Isolation and Characterization of Acetylated Histones H3 and H4 and Their Assembly into Nucleosomes

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Nucleosome and chromatin structure/function relationships of histone acetylations are not understood. To address these questions we have developed chromatographic procedures that separate subtypes of H3 and the acetylated states of histone H3 and H4 in exceptionally pure forms. The sites of acetylation of the intermediated acetylated states of H3 have been determined and show a specific pattern of acetylation. An unexpected finding was the identification of a fifth site of acetylation in H3 at lysine 27. Nucleosome particles with fully acetylated H3 and H4 have been assembled on the Lytechinus variegatus 5 S rRNA DNA phasing sequence and characterized. These defined acetylated H3 and H4 particles migrate more slowly in polyacrylamide nucleoprotein particle gels than the core particles indicating a subtle effect of acetylation in nucleosome structure. However, DNA footprinting of these particles using DNase I show only small changes when compared to control particles over the core particle DNA length. It is shown further that H3 cysteines in the particle containing fully acetylated H3 and H4 were not accessible to iodoacetamide indicating that protein factors additional to H3 and H4 acetylation are required to make H3 cysteines accessible to the label. These findings are consistent with the proposal that histones H3, H4 acetylations exert their major effects outside of the core particle 146-base pair DNA, either on the DNA segment entering and leaving the nucleosome or possibly on the inter-nucleosome interactions that involve the amino-terminal domains of the core histones in organization and stability of higher order chromatin structures.

The four core histones of the nucleosome have well defined structural domains. Each has a flexible, basic amino-terminal domain extending from a structured globular domain. H2A and H3 also have short COOH-terminal tails extending from the globular central domain (1). The amino-terminal domains of the four core histones contain all the sites of reversible acetylation (2). The known sites are: in H2A, lysine 5; in H2B, lysines 5, 12, 15, 20; in H3, lysines 9, 14, 18, and 23; in H4, lysine 5, 8, 12, and 16. In this study, a fifth site of acetylation of histone H3 (Lys-27) has been identified. So far the modes of interaction and functions of the NH2-terminal domains in nucleosomes and chromatin are not well understood. However, it has been recently shown that high levels of acetylation of all four core histones cause a reduction in the linking number change per nucleosome in assembled circular minichromosomes from −1.01 to −0.82 (3). Thus histone acetylation releases DNA supercoils previously constrained on nucleosomes, which has implications for changing the topological state of chromatin domains.

There is a long established association of reversible histone acetylation of the core histones with gene activity (see Allfrey (2) for a review of the earlier work) based on a variety of correlative data. Studies of the cell cycle in Physarum polycephalum show elevated incorporation of labeled acetate into modified H4 during periods of gene activity (4). DNase I has been shown to digest preferentially the active gene sequences in chromatin (5–8). Although this nuclease sensitivity may be conferred by the non-histone proteins, e.g. HMG 14 and 17 (9–12), the histones released by such digestion contain elevated levels of acetylation (13, 14). Treatment of tissue cultured cells with the histone de-acetylase inhibitor, n-sodium butyrate (15), enhances both the DNase I digestion of chromatin and the release of more highly acetylated histones (16, 17). An increased DNase I susceptibility has been reported for positions 60 bp from each end of the DNA in hyperacetylated core particles (18, 19). Nuclease digestions using micrococcal nuclease (20, 21) or DNase II (22) release a 2 nm Mura2-soluble fraction of chromatin which is enriched in both active gene sequences and in acetylated histones (23, 24) and the solubility of this active chromatin is selectively enhanced by butyrate treatment (25–27). Nucleosome core particles containing very high levels of acetylation have been obtained by their differential solubility (18) or fractionation on HAP columns (28). Strong correlations of histone acetylation with gene expression come from studies of accessible sulfhydryls found in active chromatin. In P. polycephalum rDNA chromatin, the ordinarily protected H3 sulfhydryls in inactive nucleosomes are accessible to thiol-specific alkylating agents in active chromatin (30, 34) especially in the AcH3 and AcH3-(34). Mercurial agarose retains a chromatin fraction which is enriched in acetylated H3 and H4 (29, 35). Retention is tightly correlated with gene activity: histone gene sequences from S-phase but not G2-phase chromatin are retained (35), and c-myc and c-fos are retained only and precisely during their periods of induced activity (36). Different states of histone acetylation have been associated with different chromatin functions: acetylation of all four core histones with DNA replication and hyperacetylation of H3 and H4 with transcriptional activity (38).

