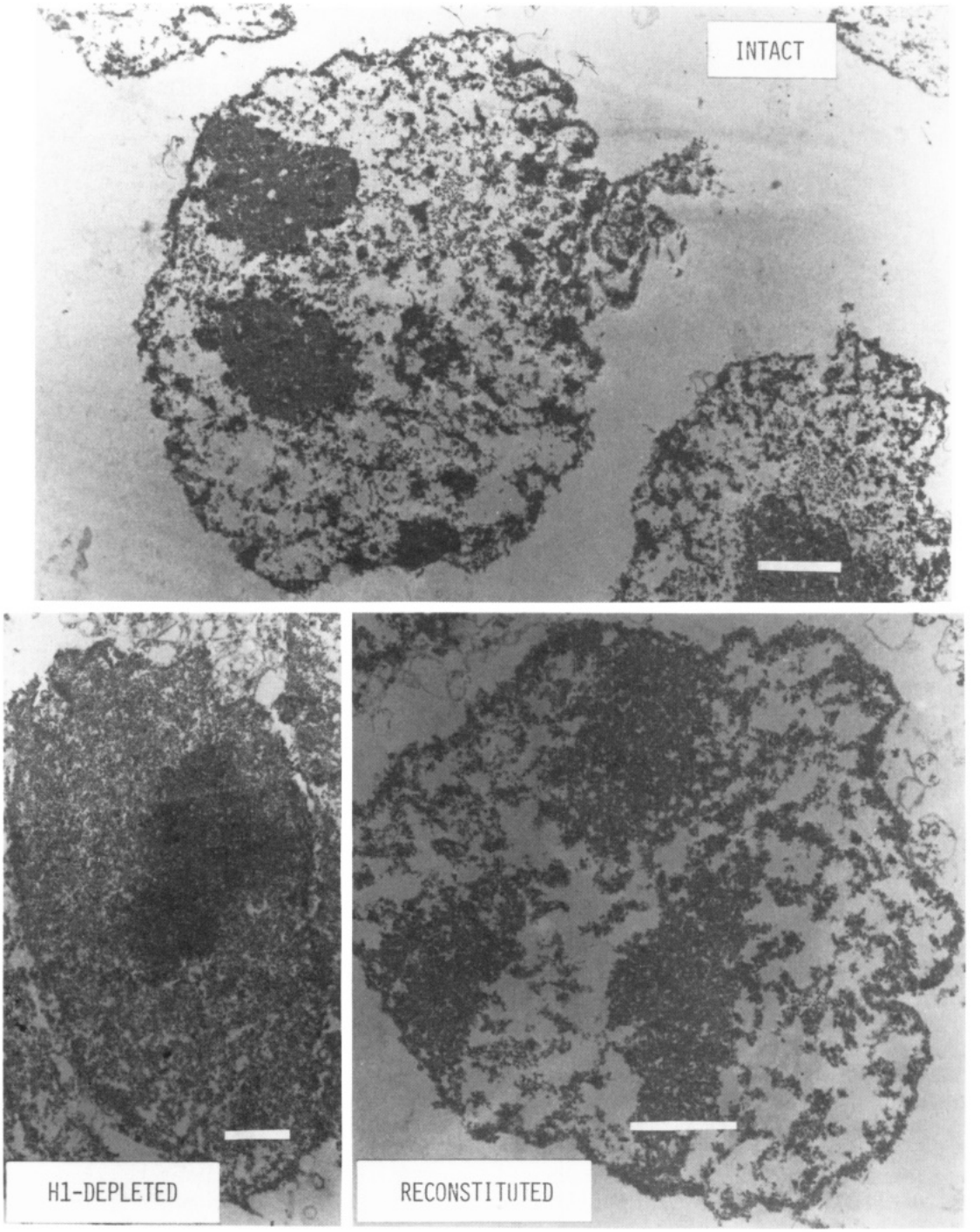

FIGURE 5: Electron micrographs demonstrating histone H1 removal and reconstitution. The top panel shows the clumped chromatin of an intact HeLa nucleus. The lower left panel shows the dispersal of chromatin that resulted from H1 histone removal. The lower right panel shows restoration of clumping of chromatin after H1 was returned to the nucleus. The white bar represent 1 μm.

