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Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences

(histone acetylation/chromatin structure/nucleosomes/transcription)

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ABSTRACT **Exposure of HeLa cells to Na butyrate leads to an accumulation of multiacetylated forms of histones H3 and H4. Our studies of histone acetylation in HeLa S-3 cells show that 7 mM butyrate suppresses the deacetylation of histones without influencing the rate of radioactive acetate incorporation. An alteration in nucleosome structure in highly acetylated chromatin is indicated by an increased rate of DNA degradation by DNase I. A close association of acetylated histones with the DNase I-sensitive sequences is confirmed by the finding that histones remaining after limited DNase I digestion are depleted in the multiacetylated forms of histones H3 and H4. DNase I treatment has also been found to selectively release [^{3}H]acetyl-labeled H3 and H4 from avian erythrocyte nuclei under conditions previously shown to preferentially degrade the globin genes in erthyrocyte chromatin. Our results are consistent with the view that histone acetylation provides a key to the mechanism for altering chromatin structure at the nucleosomal level, and that this may explain the selective DNase I sensitivity of transcriptionally active DNA sequences in different cell types.**

After the discovery of histone acetylation in 1964 (1), one of us suggested (2, 3) that this post-synthetic modification of histone structure could provide an enzymatic mechanism for modulating the interactions between histones and DNA in ways that affect the structure and function of chromatin. Numerous correlations have been noted between increased acetylation of the histones and gene activation for RNA synthesis (for recent reviews, see refs. 4–6). Acetylation of the lysine residues in the basic amino-terminal regions of the histones neutralizes their positive charges and would be expected to weaken their interactions with the phosphate groups of the DNA strand enveloping the nucleosome core.

In order to relate changes in histone acetylation to chromatin structure and function, we have investigated the effects of acetylation on the nuclease sensitivity of associated DNA sequences. This approach is based on previous observations that transcriptionally active DNA sequences are preferentially degraded during limited digestions with DNase I, but not by digestion with staphylococcal nuclease (7, 8–11). Highly acetylated chromatin has been obtained by the exposure of HeLa S-3 cells to 7 mM Na butyrate (12). We find that the accumulation of the multiacetylated forms of histones H3 and H4 under these conditions is due to a suppression of histone deacetylase activity. A consequence of increased histone acetylation is a more rapid rate of degradation of the associated DNA sequences during limited digestions with DNase I. In HeLa cells and in avian erythrocytes, the multiacetylated forms of histones H3 and H4 are preferentially released during limited DNase I digestions, further confirming their association with transcriptionally active regions of the chromatin.

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MATERIALS AND METHODS

Cell Growth and Histone Modification. HeLa S-3 cells were grown in Eagle's minimal essential medium in the presence or absence of 7 mM Na butyrate as described by Riggs *et al.* (12). Nuclei were isolated as described (13). Histones were extracted from the purified nuclei in 0.2 M H_2SO_4 and precipitated in 10 vol of acetone. The histones were analyzed by high-resolution electrophoresis in 25-cm-long, 12% polyacrylamide gels containing acetic acid and urea (4, 14, 15).

Isolation of HeLa Nucleosomes. HeLa cell nuclei were suspended at a concentration of 1 mg of DNA per ml of buffer A (10 mM Tris·HCl, pH 7.8/10 mM NaCl/3 mM $MgCl_2$) containing 1 mM $CaCl_2$ and staphylococcal nuclease at 10 units/ml (Sigma, St. Louis, MO). After 80 sec at 37°, the reaction was stopped by the addition of 100 mM EDTA, pH 7.0, to a final concentration of 10 mM. The nuclei were sheared by sonication for 3 sec at setting 2 of a Branson Sonifier and centrifuged at 2000 × *g* for 10 min. Under these conditions, 70% of the total DNA was recovered in the supernatant fraction. The chromatin subunits were separated by centrifugation at 23,000 rpm for 20 hr in a 5–20% sucrose gradient in 0.2 M EDTA, using a Beckman SW-27 rotor. The peak of monomeric nucleosomes was diluted with 5 vol of buffer A and centrifuged for 6 hr at 23,000 rpm. The pellet was extracted with 0.2 M H_2SO_4 and the histones were analyzed electrophoretically.

