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Physical properties of chemically acetylated rat liver chromatin

(acetic anhydride/nucleosomes/thermal denaturation/histones)

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ABSTRACT **The physical properties of rat liver chromatin and nucleosomes acetylated with acetic anhydride were examined in order to clarify the mechanism by which chemical acetylation of histones increases template activity *in vitro* [Marushige, K. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3937–3941]. Acetylation was found to have dramatic effects on the magnesium solubility, nuclease sensitivity, thermal denaturation, and sedimentation of chromatin and nucleosomes. The significance of the results to models of gene activation and chromatin replication is considered.**

The five principal species of histone are associated with DNA to form the eukaryotic chromosome. These basic proteins are involved in the repression of template activity of chromatin (1, 2). It has long been thought that a degree of transcriptional control might be achieved by the selective modification of certain basic residues of the histones (3, 4).

In the current model of chromatin structure, 200 base pairs of DNA are complexed with histones to form the nucleosomal subunit. Although the nucleosome is a general feature of all eukaryotic chromatin thus far examined, there appears to be heterogeneity within the nucleosomes of any single cell type (5). Some of this heterogeneity consists of different conformations of nucleosomes of active and inactive genes (6, 7), which may result from histone modification (4).

Marushige (8) has reported that chemical acetylation of calf thymus chromatin increases its template activity *in vitro* without resulting in significant removal of the histones from the DNA. These results suggest that acetylation of histones participates in activation of genes for transcription. In this report we describe the effect of chemical acetylation on the physical properties of rat liver chromatin and nucleosomes.

MATERIALS AND METHODS

Preparation of Nuclei. Rat liver nuclei were prepared from frozen rat livers (Pelfreeze). All manipulations were performed at 0° unless otherwise stated. Livers were thawed in 0.25 M sucrose/10 mM Tris·HCl, pH 7.4/6 mM KCl/5 mM $Mg(OAc)_2$/0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*-*N*′-tetraacetic acid (EGTA). Livers were transferred to three volumes of the same buffer containing 1 mM phenylmethylsulfonyl fluoride (homogenization buffer) and homogenized with five to seven strokes in a motor-driven glass/Teflon homogenizer. The homogenate was diluted to 10 volumes with homogenization buffer, strained through cheesecloth, and centrifuged at 1500 rpm in an HG-4L rotor (Sorvall RC-3 centrifuge) for 10 min. The pellet was washed twice in 10 volumes of homogenization buffer containing 0.5% Nonidet-P40 (Particle Data Laboratories, Ltd), two to four times in homogenization buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet-P40, and finally once in homogenization buffer. The nuclear pellet was resuspended in homogenization buffer and an equal volume of 5 mM $Mg(OAc)_2$/98% glycerol was added. This suspension is stored at −20° for up to 1 month.

Preparation of Chromatin. Nuclei were recovered from the glycerol suspension by centrifugation at 3000 rpm in an HB-4 rotor (Sorvall RC-2 centrifuge) and washed once in 10 mM Tris·HCl, pH 7.4/6 mM KCl/5 mM $Mg(OAc)_2$ (TKM). The nuclei were lysed by vigorous homogenization in 2.5 mM Tris·HCl, pH 7.4/2.5 mM ethylenediaminetetraacetic acid (EDTA), followed by centrifugation at 10,000 rpm in the HB-4 rotor for 10 min. The chromatin was then washed once in the same buffer and twice in 5 mM sodium borate, pH 8.2/10 mM NaCl and finally was suspended in 5 mM sodium borate, pH 8.2/10 mM NaCl at an A_{260} of 10 (all chromatin absorbances are measured in 1 M NaOH).

Novikoff hepatoma cells [line NISI-67 adapted for growth in Swimms 210 medium (Gibco)] were cultured as described by Plagemann (9). Cells were labeled with [2-^{14}C]thymidine (56 mCi/mmol) at 0.05 μCi/ml for two cell generations. Preparation of nuclei will be described elsewhere (R. B. Wallace, S. K. Dube, and J. Bonner, unpublished data); they were mixed with 4-fold excess of rat liver nuclei. Chromatin was prepared as described above.

