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Histone hyperacetylation has little effect on the higher order folding of chromatin

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ABSTRACT

HeLa cells were grown in the presence of 10 mM sodium butyrate and soluble chromatin containing hyperacetylated histones was prepared by mild micrococcal nuclease digestion and sucrose gradient fractionation. Sedimentation and electric dichroism were used to study the cation-induced folding of this acetylated chromatin from the 10 nm filament to the 30 nm solenoid conformation. Although under some conditions acetylated chromatin appears slightly less condensed than control chromatin, the major conclusion is that hyperacetylation of histones does not in itself prevent the formation of the higher order chromatin solenoid.

INTRODUCTION

Acetylation occurs postsynthetically at selected lysine residues in the N-terminal "tail" regions of nucleosome histones and has long been correlated with gene activity (reviewed in reference 1). A popular model is that histone acetylation, since it removes the lysine positive charge, might somehow "loosen" histone-DNA interactions thereby "opening up" certain genes to transcription.

The discovery (2) that sodium butyrate induces histone hyperacetylation allowed more convenient study of the physical interactions between acetylated histones and DNA. The slightly unexpected result from a number of studies was that histone hyperacetylation apparently had little effect on the stability of the nucleosome core particle (3-5). This focused attention on the possibility that histone acetylation could destabilize chromatin higher order structure. Indeed, implicit in most current studies of acetylation and gene activity is the model that histone acetylation disrupts or unwinds the thick chromatin fiber or "30 nm solenoid".

In this paper, we use electric dichroism (6, 7) and analytical centrifugation (7, 8) to monitor the cation-induced filament $\rightleftarrows$ solenoid transition of acetylated HeLa chromatin. We show that histone hyperacetylation does not prevent the formation of the chromatin solenoid, although we find

some evidence that the acetylated solenoid is slightly less condensed.

MATERIALS AND METHODS

HeLa cells strain S3 were obtained from Flow Laboratories and grown in spinner flasks in Eagle's MEM with 7% horse serum. When cells had reached mid to late log phase, half of the culture was incubated for 12 hours with 10 mM sodium butyrate (at which time pilot experiments had revealed that histone hyperacetylation was close to maximal). Butyrate-treated and control cultures were processed exactly in parallel. Cells were chilled, collected by centrifugation (2,500 g for 10 mins), washed 2-3 times in cold PBS (0.795 g/l Na_2HPO_4, 0.144 g/l KH_2PO_4, 9 g/l NaCl) + 5 mM Na butyrate, washed 3 times (1000 g x 5 mins) with 1/20-1/100 volumes (relative to the original culture volume) of 100 mM NaCl, 50 mM Tris·HCl, 5 mM Na butyrate, 3 mM $MgCl_2$, pH 8 with 0.1 mM PMSF (added freshly from a 50 mM stock solution in isopropanol), washed 5 times (3000 g for 5 mins) in the same buffer containing 0.2% Triton X-100 (incubating for 5 minutes in ice between each wash), washed three times (1000 g for 5 mins) in 100 mM NaCl, 50 mM Tris-HCl, 5 mM Na butyrate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 8.0, with 0.1 mM PMSF and finally suspended at A_{260} ~ 100 in the same buffer.

Nuclei were prewarmed at 37° for 5 minutes and incubated for a further 15 minutes at 37°C, with 0.005 to 0.025 units of micrococcal nuclease/ml (units as defined in reference 9), the digestion quenched by excess EDTA or EGTA, chromatin solubilized by overnight dialysis against two changes of 0.25 mM EDTA and insoluble material removed by centrifugation (8000 g for 20 minutes). Overall yields of soluble chromatin were in the range of 30-80%, depending on the nuclease concentrations. Variations on the above protocol (e.g. use of several different strains of HeLa cells, inclusion of the phosphatase and protease inhibitor p-chloromercuriphenylsulfonate (10) in the nuclear washes, gentle sedimentation of the digested nuclei prior to dialysis or dialysis vs. 5 mM Tris-HCl, 0.1 mM EDTA, 1 mM Na butyrate to solubilize chromatin) led to essentially identical conclusions, although yields and behaviors, etc. varied slightly.