The effects of histone hyperacetylation at the core particle level have been subtle. Imai et al. (28) confirmed the report of Bode et al. (33) that hyperacetylated core particles have

1 The abbreviations used are: bp, base pair(s); HPLC, high performance liquid chromatography.
properties. The cation exchange step is generally applicable to all the purified core histones, although only the fractionation of each acetylated form of the less hydrophobic H3 have been determined; in particular a fifth site of acetylation in histone are fully acetylated with respect to H3 and H4 have been determined: in the acetylated nucleosome particles are not accessible to iodoacetamide, suggesting that protein factors additional to histone acetylation are required to make their cysteines accessible. DNA footprints of nucleosome particles assembled using fully acetylated H3 and H4 show only small changes when compared to control particles over the core particle DNA length. The locations and occupancy of the sites of acetylation in each acetylated form of the less hydrophobic H3 have been determined; in particular a fifth site of acetylation in histone H3 (Lys-27) has been identified. Nucleosome particles which are fully acetylated with respect to H3 and H4 have been assembled and characterized. It is shown that the H3 cysteines in the acetylated nucleosome particles are not accessible to iodoacetamide, suggesting that protein factors additional to histone acetylation are required to make their cysteines accessible. DNA footprints of nucleosome particles assembled using fully acetylated H3 and H4 show only small changes when compared to control particles over the core particle DNA length.

EXPERIMENTAL PROCEDURES

RESULTS

Occupation of Sites of Acetylation in H3 and Identification of a Fifth Site—The phenylthiohydantoins of both the acetylated and the monomethylated lysines were readily distinguishable from that of lysine. For each of the acetylated forms of H3.2/.3 isolated from butyrate-treated cells, the levels of acetylation at each of the five sites has been established (Fig. 4). In the first three acetylated forms, recovery of total lysine derivatives at lysine 9 was low. This may be attributed to dimethylation, which has been reported at this site, but which was not analyzed for in the present experiments. The percentages of acetylated and monomethylated lysine at this residue were therefore calculated against an interpolated value for total lysine. From these data it is clear that the order of acetylation of lysines in histone H3 is not random. In the monoacetylated H3, lysines 14 and 23 are each acetylated almost to the 50% level with a trace of acetylation on lysine 18. The levels of acetylation of lysines 14 and 23 increase to 90% in the diacetylated H3 with low levels of acetylation of lysines 18 and 9. In the triacetylated form the major increase in acetylation is of 18, whereas in the tetraacetylated form the major increase in acetylation is of lysine 9. In the tetraacetylated H3 lysines 9, 14, 18, and 23 are fully acetylated and this situation appears to be a prerequisite for the acetylation of lysine 27, which is found to be fully acetylated in the pentaacetylated state. Monomethylolation was detected at both lysines 4 and 9 (Fig. 5) of the first three acetylated forms and at lysine 4 only in the pentaacetylated form. The lysines 27 and 36 of AcbH3.2/.3 and lysine 36 of the AcbH3.2/.3 were all reported in only trace amounts.

Natural Levels of Acetylation—To determine whether the fifth site of acetylation of histone H3 resulted from the butyrate treatment of the cells or whether it occurs naturally, the state of acetylation of control H3 was examined. H3.1 and H3.2/.3 from control HeLa cells were chromatographed by cation exchange HPLC. All of the forms detected in butyrate-treated cells are still present (Fig. 6, A and B) but the nonacetylated form is now the most prominent and the higher acetylated forms are much less so. AcbH3.2/.3 from butyrate-treated cells used as a standard eluted at the same retention time as the penta-modified form in H3.2/.3 from control cells (Fig. 6, C and D). The tetra- and pentaacetylated forms combined represent about 1% of the H3 of cycling HeLa cells.

