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Activation of chromatin by acetylation of histone side chains

(template activity/RNA synthesis/acetic anhydride/calf thymus)

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ABSTRACT **The ability of calf thymus chromatin to support DNA-dependent RNA synthesis is markedly increased by modification of the histones with acetic anhydride. The extent of chemical acetylation of histones H2a, H2b, H3, and H4 required for the increase of template activity of the chromatin is within that which can be achieved enzymatically in the cell. The evidence suggests that the acetylation of histone side chains stimulates the rate of chain elongation during transcription of chromatin.**

The ability of isolated chromatins to support DNA-dependent RNA synthesis is much less than that of deproteinized DNA (1, 2). The reduced template activity of DNA in the form of chromatin is apparently due to the presence of histones as the activity increases essentially in parallel with dissociation of the histones (2, 3). This investigation deals with the effect of chemical acetylation of histone side chains on transcription of calf thymus chromatin. It will be shown that a transcription of chromatin can be raised by modification of the histone–DNA interactions without removal of the histones from the DNA.

MATERIALS AND METHODS

Preparation of Chromatin. Chromatin was prepared from frozen calf thymus (Pel-Freez Biologicals) by the method of Marushige and Bonner (2) with a slight modification as previously described (4) and stored in 50% glycerol at −20°. Just before use, the chromatin was washed twice with 5 mM sodium borate (pH 8.2) and sheared in the same buffer as previously described (5).

Acetylation of Chromatin. The chromatin was appropriately diluted with 5 mM sodium borate (pH 8.2) and adjusted to 0.15 M NaCl at the final chromatin concentration equivalent to 300 μg of DNA per ml. Acetic anhydride was next added to the vigorously stirred chromatin suspension in an ice-bath, and the reaction was allowed to proceed for 20 min while maintaining the pH at 8.0–8.2 by addition of 0.1 M NaOH (5). The chromatin thus treated was dialyzed against 10 mM Tris·HCl (pH 7.0) for 16–24 hr.

Dissociation of Chromatin. The chromatin was appropriately diluted with 10 mM Tris·HCl (pH 7.0) and 5 M NaCl was then added dropwise while stirring to final concentrations ranging from 0.15 to 1.8 M. The chromatin concentration was equivalent to 100 μg of DNA per ml. The solution was next centrifuged at 60,000 rpm for 5 hr in a Spinco type 65 rotor. The sediment was resuspended in 10 mM Tris·HCl (pH 7.0) and examined as to its composition and template activity.

Composition of Chromatin. The chromatin was treated with 0.4 M HCl (0°, 30 min), and then by centrifugation at 10,000 × *g* for 20 min. Histones were next precipitated from the extract with 20% trichloroacetic acid and determined by the method of Lowry *et al.* (6). DNA was determined on the acid insoluble materials after hydrolysis in 5% perchloric acid by taking an A_{260} of 1 mg/ml of hydrolyzed DNA equal to 28. Histones were characterized by polyacrylamide gel electrophoresis and Bio-Gel P-60 column chromatography as previously described (5).

Template Activity of Chromatin. The ability of the chromatin to support DNA-dependent RNA synthesis was assayed using *Escherichia coli* RNA polymerase (Miles Laboratories or Sigma) (RNA nucleotidyltransferase, nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) under conditions as specified in each experiment. The reaction was terminated by the rapid addition of 0.05 ml of high-molecular-weight RNA (4 mg/ml) and then 2 ml of 5% trichloroacetic acid. The precipitate was collected on Whatman GF/C glass fiber filters and then washed three times with 10 ml of 5% trichloroacetic acid. The filters were dried, treated with 0.2 ml of NCS solubilizer (Amersham/Searle), neutralized with 10 μl of 6 M H_2SO_4 and assayed for radioactivity in a Beckman LS-250 liquid scintillation counter with toluene-based scintillation fluid.