DNase I Digestions and Analyses of Remaining Histones. Nuclei were prepared from HeLa cells grown in the presence of 7 mM Na butyrate and suspended at a concentration of 1 mg of DNA per ml in buffer A containing DNase I (Worthington, Freehold, NJ) at 20 Kunitz units/ml. After incubation at 37° for 60 sec, the reaction was stopped by the addition of 100 mM EDTA, pH 7.0, to a final concentration of 10 mM. The nuclei were sheared by pipetting and centrifuged at 2000 × *g* for 10 min. Under these conditions, *ca* 10% of the total DNA was recovered in the supernatant phase. Control nuclei, incubated in the absence of DNase I, were processed in the same way. The pellets were extracted with 0.2 M H_2SO_4 and the histones were analyzed electrophoretically.

DNA Labeling Procedures. HeLa cells were suspended at a concentration of 5×10^5 cells per ml and 1-liter aliquots were incubated for 5 hr at 37° with either 100 μCi of [*methyl*-^{14}C]thymidine (specific activity 60 mCi/mmol) or 1 mCi of [*methyl*-^{3}H]thymidine (specific activity 7 Ci/mmol; New England Nuclear, Inc., Boston, MA). The cells were washed in medium, suspended at a concentration of 3–4 × 10^5 cells per

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ml, and grown separately in nonradioactive medium for 20 hr. The ^{3}H-labeled cells were harvested and frozen in 1:1 (vol/vol) medium/glycerol. The ^{14}C-labeled cells were incubated for an additional 21 hr in medium containing 7 mM Na butyrate to increase the level of acetylation of the histones. The ^{14}C-labeled cells were harvested and combined with the ^{3}H-labeled cell preparation. Nuclei were isolated from the mixed suspension after homogenization in buffer A containing 0.5% Nonidet P-40 (British Drug House) (7, 13).

Kinetics of DNA Degradation by DNase I. Aliquots of the mixed nuclear suspension containing 1 mg of DNA per ml were incubated with DNase I at 10 Kunitz units/ml for 0–60 min. The reaction was stopped at the indicated times by adding EDTA to a final concentration of 10 mM, and acid-insoluble products were precipitated in cold 7% (wt/vol) perchloric acid. After 15 min at 0°, the samples were centrifuged at 5000 × *g* for 10 min. Aliquots of the clear supernate were withdrawn for determination of ^{3}H and ^{14}C activities by scintillation spectrometry.

Radioactive Acetate Uptake into HeLa Cell Histones. Equal aliquots (350 ml) of a HeLa cell suspension containing 5–6 × 10^5 cells per ml were incubated with either 1.5 mCi of [*methyl*-^{3}H]acetate (specific activity, 700 mCi/mmol) in Eagle's medium or 300 μCi of [*1*-^{14}C]acetate (specific activity, 4 mCi/mmol) in medium containing 7 mM Na butyrate. After 5, 10, and 15 min of incubation at 37°, 100-ml aliquots of each suspension were mixed and diluted with an equal vol of ice-cold medium. Cells were collected by centrifugation at 2000 × *g* for 10 min and nuclei were isolated. Histones were extracted in 0.2 M H_2SO_4 and precipitated in acetone. The histones were redissolved at a concentration of 1 mg/ml in 1% sodium dodecyl sulfate, and aliquots were taken for measurement of ^{3}H and ^{14}C activities.