Preparation of Nucleosomes. Nuclei were recovered from the glycerol suspension as described above, washed once in TKM, and suspended in TKM at an A_{260} of 150–200. The suspension was warmed to 37°, brought to 0.25 mM $CaCl_2$, and digested for 5 min with 0.24 μg (7.5 units) of staphylococcal nuclease (P-L Biochemicals) per A_{260} unit. The nuclei were cooled to 0° and centrifuged at 3000 rpm in the HB-4 rotor for 5 min. The nuclear pellet was homogenized in 2.5 mM Tris·HCl, pH 7.4/2.5 mM EDTA and centrifuged at 10,000 rpm in the HB-4 rotor for 10 min. The supernatant was then passed over a Sephadex G-50 column equilibrated with 5 mM sodium borate, pH 8.2/0.1 mM EGTA; the excluded fraction, representing nucleosomes, was adjusted to an A_{260} of 10. Typical preparations were 5–15% acid soluble and 50–70% monomer nucleosomes.

Acetylation of Chromatin and Nucleosomes. Chromatin and nucleosomes were acetylated with acetic anhydride as described by Wong and Marushige (10). Chromatin solutions were acetylated with 0.14 mM, 0.7 mM, and 7 mM acetic anhydride at an A_{260} of 10. Acetylated chromatin samples were dialyzed overnight against 10 mM Tris·HCl, pH 7.4. The incorporation of acetate groups was monitored by acetylating chromatin with [^{3}H]acetic anhydride (Schwartz/Mann) diluted to 0.4 mCi/mmol. The amount of acetylation achieved with the three concentrations was essentially the same as that reported by Wong and Marushige.

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; TKM buffer, 10 mM Tris·HCl, pH 7.4/6 mM KCl/5 mM $Mg(OAc)_2$; EDTA, ethylenediaminetetraacetic acid.

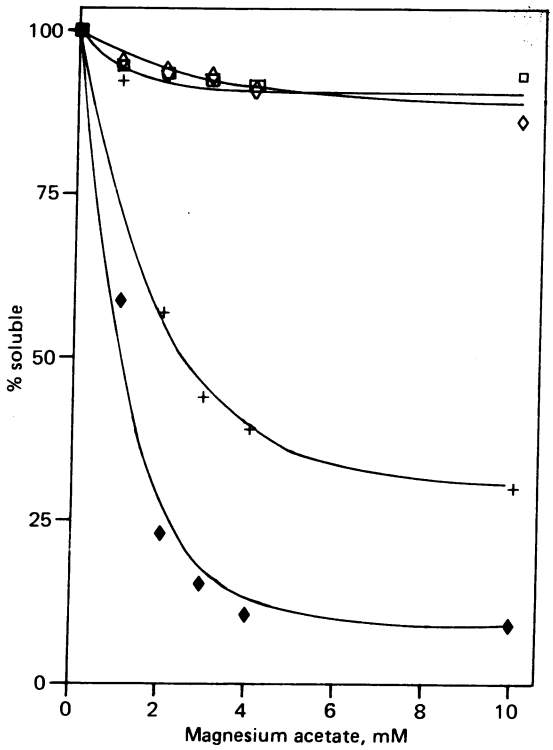

FIG. 1. Solubility of acetylated chromatin in magnesium acetate. Nucleosomes were acetylated with acetic anhydride at 0 (♦), 0.14 (+), 0.7 (◊), and 7 mM (□) (see *Materials and Methods*), dialyzed against 10 mM Tris·HCl, pH 7.4/0.1 mM EGTA, and diluted to an A_{260} of 1.0. Aliquots were adjusted to the desired magnesium acetate concentration and centrifuged at 0° for 10 min at 10,000 rpm in an SS-34 rotor (Sorvall RC-2B). The A_{260} of the supernatant was measured, and the values are expressed as percentage of total A_{260}. The lines represent nonlinear least-squares exponential fits to the data (13).

Thermal Denaturation. Acetylated and control chromatins were dialyzed exhaustively against 0.25 mM EDTA (pH 8) and adjusted to an A_{260} of approximately 1.5. Melting was performed with a Gilford 2400 spectrophotometer equipped with a thermal cuvette and digital absorbance meter under control of a Tektronix-31 programmable calculator. Absorbances were recorded every 0.4°, each point being the average of 10 readings. The data were corrected for the small hyperchromicity of the buffer and derivatized by using the least-squares method of Savitzky and Golay (11) (15-point cubic quartic polynomial first-derivative smooth). The derivative data were normalized to percentage hyperchromicity and resolved into gaussian components by using a nonlinear least-squares fitting program (12, 13).