Soluble chromatin (A_{260} = 30-70) was treated for 15 minutes at 20 ± 2°C with 1 μg/ml ribonuclease A (Sigma, dissolved at 2 mg/ml in 1 mM HCl and boiled for 5 minutes just before use), loaded on an isokinetic sucrose gradient containing 25 mM NaCl, 5 mM Tris-HCl, 0.1 mM EDTA, 1 mM Na butyrate and centrifuged (SW 40 rotor) at 4°C for 3-4 hours at 35,000 rpm. Collected fractions were dialyzed extensively against: (1) for electric dichroism,

0.13 mM cacodylic acid, 0.12 mM NaOH, 0.003 mM Na_2 EDTA, pH 7; or (2) for centrifugation, 5 mM Tris-HCl, 1 mM Na butyrate, 0.1 mM Na_2 EDTA, pH 8.

Histone acetylation was monitored on polyacrylamide gels containing acetic acid, urea and Triton X-100, essentially as described in (11). The RNA content of chromatin fractions was estimated by incubation with 0.2 M NaOH for 24-48 hours at 37°C, chilling, mixing with an equal volume of cold 2 M NaCl, 2 M perchloric acid, and centrifugation (12,000 g for 10 mins). The A_{260} of the supernatant (after correction for the nonalkali treated control) reflects the RNA content within roughly a factor of 1.5, the uncertainty in the optical hypochromicity of the contaminating RNA. In several instances, we checked that the RNA content estimated by alkaline hydrolysis agreed acceptably with the unsedimentable A_{260} resulting from ribonuclease treatment.

Measurement of electric dichroism and analytical ultracentrifugation were essentially as previously described (6, 7).

RESULTS

Removal of RNA from Chromatin Preparations

We found that both butyrate-treated and control HeLa chromatin prepared by mild micrococcal nuclease digestion invariably contained 10-30% RNA (RNA A_{260}/total A_{260}, estimated by alkaline hydrolysis as described in the Materials & Methods section) even after sucrose gradient fractionation. Such substantial RNA levels have been found in other preparations of HeLa chromatin (12, 13) and could obviously confuse the interpretations of physical measurements.

To determine conditions to remove this contaminating RNA, HeLa cells were labelled with ^{14}C-orotic acid (2 μCi/ml for 3 hours) and chromatin prepared by mild micrococcal nuclease digestion and dialysis to low ionic strength. Treatment of this soluble chromatin with 1 μg/ml (boiled) ribonuclease for 15 mins at 20 ± 2°C caused the majority of the radioactivity to become acid-soluble; 3 μg/ml ribonuclease did not lead to a significantly greater extent of digestion. Sucrose gradient fractions of chromatin pretreated with 1 μg/ml ribonuclease still contain 1-3% RNA but further RNase treatment does not change their sedimentation properties (data not shown).

The general effect of removing RNA from chromatin fractions is to increase their sedimentation coefficients by 10-20%. Lysozyme added at the same concentration has no effect, thus ruling out the possibility that the increased value of S is due simply to the addition of a polycation. Ribonuclease treat-

