

# HISTONE HYPERACETYLATION

## Its Effects on Nucleosome Core Particle Transitions

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**ABSTRACT** Effects of histone hyperacetylation on transitions of HeLa cell nucleosome core particles were studied. The transitions examined were induced by low salt concentrations, pH, temperature, and nondissociating high salt. Effects of salt dissociation were also examined. The low-salt transition was found to shift to higher ionic strength by ~ three fold for hyperacetylated particles, a change which may be due simply to the increased overall negative charge on the particles caused by acetylation of lysine residues. Some differences were also seen in the way in which core particles refold after exposure to very low salt (which induces a nonreversible change in the particles). Otherwise no significant effects of hyperacetylation were observed.

### INTRODUCTION

The DNA in eucaryotic chromatin is compacted within the nucleus through several levels of structure. First, the DNA is wrapped twice about a protein core consisting of eight inner histone molecules to form a fundamental subunit called the nucleosome. The next higher level of structure is built up by packing nucleosomes into a superhelical strand commonly called the 30 nm fiber, a process in which another histone, H1<sup>1</sup>, has been implicated (Butler, 1984). The way in which this 30 nm fiber is further compacted to form condensed chromatin or chromosomes is less well defined.

In order for a cell to replicate, transcribe, repair, or otherwise process the DNA, these ordered structures must be disrupted at least locally. Such disruptions are likely to be mediated by chemical modifications of the histones which might change the way in which they interact with the DNA or with other histones. A number of such modifications are known and include ubiquitination of inner histone H2a (and H2b to a much lesser extent), polyADP ribosylation, methylation, phosphorylation, and acetylation (Isenberg, 1979).

Post-translational acetylation of lysine residues on the core histones seems a very likely way to affect the interaction of the histones with DNA since this interaction has a major electrostatic component, presumably involving the lysine residues, and acetylation *in vivo* is found to occur in clusters within the highly charged NH<sub>2</sub>-terminal region of

the core histones (Doenecke and Gallwitz, 1982). Also, hyperacetylation of the core histones is correlated with active chromatin regions (Reeves, 1984).

Chromatin containing highly acetylated histones accumulates in tissue culture cells exposed to butyrate (Riggs et al., 1977). Simpson (1978) exploited this technique in order to compare highly acetylated chromatin and core particles with those obtained from untreated cells. Acetylated chromatin was found to have a considerably enhanced sensitivity to nuclease attack although its physical properties (circular dichroism, thermal melting, protein to DNA ratio) were essentially unchanged. Acetylated and control core particles were indistinguishable by sedimentation, but small differences in circular dichroism, thermal melting, and nuclease digestion were noted.

Bode et al. (1980) reported that hyperacetylated particles are altered in their accessibility of histone H3 cysteine side-chains and in the mode of histone displacement by protamines in addition to showing changes in the premelting region during thermal denaturation. Hyperacetylated nucleosomes were later found to move more slowly during electrophoresis (Bode et al., 1983) and to be larger and more disorganized by electron microscopy (Bertrand et al., 1984) suggesting a conformational difference from normal particles. However, Imai et al. (1986) were unable to detect any significant differences between hyperacetylated and control core particles using neutron scattering.

Making use of a method of fractionating butyrate treated HeLa chromatin developed by Perry and Chalkley (1981), Ausió and van Holde (1986) worked out a procedure for isolating core particle samples varying in degrees of hyperacetylation. Physical studies were then used to examine the effects of acetylation on nucleosome conformation and stability. The hyperacetylated cores had a lower sedimentation coefficient than control particles over

<sup>1</sup>Abbreviations: H1, H2a, H2b, H3, and H4 for the respective histones; CD, circular dichroism; bp, base pairs of DNA; CfoI, restriction endonuclease (type II); pBR322, plasmid pBR322; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; BICINE, N,N-bis(2-hydroxyethyl)-glycine; S, Svedberg unit (10<sup>-13</sup> s).

a wide range of salt concentrations. Curiously, only low levels of acetylation were required to induce this effect; changes in the acetylation level from 7 to 17 acetyl groups per particle (the control particles averaged 3) did not significantly affect the observed differences. Circular dichroism measurements on the highly acetylated particles showed an increase in ellipticity near 283 nm accompanied by loss of the negative band near 300 nm as compared with control particles. Thermal melting experiments demonstrated a decrease in the temperature for the premelt transition of hyperacetylated cores as well as a substantial increase in the proportion of DNA melting in this transition. DNase I digestion showed that hyperacetylated particles have an enhanced susceptibility to cutting at a site ~60-base pairs from the ends of the 145 base pair DNA, in agreement with Simpson's (1978) results for a lower degree of acetylation.

The studies briefly described above characterize the core particle in its "normal" conformation. In chromatin undergoing processing, the cores will have experienced a change in conformation and the role of acetylation might be to facilitate that change. While we cannot know the nature of the changes which occur *in vivo*, there are a number of transitions experienced by nucleosomes in response to changes in solution conditions. These include transitions with changing ionic strength such as dissociation of the histones from the DNA at high salt, a transition observed between 0.1 and 0.8 M NaCl independent of dissociation (Yager and van Holde, 1984), a low-salt transition (Wu et al., 1979; Libertini and Small, 1982), and a nonreversible transition at very low salt (Libertini and Small, 1987). Core particles also experience a transition with changing pH (Libertini and Small, 1984; Muller et al., 1985) and an effect of temperature which just precedes the premelt observed during thermal denaturation (Weischet et al., 1978; Simpson, 1979). Also, nucleosomes in chromatin experience a change in response to temperature as shown by nuclease digestion (Huang and Garrard, 1986).

Ausió and van Holde (1986) observed that hyperacetylation has no significant effect on the change observed between 0.1 and 0.8 M salt as measured by sedimentation and ellipticity. We report here an examination of the effect of hyperacetylation on other transitions of core particles.

## MATERIALS AND METHODS

### Preparation of Nucleosome Core Particles

Chromatin was isolated from an S-3 strain of HeLa cells. Preparation and micrococcal nuclease digestion of nuclei, fractionation of the solubilized chromatin, and digestion to core particles (and, initially, final core particle fractionation, see below) were carried out as described before (Ausió and van Holde, 1986). Only the hyperacetylated fraction "a" from butyrate-treated cell cultures, corresponding to an average degree of acetylation of ~17 acetyl groups per particle, was used in the work described here. Control core particles, having an average degree of acetylation of ~3 acetyl groups per particle, were prepared from untreated cell cultures.