Turnover of Histone Acetyl Groups in Intact HeLa Cells. Equal aliquots (500 ml) of a HeLa cell suspension containing 5–6 × 10^5 cells per ml were incubated for 15 min at 37° in medium containing either 2.5 mCi of [*methyl*-^{3}H]acetate or 500 μCi of [*1*-^{14}C]acetate. The ^{3}H-labeled cells were harvested, washed twice with warm, nonradioactive medium and resuspended in 500 ml of medium. The ^{14}C-labeled cells were harvested, washed, and resuspended in medium containing 7 mM Na butyrate. Both suspensions were incubated at 37° and samples were withdrawn at 0, 10, 20, 30, and 60 min. The ^{3}H-labeled (control) cells and ^{14}C-labeled (butyrate-treated) cells were mixed prior to isolation of the nuclei and extraction of the histones. The [^{3}H]- and [^{14}C]acetyl content of the histone samples was determined as described.

RESULTS

Multiacetylated Histones in Nucleosomes of Butyrate-Treated HeLa Cells. It has been observed that exposure of HeLa cells to Na butyrate leads to an accumulation of multiacetylated histones in the nucleus (12). The increased acetylation of the histones has now been examined in isolated nucleosomes. HeLa nuclei prepared from cells grown in the presence or absence of 7 mM Na butyrate were treated with staphylococcal nuclease and the resulting chromatin fragments were separated by sucrose density gradient centrifugation. The purified monomeric nucleosomes were extracted with 0.2 M H_2SO_4 and the histones in the extract were precipitated and analyzed by electrophoresis in polyacrylamide gels under conditions that separate the major histone classes and resolve derivatives of histones H3 and H4 that differ in their degree of internal acetylation (4, 14–16). Fig. 1 compares the densitometric tracings of the histone banding patterns from nucleosomes of control cells and butyrate-treated cells. It is clear that a major change in the level of acetylation of histones H3 and H4 resulted from butyrate treatment. The presence of multiacetylated forms of these histones in HeLa nucleosomes is in accord with the recent demonstration that multiacetylated forms of H4 occur in monomeric nucleosomes from trout testis cells (17).

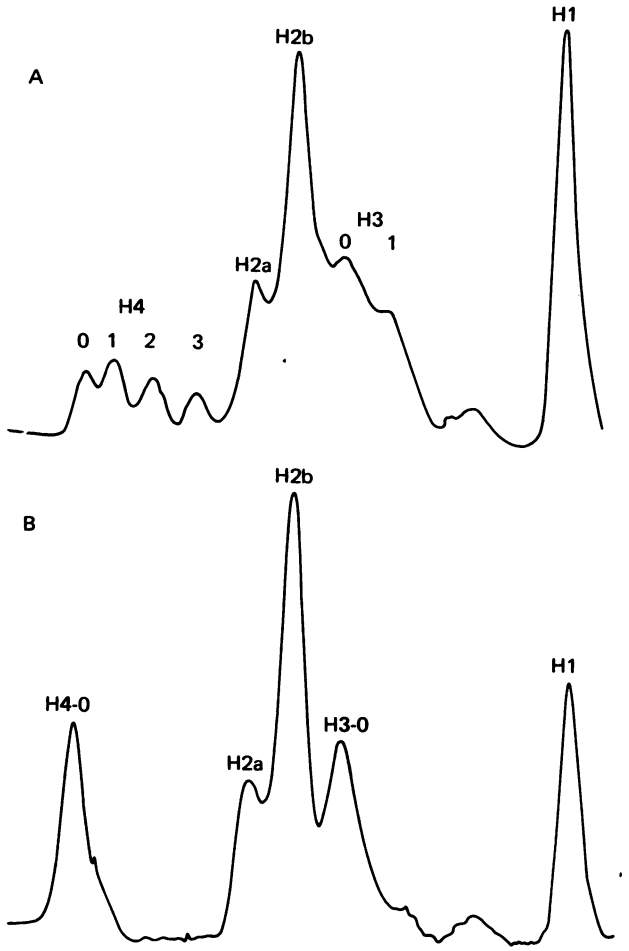

FIG. 1. Changes in acetylation of nucleosomal histones H4 and H3 following a 21-hr exposure of HeLa S3 cells to 7 mM Na butyrate. The nucleosomes of control and butyrate-treated cells were prepared by digestion of the isolated nuclei with staphylococcal nuclease, and the nucleosome monomers were purified by density gradient centrifugation. The histones were extracted and analyzed by electrophoresis. Densitometric tracings of the stained histone bands are shown for the mononucleosomes of butyrate-treated cells (*Upper*) and for the control mononucleosomes (*Lower*). The degree of acetylation is indicated by H4-0, H4-1, H4-2, etc. Note the increase in acetylated forms of histones H4 and H3 in nucleosomes from butyrate-treated cells.