Sucrose Density Gradient Centrifugation of Nucleosomes. Isokinetic sucrose gradients were prepared by the method of Noll (14), assuming a particle density of 1.44 g/cm^3 and using the following parameters: gradient volume, 11.6 ml; C_{top}, 5% (wt/wt) sucrose; C_{res}, 26.7% (wt/wt) sucrose; sample volume, 0.5 ml. The gradient buffer was 10 mM Tris·HCl, pH 8/0.1 mM EGTA. The gradients were run at 36,000 rpm in an SW41 rotor for 18 hr at 4°. The gradients were scanned by using an ISCO UA-5 ultraviolet monitor equipped with a digital absorbance meter, and data were collected by a Tektronix-31 programmable calculator. Fractions of the gradients containing nucleosomes acetylated with [^{3}H]acetic anhydride were collected and assayed for radioactivity directly in Aquasol 2 scintillation fluid (New England Nuclear).

Polyacrylamide Gel Electrophoresis. The products of DNase I digestion of control and acetylated chromatins were analyzed on 12% polyacrylamide/7 M urea gels as described by Maniatis *et al.* (15). Slab gels, 2 × 120 × 150 mm, were prepared and aged overnight. Samples were brought to 50% formamide, boiled for 3 min, and quenched on ice. Electrophoresis was at 200 V and continued until the bromophenol blue dye was 0.5 cm from the end of the gel. The gel was stained for 2 hr with ethidium bromide (1 μg/ml in 0.5 M ammonium acetate) and photographed under ultraviolet light with a yellow filter.

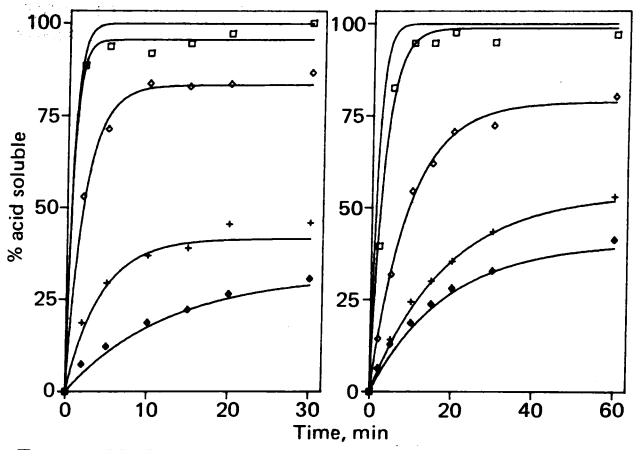

FIG. 2. Nuclease sensitivity of acetylated chromatin. Chromatin was prepared from [^{14}C]thymidine-labeled cells and acetylated as indicated in *Materials and Methods*. The samples were dialyzed against 10 mM Tris·HCl, pH 7.4, the A_{260} was adjusted to 0.4, and the sample was adjusted to 10 mM NaCl, 3 mM $Mg(OAc)_2$, and 1 mM $CaCl_2$. DNase I at 0.5 μg/ml (*Left*) or staphylococcal nuclease at 0.14 μg/ml (*Right*) was added, and the digestions were carried out at 24°. Aliquots (0.5 ml) were removed at intervals and precipitated with 1 ml of cold 0.3 M perchloric acid. Insoluble material was removed by centrifugation. The supernatant was neutralized with NaOH, adjusted to 1% sodium dodecyl sulfate, 50 mM Tris·HCl (pH 7.4), and assayed for radioactivity in Aquasol 2. After 1 hr of digestion, the remaining chromatin was adjusted to 1% sodium dodecyl sulfate, 50 mM Tris·HCl (pH 7.4), 0.2 M sodium perchlorate and assayed for total radioactivity. Acetic anhydride concentrations: 0 (♦), 0.14 mM (+), 0.7 mM (◊), and 7 mM (□). The lines represent nonlinear least-squares exponential fits to the data (13), with the uppermost line in each panel representing the digestion of deproteinized rat liver DNA (see Table 1).

RESULTS

One of the most obvious physical changes that accompanies chemical acetylation of rat liver chromatin is its solubilization. Under the conditions of the acetylation reaction (i.e., 0.15 M NaCl), chromatin is a condensed, insoluble precipitate. Chromatin is similarly condensed in the presence of 2 mM Mg^{2+}. Upon the addition of acetic anhydride at 0.7 or 7 mM, the chromatin precipitate becomes noticeably more soluble. We investigated this phenomenon by comparing the solubility of control and acetylated nucleosomes in various concentrations of magnesium (Fig. 1). The control nucleosomes were very insoluble, less than 10% remaining soluble in 10 mM magnesium. Acetylation increased the solubility of nucleosomes, the 0.7 mM and 7 mM acetylated material becoming almost completely soluble.