Electrophoretic characterization of the core particles and of their DNA and histone components is shown in Fig. 1. The results are essentially indistinguishable from the corresponding preparations studied by Ausió and van Holde (1986). The control particles contain ~146 bp of DNA and the histones show little acetylation while the hyperacetylated particles contain an average of ~151 bp of DNA and highly acetylated H2b, H3, and H4.

When the UV fluorescence of particles prepared as before was examined, it was found to be contaminated with an excessive level of tryptophan-like emission with a low anisotropy. The problem was particularly bad for the acetylated core particle preparation. To minimize the contamination, the last step in the purification (a low ionic strength sucrose gradient sedimentation) was changed to gel chromatography on Sepharose 6B-CL (Pharmacia Inc., Piscataway, NJ; 1.5 × 95 cm column, run at ~12 ml/h) in the presence of 0.35 M NaCl and 10 mM Tris/HCl, pH 7.5 (and including 5 mM butyrate for the hyperacetylated core particle preparation). This procedure greatly reduced but did not completely eliminate the impurities, particularly for the acetylated core particles. Under the instrumental conditions used earlier for tyrosine fluorescence measurements on chicken erythrocyte and calf thymus core particles (Libertini and Small, 1980, 1982), differences in the impurity levels of control and acetylated core particle preparations might mask small differences in the transitions to be examined. To minimize such effects a method was worked out which allowed a change in the emission wavelength from the 325 nm used previously to 298 nm (see below) in order to decrease the relative contributions of the tryptophan-like impurities.

### Preparation of Dimethyl Suberimidate Crosslinked Core Particles

Control HeLa core particles were transferred into 0.1 M NaCl and concentrated with a Centricon-30 microconcentrator (used as recommended by the manufacturer, Amicon Corp., Danvers, MA). The concentrate (~50  $\mu$ l) was diluted to 200  $\mu$ l with 0.1 M potassium borate, pH 10, and divided equally into two tubes. To one was added 900  $\mu$ l of 0.1 M potassium borate, pH 10 (uncrosslinked control, final core particle concentration was ~5  $\mu$ M). To the other was added 900  $\mu$ l of 5.5 mg/ml dimethylsuberimidate in 0.1 M potassium borate, pH 10, which was prepared immediately before use. The two samples were maintained at ~20°C for 1 h then concentrated and transferred into 1 mM Tris/HCl, pH 7.5, using Centricon-30 microconcentrators. SDS-gel electrophoresis showed one main band of highly cross-linked histones and there were essentially no individual histones remaining in the preparation. Native particle gel electrophoresis showed oligomer bands corresponding to intermolecularly cross-linked dimers and a very low level of trimers; velocity sedimentation analysis indicated that ~20% of the particles were present as oligomers.

### Gel Electrophoresis

SDS gel electrophoresis was done according to Laemmli (1970). Triton-urea-acetic-acid gels were performed as described by Bonner, et al. (1980) with slight modification (Ausió, 1986). Native core particles and native DNA were electrophoresed on 4% polyacrylamide as described elsewhere (Yager and van Holde, 1984).

### Physical Measurements

All physical measurements were done at 20°C. Sedimentation coefficients were determined on a Model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) run at 40,000 rpm and all results were corrected to standard conditions. Circular dichroism measurements were done on a J-41A spectrophotometer (Jasco Inc., Easton, MD) using a cell with a 1 cm path length.

Fluorescence intensity and anisotropy were measured on a computer-interfaced fluorescence anisotropy spectrometer (Ayres et al., 1974).

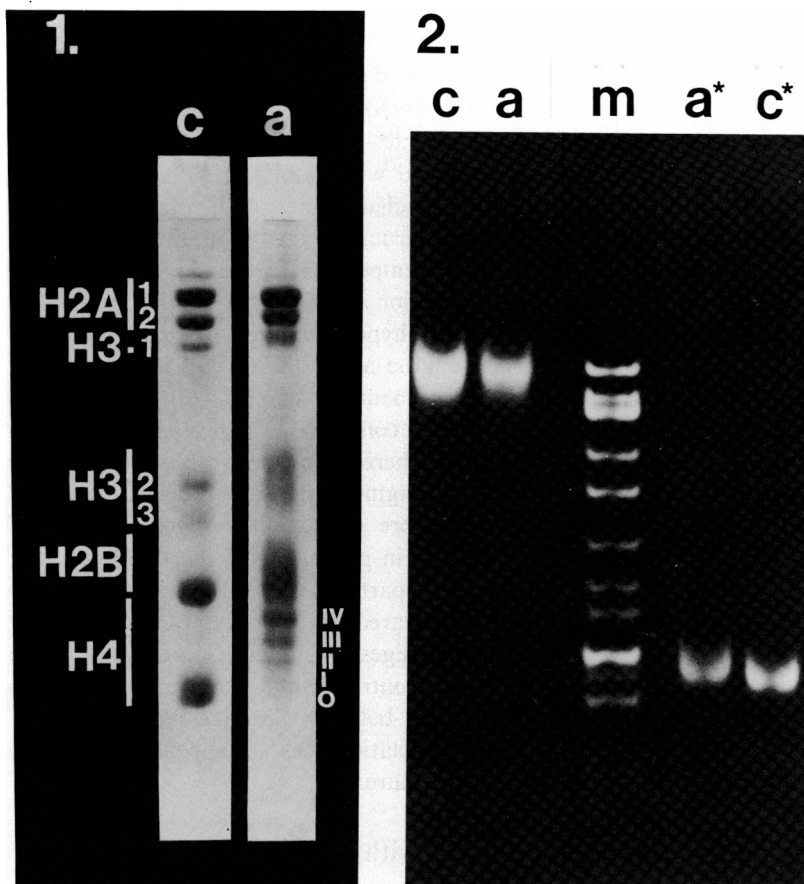


FIGURE 1 Electrophoretic characterization of the core particles used in this study. (1) Urea-triton-acetic-acid gel electrophoresis of the histones from control (lane *c*) and hyperacetylated (lane *a*) core particles. Histone variants are indicated by arabic numbers to the right of the histone designations. The number of acetyl groups per H4 molecule is indicated by the roman numerals to the right of the gel lanes. (2) Nondenaturing electrophoresis of core particles (lanes *c* and *a*) and of the corresponding DNA (lanes *a*\* and *c*\*). Lane *m* is a marker of pBR322 digested with CfoI restriction endonuclease.