RESULTS

When calf thymus chromatin is treated with acetic anhydride, all five major histone fractions become acetylated at a limited number of sites (5). The maximum acetylation of all of the histone fractions occurs essentially at 7 mM acetic anhydride under the conditions employed in the present experiments. As shown in Fig. 1 (●), the ability of calf thymus chromatin to support DNA-dependent RNA synthesis is increased sharply by treatment at 0.7 mM acetic anhydride and gradually thereafter at higher concentrations of the reagent. As also seen in Fig. 1 (○), little histone is dissociated from the chromatin by acetylation at 0.7 mM acetic anhydride which indicates that the increase of template activity has occurred without removal of any of the major histone fractions. Approximately 25% and 70% of the total histones are dissociated by treatment at 1.4 mM and 3.5 mM or higher, respectively. Electrophoretic and chromatographic analyses have shown that histone H1 becomes dissociable first by treatment at 1.4 mM acetic anhydride and histones H2a and H2b are next dissociated at 3.5 mM, while essentially all the histones H3 and H4 are still bound to the DNA after acetylation at 7 mM acetic anhydride (5, 7).

To differentiate the effect of acetylation of individual histone fractions on the template activity of chromatin, I acetylated calf thymus chromatin with 0.7 mM and 7 mM acetic anhydride as well as control (untreated) chromatin which were then both dissociated with various concentrations of NaCl. As previously reported (3), the increase of template activity in the untreated chromatin essentially parallels the removal of the histones (Fig. 2A and B, ●). In this chromatin, histone H1 completely dissociates in 0.6 M NaCl and the major portion of histones H2a and H2b is removed between 0.6 M and 1.2 M, while dissociation of histones H3 and H4 mainly occurs from 1.2 to 1.8 M. When calf thymus chromatin is treated with 0.7 mM acetic anhydride, histones H1, H2 (mixture of H2a and H2b), H3, and H4 are modified at an average of 7.9, 2.3, 1.4, and 0.8 sites per molecule, respectively (5). In the chromatin thus acetylated, disso-

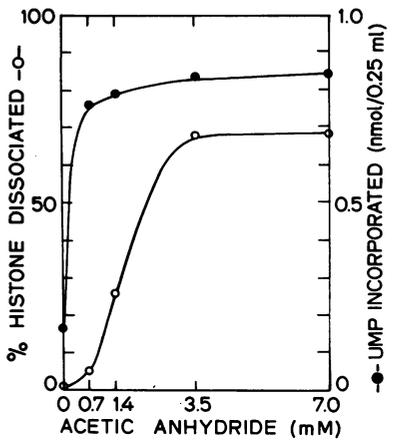

FIG. 1. Acetylation and template activity of chromatin. Calf thymus chromatin treated with 0, 0.7, 1.4, 3.5, or 7 mM acetic anhydride was examined for its ability to support DNA-dependent RNA synthesis (●). Reaction mixtures (0.25 ml) contained 40 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 0.15 M NaCl, 0.5 mM each of ATP, GTP, and CTP, 0.5 mM [^{3}H]UTP (10 μCi/μmol), 100 μg of bovine serum albumin, 5 μg of DNA as chromatin, and 1.9 units of RNA polymerase. One unit of RNA polymerase equals that amount catalyzing the incorporation of 1 nmol of labeled ATP into acid-insoluble material in the presence of calf thymus DNA in 10 min at 37°. Incubation was carried out at 37° for 10 min. Chromatins similarly treated with various concentrations of acetic anhydride were sedimented in 0.15 M NaCl and analyzed for histones remaining bound to DNA, and the percentages of histones dissociated then calculated (○).