Mechanism of Induction of Histone Hyperacetylation by Butyrate. Acetate uptake into histones *in vivo* is known to be reversible, and the overall level of acetylation involves an equilibrium between acetate uptake, as catalyzed by acetyl transferases, and acetate removal, as catalyzed by histone deacetylases (5, 6). The effect of butyrate on both of these processes has now been investigated in intact HeLa cells. Comparison of the kinetics of radioactive acetate uptake into the histones of cells grown in the presence or absence of 7 mM Na butyrate shows that butyrate has little or no effect on the rate of acetylation of nucleosomal histones (Table 1). Thus, the increased level of histone modification seen in butyrate-treated

Table 1 Effects of Na butyrate on acetate uptake and release from HeLa cell histones

Time, min	Specific activities of histones labeled with [^{3}H]-Acetate,* cpm/mg	[^{14}C]-Acetate,† cpm/mg	Ratio ^{14}C/^{3}H‡
	Acetate uptake		
5	3580	3610	1.01
10	4590	4410	0.96
15	7990	7360	0.92
	Acetate retention§		
0	5710	6190	1.08
10	4280	5610	1.31
20	3460	5260	1.52
30	3060	5370	1.75
50	2420	5200	2.15

* Control cells in butyrate-free Eagle's minimal essential medium.
† Cells in medium containing 7 mM Na butyrate.
‡ The rates of uptake of [^{3}H]acetate and [^{14}C]acetate were essentially linear over this brief period, and plots of histone ^{3}H or ^{14}C activity vs. time (not shown) had equal slopes. This accounts for the near constancy of the ^{14}C/^{3}H uptake ratio.
§ HeLa cell histones were prelabeled with either [^{3}H]acetate or [^{14}C]acetate. The cells were washed and suspended in nonradioactive medium in the presence (^{14}C) or absence (^{3}H) of 7 mM Na butyrate during the "cold chase."

cells cannot be attributed to stimulation of acetyl transferase activities.

Butyrate has a marked inhibitory effect on the "turnover" of previously incorporated acetyl groups, as indicated by the experiments summarized in the lower half of Table 1. Butyrate suppressed the deacetylation of histones. Since it had no appreciable effect on the uptake of acetate, the net effect was a progressive accumulation of the acetylated forms of histones H3 and H4, as observed in the isolated nucleosomes (Fig. 1) and in whole nuclei of butyrate-treated cells (12).

Enhanced DNase I Sensitivity of Chromatin Containing Multiacetylated H3 and H4. We have compared the chromatins of control and butyrate-treated HeLa cells for their sensitivities to DNase I and staphylococcal nuclease. The DNA in the two cell populations was differentially labeled and the cells were mixed prior to isolation of the nuclei. The kinetics of DNase I degradation of the nuclear DNAs are compared in Fig. 2, which plots the time course of acid solubilization of the control (^{3}H) and highly acetylated (^{14}C) chromatins and also shows the ratio of ^{14}C/^{3}H activities at each time point during the DNase I digestion. The change in the ^{14}C/^{3}H ratio at early times is particularly revealing, because it indicates that chromatin containing the highly acetylated histones is more susceptible to DNase I attack.

Similar experiments using staphylococcal nuclease in place of DNase I did not show this striking difference in the rates of degradation of chromatins from control and butyrate-treated cells (data not shown). This result, taken together with the knowledge that staphylococcal nuclease selectively cleaves the DNA strands between the nucleosomes whereas DNase I can attack DNA enveloping the nucleosome core (18), strongly supports the view that acetylation of the nucleosomal histones H3 and H4 alters DNA interaction with the amino-terminal arms of the core histones and increases its susceptibility to DNase I attack.