Excitation was with the 280-nm line of a mercury-xenon arc isolated through two grating monochrometers and polarized with a double Glan-Taylor prism polarizer (Karl Lambrecht, Corp., Chicago, IL). Emission was detected at right angles to both the propagation and polarization directions of the exciting light. Emission at 298 nm was isolated through a grating monochrometer after passage through a double Glan-Taylor prism polarizer oriented either parallel ( $F_1$ ) or perpendicular ( $F_2$ ) to the excitation polarizer. Stray scattered excitation light was removed with a 2 mm WG 295 cutoff filter (Schott Glass Technologies, Inc., Duryea, PA). After sensitivity correction (Ayres et al., 1974), fluorescence intensity was calculated as  $F = F_1 + 2F_2$  and the anisotropy was calculated as  $r = (F_1 - F_2)/F$ .

The use of 298 nm fluorescence emission, dictated by the presence of tryptophan-like impurities, is a departure from previous work of this laboratory on nucleosomes (Libertini and Small, 1980; 1982; 1984; 1987) and that of Isenberg's laboratory on histones (Isenberg, 1979). These earlier studies made use of CS 0-54 cutoff filters (Corning Glass Works, Corning, NY) and 325 nm emission. Measuring the weak tyrosine fluorescence of core particles at 298 nm is made possible by the Schott Glass WG 295 filter which has a sharp cutoff wavelength with a half-transmittance near 295 nm. The wavelength 298 nm was chosen because it falls between the major water Raman bands for 280 nm incident light. Effects due to residual Raman scattering can be easily corrected.

Although use of the shorter emission wavelength brings with it an increased possibility of contamination by stray scattered light, there are a number of significant advantages. Since tyrosine fluorescence has an unusually small Stoke's shift, measuring its emission very close to the excitation wavelength greatly reduces the relative fluorescence contribution by impurities with longer Stoke's shifts. Such impurity fluorescence may derive from solvent impurities, from tryptophan containing impurities, from excited state processes such as proton transfer (observed for example in histone H1; Libertini and Small, 1985), or from photochemi-

cal reactions of tyrosine (see for example Malencik and Anderson, 1987; Small and Anderson, 1987). Also, the steady state tyrosine anisotropy is higher at shorter emission wavelengths, presumably due to less depolarization by vibrational relaxation (Libertini and Small, 1985). Measurements of higher anisotropy should have intrinsically higher signal-to-noise.

## RESULTS AND DISCUSSION

### Nuclease Digestion

Simpson (1978) noted that, while the rate for micrococcal nuclease digestion of bulk chromatin from HeLa cells was unaffected by butyrate treatment, monomer nucleosomes were preferentially excised from hyperacetylated regions of the chromatin in the early stages of digestion. He further noted that  $^{32}\text{P}$  is removed from 5'-end-labeled hyperacetylated particles two- to three-fold faster than from control. (It is not possible to determine from his data whether this difference might be due to extended ends on his acetylated core particles as was observed here.) An increased susceptibility to digestion of linker DNA in hyperacetylated regions was further indicated during preparation of the core particles studied here. When fractionated chromatin which had been stripped of H1 and most nonhistone chromosomal proteins was digested, the optimal time required to give a good yield of core particles was two- to three-fold shorter for hyperacetylated chromatin than for control.

The shorter digestion time, however, resulted in a somewhat longer average DNA length—151 bp for hyperacetylated particles as compared with 146 bp for the control. The criteria used to determine the time of digestion to core particles are a narrow range of DNA lengths without excessive production of DNA fragments shorter than 146 bp. One explanation for a longer average DNA length would be that the sensitivity of the intra-core DNA to digestion is enhanced by hyperacetylation even more than for the linker. This would lead to termination of the digestion before all the linker DNA was eliminated. To test this possibility we compared the rate of production of sub-146 bp DNA fragments when purified core particles were further digested with micrococcal nuclease. Results are presented in the left column of Fig. 2. The most obvious difference is the rapid reduction of the longest DNA in the hyperacetylated particles from 151 bp to ~146 bp. Otherwise the two results are very similar. (There may be a slightly enhanced production of a 130 bp fragment and a slightly decreased production of a fragment of ~90 bp from hyperacetylated core particles, but the differences are too small to be important to the arguments made here). We conclude that hyperacetylation has no major effect on the micrococcal nuclease sensitivity of core particle DNA.

Certainly the minor differences which may exist are not sufficient to account for the longer average DNA length of the hyperacetylated cores. Also it seems unreasonable to suggest that the last few base pairs on incompletely digested particles are more difficult to remove from hyperacetylated particles. Thus the source of the difference remains unknown.

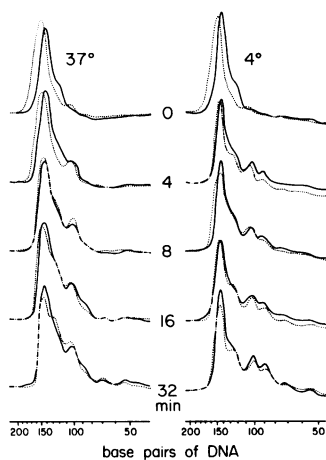


FIGURE 2 Comparison of the relative rates of micrococcal nuclease digestion and the effect of temperature on products of digestion for control (solid lines) and hyperacetylated (dotted lines) chromatin core particles. The core particle concentration during digestion was  $\sim 4 A_{260}$  units per ml in 0.01 M Tris/HCl, pH 7.5, 0.025 M NaCl and 1 mM  $CaCl_2$ . The nuclease concentration was 4.5 units/ml at 37°C (left column) and 45 units/ml at 4°C (right column). The reaction was started by adding the nuclease. At the times indicated in the figure, digestion

was stopped by diluting aliquots of the reaction mixture 1:1 with 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, 20% glycerol, 0.4% SDS and 0.3% bromophenol blue (pH 7.2). After warming the samples to 37°C for at least 5 minutes, aliquots were electrophoresed at 4°C on 4% polyacrylamide gels (the volume loaded was increased with digestion time), stained with ethidium bromide, and photographed under ultraviolet illumination. The figure shows densitometer scans of the resulting negatives. Logarithmic scales for the DNA base-pair axes were calibrated with fragments from pBR322 DNA which had been digested with CfoI restriction endonuclease.