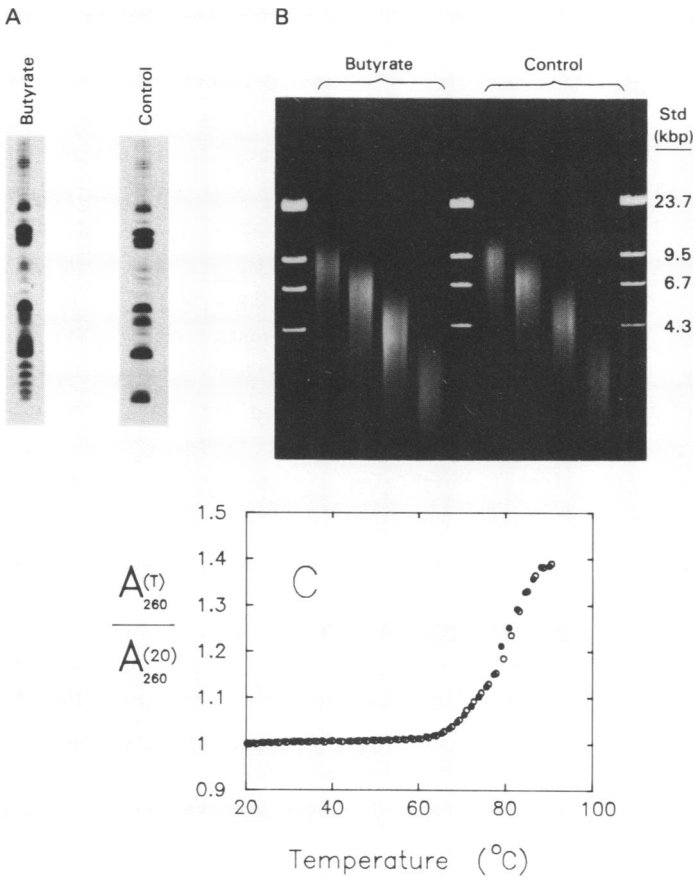

Fig. 1A. Triton-acetic acid urea gels of extracts of soluble chromatin prepared from HeLa cells, grown for 12 hours in the presence (left) or absence (right) of 10 mM Na butyrate. Acetylated histone H4 is the fastest migrating series of bands.

B. DNA content of chromatin in successive sucrose gradient fractions isolated from HeLa cells grown in the presence (left) or absence (right) of 10 mM Na butyrate; 1% agarose, stained with ethidium bromide.

C. Thermal denaturation profiles of typical chromatin fractions; (●) = hyperacetylated; (○) = control. Solvent = 5 mM Tris-HCl, 1 mM Na butyrate, 0.1 mM EDTA, pH 8.

ment of rat liver chromatin and CHO cell chromatin also causes an increase in S (data not shown). We do not know the source of the RNA contamination or the details of the RNA size and conformation. We have not further investigated why RNA removal should cause an increase in sedimentation rate. However, we question the accuracy of physical studies, especially optical measurements,

made on chromatin preparations in which the RNA content is undefined, and possibly large.

Characterization of Ribonuclease Treated Chromatin

Figure 1A shows Triton-acetic acid urea gels (11) on HCl extracts of soluble chromatin isolated from HeLa cells grown either in the presence or absence of 10 mM Na butyrate. The butyrate-enhanced acetylation is most apparent for histone H4, (the lowest band on the control gel). As measured from densitometer traces, butyrate treatment increases the average acetylation content of histone H4 from ~ 0.5 to ~ 2.5 acetyl group/molecule, similar to previously reported changes (2-5). The level of histone acetylation is unchanged in chromatin fractionated on sucrose gradients or retrieved from the analytical ultracentrifuge (data not shown).

Electrophoresis of total nuclear extracts on polyacrylamide gels containing SDS confirmed that butyrate treatment does not lead to significant changes in the overall protein composition of the chromatin (data not shown); in particular, we found no evidence for histone proteolysis or for significant concentrations of H1° (less than one molecule/20 nucleosomes as judged from overloaded SDS gels of perchloric acid extracts of nuclei).

Figure 1B shows the size distribution of DNA purified from the ribonuclease treated chromatin of adjacent sucrose gradient fractions. In a particular gradient fraction, the DNA sizes of the hyperacetylated and normal chromatin are similar; any difference in DNA size is within the variations found between replicate gradients. This observation in itself indicates that butyrate treatment does not cause large changes in chromatin conformation. We agree with previous reports (3, 14) that butyrate treatment has little effect on chromatin sensitivity to micrococcal nuclease digestion and on the resulting overall distribution of DNA sizes, either double or single stranded.

Figure 1C shows thermal denaturation profiles of typical (ribonuclease treated) hyperacetylated and control chromatin fractions. Butyrate treatment has no significant effect on the chromatin melting profiles, whether measured in the solvent used for centrifugation or in the solvent used for electric dichroism. This lack of Tm shift corroborates evidence cited in the Introduction (3-5) that histone hyperacetylation has little effect on the intrinsic thermal stability of chromatin DNA. Our results do not agree with the report of Reczek et al. (15) who studied the melting of unfractionated and rather short chromatin fragments that were of unreported RNA content.