Template active regions of chromatin are more sensitive to DNase I (6, 7) than are inactive regions. If acetylation is responsible for gene activation *in vivo, in vitro* acetylated chromatin might be expected to show greater nuclease sensitivity than unacetylated chromatin. Fig. 2 *left* and Table 1 show the kinetics of digestion of acetylated ^{14}C-labeled chromatin with DNase I. Increased acetylation of chromatin dramatically al-

Table 1. Kinetic parameters from Fig. 2

	DNase I			Staphylococcal nuclease		
	k*	M†	R‡	k	M	R
Control	0.079	32.0	1.0	0.054	40.5	1.0
Acetylated chromatin:						
0.14 mM	0.237	41.6	3.9	0.054	53.9	1.3
0.7 mM	0.458	83.3	15.2	0.110	78.9	3.9
7 mM	1.300	95.6	49.4	0.322	98.8	14.4
DNA	1.290	100.0	51.3	0.653	100.0	29.6

* First-order rate constant (min^{-1}).
† Maximal percentage digested.
‡ Relative initial rate of reaction. The initial rate for control chromatin was 0.01 A_{260} unit/ml per min with DNase I and 0.0088 A_{260} unit/ml per min with staphylococcal nuclease.

tered the sensitivity of the DNA to the nuclease. Chromatin acetylated with 0.14 mM acetic anhydride was digested 4 times faster than control chromatin, and that acetylated at 0.7 mM was digested 15 times faster. Maximally acetylated chromatin (7 mM) was as sensitive as deproteinized DNA to DNase I. Fig. 2 *right* shows the results of a similar experiment with staphylococcal nuclease. It can be seen that 0.14 mM acetylated chromatin was slightly more sensitive than control chromatin to staphylococcal nuclease, and 0.7 mM acetylated chromatin was digested 4 times as fast as control chromatin; 7 mM acetylated chromatin was digested approximately half as fast as deproteinized DNA. Thus, chemically acetylated chromatin is moderately sensitive to staphylococcal nuclease but extremely sensitive to DNase I.

Weintraub and Groudine (6) reported that, during the digestion of active genes with DNase I, the digested DNA appears as multiples of 10 nucleotides. Fig. 3 shows that 0.14 mM and 0.7 mM acetylated chromatins were digested by DNase I to the same 10-nucleotide repeat pattern as control chromatin. This result suggests that the histone–DNA interaction that produces this periodic pattern is not disrupted by the acetylation. On the other hand, the 7 mM acetylated chromatin did not have this repeat pattern (although faint bands are apparent at 10-nucleotide intervals).

The DNA of the transcriptionally active regions of chromatin

Table 2. Summary of melting transitions from Fig. 4

	Tm1	%	Tm2	%	Tm3	%	Tm4	%
				Nucleosomes				
Control	64.5	34.4	76.3	60.6	81.1	5.0	—	—
Acetylated:								
0.14 mM	60.2	39.3	73.8	52.0	79.7	8.8	—	—
0.7 mM	48.3	48.0	65.7	39.0	75.7	13.0	—	—
7 mM	42.1	55.2	52.2	31.6	69.7	13.2	—	—
DNA	40.8	100.0	—	—	—	—	—	—
				Chromatin				
Control	62.0	29.1	71.1	18.7	77.1	23.4	83.4	28.8
Acetylated:								
0.14 mM	53.3	20.8	65.0	26.4	73.6	20.5	81.3	32.3
0.7 mM	47.6	44.9	63.3	28.7	—	—	79.8	26.4
7 mM	43.7	83.7	50.7	6.4	—	—	75.6	9.9
DNA	40.8	100.0	—	—	—	—	—	—

Temperatures are in degrees Celsius. Transitions are numbered in order of increasing temperature. Percentages refer to the fraction of the total hyperchromicity of each sample in a given transition.