## Core Particle Changes with Temperature

When chromatin is digested at 4°C with micrococcal nuclease, there appears to be an enhanced sensitivity of the intra-core DNA at a site  $\sim 20$  bp from the center of the core DNA when compared with results at 37°C (Huang and Garrard, 1986). This change presumably indicates a temperature-dependent structural transition of the chromatin subunit within this temperature range. Fig. 2 includes a time course for digestion at 4°C (right-hand column). For both the control and hyperacetylated particles, enhanced cutting  $\sim 20$  bp from the center of the DNA is suggested by a well defined fragment of length  $\sim 90$  bp which is distinctly increased as compared with results at 37°C (left column). Curiously, there is no comparable enhancement of a corresponding fragment at  $\sim 50$ – $60$  bp; perhaps it is released from the core and quickly digested. Also of interest is a decrease in proportion of the fragment near 130 bp at 4°C which, particularly for the control, appears not to change much in area relative to the 146 bp peak over the entire range of digestion times. Comparison of the hyperacetylated and control results, however, indicates no significant differences between the digestion patterns in Fig 2; thus hyperacetylation does not appear to affect the changes with temperature.

## Transition with pH

Chicken erythrocyte core particles undergo a relatively weak transition with pH near pH 7 over a wide range of ionic strengths including near physiological (Libertini and Small, 1982, 1984). This transition results in small changes in tyrosine fluorescence anisotropy and in the CD near 283 nm. Both suggest a loosening of the structure at higher pH. No significant changes in sedimentation coefficient with pH were detected.

Control and hyperacetylated HeLa core particles both experience a transition with increasing pH as evidenced by the small tyrosine anisotropy changes shown in Fig. 3. The shape of the changes observed as well as the overall magnitude of the anisotropy drop with increasing pH are comparable to those seen for erythrocyte particles and are consistent with titration of groups with a  $pK_a$  of  $\sim 7$ . The apparent downward shift of the curve for the hyperacetylated particles relative to that for the control may indicate a slightly looser structure for the hyperacetylated cores over the entire pH range; however, it may also arise from a somewhat higher level of tryptophan-like fluorescent impurities in the hyperacetylated preparation (see Materials and Methods). Otherwise the two results are indistinguishable, again indicating no measurable effect of hyperacetylation on this transition.

## Effect of High Salt Concentrations

The effects of increasing ionic strength above 10 mM on the tyrosine fluorescence intensity and anisotropy of con-

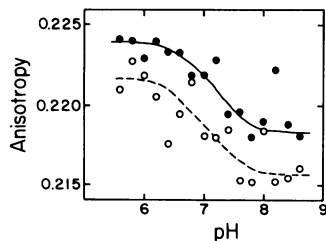


FIGURE 3 Effect of hyperacetylation on the pH transition of chromatin core particles. Core particles were first adjusted to about  $6 \mu\text{M}$  in  $100 \text{ mM}$  NaCl without buffer using a Centricon-30 microconcentrator. Samples were prepared by diluting this stock into solutions contain-

ing  $100 \text{ mM}$  NaCl and  $10 \text{ mM}$  buffer at the given pH's. The final core particle concentration was  $0.4 \mu\text{M}$ . Buffers used were MES (pH 5.6–6.6), PIPES (pH 6.2–7.2), MOPS (pH 6.8–8.0) and BICINE (pH 7.6–8.6). Tyrosine fluorescence was measured 1 h after sample preparation. When results were obtained at the same pH for two different buffers, the resulting anisotropy values were averaged for plotting. Results are shown for control (filled circles) and hyperacetylated (open circles) core particles.

control and hyperacetylated core particles are shown in Fig. 4. The changes observed in these parameters arise from a number of factors.

Since tyrosine fluorescence is partially quenched by radiationless energy transfer to DNA bases (Libertini and Small, 1982), intensity measurements can report on changes in the proximity of tyrosine residues and the DNA. Thus the small decrease in intensity between 0.01 and 0.2 M salt suggests a contraction in the size of core particles with increasing ionic strength. A similar suggestion has been made based on sedimentation measurements (Burch and Martinson, 1980). No measurable effects of acetylation in this concentration range are observed.

Between 0.2 and 0.8 M NaCl the tyrosine intensity increases while the anisotropy drops slightly. The histones do not dissociate extensively from the DNA within this ionic strength range (Germond et al., 1976); however, free core particle DNA can be detected in amounts which increase somewhat with increasing salt concentration (Ausió et al., 1983; Yager and van Holde, 1984) and the observed changes may arise from this partial dissociation. It is also possible that the fluorescence changes from 0.2 to 0.8 M salt represent a swelling of the particle with increasing salt, which would increase the average tyrosine-DNA base distance and could loosen the internal structure (thus increasing the average mobility of the tyrosines). Such a swelling is evidenced by a decrease in sedimentation coefficient with increasing salt and may be responsible for a corresponding increase in circular dichroism in the vicinity of 283 nm (Ausió and van Holde, 1986). However, none of the observed changes indicate any measurable difference in the manner in which the control and hyperacetylated particles react to increasing ionic strength.

When the salt concentration is raised enough to disassociate the histones from DNA (above 0.8 M salt), the quenching is relieved, which results in the observed sharp increase in intensity. An accompanying sharp drop in anisotropy results from two factors, partially from the increased average decay lifetime and secondly from

changes in tyrosine mobility when the histones dissociate as H2a-H2b dimers and  $(\text{H3-H4})_2$  tetramers. Again these changes are virtually identical for the two preparations, indicating that hyperacetylation does not significantly affect the way in which the histones interact electrostatically with and dissociate from the DNA.

Above 1.4 M salt, where the intensity is relatively constant, the anisotropy shows a slow increase with increasing ionic strength. This change, which can also be observed with histone complexes in the absence of DNA (Libertini, unpublished observations), most likely reflects reassociation of the H2a-H2b dimers and H3-H4 tetramers to form hexamers and the complete octamer, a process which is known to be enhanced by increasing ionic strengths (Eickbush and Moudrianakis, 1978; Godfrey et al., 1980). Once more the changes above 1.4 M salt suggest that reassociation of the core histones to form larger complexes at high salt is minimally affected by histone hyperacetylation.