Selective Release of Acetylated Histones during Limited Digestions with DNase I. To test the assumption that the DNA

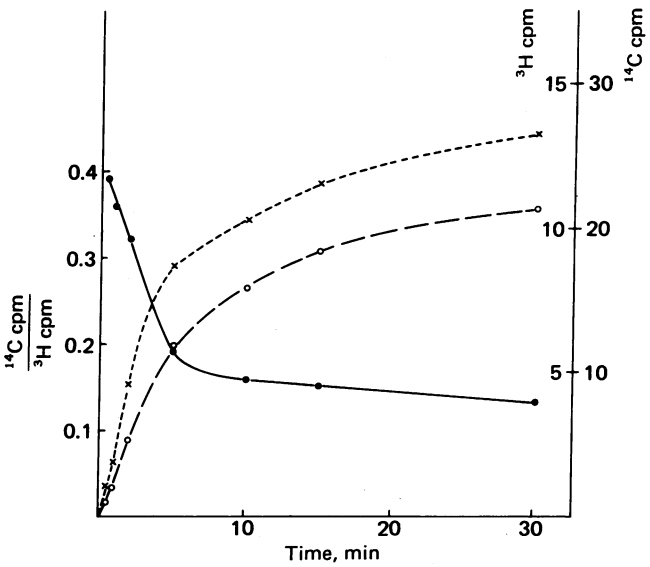

FIG. 2. Comparative kinetics of DNase I digestion of HeLa chromatins differing in their content of acetylated histones. Cells were labeled with [^{3}H]thymidine or [^{14}C]thymidine. The ^{3}H-labeled cells were harvested, and the ^{14}C-labeled cells were incubated in 7 mM Na butyrate to increase the level of histone acetylation. Both cell populations were then mixed and the nuclei were isolated. The mixed nuclear suspension was incubated with DNase I and the kinetics of release of [^{3}H]DNA (o---o, cpm × 10^{-3}) fragments and [^{14}C]DNA (×---×, cpm × 10^{-2}) fragments were compared. The ratio of ^{14}C/^{3}H was calculated for each time point. The early decrease in this ratio (●—●) indicates a preferential attack by DNase I on the highly acetylated chromatin in butyrate-treated cells. This result has been confirmed in eight separate experiments.

sequences that are most rapidly degraded by DNase I are associated with highly acetylated forms of histones H3 and H4, we have examined the proportions of modified molecules remaining in the nuclei of butyrate-treated HeLa cells after a brief incubation in the presence or absence of DNase I. The histone electrophoretic patterns are compared in Fig. 3, which shows that the proportions of the multiacetylated forms of histone H4 (relative to the content of the nonacetylated form) are significantly lower after removal of only 10% of the DNA. It follows that the most highly acetylated H4 molecules must have been released together with the most rapidly degraded DNA sequences.

Similar conclusions have been drawn from studies of histone release during limited DNase I digestions of avian erythrocyte nuclei containing [^{3}H]acetyl-labeled histones. Equal aliquots of a nuclear suspension were incubated in the presence or absence of DNase I and the specific activities of the residual histones were compared. The specific ^{3}H activities of H3 and H4 in the control nuclei were 67,000 and 70,000 cpm/mg, respectively; in the DNase I-treated nuclei the corresponding ^{3}H activities were 43,000 cpm/mg of histone H4 and 50,000 cpm/mg of histone H3. Thus, the specific activities of both H3 and H4 remaining in the chromatin after removing only 11–12% of the DNA were about 32% lower than the ^{3}H activities of histones in nondigested chromatin. It follows that the acetylated forms of the histones were preferentially released by DNase I under conditions known to selectively degrade the transcriptionally active DNA sequences of the erthrocyte nucleus (7). These results are consistent with early suggestions that acetylation of the histones plays a role in gene activation (1–3), presumably by releasing structural constraints upon associated DNA sequences.