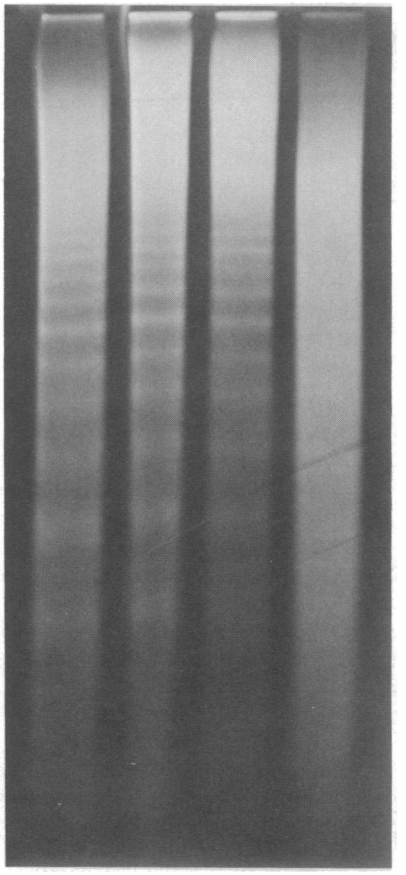

FIG. 3. Polyacrylamide gel electrophoresis of DNase I-digested chromatin DNA. Control and acetylated chromatins were prepared as described in *Materials and Methods*. The samples were dialyzed against 10 mM Tris·HCl (pH 7.4) and adjusted to A_{260} of 10. Each was brought to 10 mM NaCl, 3 mM $Mg(OAc)_2$, and 1 mM $CaCl_2$, and DNase I, 10 μg/ml, was added. Each sample was digested for various times at 24° such that the percentage of acid-soluble A_{260} was 25, 30, 15, and 15 for control, 0.14 mM, 0.7 mM, and 7 mM acetic anhydride, respectively. Reactions were stopped by bringing each to 1% sodium dodecyl sulfate; then the samples were phenol/chloroform extracted as described by Marmur (29). DNA was precipitated with ethanol and dissolved in gel buffer (15); 25 μg of each was brought to 50% formamide, boiled for 3 min, and cooled on ice. Electrophoresis was performed as described in *Materials and Methods*. From left to right: DNA from unacetylated chromatin and 0.14 mM, 0.7 mM, and 7 mM acetylated chromatin.

is believed to have a lower melting temperature than the DNA of the inactive regions. This belief is supported by the thermal fractionation experiments of McConaughy and McCarthy (16). In addition, DNase II-fractionated template-active chromatin melts at a lower temperature than does unfractionated chromatin (ref. 17; unpublished data).

The thermal denaturation of control and acetylated chromatins and nucleosomes, as well as deproteinized DNA, is shown in Fig. 4. The data are presented as first-derivative melting profiles, fitted to three or four gaussian components to quantitate the transitions observed. Table 2 summarizes the melting transitions and the contribution of each to the total hyperchromicity. The most obvious effect of chemical acetylation on thermal denaturation is the dramatic shift to lower melting temperatures of all the observed transitions. This is true for both chromatin and nucleosomes, although the melting profiles of control and acetylated chromatin were more complex.

Fig. 5 shows the effect of acetylation on the sedimentation behavior of nucleosomes. The sedimentation of acetylated