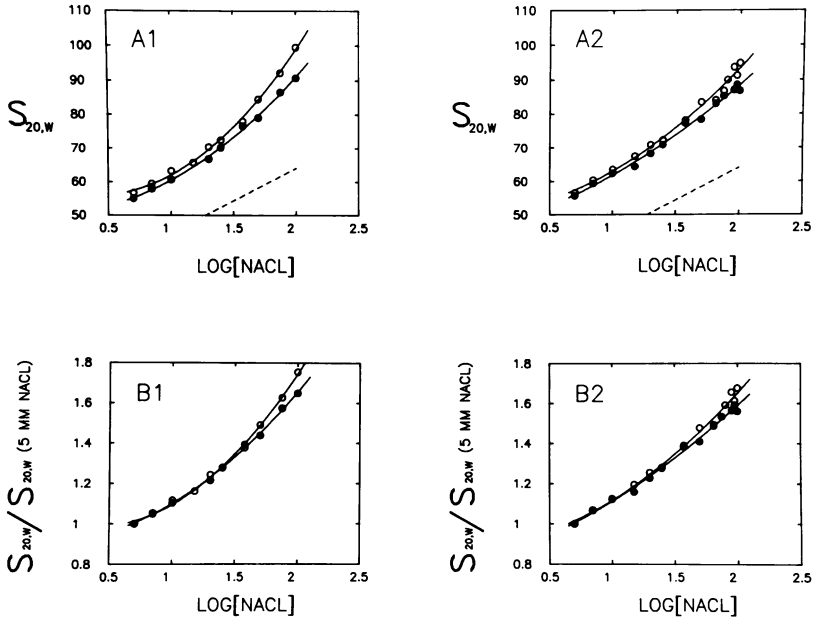

Fig. 2A. Plot of $s_{20,w}$ vs. logarithm of the added NaCl concentration (millimolar) for pairs of chromatin fractions, each ~ 35 nucleosomes in length. (●) = hyperacetylated; (○) = control chromatin. All samples also contained 5 mM Tris-HCl, 1 mM Na butyrate, 0.1 mM EDTA, pH 8. Solid lines are best fit quadratics; dashed line is behavior expected for chromatin stripped of the lysine-rich histones (8). Figures 2A1 (left) and 2A2 (right) represent data from complete duplicate experiments, starting from different cell batches.

B. Sedimentation data from Figure 2A, normalized to the $s_{20,w}$ measured at 5 mM NaCl, to correct for any possible DNA size differences between hyperacetylated and control chromatins.

Filament-Solenoid Transition in Hyperacetylated Chromatin

The reversible transition from the 10 nm filament (beaded string) to the condensed 30 nm fiber (solenoid) can be induced by addition of monovalent ions and monitored by increased sedimentation rate (7, 8), or induced by added divalent ion and monitored by changes in electric dichroic properties (6, 7). We now describe the use of both these physical techniques to compare the folding of hyperacetylated chromatin with control chromatin.

a) Sedimentation. Chromatin was isolated from both butyrate-treated and control cells, the majority of the contaminating RNA removed as discussed above, and sucrose gradient fractions containing closely similar DNA sizes were selected. Figure 2A shows the ionic strength dependence of $s_{20,w}$ measured for pairs of fractions of hyperacetylated and control chromatins. Figure 2A1

and 2A2 contain data from duplicate experiments (beginning from separate batches of cells) and show the reproducibility of the measurements. Over the range of 5-100 mM NaCl, $s_{20,w}$ for both acetylated and control chromatins increases by 60-70%. The dashed line in Figure 2A shows the sedimentation properties of H1-stripped rat liver chromatin (taken from reference 8 and adjusted for size differences) and is roughly the hydrodynamic behavior that might be expected if condensation into a higher order solenoid were prevented. The most obvious conclusion to be drawn from Figure 2 is that histone hyperacetylation does not prevent the chromatin condensation induced by Na^+ ions. We have measured chromatin sedimentation properties from 100 mM to 600 mM NaCl and also do not find a major difference between hyperacetylated and control chromatin (data not shown).