### Effect of Low Salt

When core particles or nucleosomes are taken to low ionic strength ( $<10 \text{ mM}$  monovalent salt), they undergo a transition to a more open conformation. This transition is accompanied by changes in a number of physical parameters. Wu et al. (1979), on the basis of transient electric dichroism measurements, suggest that the low-salt form of core particles is dramatically different than the normal moderate-salt form. This is supported by sedimentation measurements which show that the sedimentation coefficient of core particles may be decreased from the usual

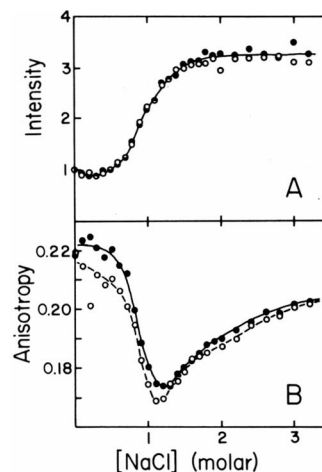


FIGURE 4 Effect of hyperacetylation on high-salt dissociation of chromatin core particles as monitored by tyrosine fluorescence intensity (A) and anisotropy (B). Core particles were first adjusted to  $1.5 \mu\text{M}$  in  $10 \text{ mM}$  Tris/HCl,  $0.1 \text{ mM}$  EDTA, pH 7.5. Samples were prepared by diluting this stock into solutions containing  $10 \text{ mM}$  Tris/HCl, pH 7.5 and sufficient NaCl to give the indicated final concentrations and  $0.3 \mu\text{M}$  core particles. Tyrosine fluorescence was measured  $\frac{1}{2}$  h after sample preparation. Results are shown for control (filled circles) and hyperacetylated (open circles) core particles.

10-11 S to a value as low as 6 S at very low salt (Libertini and Small, 1987, but see results on crosslinked particles below).

The low-salt transition is thought to be driven by electrostatic repulsion arising from the high overall negative charge ( $>140$ ) which can be calculated for particles near pH 7. This is suggested by the fact that, whereas core particles undergo the low-salt transition at or below 1 mM ionic strength (Schlessinger, et al., 1982), nucleosomes containing 170 bp or more of DNA, with their much higher charge, experience a transition at  $\sim 3$  mM ionic strength (Burch and Martinson, 1980; Schlessinger, et al., 1982). Also, the low-salt transition shifts to higher salt with increasing pH (Libertini and Small, 1982); titration of this effect suggests that it results from an increase in overall negative charge caused by deprotonization of lysines and other groups on the particle (Libertini and Small, 1984).

The effect of low salt concentrations on the sedimentation coefficient of control and hyperacetylated core particles is shown in Fig. 5. As noted previously (Ausio and van Holde, 1986), above 10 mM salt the hyperacetylated particles have an S-value significantly smaller than that of the control; this comparison holds up to an ionic strength of 0.75 M, just below that which begins to dissociate the particles. Below 10 mM a relatively sharp transition occurs in which the S-value decreases dramatically to below 6 S. The results for control HeLa core particles are very similar to those obtained for chicken erythrocyte cores, while those for the hyperacetylated particles follow a similar curve but are shifted to  $\sim 2$ -fold higher ( $M^+$ ).

Circular dichroism at wavelengths above 250 nm is sensitive to the overall conformation of the DNA in core particles. The ellipticity of DNA near 280 nm decreases dramatically, from  $\sim 9,000$  deg-cm/decimole phosphate when in solution to below 2,000 when it is incorporated into core particles (Cowman and Fasman, 1978; see Fig 6 B). The effect of low ionic strength on the ellipticity at 283 nm of HeLa core particles is plotted in Fig. 6 A. Again, the results for control HeLa particles are indistinguishable from those for erythrocyte cores and show a transition, centered just above 0.1 mM ( $M^+$ ), characterized by a

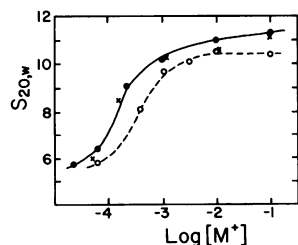


FIGURE 5 Effect of hyperacetylation on the low-salt transition of HeLa chromatin core particles as measured by sedimentation. The filled circles and solid line represent results for the control particles and the open circles and dotted line represent data for the hyperacetylated core particles. For comparison, results obtained for chicken erythrocyte core particles are included (small x's, Libertini and Small, 1987). Core particles were first adjusted to a concentration of  $\sim 20 \mu M$  in 1 mM Tris/HCl, pH 7.5 using a Centricon-30 microconcentrator. Samples were prepared by diluting this stock into unbuffered salt solutions to give the indicated total  $[M^+]$ . The final core particle concentration was  $0.5 \mu M$ . The final pH was  $\sim 6$ .

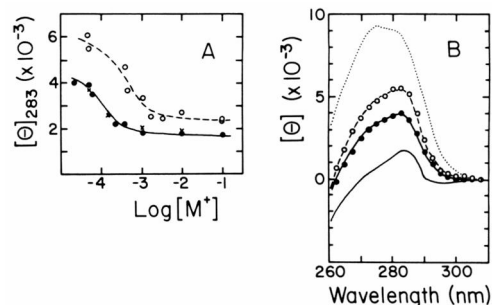


FIGURE 6 Effect of hyperacetylation on the low-salt transition of HeLa chromatin core particles as measured by circular dichroism. (A) Effect of salt concentration on the ellipticity near 283 nm. Filled circles (solid line) and open circles (dotted line) represent results for the control and hyperacetylated core particles, respectively. For comparison, results for chicken erythrocyte core particles are included (small x's, Libertini and Small, 1987). (B) Comparison of the CD spectra of DNA (dotted curve, Cowman and Fasman, 1978), control HeLa core particles in 10 mM NaCl (solid line without symbols), control HeLa core particles at very low salt ( $\sim 50 \mu M [M^+]$ , filled circles) and hyperacetylated core particles at very low salt ( $\sim 50 \mu M [M^+]$ , open circles). The lines drawn through the latter two sets of data are linear combinations of the core particle at 10 mM salt and DNA curves (see text). Samples were prepared as in the caption to Fig. 5. The units for ellipticity are deg-cm<sup>2</sup>/decimole phosphate.

doubling of the ellipticity as the ionic strength is lowered. Hyperacetylated particles show a somewhat larger increase in ellipticity in a transition which is again shifted to higher salt concentrations than for the control.

The shape of the CD spectrum is affected at very low salt (Fig. 6 B) in a manner which suggests that much of the DNA is changing to a conformation more similar to that of free DNA in solution. This is shown by the agreement between the experimental data (the filled and open circles were obtained for control and hyperacetylated particles at very low salt) and the lines drawn through the data. These lines are linear combinations of the spectra shown for DNA and for core particles at moderate salt. For control particles at very low salt the best fit, determined by a simple least squares procedure, consisted of 31% free DNA and 69% folded core particle spectra. For the hyperacetylated cores the percentage of free DNA spectrum required increased to  $\sim 37\%$ . (We note that, examination of the resulting deviation functions suggests that the two basis spectra used free DNA and core particles at moderate salt, are not sufficient to adequately fit the data at very low salt; however, such a conclusion would require a much more sophisticated analysis of higher quality data.) Thus acetylation appears to decrease the proportion of DNA which is modified in ellipticity by its binding to the histone core. (Note that, without a knowledge of the correct basis spectrum for the "modified" DNA, no more detailed quantitation is possible.)