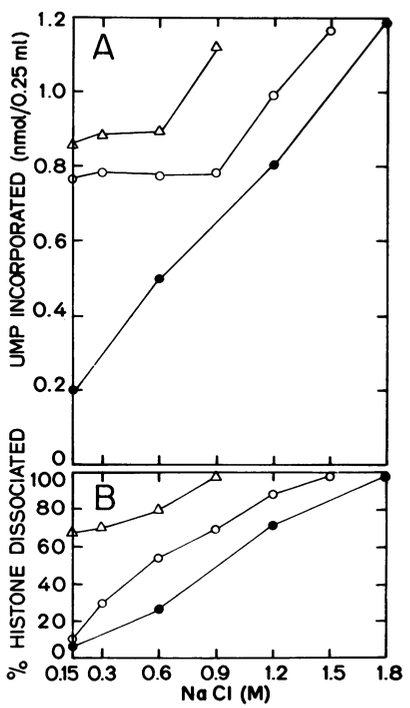

FIG 2. Dissociation and template activity of acetylated chromatin. Control (unacetylated) calf thymus chromatin (●), the chromatins acetylated with 0.7 mM (○), and 7 mM acetic anhydride (△), were sedimented in various concentrations of NaCl and assayed for template activity (A) and histones (B). Incubation mixture and other conditions for RNA synthesis as given in Fig. 1. Reaction mixtures (0.25 ml) contained 4 μg of DNA as chromatin and 2.0 units of RNA polymerase.

ciation of histones H1, H2a and H2b, and H3 and H4 has been found to occur at 0.15–0.3 M, 0.3–0.9 M, and 0.9–1.5 M NaCl, respectively. As seen in Fig. 2 (○), the template activity of this chromatin is approximately four times that of the untreated chromatin, and is, in turn, similar to that of the untreated chromatin, whose histones H1, H2a, and H2b are removed by 1.2 M NaCl. Furthermore, in this acetylated chromatin, there is virtually no change of template activity up to 0.9 M NaCl at which concentration histones H1, H2a, and H2b are removed, while the activity increases concurrently with dissociation of histones H3 and H4 between 0.9 and 1.5 M NaCl. It seems thus clear that the inhibition of RNA synthesis by histones H1, H2a, and H2b is essentially completely abolished by acetylation of their side chains to even a limited extent, whereas acetylation of histones H3 and H4 at this concentration of acetic anhydride causes little change in their abilities to suppress DNA-dependent RNA synthesis.

The treatment of calf thymus chromatin with 7 mM acetic anhydride results in the modifications at an average of 23.2, 5.7, 9.5, 5.5, and 2.7 sites in histones H1, H2a, H2b, H3, and H4, respectively (5). In this acetylated chromatin, dissociation of histones H1, H2a, and H2b is essentially complete at 0.15 M NaCl and histones H3 and H4 are removed between 0.6 M and 0.9 M NaCl (5, 7). The template activity of this chromatin increases as histones H3 and H4 are removed between 0.6 M and 0.9 M NaCl (Fig. 2, △). As also seen in this figure, the DNA associated with histones H3 and H4 acetylated to this extent (△, 0.15–0.6 M NaCl) supports significantly greater RNA synthesis than that supported by the DNA associated with untreated H3 and H4 (●, 1.2 M NaCl) or with those histones acetylated to a lesser extent (○, 0.9 M NaCl) which suggests that the extensive modifications of histones H3 and H4 have made them less inhibitory to DNA-dependent RNA synthesis.

As clearly seen in Fig. 2B, the control chromatin treated with 0.6 M NaCl and the chromatin acetylated with 0.7 mM acetic anhydride and then treated with 0.3 M NaCl, both contain identical amounts of histones (all the histones H2a, H2b, H3, and H4). It seems, therefore, likely that the observed difference of template activities between these templates (Fig. 2A), and, similarly, the difference between the control chromatin treated with 1.2 M NaCl and the chromatin acetylated with 7 mM acetic anhydride and then treated with 0.3 M NaCl (both of which contain essentially all of the histones H3 and H4) are not due to differences in availability of the sites for RNA chain initiation but are attributable to differing rates of RNA chain elongation. Effects of histone acetylation on the initiation and elongation of RNA chains were then examined, by using the four kinds of templates described above as well as the chromatin which had been treated with 2 M NaCl (chromatin minus histones). Because acetylation of internal lysyl residues of histone H1 does not occur in the cell, this histone has been excluded from consideration in this experiment. As shown in Table 1, incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP is not appreciably different between various templates tested which indicates that stimulation of the rate of RNA synthesis by the acetylation as well as the removal of histones is, in fact, not due to an increase of the rate of chain initiation. Estimation of average chain lengths on the basis of the incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP and of [^{3}H]UMP thus shows that the rate of chain elongation on the DNA associated with unmodified histones H2a, H2b, H3, and H4 is approximately 45% of that on the dehistonized DNA. The acetylation of these histones at 0.7 mM acetic anhydride increases the rate of chain elongation to a level similar to that on the DNA template containing only unmodified histones H3 and H4, while a further increase of the elongation rate is seen upon acetylation of histones H3 and H4 at 7 mM acetic anhydride.