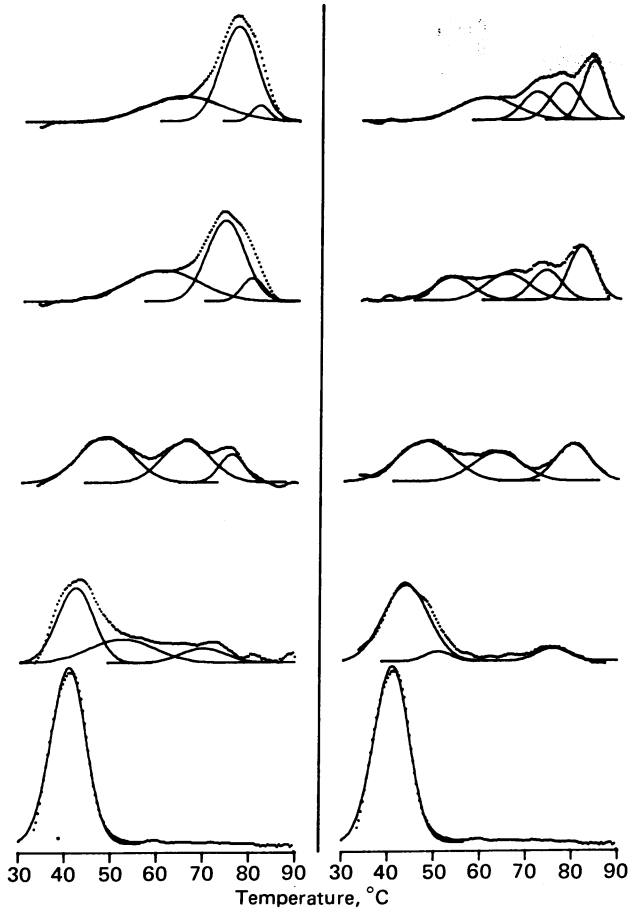

FIG. 4. First-derivative melting profiles (see *Materials and Methods*). Full scale for each profile is 3% change in hyperchromicity per degree. From top to bottom: unacetylated material, 0.14 mM, 0.7 mM, and 7 mM acetylated material, and deproteinized rat liver DNA. (*Left*) Nucleosomes. (*Right*) Chromatin. ···, dH/dT. —, Gaussian components.

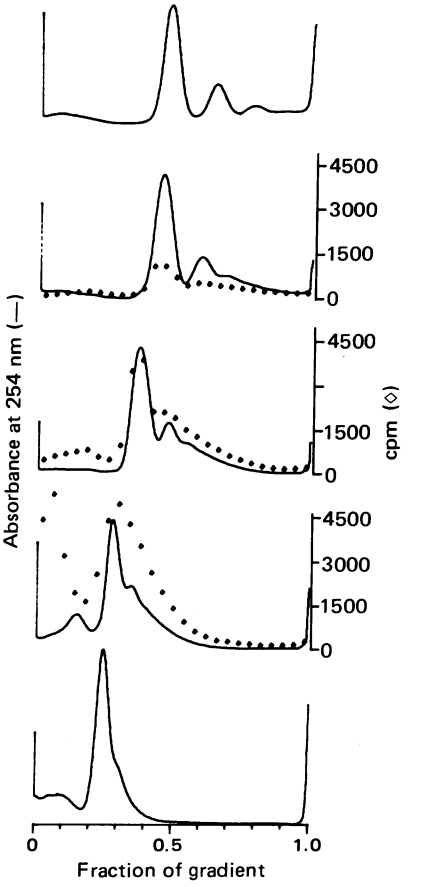

FIG. 5. Sucrose gradient sedimentation of nucleosomes. Sedimentation is from left to right. From top to bottom: nucleosomes, 0.14 mM acetylated nucleosomes, 0.7 mM acetylated nucleosomes, 7 mM acetylated nucleosomes, and sodium dodecyl sulfate-treated nucleosomal DNA (30). The sedimentation coefficients of the monomer peak are 11.3, 10.6, 8.6, 6.2, and 5.4 S, respectively. The specific activity of the labeled samples was 19.1, 67.4, and 119 acetates per 200 base pairs of DNA for 0.14 mM, 0.7 mM, and 7 mM acetylated nucleosomes, respectively. Under the conditions of acetylation, approximately half as much acetate was incorporated into nonhistones as into histones, and no detectable acetate was incorporated into DNA.

nucleosomes was dramatically retarded compared with that of control nucleosomes and approached that of sodium dodecyl sulfate-treated control nucleosomes. To determine whether all of these nucleosomes were acetylated equally and whether all of the acetate remained bound to the nucleohistone, the acetylation reaction was carried out with [^{3}H]acetic anhydride. Fig. 5 also shows the distribution of [^{3}H]acetate across the gradients. From 0.14 to 0.7 mM acetic anhydride there was a 4-fold increase in acetate incorporation per nucleosome whereas from 0.7 to 7 mM acetic anhydride there was only a 2-fold increase. This demonstrates the limit acetylation reported by Wong and Marushige (10). All nucleosomes, monomer through trimer, were acetylated. At 7 mM acetic anhydride, [^{3}H]acetate counts were found at the top of the gradient, perhaps representing dissociated histone H1.

DISCUSSION

We have demonstrated that chemical acetylation of nucleohistone *in vitro* results in dramatic changes in its physical properties. The results obtained suggest possible functions for acetylation *in vivo*.

The chemical acetylation of nucleosomes was found to increase their solubility in the presence of Mg^{2+}. Previous studies from this laboratory have shown that DNase II digestion followed by precipitation with 0.15 M NaCl (18) or 2 mM $MgCl_2$ (19) yields a fraction of rat liver chromatin enriched in sequences transcribed *in vivo* (19).