As reported for erythrocyte core particles (Libertini and Small, 1987), no significant changes in the CD below 250 nm are observed for hyperacetylated core particles at very low salt. Since this region of the spectrum is dominated by

the histones (Zama et al., 1978), this result indicates that secondary structure of the histones is relatively unaffected during the low salt transition.

A more detailed examination of the low-salt transition using tyrosine fluorescence intensity and anisotropy is shown in Fig. 7. Results for control HeLa particles are again very similar to those reported for chicken erythrocyte cores (Libertini and Small, 1982, 1987) although the anisotropy values in Fig. 7 B are larger and vary over a somewhat wider range than for the erythrocyte cores because of the different emission wavelength used here to monitor the fluorescence. However, the overall change in the intensity, normalized to the result at 10 mM ( $M^+$ ), is essentially the same. The low-salt transition for hyperacetylated particles is similar in shape to that for the control, but broadened somewhat and shifted to a higher salt concentration by ~three-fold (as measured from the estimated midpoint of the anisotropy transition), in agreement with the sedimentation and CD results. The anisotropy at moderate salt is lower for the hyperacetylated cores but, as discussed in reference to Fig. 4, this difference is of uncertain origin. The lower anisotropy at very low salt may be due at least partially to a more complete low-salt transition at the minimum ( $M^+$ ) attainable, resulting from the shift of the transition to higher salt. This suggestion is supported by the much higher relative intensity attained for the hyperacetylated cores at very low salt, by the observation that the transition for hyperacetylated particles is clearly beginning to plateau at very low salt while the control transition is not, and by the larger increase in ellipticity found for the hyperacetylated cores at very low salt (Fig. 6 A).

The higher charge on acetylated core particles might be directly responsible for the observed shift of the low salt transition to higher salt. Calf thymus core particles should have an overall negative charge of ~124 at pH 6 in the absence of acetylation—218 lys + arg, 22 his, 74 glu + asp, and 290 DNA phosphates (McGhee and Felsenfeld, 1980). Assuming HeLa particles to have the same compo-

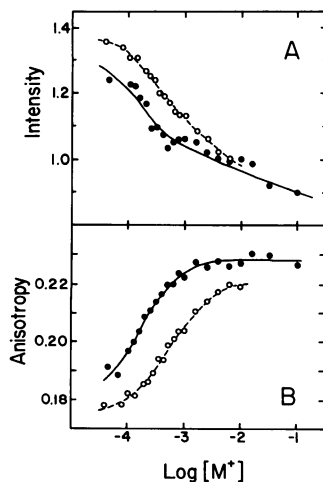


FIGURE 7 Effect of hyperacetylation on the low-salt transition of chromatin core particles as measured by tyrosine fluorescence intensity (A) and anisotropy (B). Filled circles and solid lines, control core particles; open circles and dotted lines, hyperacetylated core particles. Samples were prepared as described in the caption to Fig. 5.

sition, the average control particle would have an overall charge of  $-127$  while that of the average acetylated particle (taking into account that the average DNA length for the acetylated cores is 5 bp > for the control) would be  $-151$ , or ~20% higher overall charge. The electrostatic repulsion within acetylated core particles thus may be sufficiently greater at each salt concentration that the disruptions leading to the low salt transition occur at a higher ionic strength.

Since the low-salt transition certainly involves disruption of histone-histone interactions within the particle (histone crosslinking prevents the transition; Wu et al., 1979; Burch and Martinson, 1980; and see below), the shift of the transition to higher ionic strength might also indicate that the affected interactions are weakened by hyperacetylation. The strength of the electrostatic repulsion required to disrupt them would thus be less and could be attained at higher ionic strength. However, changes in the strength of histone-histone interactions might also be expected to affect reassociation of histones to the octamer at high ionic strengths; but as noted in reference to Fig. 4, there is no evidence of any differences over the range of salt concentrations where such interactions are expected (above 1.4 M salt). Alternatively, a small number of critical histone-DNA interactions might be disrupted by hyperacetylation and loss of these interactions could facilitate opening of the particle.

#### Effect of Protein Crosslinking on the Low-Salt Changes

The extraordinarily large change in sedimentation coefficient for core particles at very low salt has not been observed previously; the lowest values reported for core particles or nucleosomes were ~9 S (Burch and Martinson, 1980; Gordon et al., 1978; Libertini and Small, 1980; Simpson, 1981). This difference probably results from the fact that the salt concentrations obtained here are much lower than were reached previously. At salt concentrations in the range used by the above mentioned researchers, our results are generally comparable to theirs. The core particle DNA alone at moderate ionic strength has an S-value above five (Cotton and Hamkalo, 1981) and a similar value has been reported for core particles in 8 M urea, a condition which does not dissociate the protein from the DNA but which does disrupt both histone secondary structure and the DNA tertiary structure as indicated by large changes in the CD spectra (Olins et al., 1977). It is reasonable to ask whether the effects of low ionic strength could be as dramatic as those of 8 M urea.

One likely alternative is that part of the decrease in S-value may be artifactual due to effects of low salt in addition to the low salt transition. In order to investigate this possibility, we examined the effects of low salt concentrations on core particles which had been treated with a protein crosslinking agent, dimethyl suberimidate. Dimethyl suberimidate crosslinking of the histone component



of core particles has been reported to prevent the low-salt transition (Wu et al., 1979; Burch and Martinson, 1980), an observation which suggests that histone-histone interactions are disrupted at such low ionic strengths. Results are presented in Fig. 8. In Fig. 8 *A* the *S*-values of crosslinked control HeLa particles are compared with those of untreated particles. Both show large changes in sedimentation coefficient with decreasing ( $M^+$ ) although the *S*-values for untreated control are consistently lower than those for the crosslinked particles. In contrast, Fig. 8 *B* shows that crosslinked particles at very low salt (triangles) do not experience the large increase in ellipticity of the DNA which characterized the low salt transition for untreated particles, while particles which were treated identically but without addition of dimethyl suberimidate behave normally (circles). Similarly, Figs. 8 *C* and *D* show that the tyrosine fluorescence intensity and anisotropy are independent of ( $M^+$ ) over the range characteristic of the low-salt transition, in contrast to results for identically treated but uncrosslinked controls.