of the outer portions of nucleosomal DNA, in agreement with studies on core particles by Weischet et al. (1978), Simpson (1979), and Bina et al. (1980). However, although Reczek et al. found this transition to be unaffected by a change in salt concentration from 0 to 5 mM NaH_2PO_4, Riehm and Harrington found that this transition decreased in magnitude by a factor of 2 when the sodium ion concentration was raised from 0.2 to 5 mM. Simple extrapolation of the latter suggests that the second transition observed in low salt would not even occur at salt concentrations much higher than 5 mM, while extrapolation of the former suggests a substantial magnitude for that transition. The discrepancies between these results show the sensitivity of this transition to subtleties of experimental conditions in the low salt range and reveal the danger of extended extrapolations from the data obtained at <10 mM salt concentrations to those obtained at 133 mM.

An identification of thermal transitions under approximately physiological salt conditions was made in recent studies by Almagor and Cole (1987), without extensive extrapolation of data from low ionic strength experiments. HeLa nuclei and chromatin fragments were digested with either proteinase K or DNase I, and the digested samples were scanned calorimetrically in 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Complete digestion of all five histones with proteinase K resulted in the total loss of Tn-7 in both nuclei and chromatin, but Tn-8 was unaffected. Since Tn-7 was completely destroyed

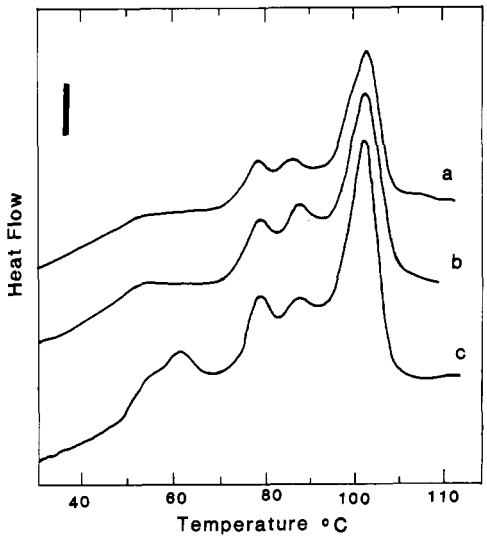

FIGURE 6: Effect of histone H1 removal on the melting profile of HeLa nuclei. Differential scanning calorimetry was done as in Figure 1 on intact isolated nuclei (c), nuclei from which H1 had been removed (b), and nuclei from which H1 had first been removed and then restored (c). The DNA contents of the samples of nuclei were the following: (a) 0.21 mg; (b) 0.18 mg; (c) 0.29 mg. The bar indicates 0.01 mcal/s.

by digestion of protein, it could not have been due to the melting of naked, linker DNA. Digestion with DNase resulted in the loss of Tn-8 and a shift in melting temperature from 73 to 58 °C for Tn-7. Since Tn-7 depended on the integrity of both protein and DNA, it was concluded that Tn-7 was not due to the melting of linker DNA but rather to the denaturation of the protein components of the nucleosome, which were stabilized by nucleosomal DNA. The temperature of Tn-7, moreover, matches the major melting temperature of core particles as reported by Weischet et al. (1978), Seligy and Poon (1978), and Simpson and Bergman (1980). As discussed below, Tn-8 was thought to arise from the melting of unconstrained DNA, since it did not depend on the presence of histone.

Further evidence that Tn-7 in nuclei is not due to the melting of linker DNA derives from the observation that the melting temperature of Tn-7 was not increased as the NaCl concentration was raised. As shown in Figure 1, increases in salt concentration resulted in a slight increase in the melting temperature of Tn-8, and a lowering of the melting temperature of Tn-7. Tn-7 therefore cannot be considered to be due to naked linker DNA since it is well established that the melting temperature of DNA is raised with increasing ionic strength. Subirana (1973) reported melting temperatures for sheared sea urchin DNA as 48, 65, and 84 °C at 1, 10, and 100 mM NaCl, respectively. Furthermore, at ionic strengths approximately physiological, linear DNA and relaxed DNA melt at 89 °C and supercoiled DNA at 104–107 °C (Vinograd et al., 1968). Tn-7 matches neither of these melting temperatures, and to accept the notion that it is the melting of linker DNA, we would have to postulate that the linker DNA was substantially destabilized by the binding of histones to the chromatin.

