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CHEMICALLY INDUCED GENE ACTIVATION: SELECTIVE INCREASE IN DNAase I SUSCEPTIBILITY IN CHROMATIN ACETYLATED WITH ACETYL ADENYLATE

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SUMMARY

Treatment of calf thymus chromatin with acetyl adenylate under appropriate conditions produces acetylation of histones. The pattern of histone acetylation obtained is similar to that produced in vivo. The acetylated chromatin shows increased sensitivity to DNAase I but no increased sensitivity to Staphylococcal nuclease. These digestion patterns are similar to those observed in active genes.

INTRODUCTION

The study of the molecular mechanisms involved in the control of gene expression is an area of intense interest in molecular biology. Identification of the active areas of the genome is necessary. Many probes have been tried and it has been demonstrated that DNAase I digests an active fraction of chromatin isolated on sucrose gradients faster than an inactive fraction (1). This finding was strengthened when it was shown that DNAase I selectively digested activated globin genes in red blood cell nuclei but did not selectively digest the inactive globin gene in the fibroblast nuclei (2). Similar results have been obtained for the ovalbumin gene (3). In these cases, however, no selective digestion is seen with Staphylococcal nuclease (2). This gene activation is probably mediated thru non-histone proteins and histone modification (4-6). Of the possible histone modifications, acetylation has been studied extensively and the extent of acetylation has been found to be increased during early stages of gene activation (7). We report here a chemical acetylation procedure that produces a limited acetylation of histones H3 and H4 similar to that seen in vivo. The chromatin thus acetylated shows a dramatic increase in susceptibility to DNAase I, but little increase in susceptibility to

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Staphylococcal nuclease, similar to the results observed with the globin and ovalbumin genes (2,3). These results also correspond favorably to those obtained recently in HeLa cell nuclei in which H3 and H4 acetylation levels had been increased by n-butyrate treatment of the cells (8). This chemical acetylation technique has significant potential usage in the study of the effect of histone acetylation on chromatin structure and its possible role in genetic control.

MATERIALS AND METHODS

Preparation of chromatin. Nuclei were prepared from frozen calf thymus tissue by following the procedure described in (9), however homogenization in the EDTA solution was omitted. The grinding medium used contained 0.1mM PMSF (phenylmethanesulfonylfluoride) rather than 0.05M $NaHSO_3$. The intact nuclei thus obtained were washed once in a 50X volume of a slightly modified 0.34M sucrose-buffer A (10) containing 0.34M sucrose, 60mM KCl, 15mM NaCl, 15mM BME, 0.1mM PMSF, 0.45mM spermine, 1.5mM spermidine, and 15mM Tris-HCl pH 7.4. The nuclei were collected by spinning at 480g for 10 min. and native chromatin prepared by brief nuclease digestion (11) except that the slightly modified 0.34M sucrose-buffer A described above was used.

Acetylation of chromatin. Acetyl adenylate prepared according to (12) was made fresh prior to each usage. 1mg/ml chromatin solutions in 0.16M Tris-Base pH 8.9, 0.05M $NaHSO_3$ were made .08mM in acetyl adenylate and were incubated at 37° C for 1 hour, during which time the reaction mixture was maintained at pH 8.9 with a pH Stat. The resulting acetylated chromatin was then dialyzed exhaustively against 0.2mM EDTA pH 7.0, 0.1mM PMSF. Acetylation levels were checked by acid precipitating an aliquot of the acetylated chromatin with 0.4N H_2SO_4. The histones thus obtained were then analyzed on a 6.25M urea acid-urea gel prepared according to (13) but run vertically for 48 hours at 15V/cm. Densitometric scans of gel negatives were run on a Joyce microdensitometer (Joyce-Loebl, England). Integrity of the histones was monitored on 18% SDS gels prepared as described in (14) with the acrylamide:bis ratio raised to 30:0.4.