The CD and fluorescence results strongly suggest that the change in *S*-value for the crosslinked particles at very low salt is not directly related to the low-salt transition. A likely alternative explanation is a direct effect of the very low salt on the *S*-value as has been found for polyelectrolytes at low ionic strength (for a theoretical treatment of this effect, see Eisenberg, 1976). For untreated particles the larger overall change in sedimentation coefficient at low salt would derive from two factors: this "primary charge effect" combined with the effects of changes in core particle shape arising from the low-salt transition.

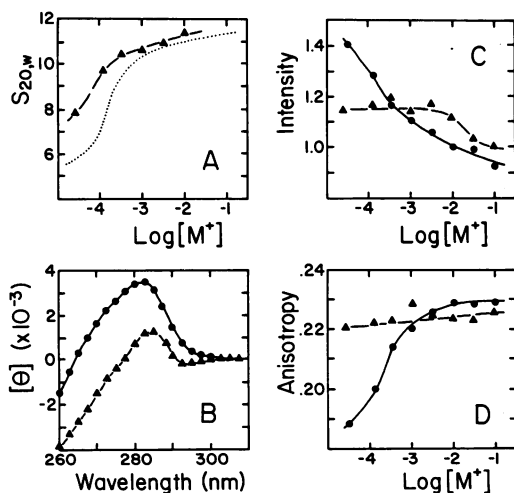


FIGURE 8 Effect of protein crosslinking with dimethyl suberimidate on the low-salt changes for control HeLa core particles at low salt. (*A*) Sedimentation. The dashed line represents results for untreated core particles taken from Fig. 5. The triangles show results for crosslinked particles prepared as described in Materials and Methods. (*B*) CD spectra of crosslinked core particles (triangles) and of particles treated identically without addition of dimethyl suberimidate (circles) which had been diluted to very low salt ( $\sim 50 \mu M [M^+]$ ). (*C* and *D*) Tyrosine fluorescence. Data symbols are as in *B*.

## Irreversibility of the Low-Salt Transition

When chicken erythrocyte core particles are exposed to very low salt concentrations ( $[M^+]$  below 0.2 mM) they experience a nonreversible secondary transition (Libertini and Small, 1987). Once exposed to such low ionic strength, the lack of reversibility shows up when the salt concentration is subsequently increased (which we will term a forward titration): the particles require a much higher salt concentration to begin refolding to the high-salt form than was required to unfold them with decreasing salt concentration (a back titration). Although very low ionic strength is required to attain those metastable conformations involved in the nonreversible effects, such conformations persist at relatively high ionic strengths. The low-salt transition is of interest because those histone-histone or histone-DNA interactions which are disrupted will be the weakest interactions within the core particle at any ionic strength. During replication and other chromatin processing it is likely to be these same interactions which are subject to disruption by other mechanisms. Similarly, the metastable forms arising from the nonreversibility are of interest because they may be representative of important conformations or intermediates in the rearrangements which occur *in vivo*.

HeLa control and hyperacetylated core particles also experience nonreversible effects as is illustrated in Fig. 9 (which includes the back titration curves from Fig. 7 for comparison). In contrast to the single step observed for the back titration, the forward titration for control HeLa particles (Figs. 9 *A* and *B*) results in three distinct regions indicating changes in the particles with increasing ( $M^+$ ): transition 1, a relatively small increase in anisotropy

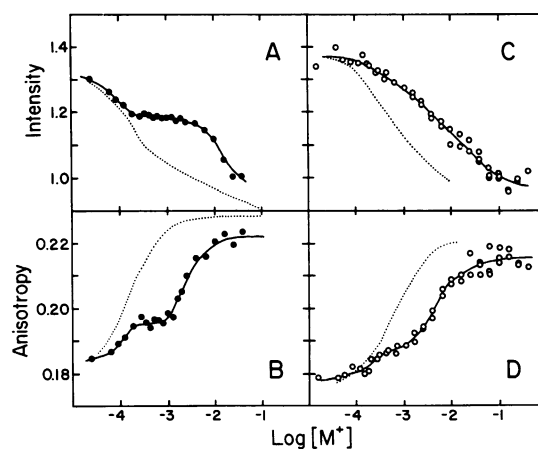


FIGURE 9 Nonreversible effects of exposure to very low ionic strength on the low-salt transition of control (*A* and *B*) and hyperacetylated (*C* and *D*) HeLa core particles. The dotted curves are redrawn from Fig. 7 for comparison. Data represented by circles were obtained by first diluting the core particle stock (prepared as in the caption to Fig. 5) into water to  $0.55 \mu M$  and an estimated  $[M^+] < 30 \mu M$ . At least 1 h later, aliquots of this diluted stock were mixed with  $1/5$  vol of salt solutions of concentration adequate to give the indicated final  $[M^+]$ . Fluorescence measurements were then made 1 h after the sample was completed.



accompanied by a similarly small decrease in intensity over an ( $M^+$ ) range near the lower part of the back titration results; transition 2, a larger increase in anisotropy with little change in intensity centered at about 10-fold higher ( $M^+$ ) than the back titration results; and transition 3, a relatively large decrease in intensity with little change in anisotropy centered at nearly 100-fold higher ( $M^+$ ) than the back titration. These results are indistinguishable from those obtained for chicken erythrocyte particles (Libertini and Small, 1987) except that, for the latter, the anisotropies near 0.1 M salt were the same for both titration directions.

For hyperacetylated core particles, anisotropy changes in the forward titration (Fig. 9 D) are similar although transitions 1 and 2 are both shifted to noticeably higher ( $M^+$ ) (~ two-fold). Also, transition 2 is noticeably broadened compared with the control, similar to the back titration comparison. In contrast to the control result, the intensity (Fig. 9 C) suggests one continuous change over the entire range of ( $M^+$ ); unlike the control, there is no plateauing of intensity over the range of transition 2 and no distinct transition 3 (although the intensity is still dropping, somewhat less sharply than for the control, over the corresponding range of [ $M^+$ ]). For hyperacetylated cores, it appears that either transition 2 affects the intensity as well as the anisotropy or transition 3 has shifted to lower ionic strength and is much broader than for control particles.