Balbi et al. proposed that Tn-8, at 88–90 °C, is due to melting of the nucleosomes placed within an expanded loop. This proposal cannot be reconciled to the observation that Tn-8 is unaffected by the complete destruction of the chromosomal proteins. Our interpretation is that Tn-8 is a result of the unstacking of base pairs in unconstrained DNA. The latter interpretation is in agreement with the data of Vinograd et al. (1968), who found the T_m of relaxed DNA to be 89 °C under salt conditions similar to ours. The approximate agreement between the T_m for relaxed DNA in chromatin and the T_m for relaxed naked DNA is explained by the experiments of Subirana (1973), who showed that while the presence of proteins associated with DNA affected the melting temperature of DNA at low ionic strengths, as the sodium chloride concentrations were increased the nuclear proteins lost their effect on the melting temperature of DNA. Therefore, although Vinograd et al. (1968) studied naked supercoiled and nicked polyoma DNA, their results are comparable to nuclear DNA at physiological ionic strengths. Thus, it seems likely that Tn-8, observed in our studies, represents the melting of unconstrained DNA.

Tn-10 (105–107 °C) was attributed by Balbi et al. to the 300-Å fiber, but the present results rule out this possibility. Previously, we observed that the major enthalpic transition in intact HeLa nuclei occurred at 105 °C, while nuclei subjected to light nuclease treatment melted predominantly at 89 °C. This was confirmed by Balbi et al. (1989) using liver nuclei. Balbi et al. interpreted the loss of Tn-10 upon nuclease digestion to mean that the chromatin has been digested to such small fragments that solenoids could not be formed. However, we observed (Touchette & Cole, 1985) loss of Tn-10 when digestion of HeLa nuclei had proceeded only far enough to generate fragments in the 20 kbp range, which was shown by Thoma et al. to be of sufficient length to permit solenoid formation. Thus, the disappearance of Tn-10 did not correlate with the disappearance of the 300-Å fiber.

Thoma et al. (1979) established that solenoid formation is dependent upon the presence of histone H1 (or other linker histone) and the presence of either monovalent cation (75–100 mM NaCl) or lower levels of $MgCl_2$. Thus, if Tn-10 were due to the solenoid form of chromatin, it should not occur for chromatin in the absence of either histone H1 or salt, but might be expected to reappear when the appropriate linker histone and cations are restored. In contrast to the behavior of the solenoid, as shown in Figure 1, Tn-10 was observed in the presence of 5 mM Tris and 0.2 mM EDTA, even when neither NaCl nor $MgCl_2$ was added. Moreover, as the salt concentration was raised to 200 mM, there was only a slight increase in the melting temperature and no change in the magnitude of Tn-10. The small increase in melting temperature is fully explained by the salt stabilization of the phosphate groups through electrostatic interactions (Record et al., 1976).

When histone H1 was selectively displaced from HeLa nuclei, marked changes in the appearance of the chromatin were evidenced by electron microscopy (Figure 5), but no perceptible change was seen in the thermal melting profiles (Figure 6). In the absence of histone H1, nuclear chromatin took on a dispersed, disaggregated appearance, but appeared recompacted upon reconstitution with histone H1. However, both the melting temperature and the magnitude of Tn-10 were unaffected by either histone H1 depletion or reconstitution. Because the notion that Tn-10 represents the melting of the solenoid would call for a conversion from Tn-10 to Tn-8 with histone depletion, Tn-10 cannot represent the melting of solenoidal structures. A more plausible idea is that Tn-10 (105 °C) is the melting of the supercoiled DNA that was constrained in looped domains of chromatin bound to the protein scaffold (Cook & Brazell, 1976; Cook et al., 1976).