Table 1. Effect of acetylation and removal of histones on the chain initiation and elongation of chromatin-directed RNA synthesis

Treatment of chromatin			Incorporation (pmol)			
Acetic anhydride (mM)	NaCl (M)	Histones remaining bound to DNA	[γ^{32}-P]ATP*	[γ^{32}-P]GTP*	[^{3}H]UMP	Average chain length†
0	0.6	H2a, H2b, H3, H4	0.68	0.56	88	241
0.7	0.3	H2a, H2b, H3, H4	0.55	0.45	100	340
0	1.2	H3, H4	0.63	0.48	99	303
7	0.3	H3, H4	0.56	0.50	133	427
0	2.0		0.56	0.43	162	540

Chromatins treated with designated concentrations of acetic anhydride and NaCl were incubated (37°, 10 min) with either [γ^{32}-P]ATP (175 cpm/pmol), [γ^{32}-P]GTP (217 cpm/pmol), or [^{3}H]UTP (10 μCi/μmol). Reaction mixtures (0.1 ml) contained 0.1 μmol each of four ribonucleoside triphosphates, chromatin equivalent to 1.25 μg DNA, and 3.2 units of RNA polymerase. Other conditions as given in Fig. 3. After incubation RNA was precipitated, washed essentially as described by Millette and Trotter (8), and assayed for radioactivity.

* Incorporation by enzyme alone (0.08–0.17 pmol for ATP and 0.35 pmol for GTP) subtracted.

† Calculated from pmol UMP incorporated/0.295 × pmol ([γ^{32}P]ATP + [γ^{32}P]GTP) incorporated. The 0.295 = mole fraction of A in calf thymus DNA.

The effect of the histone acetylation on chromatin-directed RNA synthesis has been further investigated using rifampicin. Sippel and Hartmann (9) have shown that the attachment of *E. coli* RNA polymerase to certain sites on a DNA template leads to the formation of a "preinitiation complex" which can be assayed by its resistance to rifampicin. Using this assay, Bautz and Bautz (10) have estimated the number of promoter sites per genome for several DNA phages. When calf thymus chromatin variously treated with acetic anhydride and NaCl as described in Table 1 is incubated with RNA polymerase prior to the simultaneous addition of rifampicin and four ribonucleoside triphosphates, rifampicin-resistant incorporation of the nucleotides increases with increasing concentrations of the enzyme and attains a plateau (Fig. 3) which suggests that calf thymus DNA, too, possesses a limited number of *E. coli* RNA polymerase binding sites which can render the enzyme molecules rifampicin-resistant. As also seen in Fig. 3, all the templates tested attain their plateaus at essentially the same concentration of RNA polymerase which further suggests that they possess essentially the same number of such binding sites. The different plateau levels observed in Fig. 3 would thus be attributable to varying rates of elongation of RNA chains initiated by the rifampicin-resistant enzyme molecules on the various templates. The plateau obtained for the DNA associated with unmodified histones H2a, H2b, H3, and H4 (■) and that for the DNA associated with unmodified histones H3 and H4 (□) are, respectively, about one-seventh and one-fourth that obtained for the chromatin depleted of histone (○). The DNA associated with histones H2a, H2b, H3, and H4 acetylated at 0.7 mM acetic anhydride (▲) and that associated with histones H3 and H4 acetylated at 7 mM acetic anhydride (●) give plateaus considerably higher than that obtained for the templates containing corresponding unmodified histones. These results are consistent with the earlier observations (Table 1) in showing that the acetylation of histones increases the rate of chain elongation in chromatin-directed RNA synthesis. It is, however, noted that relative activities of the various templates found in Fig. 3 are markedly different from those obtained for the same templates in Table 1. In order to clarify this discrepancy, the chromatins variously treated with acetic anhydride and NaCl as described in Table 1 and Fig. 3 were incubated with RNA polymerase, followed by the addition of the substrates with and without rifampicin (Table 2). The data show that the differences between the activities of these templates are much greater when the pre-incubated templates are assayed in the presence of rifampicin than when assayed in its absence, and that less active templates exhibit greater differences in RNA synthesis between the two different conditions. Consequently, the proportions of RNA synthesis which is resistant to rifampicin (+Rif/−Rif in Table 2) vary from 0.08 for the H1-depleted chromatin to 0.23 for the dehistonized DNA. The RNAs synthesized in the absence of rifampicin are most likely to include those initiated by RNA polymerase molecules which are bound to internal single strand breaks, if any, as well as to the sheared ends of the DNA molecules in these templates (DNA isolated from calf thymus chromatin which is prepared in the present manner possesses a molecular weight of about 2 to 4 × 10^6). It has been shown that the enzyme molecules bound to such strand interruptions do not undergo the conformational changes which render them rifampicin-resistant (10). The frequencies of chain initiation