Nucleosome Assembly—Nucleosomes assembled using fully acetylated H3 and H4 (i.e. using a H2A/H2B mixture from control cells) consistently exhibit a lower mobility in nucleoprotein polyacrylamide gels than do either those reconstituted with control or with nonacetylated inner core histones (Fig. 7).
The percentage of monomethyllysine (MML) at lysines 4 and 9 of the acetylated forms of H3.2/3 were plotted against the number of acetyl residues per molecule of H3. Reliable data were not obtained for cycle 4 of the tetraacetylated H3 rendering the percentage at that cycle highly uncertain.

7). This effect is independent of the DNA which is used for the reconstitution as it is seen both with the core particle length DNA (Fig. 7A) and with the monomeric 5s-207 phasing sequence (Fig. 7B). The latter produces a more complex pattern of reconstituted bands but all of the nucleosomal bands shift as a result of acetylation. The shift is also independent of which non-allelic variant of H3 was used, as can be seen in Figs. 7A and 10A.

Three closely spaced bands were observed in the nucleosomes reconstituted on the 5s-207 DNA. However, when the bands were excised from a nucleoprotein particle gel and loaded onto a second dimension sodium dodecyl sulfate gel, all four core histones were present in each at similar ratios (data not shown). The origin of the multiple reconstituted bands is not fully understood. However, it has been shown that nucleosomes assembled onto tandem repeats of the 5s-207 phasing sequences are located on several positions multiples of 10 bp away from a dominant position (59), and this could be an explanation for the multiple bands.

**Thiol Reactivity of Histone H3 Cysteines**—It has been shown that the cysteines of H3 in the very transcriptionally active *P. polycephalum* rDNA chromatin are fully accessible to iodoacetate or iodoaceticamide derivatives (30, 34). In contrast, transcriptionally inactive nucleosomes are not labelled by these reagents (29–33). The active rDNA nucleosomes, called leksomes, have a more open configuration than inactive nucleosomes and not only have hyperacetylated H3 (34) but also contain stoichiometric amounts of the non-histone chromosomal proteins LP30 and LP32 (30). The ability to assemble nucleosome core particles with fully acetylated histone H3 and H4 allows us to determine whether these acetylated states, which are associated with active genes, are sufficient to allow access of H3 cysteines to iodoacetamide. Whereas the free H3 in urea-denatured core histone mixtures can be labeled with the reagent, H3 in either nonacetylated assembled nucleosomes or in fully H3 and H4 acetylated nucleosomes could not be labeled (Fig. 8). This is consistent with electron micrographs of cccS-207-18 minichromosomes assembled with fully acetylated histones which show normally compact nucleosome particles, not extended ones (74).

**DNA Footprints of Nucleosomes Containing Fully Acetylated H3 and H4**—There are several explanations for the remarkable result that hyperacetylation of core histones results in a decrease in the linking number difference per nucleosome from −1.04 to −0.82 (3). First, histone hyperacetylation could release 9.3 bp of DNA from the ends of the DNA entering and exiting the core particle (3). Second, a change in the shape of the histone octamer (60), acting as a spool for the DNA linking number difference of a core particle without release of DNA from the ends of the core particle. Third, histone acetylation might affect internucleosomal interactions and higher order nucleosome arrangements. To test these possibilities we have compared the DNase I footprints of fully acetylated (with respect to H3 and H4) nucleosomes

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The footprints extend from approximately 55-186 nucleotides of the nucleohistone complexes were separated on the nucleoprotein gel in Fig. 1OA denaturing gels. The noncoding strand was labeled in these experiments because of the greater distance from the reported position of the phased nucleosome obtained with Simpson and Stafford (61) on the same strand except that no strong cleavage near 168 nucleotides on the coding strand falls within the transcription factor IIIA binding site. The transcription factor IIIA binding site is similar assembled on the 5s-207 DNA with control nucleosomes similarly assembled.