The data from Figure 2A1 and 2A2 do, however, show a small but reproducible difference between the sedimentation behavior of hyperacetylated and control chromatins. When measured at low ionic strength, sedimentation coefficients of the two chromatins are closely similar. However, when measured at 100 mM NaCl, $s_{20,w}$ for hyperacetylated chromatin is 5-10% lower than for control chromatin. As shown in Figure 2B1 and 2B2, this difference persists after normalization to correct for any slight differences in size of the starting material. For a particle of the dimensions of the chromatin solenoid, equations 73 and 77 of Garcia de la Torre and Bloomfield (16) can be used to calculate that, for the same DNA size, hyperacetylated chromatin appears 10-20% longer (i.e. less compact) than control chromatin. Alternatively, this small difference in sedimentation rate could reflect a decreased tendency for acetylated solenoids to interact with each other. Although we found no evidence for any differential precipitation effects, it is well known that acetylated chromatin is more soluble than normal chromatin (see for example 14).

b) Electric dichroism. Figure 3A plots values of ρ (the reduced dichroism) vs. the reciprocal electric field strength (1/E) for a typical butyrate-treated and a control chromatin fraction (from both of which the majority of RNA has been removed). Within the variation found in replicate experiments, the two samples behave identically, both in the filament conformation (i.e., in the absence of Mg^{++}) and in the solenoid conformation (i.e., in the presence of 2 Mg^{++}/DNA phosphate). Extrapolation to 1/E = 0 (i.e., infinite field strength and hence complete molecular orientation) can be made with little ambiguity. The value of this intercept (ρ_∞) for the filament conformation ($\rho_\infty \cong -0.45$) corresponds closely to the value of -0.49 predicted

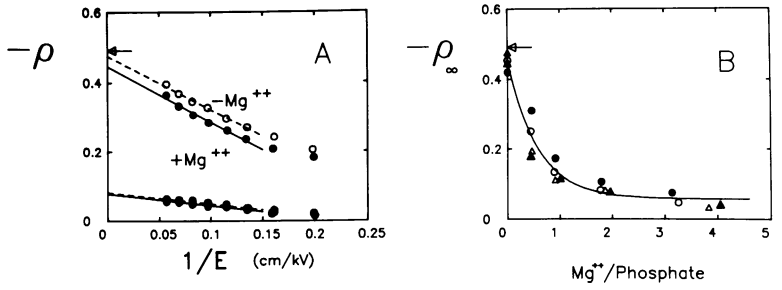

Fig. 3A. Plot of reduced dichroism, $\rho = (A_{\parallel}-A_{\perp})/A$ measured at 260 nm vs. the reciprocal electric field strength (1/E) for two chromatin fractions, ~ 30 nucleosomes in length. (●, ▲) = hyperacetylated chromatin (solid lines); (○, △) = control chromatin (dashed lines); (●, ○) = ρ measured in absence of divalent ions and hence chromatin in 10 nm filament conformation; (▲, △) = ρ measured in presence of 2 Mg^{++}/DNA phosphate and hence chromatin in the 30 nm solenoid conformation. All solutions contain 0.13 mM Na^{+} ions. Intercepts with ordinate axis (ρ_{∞}) represent linear extrapolations to 1/E = 0 and contain information about DNA arrangement in completely oriented particles. The arrow represents ρ_{∞} expected for the 10 nm filament conformation if the chromatosome faces as well as the spacer DNA are parallel to the filament long axis.

B. Plot of ρ_{∞} vs. Mg^{++}/DNA phosphate, showing the drop of dichroism from a value characteristic of the 10 nm filament conformation ($\rho_{\infty} \simeq -0.45$) to a value characteristic of the 30 nm solenoid conformation ($\rho_{\infty} \simeq -0.07$), at Mg^{++}/DNA phosphate > 2. (●, ▲) = hyperacetylated chromatin; (○, △) = control chromatin.

for a model in which the chromatosome faces are parallel to the direction of orientation (see reference 6 for details of the possible models).