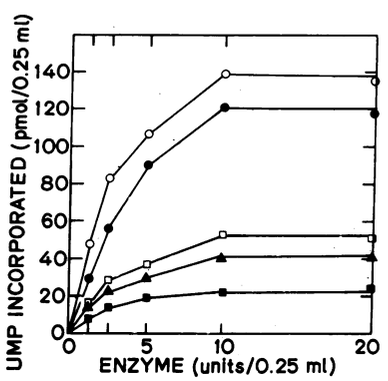

FIG. 3. Effect of acetylation and removal of histones on the formation of chromatin-RNA polymerase complex resistant to rifampicin. Calf thymus chromatin sedimented in 0.6 M (■), 1.2 M (□) or 2.0 M NaCl (○), the chromatin treated with 0.7 mM acetic anhydride and then sedimented in 0.3 M NaCl (▲), and the chromatin treated with 7 mM acetic anhydride and then sedimented in 0.3 M NaCl (●), each equivalent to 1.25 μg of DNA, was incubated (19°) with various amounts of RNA polymerase in a reaction mixture (225 μl) containing 10 μmol of Tris·HCl (pH 8.0), 2.5 μmol of $MgCl_2$, and 12.5 μmol of 2-mercaptoethanol. After 20 min, a solution (25 μl) containing 0.1 μmol each of ATP, GTP, CTP, and [^{3}H]UTP (10 μCi/μmol) and 2.5 μg of rifampicin was added and incubation was continued at 37° for an additional 10 min.

Table 2. Effect of rifampicin on the RNA synthesis directed by various templates pre-incubated with RNA polymerase

Treatment of chromatin			UMP incorporated (pmol)		
Acetic anhydride (mM)	NaCl (M)	Histones remaining bound to DNA	−Rif*	+Rif*	+Rif/−Rif
0	0.6	H2a, H2b, H3, H4	146 (40.4)	11.7 (14.1)	0.08
0.7	0.3	H2a, H2b, H3, H4	184 (51.0)	22.1 (26.8)	0.12
0	1.2	H3, H4	197 (54.6)	27.7 (33.6)	0.14
7	0.3	H3, H4	314 (70.0)	54.4 (66.0)	0.17
0	2.0		361 (100)	82.4 (100)	0.23

Chromatins treated with designated concentrations of acetic anhydride and NaCl (each equivalent to 1.25 μg of DNA) were pre-incubated (19°, 20 min) with 2.5 units of RNA polymerase and then incubated at 37° for 10 min with the four nucleotides in the presence (+Rif) or the absence of rifampicin (−Rif). Incubation mixture and other conditions as given in Fig. 3.