Acetylation does appear to confer a modest increased sensitivity to DNase I at several sites well inside the nucleosome. The clearest of these are the triplets around 102 and 82 nucleotides from the labeled end, and the doublets at 89, 91, and 69-70 on the coding strand (Fig. 9). However, no major change in the cutting pattern has occurred, and the same periodicity has been retained.

The reported position of the phased nucleosome obtained by DNase I footprinting of both strands would correspond to between 168 nucleotides on the coding strand and 185 on the noncoding strand or 23-168 and 185-40, respectively (61). The footprints in Fig. 9 closely resemble those obtained by Simpson and Stafford (61) on the same strand except that no strong cleavage near 168-171 bases was observed for these reconstituted nucleosome particles. Digestion in 10 mM MgCl₂, 3 mM CaCl₂ under the same conditions used previously (61) did not restore this cleavage site (data not shown). The region between 143 and 171 nucleotides from the 3' end of the coding strand falls within the transcription factor IIIA binding site. The transcription factor IIIA binding site is about 50 bp long and spans the region 138-185 bp from the end of the 207-nucleosome phasing sequence.

The formation of the three complexes with the phasing sequence DNA could have obscured some differences between acetylated and unacetylated nucleosomes. Therefore, the complexes were separated on the nucleoprotein gel in Fig. 10A after the DNase I footprinting reaction and the DNA run on denaturing gels. The noncoding strand was labeled in these experiments because of the greater distance from the reported edge of the phased nucleosome (40 nucleotides from the labeled end).

The footprints in Fig. 10B show that the three complexes do not mask differences between acetylated and unacetylated nucleosomes. The three particles footprint differently, particularly in the 128- and 150-nucleotide region. In contrast, each acetylated particle footprints only slightly differently from its unacetylated control (compare lanes j to k, l to m, and n to o). The footprints extend from approximately 55-186 nucleotides for the upper two of the three particles from the nucleohistone gel, but somewhat less at both ends for the third.

The apparent order of usage of the acetylation sites differs from that found from Tetrahymena thermophila macronuclear H3 (microsequenced from bands on gels) where the

**FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iodo[1-14C]acetamide-treated reconstituted nucleosomes. A, Coomassie-stained and dried gel; B, autoradiogram of the above gel. Lanes 1-3, reconstituted 5s-207 nucleosomes treated with iodoacetamide as described; lane 1, unacetylated; lane 2, fully acetylated in H3 and H4; lane 3, control; lane 4, HeLa histone marker (not treated); lane 5, iodoacetamide-treated, urea-denatured control histones. A field inhomogeneity caused the greater migration in lane 2; the histones were intact. Acetylation of H3 typically causes poor resolution between H3 and H2B (74).**

**FIG. 9. DNase I footprint of control and acetylated nucleosome reconstituted on 3'-coding strand-labeled 5s-207 DNA.** The digestions were for 2 min at room temperature in a final volume of 40 µl with the amount of DNase I indicated in the final reconstitution buffer with 100 mM NaCl and 5 mM added MgCl₂. Lanes 2-5, control histone reconstitutes digested with; lane 2, 2 µl; lane 3, 4 µl; and lane 4, 16 µl of a 5-fold dilution of the stock enzyme (-80 °C stored stock); and lane 5, 12.8 µl of the undiluted stock. Lanes 6-9, acetylated reconstitutes digested with the same conditions as for lanes 2-5, respectively. Lanes 10-13, the free labeled 5s-207 DNA digested with; lane 10, 2 µl; lane 11, 4 µl; lane 12, 16 µl of a 250-fold dilution of the stock enzyme; and lane 13, 1.28 µl of a 5-fold dilution. Lane G, (CH₃)₂SO₄/piperidine-cleaved, labeled 5s-207.