DNAase I digestion studies. The DNAase I digestions were done under conditions similar to those under which DNAase I was shown to selectively digest active genes (2,3). Chromatin in 0.01M Tris-HCl pH 7.4, 0.01M NaCl, 3mM $MgCl_2$, 0.1mM PMSF at $A_{260} \sim 1$ was digested for various times with DNAase I (Worthington) at a concentration of 60U/ml at 37° C. Reactions were terminated by addition of cold 0.1M EDTA pH 7.0 to a final concentration of 0.01M and chilling on ice. Digestion of the samples was monitored by perchloric acid precipitation (15) and percent acid solubility calculated as in (16). To analyze the DNA digestion products the DNA was extracted (17) and the lyophilized product analyzed on 12% polyacrylamide gels (18). The gels were stained in 0.5mg/ℓ ethidium bromide for 1 hour and photographed through a Tiffen 15 Orange filter with UV illumination. Gel negatives were scanned on a Joyce midrodensitometer (Joyce-Loebl, England).

Staphylococcal nuclease digestion studies. Chromatin in 0.01M Tris pH 8.0, 2mM $CaCl_2$, 0.1mM PMSF at $A_{260} \sim 1$ was digested for various times with 5U/ml Staphylococcal nuclease (Worthington). The reaction was terminated by addition of cold 0.1M EDTA pH 7.0 to a final concentration of 0.01M and

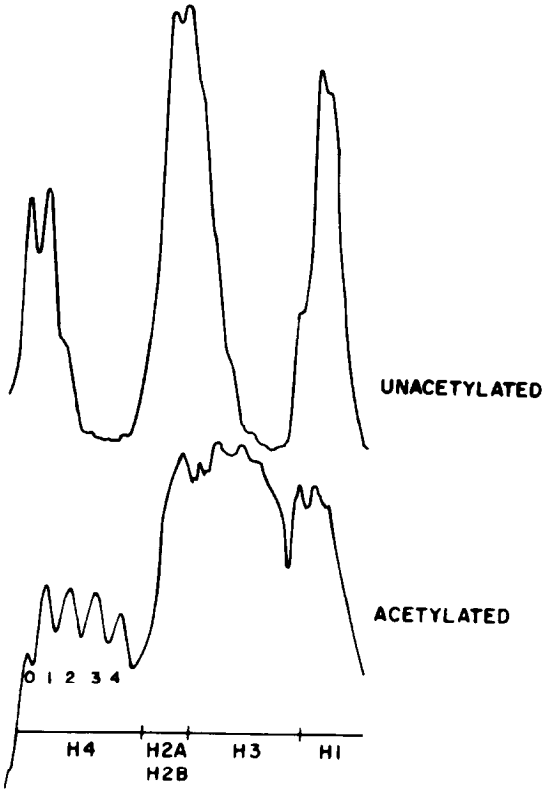

Figure 1. Densitometric scans of acid-extracted histones from acetylated and unacetylated chromatin analyzed on an acid-urea gel (13).

chilling on ice. Kinetics were followed by perchloric acid precipitation as described for the DNAase I studies.

RESULTS

The incubation of chromatin with acetyl adenylate leads to extensive, but discrete, acetylation of histones H3 and H4. This can be seen on a scan of an acid-urea gel, Figure 1. The mono-, di-, tri- and tetra-acetylated forms of histone H4 are present. From the densitometer scan, it can be estimated that between 70-75% of histone H4 has been acetylated by the acetyl adenylate procedure, while 25-30% remains unacetylated. Extent of acetylation of histone H3 is difficult to determine on the 6.25M urea acid urea gel system used (19). Mono- and di-acetylated forms of histone H3 appear to be present