Figure 3B plots the values of ρ_{∞} as a function of the number of Mg^{++} ions added per DNA phosphate and shows that both acetylated and control chromatins fold at equivalent Mg^{++} concentrations. For the fully condensed solenoid at 2-4 Mg^{++}/phosphate, ρ_{∞} can be interpreted to indicate that faces of the chromatosome discs are tilted ~ 30° from the solenoid axis (reference 6 and manuscript in preparation). Overall, the dichroism measurements confirm the above sedimentation results, namely that hyperacetylated chromatin is still capable of condensing into a higher order structure.

The field-free relaxation time is a much more sensitive reflection of overall particle dimensions than is the actual particle dichroism. With the addition of Mg^{++} to induce the formation of solenoids, the average relaxation times for both acetylated and control chromatin drop roughly fivefold. We find only a small and not entirely reproducible difference between hyperacetylated and control chromatins. In the presence of ~ 2 Mg^{++} ions added per DNA phosphate (i.e. when the chromatins are condensed into solenoids),

relaxation times can be interpreted to suggest that acetylated solenoids are 0-30% longer (less condensed) than control solenoids.

DISCUSSION

Histone acetylation has long been correlated with gene activity, and has generally been interpreted in terms of a "loosening of chromatin structure". However, the results described above eliminate the extreme model in which histone acetylation completely prevents the transition of the chromatin 10 nm filament into the more condensed 30 nm fiber or solenoid. Both by the criteria of increased sedimentation rates and decreased dichroisms and relaxation times, hyperacetylated chromatin can still be condensed by monovalent or divalent ions. We must, of course, offer the ritual disclaimer that histone acetylation levels are assumed to be the only differences between our butyrate-treated and control chromatins. Nonetheless, other known effects of Na butyrate, such as inhibition of histone phosphorylation (17), might also be expected to lead to a chromatin decondensation, which was not observed.

While the extreme model that histone acetylation causes chromatin unfolding could have been conceivable in terms of, for example, very specific protein-protein interactions, such a model seems less likely in terms of more general energetic considerations. Cell growth in butyrate reduces the average number of histone positive charges by only 4 to 8 per nucleosome and this must be compared to a net negative charge per nucleosome of somewhere in the neighborhood of 200. Even if a sizeable fraction of these excess negative charges were neutralized by condensed counterions (18), hyperacetylated histones are unlikely to change the net nucleosome charge by more than 10 percent. While it is entirely possible that this magnitude of charge change could cause dramatic conformational changes under certain conditions (recall the abrupt collapse of DNA induced by polyamines; references 19, 20), it is perhaps not surprising that under most solvent conditions, we detect much more modest changes in behavior.

We had proposed earlier (6) that the chromatin solenoid might be stabilized by internucleosome histone-DNA bridges, the strength of which could thus be modulated by histone acetylation. The relatively minor increases (10-20%) in solenoid length that we have found to be associated with histone hyperacetylation is perhaps of the magnitude expected from this model. Even with the highest level of histone acetylation obtained in the present study, the histone tails, on the average, still retain more than 80% of their positive charge. The model has recently received more direct support from experiments

in which the postulated histone bridges have been completely removed by proteolysis (21).

In summary, our results certainly do not impugn the many excellent correlations that have been formed between histone acetylation and gene transcription. Our experiments use butyrate treatment of otherwise "normal" cells and thus do not address the possibility that in certain biological circumstances, such as histone acetylation prior to protamine substitution, chromatin structure can indeed be disrupted (22, 23). Our results do imply, however, that acetylation of the histones in otherwise normal chromatin is not by itself sufficient to cause gross disruption of the chromatin solenoid. It should be pointed out that, with exceptions such as very actively transcribing ribosomal genes, it has not yet been clearly demonstrated that the solenoid structure of the average differentiated gene has indeed been opened up or otherwise disrupted. On the contrary, we have presented evidence (7, 24, 25) that the chromatin of the chicken β-globin genes is still capable of folding into an apparently normal solenoid.

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