Location of Histone Lysyl Residues Modified by in Vitro Acetylation of Chromatin†

Lois O. Tack‡ and Robert T. Simpson*

ABSTRACT: Calf or rabbit thymus chromatin was modified in vitro with varying excesses of acetic anhydride to acetylate up to 70% of the total histone lysyl residues. Peptide analysis of the modified histones demonstrated that the major sites for acetylation were (1) essentially all the lysyl residues of histone H1, (2) the lysyl residues in the amino-terminal third of histones H2A, H2B, H3, and H4, (3) a pair of lysyl residues near the center of H2A, and (4) lysyl residues near the carboxyl termini of H4 and H2B. This distribution of modification is largely consistent with other studies showing the presence of the amino-terminal regions of these inner histones near the surface of the nucleosome. Further, it suggests that several other sites in these histones may be on the surface of the histone octamer in the core particle, possibly providing DNA-binding sites. Very little acetylation of tyrosyl residues was detected. When modifications were performed to yield varying levels of substitution, essentially no differences were found in the actual sites of modification or the relative extents of modification for one site relative to another for the four smaller histones. We conclude that in vitro acetylation of chromatin is not a valid model for the structural effects of in vivo acetylation since numerous positions not modified in vivo are modified by acetic anhydride in vitro.

This laboratory first became interested in in vitro acetylation of chromatin with acetic anhydride as a method for mapping the availability of lysyl and tyrosyl residues of histones when complexed with DNA (Simpson, 1971). The assumption was that, as is the case for globular proteins, surface lysyl and tyrosyl residues would be more readily modified than those buried in interior regions of the nucleoprotein complex. Thus, by isolation, identification, and quantitation of peptides from the modified histones, one should be able to gain some insight into which regions of the five different histones were at the surface of the chromatin complex and which were interior. This question becomes at once more easily interpreted and also more significant in the light of the evolving understanding of the repetitive subunit organization of chromatin.

In vivo acetylation of chromatin histones, primarily H3 and H4, has been recognized for a number of years. Spatial and temporal correlations of acetylation (deacetylation) with either gene activation (inactivation) or DNA replication have led to postulated roles for the in vivo modification in transcriptional regulation and/or histone deposition (Ruiz-Carillo et al., 1975; Dixon et al., 1975; DeLange & Smith, 1975). Several attempts have been made to acetylate chromatin in vitro, using acetic anhydride, and use the modified nucleoprotein as a model to study the possible functional consequences of in vivo acetylation.

Such in vitro acetylation was found by Marushige and co-workers to increase the susceptibility of H3, H2B, and H2A to a protease in calf thymus chromatin. Acetylation also led to a decrease in the salt concentration necessary for displacement of the histones from their complex with DNA (Wong & Marushige, 1976; Marushige et al., 1976). Marushige (1976) showed that acetylation of chromatin in vitro increased the template activity of the nucleoprotein when assayed with *Escherichia coli* DNA-dependent RNA polymerase; this was related to previous observations by Allfrey et al. (1964) showing a correlation of in vivo acetylation with increased RNA synthesis in several systems. The proposal was made that acetylation partially neutralized the strong basic charge of the amino-terminal regions of the histones, leading to localized displacement of the proteins from DNA or some type of conformational change in the chromatin subunit, thereby allowing transcription (Marushige, 1976).

Wallace et al. (1977) have investigated some of the physical properties of both chromatin and nucleosomes acetylated with acetic anhydride at varying excesses. The acetylated products had a lowered melting temperature, increased susceptibility to nucleases, and increased solubility in 2 mM $MgCl_2$, when compared to control chromatin. These characteristics are those of putatively transcribed chromatin (Gottesfeld et al., 1975; Weintraub & Groudine, 1976; Garel & Axel, 1976; Pages & Alonso, 1978).

Wong & Marushige (1976) partially characterized the extent and location of in vitro acetylation of chromatin histones at varying excesses of acetic anhydride; they found a limit of about 50% of histone lysyl residues modified in 0.15 M NaCl at the highest concentration of acetic anhydride used. Modification occurred primarily in H1 (23 to 24 sites on the average) and H2B (9 to 10 sites) while H3 (5 to 6 sites), H2A (5 to 6 sites), and H4 (3 to 4 sites) were acetylated to a lesser degree. The exact location and extent of modification of any given lysyl residue were not determined in this study. In contrast, in vivo acetylation sites have been determined for several tissues [summarized in Dixon et al. (1975)]. Generally, three to four sites near the amino termini of H3 and H4 can be modified, up to four different sites in the same area of H2B can be modified, and one site is occasionally found acetylated in H2A.

Here, we have attempted to define the sites of modification of chromatin histones by acetic anhydride in vitro and establish relative reactivities for the different sites. By so doing, we can judge the suitability of in vitro acetylation as a model for the in vivo modification and can also begin to map the availability of histone regions to solution components in chromatin.