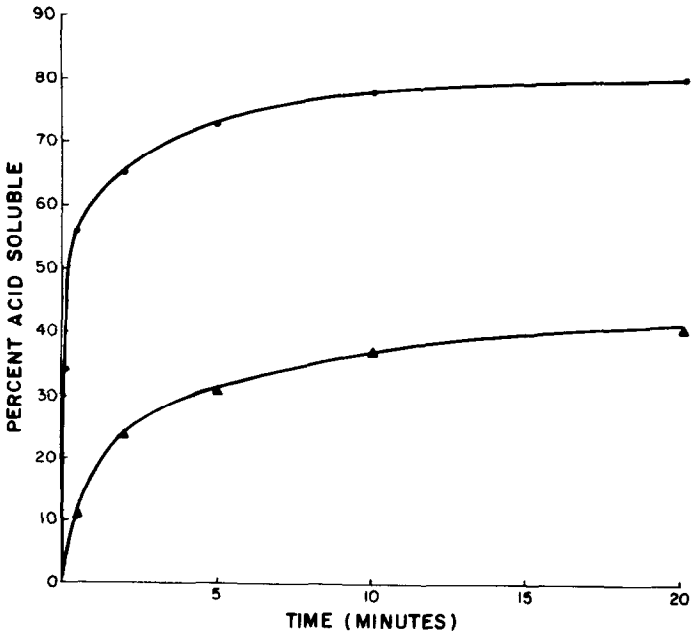

Figure 2. Time course of DNAase I digestion of unacetylated (Δ) and acetylated (o) chromatin. Chromatin (A_{260} 1) was digested at 37° C in 0.01 M Tris-HCl pH 7.0, 0.01 M NaCl, 3mM $MgCl_2$, 0.1 mM PMSF at a DNAase I concentration of 60U/ml. Reactions were stopped by addition of cold 0.1 M EDTA pH 7.0 to a final concentration of 0.01 M and chilling on ice.

and some tri-acetylated forms may also be present. Integrity of the histones following the acetylation procedure was routinely monitored on 18% SDS gels and no proteolysis was observed.

Active genes show an increased susceptibility to DNAase I but not to Staphylococcal nuclease (2,3). DNAase I and Staphylococcal nuclease digestion studies were performed to ascertain if the described chemical histone acetylation produces similar effects. As can be seen in Figure 2, the acetylation produces a dramatic increase in DNAase I susceptibility. Figure 3 shows that the DNAase I digestion of both acetylated and unacetylated chromatin produced characteristic DNA fragments that are multiples of ten base-pair units (17). The Staphylococcal nuclease digestion patterns

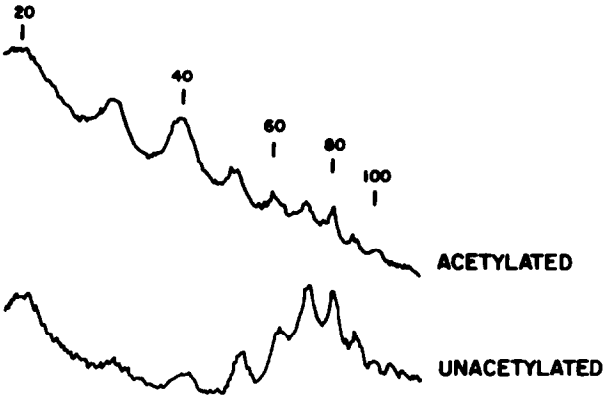

Figure 3. Densitometric scan of single-stranded DNA fragments produced by DNAase I digestion of acetylated and unacetylated chromatin. DNA was extracted (17), analyzed on 12% polyacrylamide gels (18), and stained with 0.5 mg/l ethidium bromide. Numbers indicate sizes of fragments in base-pairs. The unacetylated sample showed 23% acid-solubility (2 min) and the acetylated sample showed 35% acid-solubility (10 sec).

(Figure 4) of acetylated and unacetylated chromatin are initially the same, however, the acetylated chromatin becomes slightly more sensitive to Staphylococcal nuclease with time.

DISCUSSION

Acetylation with acetyl adenylate leads to mono-, di-, tri- and tetra-acetylated forms of histone H^4, and mono-, di- and perhaps tri-acetylated forms of histone H3. This acetylation pattern is similar to that produced *in vivo* in cells stimulated with n-butyrate (8). These same levels of histone acetylation have been observed *in vivo* in radiolabeling studies (7,20). Interestingly, this same level of histone acetylation is present in wild type SV 40 (21). In cells stimulated with n-butyrate, 70% of the histone H^4 already deposited on the chromatin can be acetylated (22); this correlates well with the 70-75% modification obtained with acetyl adenylate.