The sensitivity of chromatin to DNase I and, to a lesser extent, to staphylococcal nuclease is significantly increased after treatment with acetic anhydride. Similar nuclease sensitivity properties have been reported for transcriptionally active sequences (6, 7, 20), although the biochemical basis for this phenomenon has not been determined. Marushige (8) reported that the derepression of template activity by chemical acetylation with 0.7 mM acetic anhydride was a result of the acetylation of histones H2A and H2B, with H3 and H4 not contributing significantly. H3 and H4 have been implicated in the protection of multimers of 10 nucleotides of DNA from digestion by DNase I (21). Fig. 3 demonstrates that the 10-nucleotide repeat pattern is observed in 0.7 mM acetylated chromatin even though the rate of digestion by DNase I is dramatically accelerated (Fig. 2 *left*). This suggests that the interaction of H3 and H4 with the DNA has not been greatly perturbed, whereas the template activity measurements by Marushige suggest that the interaction of H2A and H2B with DNA has been functionally altered by acetylation. Higher levels of acetylation result in a disruption of the 10-nucleotide repeat pattern (Fig. 3).

Thermal denaturation of nucleohistone demonstrates the

dramatic stability of the histone–DNA complex. In order to make DNA sequences available for transcription (or replication), it might be necessary to decrease the stability of this complex (22). Although no direct correlation of nucleohistone thermal denaturation behavior with functional state has been demonstrated, isolated template-active chromatin has a lower melting temperature than does the bulk of chromatin (17), and active sequences of chromatin elute from hydroxylapatite at lower temperatures (16). The results presented in Fig. 4 and Table 2 demonstrate that chemical acetylation lowers the melting temperature of nucleohistone, as might be expected if acetylation were correlated with transcriptional activation. The transitions observed in the first-derivative melting profiles are similar to those reported by others (23–25). We have resolved the transitions into gaussian components in order to quantitate the contribution of each transition to the total hyperchromicity. From previous work (2, 23), as well as unpublished observations in our laboratory, tentative assignments may be made to the transitions of control nucleosomes. The two transitions at the highest temperatures are due to the denaturation of the histone octamer–DNA complex. The lowest transition is due to a more heterogeneous set of interactions as reflected by the breadth of this transition. One of these contributing interactions is probably H1–DNA complexes (26). Acetylation of nucleosomes with 0.14 mM acetic anhydride had little effect on the profile. The relative contribution of each transition remained unchanged, and the transitions were shifted to lower temperatures, T1 being shifted by the largest amount. This is consistent with the role of H1 in the T1 transition, in view of the extensive acetylation of this histone by acetic anhydride (10). The acetylation of nucleosomes with 0.7 mM acetic anhydride resulted in dramatic changes in the melting profile: all three transitions were shifted to lower temperatures and the relative contributions of T1 and T2 were increased at the expense of T3. This may reflect the differential effect of acetylation of H2A/H2B and H3/H4 as discussed above. Acetylation with 7 mM acetic anhydride virtually abolished the stabilization of DNA melting by histones, even though histones remain associated with the DNA (refs. 8 and 10; Fig. 5). The thermal denaturation profiles of chromatin show the same general features as those of nucleosomes.

At the lowest level of acetylation used (0.14 mM acetic anhydride), there were dramatic changes in magnesium solubility (Fig. 1) and DNase I sensitivity (Fig. 2 *left*). However, staphylococcal nuclease sensitivity (Fig. 2 *right*), DNase I digestion pattern (Fig. 3), and thermal denaturation (Fig. 4 and Table 2) were only slightly perturbed by the acetylation. Although the sites of chemical acetylation have not been determined, it is possible that this low level of acetylation destabilizes interactions that are qualitatively different from those affected by higher levels.

The demonstration of a direct role of acetylation of histones in gene activation, as suggested by Allfrey *et al.* (3), has been elusive. Jackson *et al.* (27) argued that histone acetylation is too extensive to be a specific mechanism of gene regulation. However, histone modification may be important in DNA replication and histone deposition, as well as in gene activation (4, 28). The many functions of acetylation might be accomplished through different classes of sites for acetylation on the histones. For gene activation, it may be sufficient to alter histone–histone or histone–DNA interactions slightly as in the 0.14 mM acetylation. This could result in the conversion of nucleosomes to a conformation more conducive to transcription of the associated DNA. On the other hand, DNA replication or histone deposition might require a more extensive destabilization of these histone–DNA interactions, as in the higher levels of acetylation reported here.

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