The arguments regarding the identification of the major transitions in the melting of chromatin in isolated nuclei may be summarized as follows. The transition at 76 °C cannot be linker DNA because relaxed DNA is known to melt at 89 °C

under the present salt conditions, and because it was not stabilized by increasing salt conditions. The transition at 76 °C is most likely the melting of the nucleosome, since it depends on the integrity of both the DNA and the core histones. The transition at 89 °C cannot be the melting of nucleosome on distended chromatin loops because it was insensitive to the destruction of core histones (Almagor & Cole, 1987) and its magnitude did not decrease as salt conditions were changed to favor the formation of the 300-Å fiber. The transition at 89 °C is most likely the unstacking of bases in relaxed DNA, since it is indifferent to the presence of histones and because its T_m matches that published previously for relaxed DNA. The transition at 105–107 °C cannot be melting of nucleosomes in the 300-Å fiber, since it is indifferent to the presence of H1 histone and to changes in salt concentration that induce or destroy the solenoid. Instead, it is probably the unstacking of bases in DNA that is supercoiled after its release from the nucleosome; its T_m matches that published previously for supercoiled DNA.

It is of interest to note the effects of NaCl concentration on the stability of the nucleosome in the absence of polyamine or divalent cations. Raising the [NaCl] lowered the melting temperature of Tn-7, but as would be expected for naked DNA, the salt increase elevated the melting temperature of Tn-8 by 5 °C. These observations can be explained as follows. At low salt concentrations and in the absence of NaCl, the nucleosome consists of a tight complex between histone octamers and DNA. As the salt concentration is increased, the DNA is stabilized through electrostatic screening of its phosphate charges by Na^+, while the interactions between core histone proteins and the DNA strand are weakened. This is consistent with the polyelectrolyte theory developed by Manning (1978), where it was shown that in complexes of histone H1 and DNA, the DNA was stabilized by increasing concentrations of NaCl, while the interactions between histone and DNA were weakened. The weakening of the histone–DNA interactions results in a lowering of the melting temperature of Tn-7, which is due to the denaturation of the nucleosome. An analogous rationale was given by McMurray and van Holde (1986) for the effects of intercalation drugs on core particles. The stiffening of DNA by intercalation was considered to be the cause of the lowering of the T_m for the core particle. In the case of chromatin, Almagor and Cole (1989b) observed the stabilization of DNA by intercalators as increased temperatures for TN-8 and Tn-10, in addition to the destabilization of the nucleosome as a lowering of the temperature for Tn-7.

We had previously reported a correlation between the presence of Tn-10 and cell growth. Tn-10 was dominant in rapidly dividing mouse neuroblastoma cells, but disappeared when these cells were induced to differentiate. Almagor and Cole (1987) subsequently discovered that Tn-10 was correlated with the potential for cell growth; as cells progressively lost the structure melting at 105 °C, they lost progressively their capacity to be induced to proliferate. Balbi et al. (1989) contend that Tn-10 is representative of quiescent rather than cycling cells, but in preparing "quiescent" cells, they used fractionated liver cells from hepatectomized rats, in which DNA replication would be induced 20 h after partial hepatectomy. The observation of Rice et al. (1988) that Tn-10 is maximal in the G_1 phase suggests that the cells fractionated from liver after hepatectomy had been induced to enter G_1; according to Rice et al., such cells would be expected to show dominance of Tn-10.

The idea that Tn-10 represents the denaturation of DNA constrained by the protein scaffold and is correlated with cell growth or the potential for proliferation is consistent with studies by Cook et al. (Cook & Brazell, 1976; Cook et al., 1976), who noted a decrease in supercoiling in nucleoids isolated from chicken erythrocyte during embryonic development. Since DNA must be supercoiled in order for replication to occur, one would predict that supercoiling would be greatest prior to S phase and that proliferating cells would be characterized by a higher degree of supercoiled or constrained DNA than cells that have lost their proliferative potential. The increase in Tn-8 at the expense of Tn-10 as cells became differentiated must represent an accumulation of nicks in the DNA. In other words, the state of repair of the DNA drops as differentiation occurs. This is presumably because the gross aggregation of the majority of the chromatin in terminal stages of differentiation would make its DNA inaccessible to repair enzymes. Since this DNA is committed to inactivity, its poor state of repair would not be deleterious to the health of the cell. Indeed, the irreversibility of committed differentiation might be based in large part on the poor state of repair of DNA.