For both the control and hyperacetylated particles, the fluorescence parameters do not reach their expected values in the forward titration even at 0.1 M NaCl. For control cores the ellipticity at 283 nm was found to show a similar behavior, reaching a value of only 2,300 units ( $\text{deg}\cdot\text{cm}^2/\text{dmol}$  DNA phosphate) on forward titration to 0.1 M salt compared with 1,700 units when diluted directly to that ionic strength. Similarly the sedimentation coefficient for both control and hyperacetylated particles on forward titration to 0.1 M salt (10.9 and 10.2 S, respectively) were somewhat smaller than those obtained on direct dilution (11.3 and 10.4 S, Fig. 5). Thus, even near physiological salt the core particles do not appear to have refolded completely.

A likely explanation of the nonreversible effects (Libertini and Small, 1987) is disruption of ionic bonds between the histones and the DNA due to strong electrostatic repulsion at very low salt. This process could be followed by rearrangements which allow formation of alternate ionic bonds involving some of the same groups. Then, as the salt concentration is increased in a forward titration, the particles would be constrained from assuming their normal conformation until the new ionic bonds become labile. Hyperacetylation could be expected to decrease the number of alternate ionic bonds and thus decrease the salt concentration required to labilize them. Furthermore, heterogeneity in the degree of acetylation could explain a broadening of transitions 2 and 3 as compared with the

control particles. In this context it is of interest that transition 2 is shifted to somewhat higher salt for hyperacetylated cores. Thus this transition may not require breaking of ionic bonds or the bonds involved are not weakened by hyperacetylation. (It has been suggested that transition 1 represents refolding of a small proportion of core particles which did not undergo the changes which lead to nonreversibility; Libertini and Small, 1987).

Hyperacetylation might also weaken the overall ionic interaction which must be broken to give the nonreversible effects. This could be reflected in a difference in the salt concentration required. By varying the minimum salt concentration to which the particles are exposed before adjusting them to ~1 mM [ $M^+$ ] and measuring the anisotropy, one can estimate the range required for the nonreversible effects. This is shown in Fig. 10. The resulting anisotropy is observed to increase up to ~0.2 mM [ $M^+$ ] where it levels off to a value comparable to that obtained when the particles are diluted directly to 1 mM salt. The cutoff value of ~0.2 mM [ $M^+$ ] is the same as that found for chicken erythrocyte core particles (Libertini and Small, 1987). Thus, in this case again, hyperacetylation appears to have little or no effect.

#### SUMMARY

Most comparisons of the physical properties of acetylated and control core particles found at most only minor differences. The sedimentation coefficient was ~10% lower for acetylated particles, a significant and reproducible change, but certainly not a major one. Circular dichroism suggested a somewhat looser structure for the DNA (Ausio and van Holde, 1986). DNA melting profiles gave an increased proportion of the premelt transition and comparative DNase I digestions indicated that acetylation enhanced nicking at a location 60 bp from the 5'-end of the

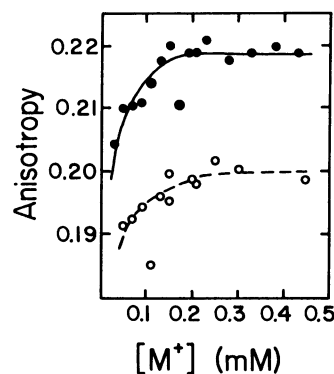


FIGURE 10 Effect of salt concentration during unfolding on reversibility for control (filled circles) and hyperacetylated (open circles) HeLa core particles. A concentrated core particle stock solution prepared as in the caption to Fig. 5 was diluted into unbuffered NaCl solutions to the indicated [ $M^+$ ] (including that estimated as contributed by the stock) and held at 20°C for ~1 h. Then 1/5 vol of a salt solution sufficient to raise the total [ $M^+$ ] to 1 mM was added and the tyrosine fluorescence measured ~1 h later.

DNA (Simpson, 1978; Ausió and van Holde, 1986). Also, micrococcal nuclease attacked the 5'-end of the particle about twice as fast for acetylated cores (Simpson, 1978). When examined by neutron scattering no differences were apparent (Imai et al., 1986).

The results presented here on effects of acetylation on core particle transitions similarly indicate little difference. Only the shift of the low-salt transition to higher ionic strength and differences in the nonreversible effects of very low salt suggest any change due to acetylation. The first very likely arises from the increased total charge on acetylated particles. The origin of the latter difference is uncertain because of the complexity of the effects observed.

The absence of a change in high-salt dissociation suggests that the more important ionic interactions between the histones and 145 bp DNA are not significantly modified by acetylation, in agreement with earlier conclusions (Ausió and van Holde, 1986). Also, the lack of difference under high salt conditions known to induce association of the histones to the free octamer suggests that the principal interactions between the histones are not affected.

However, hyperacetylated regions are solubilized by micrococcal nuclease more rapidly than the remaining chromatin (Simpson, 1978). Also, we have found that the linker region is attacked more readily in hyperacetylated stripped chromatin. Taken together with the minimal effect of acetylation on core particles, these observations clearly suggest that many of the lysines which are subject to acetylation may be involved in interactions with the linker DNA. The lower rate for digestion of the linker in control chromatin would follow from the postulated larger number of lysines interacting with it. Thus a comparison of the physical properties of acetylated and control nucleosomes which contain linker DNA might be instructive. It is perhaps significant in this context that studies in which rather dramatic differences between control and acetylated particles were reported were done on particles containing linker DNA (Bode et al., 1983; Bertrand et al., 1984).

In control chromatin, removal of the linker DNA would free any lysines (and arginines) which were interacting with it. Present structural models of the core particle indicate that the DNA is wound 1.75 times around the histone core. Thus the linker DNA would be expected to lie on either side of the central portion of the 145 bp core DNA (and, of course, near the ends). In core particles, those charged residues which would have otherwise bound to the linker could be expected to interact now with the core DNA, particularly at the ends and near the middle. If as was suggested above, the lysines which are acetylated *in vivo* are primarily those which interact with the linker, there would be a larger number of extra interactions in control core particles. These extra interactions might thus be responsible for the observed decrease in nuclease sensitivity of the site 60 bp from the 5' end (i.e., near the middle

of the core DNA) and for the decreased sensitivity of the DNA ends observed with control core particles. Furthermore, they might affect the strength with which the DNA, particularly near the ends, is bound by the histone core and thus change the premelting behavior or induce the small differences observed between control and hyperacetylated core particles in the sedimentation coefficient and molar ellipticity of the DNA. It is interesting to note that such "extra interactions" could mean that the core particle is not a good model for the corresponding structure in chromatin.

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