**DISCUSSION**

A two-column HPLC procedure has been developed for the fractionation and purification of all of the acetylated states of histones H3 and H4 essentially to homogeneity as shown in Figs. 2 and 3 in the “Miniprint.” It is well known that there are four sites of acetylation in histone H4 and four acetylated states of H4 have been obtained. However, for histone H3 we have established that there are five sites of acetylation in both butyrate-treated and control cells. Lysine 27 is the fifth site. HeLa cell acetyltransferase clearly uses this and the other four sites of acetylation in histone H4 and four acetylated states of H4 have been observed. However, for histone H3 we have established that there are five sites of acetylation in both butyrate-treated and control cells. Lysine 27 is the fifth site. HeLa cell acetyltransferase clearly uses this and the other four sites of acetylation in histone H3 in a nonrandom order (Fig. 4). Lysines 14 and 23 are acetylated first, followed by lysines 18 and 9, and, finally, lysine 27. With increased acetylation of H3, monomethylation of lysine 9 decreases while that of lysine 4 increases (Fig. 5), so that in the pentaacetylated state the only monomethylated site is lysine 4, which is largely occupied. In the first three acetylated forms of H3 the dimethylation of lysine 9, estimated from the under recovery of total lysine at that residue, was quite substantial, about 75, 45, and 55% for Ac₁, Ac₂, and Ac₃H3, respectively. A possible role for methylation is to limit acetylation to the correct sites in the amino-terminal domain of H3.
order in the presence or absence of butyrate was 9 and 14 followed by 18 (62). Either the mono- or diacetylated forms of H3 in the butyrate-treated HeLa cells represent largely the replication related forms or alternatively the utilization patterns of the sites of acetylation in the two organisms are different. The methylation of lysine 9 suggests the latter interpretation because the cells appear to maintain the intermediate acetylated states in a class of chromatin. Two studies show different patterns of acetylation of H4 for transcriptionally active genes. We have assembled 147 different. The methylation of lysine 9 suggests the latter interpretation because the cells appear to maintain the intermediate acetylated states in a class of chromatin. Two studies show different patterns of acetylation of H4 for transcriptionally active genes. We have assembled 147
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been localized in the region of the dyad axis and introduce factor IIIA binding site (the internal control region) by the ends of the DNA coiled on the nucleosome particle. The kind gift of p-207-18; Dr. of the University of California at Davis for protein sequencing; and also for the quantitative amino acid analyses; and Alan J. Smith, director, for advice on the quantitation.

The reported position of the the phased nucleosome on the Lytechinus variegatus 58 gene (61) overlaps the the transcription factor IIIA binding site (the internal control region) by about 30 bp (70) It is over much of this region that the nucleosomal footprints become less distinct. (The internal control region spans 138-185 nucleotides from the 3'-labeled end in Fig. 9 and 72-25 nucleotides from the 3'-labeled end in Fig. 10B). The situation resembles somewhat that of the positioned nucleosome in the promoter of β-globin gene of chickens in which 32 bp in the downstream side of the nucleosome footprints poorly because of the presence of sites for the binding of the G-string binding protein and transcription factor Sp1 (71).

The major effect of hyperacetylation of the core histones is a reduction in the linking number difference of the nucleosome particle (3). It is possible that this is caused by flanking DNA segments external to core particle DNA unwinding from the ends of the DNA coiled on the nucleosome particle. The possibility that histone acetylation induces a polymorphic change in the shape of the histone octamer such as promoting a more facile cylinder to ellipsoid transition of the type proposed by White et al. (60) cannot be excluded. This may explain the small differences observed in the DNA footprints, but, overall, the evidence in support of this model is not strong. The possibility remains that the amino-terminal domains of the core histones are involved in inter-nucleosomal interactions in chromatin and that histone acetylation modifies this interaction and changes the writh of the DNA linker regions.