The results of the DNAase I digestion studies are similar to those seen in activated genomes (2,3) and to those obtained in cells whose histone acetylation levels have been increased by treatment with n-butyrate (8,23). n-Butyrate

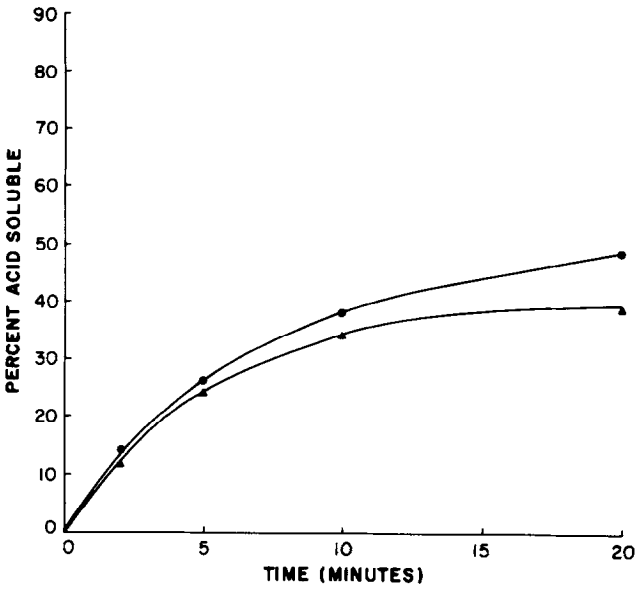

Figure 4. Time course of Staphylococcal nuclease digestion of unacetylated (Δ) and acetylated (o) chromatin. Chromatin (A_{260} 1) was digested at 37° C in 0.01 M Tris pH 8.0, 2mM $CaCl_2$, 0.1 mM PMSF at a Staphylococcal nuclease concentration of 5U/ml. Reactions were stopped by addition of cold 0.1 M EDTA pH 7.0 to a final concentration of 0.01 M and chilling on ice.

treatment may lead to induction of non-histone proteins (24) as well as histone acetylation and these non-histone proteins could have been responsible for the increased DNAase I sensitivity seen in n-butyrate treated cells (8). Production of this same effect in chromatin which has been chemically acetylated to in vivo levels supports the contention that the histone acetylation in n-butyrate treated cells is responsible for the increased DNAase I sensitivity.

Staphylococcal nuclease digestion studies using nuclei showed no increased sensitivity to this enzyme in active regions of the genome (2). Similarly, nuclei with increased acetylation levels due to n-butyrate, also show no increased sensitivity to Staphylococcal nuclease (8). However, nucleosomes isolated from n-butyrate treated cells do show an increased sensitivity to Staphylococcal nuclease (8). Therefore, the slight increase in sensitivity with time seen in Figure 3 is probably due to the release of nucleosomes from the 300-3000 base-pair native chromatin employed (11), allowing further digestion as observed by (8).

Chemical acetylation of histones while still bound to the DNA in chromatin has been accomplished previously with acetic anhydride (25). This treatment leads to acetylation of histones H1, H2A and H2B as well as histones H3 and H4. The levels of acetylation obtained in histones H1, H2A and H2B are greater than those observed in vivo (26). Although acetylation with acetic anhydride leads to increased levels of DNAase I sensitivity it also leads to a substantial increase in Staphylococcal nuclease sensitivity (27) which may arise from acetylation of histones H1, H2A and H2B to levels not observed in vivo. Chemical acetylation with acetyl adentylate was previously applied only to histones in solution (12) where it was observed to cause non-specific acetylation but not adenylation of histones. By performing the reaction on chromatin, with appropriate choice of pH, an acetylation pattern similar to that produced in vivo has been obtained.

Acetylation of histones has been linked to gene activation in numerous investigations (7). Early studies indicated a close association between levels of histone acetylation and levels of RNA synthesis. Specifically, in lymphocytes stimulated with mitogens, an increase in histone acetylation preceded an increase in RNA synthesis (28). In a study on erythrocytes at various stages of maturation (29), it was demonstrated that both histone acetylation and RNA synthesis decrease with increasing ages of erythrocytes. More recently it has been shown (21) that histones H4 and H3 associated with the SV 40 minichromosome are highly acetylated but those associated with the minichromatin of a non-transforming mutant lack extensive acetylation. The findings presented herein demonstrate that acetylation of histones with acetyl adenylate produces an acetylation pattern similar to that produced in vivo. This chemical acetylation procedure therefore should be very advantageous in studies on the effects of acetylation on the physical parameters involved in histone-DNA interactions. It should also be very useful in attempts to elucidate the possible role of acetylation of histones in gene activation.

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