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^{31}P NMR Spectra of Oligodeoxyribonucleotide Duplex *lac* Operator–Repressor Headpiece Complexes: Importance of Phosphate Ester Backbone Flexibility in Protein–DNA Recognition†

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Received September 9, 1991; Revised Manuscript Received November 15, 1991

ABSTRACT: The ^{31}P NMR spectra of various 14-base-pair *lac* operators bound to both wild-type and mutant *lac* repressor headpiece proteins were analyzed to provide information on the backbone conformation in the complexes. The ^{31}P NMR spectrum of a wild-type symmetrical operator, d(TGTGAGCGCTCACA)$_2$, bound to the N-terminal 56-residue headpiece fragment of a Y7I mutant repressor was nearly identical to the spectrum of the same operator bound to the wild-type repressor headpiece. In contrast, the ^{31}P NMR spectrum of the mutant operator, d(TATGAGCGCTCATA)$_2$, wild-type headpiece complex was significantly perturbed relative to the wild-type repressor–operator complex. The ^{31}P chemical shifts of the phosphates of a second mutant operator, d(TGTGTGCGCACACA)$_2$, showed small but specific changes upon complexation with either the wild-type or mutant headpiece. The ^{31}P chemical shifts of the phosphates of a third mutant operator, d(TCTGAGCGCTCAGA)$_2$, showed no perturbations upon addition of the wild-type headpiece. The ^{31}P NMR results provide further evidence for predominant recognition of the 5′-strand of the 5′-TGTGA/3′-ACACT binding site in a 2:1 protein to headpiece complex. It is proposed that specific, strong-binding operator–protein complexes retain the inherent phosphate ester conformational flexibility of the operator itself, whereas the phosphate esters are conformationally restricted in the weak-binding operator–protein complexes. This retention of backbone torsional freedom in strong complexes is entropically favorable and provides a new (and speculative) mechanism for protein discrimination of different operator binding sites. It demonstrates the potential importance of phosphate geometry and flexibility on protein recognition and binding.

How do proteins recognize DNA? Most attention on understanding the binding specificity between amino acid sequences and DNA sequences has centered on hydrogen-bonding to the acceptor/donor groups on the Watson–Crick base-pairs in the major groove [cf. Landschulz et al. (1988)]. At present we do not understand this "second genetic code" of protein–DNA recognition. Perhaps one reason for the inability to dissect the basis for this specificity is the emphasis on base-pair interactions alone. Localized, sequence-specific conformational variations in DNA are quite likely another important component of a protein's recognition of specific sites on the DNA (Landschulz et al., 1988; Matthews, 1988). Thus, although the *lac* repressor protein does not recognize an alternating AT sequence as part of the *lac* operator DNA sequence, the repressor protein binds to poly[d(AT)] 1000 times more strongly than to random DNA (Saenger, 1984). Repressor protein is quite likely recognizing the alternating deoxyribose–phosphate backbone geometry of the two strands (Klug et al., 1979), rather than the chemical identity of the AT base pairs.

The *lac* repressor system is ideal for studying DNA–protein interactions by NMR (Buck et al., 1978, 1980, 1983; Hogan et al., 1981; Nick et al., 1982; Scheek et al., 1983; Wade-Jardetzky et al., 1979; Wemmer & Kallenbach, 1983; Zuiderweg et al., 1985). It appears to be possible to duplicate the basic *lac* operator–*lac* repressor protein interaction by using the smaller *lac* repressor headpiece N-terminal domain fragment (Adler et al., 1972; Buck et al., 1978; Wade-Jardetzky et al., 1979; Wemmer & Kallenbach, 1983; Zuiderweg et al., 1985). Kaptein and co-workers (Boelens et al., 1987; Zuiderweg et al., 1985) have assigned many of the ^{1}H signals of

† This work was supported by the NIH (AI27744), the Purdue University Biochemical Magnetic Resonance Laboratory, which is supported by the NIH designated AIDS Research Center at Purdue (AI727713), and the NSF Biological Facilities Center on Biomolecular NMR, Structure and Design at Purdue (Grants BBS 8614177 and 8714258 from the Division of Biological Instrumentation).

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0006-2960/92/0431-1849$03.00/0