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REFERENCES


The idea that the histone tails bind in this region.
The presence of two EcoRI sites per monomer phasing sequence noted by Hansen et al. (59) requires an actual length for the monomer unit of 208 bp.
**Nucleosomes Containing Fully Acetylated H3 and H4**

**Thin Acetates.** Nucleosomes were reconstituted as above at a titration to DNA ratio of 0.02 mmol/g. The final concentration of 0.02 mmol/g was achieved by mixing 0.1 mmol/g of DNA with 9 volumes of 50 mm Tris-HCl, pH 7.6, and 10 volumes of 0.02 mmol/g of acetic acid. The mixture was incubated at 37°C for 30 min. The DNA was then removed by centrifugation and the supernatant was used for the next experiment. The final concentration of 0.02 mmol/g was achieved by mixing 0.1 mmol/g of DNA with 9 volumes of 50 mm Tris-HCl, pH 7.6, and 10 volumes of 0.02 mmol/g of acetic acid. The mixture was incubated at 37°C for 30 min. The DNA was then removed by centrifugation and the supernatant was used for the next experiment.

**Electrophoresis.** The DNA was electrophoresed in a 0.8% agarose gel, stained with ethidium bromide, and visualized under UV light. The gels were scanned and analyzed using ImageJ software. The data were normalized to the total DNA content in each sample. The results showed that the incubation time with acetic acid had a significant effect on the mobility of the DNA fragments. The DNA fragments migrated more slowly with increasing incubation time.

**Figure 1:** Preparative Reversed Phase HPLC of Core Histones

- **Panel A:** Gel filtration chromatography of the DNA samples before and after incubation with acetic acid. The samples were run on a Superdex 200 column and the eluate was collected in 10-min fractions. The DNA fragments were detected by UV absorbance at 260 nm.
  - **Lane a:** DNA from untreated nuclei, 20 min.
  - **Lane b:** DNA from nuclei incubated with acetic acid for 10 min.
  - **Lane c:** DNA from nuclei incubated with acetic acid for 30 min.
  - **Lane d:** DNA from nuclei incubated with acetic acid for 60 min.

- **Panel B:** SDS-PAGE analysis of the core histones before and after incubation with acetic acid. The gels were stained with Coomassie Blue. The protein bands were analyzed using ImageJ software.
  - **Lane H3:** Histone H3 from untreated nuclei.
  - **Lane H3:** Histone H3 from nuclei incubated with acetic acid for 10 min.
  - **Lane H3:** Histone H3 from nuclei incubated with acetic acid for 30 min.
  - **Lane H3:** Histone H3 from nuclei incubated with acetic acid for 60 min.

The results showed that the incubation time with acetic acid had a significant effect on the mobility of the DNA fragments. The DNA fragments migrated more slowly with increasing incubation time. The SDS-PAGE analysis showed that the core histones were not significantly altered by the incubation with acetic acid.
Nucleosomes Containing Fully Acetylated H3 and H4

Figure 2: Ion Exchange HPLC of Isolated Histones
Profiles for the separation of isolated basic forms of H3 and H4 on a 250 x 8 mm Shodex DA5000 column in an appropriate buffer, pH 7.0, with a 100 to 300 mM gradient of NaCl at a flow rate of 1.5 ml/min. A, H4; B, H3. The peaks are numbered according to the level of acetylation. Typical recoveries were 80-85% as detected by dye binding. For preparative work a 250 x 10 mm column was used at 3 ml/min with all the other parameters mentioned above. Typical loadings for the larger column were 10 to 15 mg.

Figure 3: Electrophoresis of H3 and H4 Acetylated Forms
A: 15 cm long x 16 cm x 1.5 mm, 10% acrylamide, 0.1% methylene bisacrylamide, 0.8 M urea acid, and 8 charged gel. The numbered lanes correspond to the numbered peaks in the H4 chromatogram (Figure 2A):
Lane 1, H4 purified from budding yeast; lane 2, H4 from control Hela cells. B: 8.25 cm long gel of the same type as above. The numbered lanes correspond to the numbered peaks in the H3 chromatogram (Figure 2B). The standards, S, are HRF-HPLC purified H3.0.3 from budding yeast (Hela cells).
C: 15 cm acid ura gel as in A. The numbered lanes correspond to the numbered peaks in the H3.1 chromatogram (Figure 2C). Lane S, a mixture of all levels of acetylation; lane U, unacetylated H3.0.3.
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