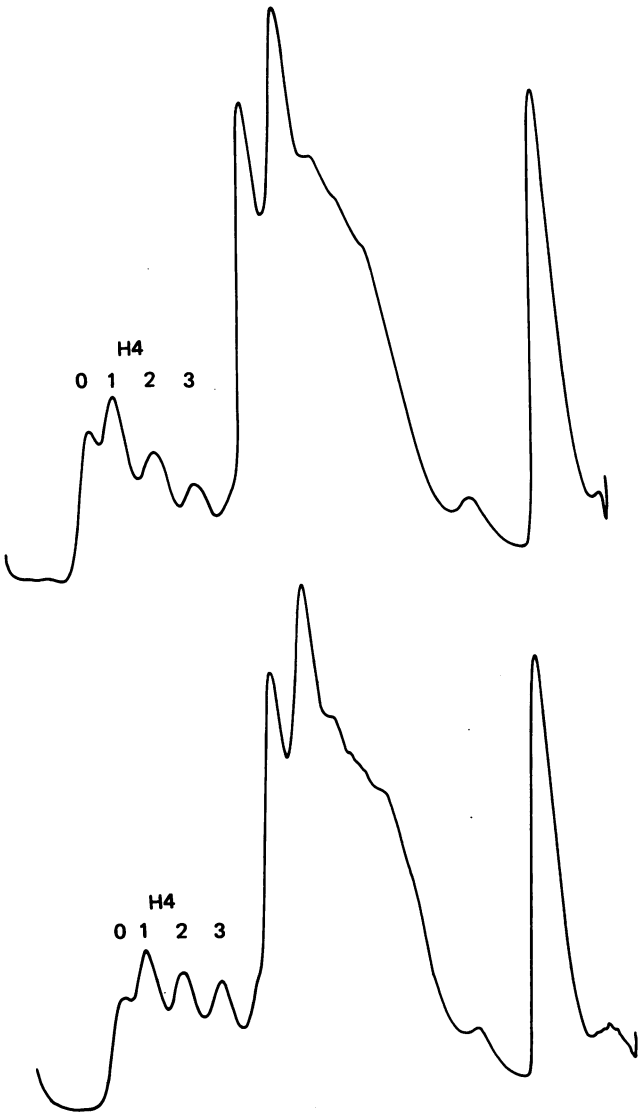

FIG. 3. Selective release of multiacetylated forms of histone H4 during limited digestion of HeLa cell nuclei by DNase I. Aliquots of a nuclear suspension from butyrate-treated cells were incubated with DNase I (*Upper*) or in nuclease-free medium (*Lower*). After digestion to solubilize *ca* 10% of the total nuclear DNA, the histones were extracted from the residual chromatin and analyzed electrophoretically. Note that the proportions of the di- and tri-acetylated forms of H4 (relative to the nonacetylated form, H4-0) are much reduced after DNase I treatment, compared to the corresponding proportions in control nuclei incubated without DNase I. Quantitative densitometry of the banding patterns indicates an H4-3/H4-0 ratio of 0.9 in the residual chromatin after DNase I digestion, compared with a ratio of 1.9 in the control chromatin.

DISCUSSION

The enhanced DNase I sensitivity of transcriptionally active DNA sequences (7, 8–11) appears to depend upon their association with multiacetylated forms of the histones. This view is supported by a number of independent observations. The diffuse, extended regions of the chromatin, which are the predominant sites of chromosomal RNA synthesis (19), show the highest levels of histone acetylation (2–5, 20) and are digested more rapidly by DNase I than are the transcriptionally inert, condensed areas of chromatin (21). The increase in histone acetylation that precedes the induction of new RNA synthesis in mitogen-stimulated lymphocytes (22) is accompanied by an increased accessibility of DNA to acridine orange (23) and actinomycin D (24). Conversely, the suppression of transcription in maturing avian erythrocytes is paralleled by a decreased acetylation of histones H3 and H4 (25). In the mature sperm of *Arbacia lixula*, where no RNA synthesis takes place, these histones appear only in their nonacetylated forms (16); after fertilization, the acetylated forms of H3 and H4 reappear with the resumption of RNA synthesis at the blastula stage (16).