* The values in parentheses represent the percent activities relative to the activity of the chromatin treated with 2 M NaCl (chromatin minus histone).

do not appear to differ appreciably between these templates either in the presence of rifampicin after their pre-incubation with the enzyme (Fig. 3) or in the absence of rifampicin (Table 1). The finding that the DNA templates associated with histones are relatively more active without this inhibitor may thus be explained by postulating that the presence of histones on a DNA template becomes less inhibitory when RNA synthesis is initiated at strand breaks. Comparison of the rate of RNA synthesis directed by the rifampicin-resistant preinitiation complexes between the templates might therefore provide a better measure for the intrinsic ability of histones to suppress DNA-dependent RNA synthesis. The data of Table 2 as well as those of Fig. 3 thus indicate that the DNA complexed with unmodified histones H2a, H2b, H3, and H4 and the DNA with unmodified histones H3 and H4 are transcribed at approximately one-seventh and one-third the rate of free DNA, respectively. It is again clear from these results that the limited acetylation of the side chains of these histones markedly increases the rate of transcription of such histone-DNA complexes.

DISCUSSION

The present results have shown that chemical acetylation of histone side chains has a dramatic effect on the ability of isolated chromatins to support DNA-dependent RNA synthesis (Figs. 1 and 2), and that such modifications of the histone binding to DNA appear to facilitate the movement of RNA polymerase along the chromatin fiber (Fig. 3; Tables 1 and 2). Enzymatic acetylation of internal lysyl residues of histones H2a, H2b, H3, and H4, but not histone H1, is known to occur in the cell (11–17), and the number of sites of the modification have been reported to be one for H2a, four for H2b, four for H3, and four for H4 (14–16). In this investigation, the abilities of histones H2a and H2b to inhibit RNA synthesis have been found to be essentially completely abolished by acetylation of chromatin at 0.7 mM acetic anhydride (Figs. 1 and 2). At this acetic anhydride concentration, histones H2a and H2b are acetylated at approximately one and three sites per molecule, respectively (5). Stimulation of RNA synthesis as a result of acetylation of histones H3 and H4 has been clearly seen in the chromatin treated with 7 mM acetic anhydride (Fig. 3; Tables 1 and 2). In this acetylated chromatin, histone H3 is acetylated at five to six sites per molecule, one of which may be NH_2-terminal alanine, and the acetylation of histone H4, including the ones present *in situ*, occurs at three to four sites per molecule (5). Although the location of chemically modified sites on the histone molecules has not been investigated in the present study, the internal lysyl residues of the histones that are accessible to enzymatic acetylation within the chromatin structure are probably also available for chemical acetylation. A comparison of the extents of chemical acetylation of histones H2a, H2b, H3, and H4 required for the stimulation of chromatin-directed RNA synthesis with those of enzymatic acetylation which can be achieved *in vivo* thus suggests that such an activation of chromatin as a result of acetylation of the histone side chains could also occur in the cell.

Since Allfrey *et al.* (18) first suggested that acetylation of histones might modulate template function of chromatin in RNA synthesis, there have been many reports, as recently reviewed by Ruiz-Carrillo *et al.* (19), in which increased histone acetylation is found to precede an increase in RNA synthesis. Whether and how the histone acetylation can be involved in activation of specific genes has not been well understood. The template-active portion of rat liver chromatin is not particularly enriched with acetylated histones (20, 21). Activation of specific genes does not appear, therefore, to be achieved in such a way that the histones associated with certain stretches of DNA become specifically acetylated in a metabolically stable fashion. Jackson *et al.* (22) have reported that acetyl groups of internal lysyl residues of the histones turn over rather rapidly and, on the basis of the extent to which the population of histones is acetylated during a short pulse, have argued against the involvement of histone acetylation in specific gene activation. Acetylation of histones is known to occur not only in dividing cells (19, 22, 23) but also in nondividing cells containing template-inactive nuclei (23, 24). The acetylation may thus represent a general cellular activity which would function differently in different cell types as well as at different phases of the cell-cycle. It seems entirely possible that the transcription rate of the genes which are specifically turned on by some other mechanisms is generally controlled by the level of histone acetylation.

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