Our observation that increased acetylation of HeLa histones labilized the associated DNA sequences to DNase I digestion suggests that a significant structural change has occurred at the nucleosomal level. Yet comparisons of the physical properties of nucleosome monomers prepared from butyrate-treated and control cells show no appreciable differences in sedimentation coefficient, DNA melting curves, or circular dichroism (data not shown). Failure to detect such differences may simply be due to the fact that the nucleosome fractions analyzed represent populations of particles differing widely in their degree of acetylation, and only a small fraction of the total population may exhibit the changes accounting for increased lability to DNase I. The DNA degradation kinetics shown in Fig. 2 clearly indicate that only some of the nucleosomes in the butyrate-treated cells are subject to preferential attack, presumably determined by an unusually high content of multiacetylated forms of the histones (Fig. 3).

The distribution of ϵ-*N*-acetyllysine residues is not random; all of the modifiable lysines in histones H3 and H4 occur in the amino-terminal portion of the polypeptide chain, which by virtue of its clustering of the basic amino acids—lysine, arginine, and histidine—carries a high net positive charge. Similar structural considerations apply to histones H2A and H2B. Thus, all histone classes in the octet comprising the nucleosome core (18) are structured to favor DNA binding through their amino-terminal regions. These positively charged regions would be expected to interact electrostatically with the negatively charged phosphate groups of the DNA helix which envelops the nucleosome core (26). There are many indications that the amino-terminal arms of the histones are responsible for DNA binding (27–33). The selective digestion of the arms by trypsin (30) increases the rate of nuclease attack on the DNA of the nucleosome particle (30–32) and alters the cutting pattern of the 140-nucleotide-pair DNA fragment by DNase I, changing the accessibility of certain sites without destroying the 10-nucleotide spacing of the cuts (31, 32).

We believe that similar changes in DNase I sensitivity, reflecting conformational changes in chromatin structure, are brought about by acetylation of the amino-terminal regions of the histones. The relatively high rate of acetylation of histones H3 and H4, in comparison with other histone classes (1, 25, 34), is consistent with the key role played by H3 and H4 in nucleosome organization (35–37). The release of constraints on the enveloping DNA helix may result in a relaxation of DNA twists within the nucleosome to favor a more extended configuration of the template. This structural change, which clearly influences DNA accessibility to DNase I, may permit DNA interactions with other enzymes and regulatory proteins involved in transcription. This would account for the many spatial and temporal correlations between histone acetylation and RNA synthesis (5, 6) and for observations that chemical acetylation of the histones increases the transcriptional activity of cell-free systems (1, 38).

While the binding of histones to DNA in isolated nucleosomes may be largely restricted to the 140-nucleotide-pair segment, higher orders of organization in chromatin may also involve the amino-terminal arms of the histones, perhaps by affecting DNA

structure in the internucleosomal "linker" regions. If so, the modification of the histones by acetylation could have long-range effects on chromatin coiling.

Of particular interest in relation to acetylation as a structural control in transcriptionally active DNA are observations on the high level of acetylation of histones in viral chromatins (39) and evidence that the transcribing segments of integrated viral genomes are selectively attacked by DNase I (10). Recent experiments (G. Vidali and C. Vesco, unpublished data) have shown that the accumulation of multiacetylated forms of histones H3 and H4 in simian virus 40 chromosomes is due to a suppression of histone deacetylation in the viral, but not in the host, chromatin. This emphasizes the important role of the histone deacetylases in the control of chromatin structure and activity. It is known that liver nuclei contain deacetylases specific for H3 and H4 (40) and that the activity of the deacetylases immediately increases when hepatic RNA synthesis is suppressed by aflatoxin B_1 (34). A hierarchy of controls over histone deacetylation is indicated by changes in enzyme activity during muscle development in the chicken embryo (41) and by the apparent loss of an inhibitor during purification of the enzyme from thymus nuclei (40). In view of the accumulating evidence that histone acetylation does affect the organization and function of chromatin, the control of deacetylase activities warrants further investigation.

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