Experimental Section

Materials. [^{14}C]Acetic anhydride (carrier-free), specific activity 10–40 mCi/mmol, was purchased from Amersham/Searle. Pyridine (Sequanal grade) and acetic acid (pHix grade) were products of Pierce Chemical Corp. Spectral grade acetone was obtained from Eastman and fluorescamine was from Roche Diagnostics. Rabbit thymus was a product of Pel-Freez Biologicals.

† From the Developmental Biochemistry Section, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205. *Received February 28, 1979.*

‡ Present address: Laboratory of Molecular Biology, NIAMDD, National Institutes of Health, Bethesda, MD 20205.

This article not subject to U.S. Copyright. Published 1979 by the American Chemical Society

Chromatin Preparation and Characterization. Sheared chromatin was prepared from frozen calf or rabbit thymus by using EDTA saline buffers (Zubay & Doty, 1959) or by the method described by Simpson & Sober (1970) from Triton X-100 washed nuclei and dialyzed extensively against 0.25 mM EDTA, pH 7.0, at 4 °C. DNA concentrations were measured by assuming the absorbance of a 1 mg/mL solution at 260 nm to be 20 cm^{-1}. Protein concentrations were measured by the Lowry method as described by Layne (1957). The protein to DNA mass ratios for chromatins used in the modification studies were (1.3–1.5):1. Discontinuous $NaDodSO_4$[1] slab gel electrophoresis (LeStougeon & Rusch, 1973) was used to analyze chromatin and histones throughout the various isolation and modification procedures. Labeled histones were also analyzed on acid–urea gels (Panyim & Chalkley, 1969) and acid–urea–Triton X-100 gels (Alfageme et al., 1974). These gels were processed for fluorography by using the method of Bonner & Laskey (1974).

Acetylation was performed essentially as previously described (Simpson, 1971). All manipulations were carried out on ice. Unlabeled acetic anhydride was mixed with the ^{14}C compound (0.25–1.0 mCi) and quickly added to 50 mL of vigorously stirred chromatin at a DNA concentration of 1 mg/mL in 50 mM sodium borate, pH 9.0. The final concentration of acetic anhydride in the reaction mixture corresponded to a molar excess of acetic anhydride to DNA base pairs of four. The reaction was allowed to proceed for 20 min; histones were then extracted with cold 0.4 N H_2SO_4 and centrifuged at 12000*g* for 20 min to remove DNA and acidic proteins. The histones were dialyzed extensively against 1% acetic acid and then lyophilized. Recovery of histone was 90–95% of the theoretical amount, based on 1 g of histone per g of DNA. The isolated, [^{14}C]acetyl-labeled histones were then dissolved in 4 M Gdn·HCl and 50 mM sodium borate, pH 9.0, and acetylated with a 15-fold molar excess of unlabeled acetic anhydride. The pH was maintained at 8.5–9.0 by addition of NaOH, and the reaction was allowed to proceed for 30 min. The "hot–cold" histones were then again dialyzed and lyophilized. "Cold–hot" acetylations were done in an analogous fashion to that just described, except that the initial modification was performed with a 4-fold molar excess of cold acetic anhydride and the modification in Gdn·HCl was performed by using a 15-fold molar excess of unlabeled acetic anhydride plus 1 mCi of [^{14}C]acetic anhydride.

The extent of acetylation was determined both by measuring the [^{14}C]acetate incorporation and by assaying free lysyl residues using TNBS (Tack & Simpson, 1977).

Proteolytic Digestion. Modified histones (15–20 mg, 250–300 nmol) were suspended in water at a concentration of 4 mg/mL and digested at pH 8.0 and 37 °C with 1% (w/w) TPCK–trypsin (Worthington Biochemical Corp.). A pH stat was used to maintain the pH and monitor the progress of the reaction. After a 2-h digestion, base consumption had ceased and no further digestion was observed on addition of an additional aliquot of trypsin. Calculation of the numbers of bonds cleaved indicated that over 90% of the theoretical arginyl peptide bonds had been cleaved.

The digest was made 10% in acetic acid, whereupon 25–30% of both dry weight and ^{14}C counts of the digest became insoluble. The precipitate was removed by centrifugation and washed twice with cold water. The washed precipitate was suspended in 0.1 M NH_4HCO_3, pH 8, and digested with 1% (w/w) thermolysin (Calbiochem) for 12 h at 25 °C or 5 h at 37 °C. The material remaining insoluble after thermolysin digestion was further digested with β-subtilisin (BPN, Sigma Protease Type VII) under conditions identical with those utilized for thermolysin digestion. Soluble peptides from all digests were lyophilized and used for further fractionation. The combination of three proteases used for digestion routinely resulted in solubilization of about 97% of the total incorporated radioactivity.

Peptide Fractionation. The soluble tryptic digest in 10% acetic acid was initially fractionated by gel filtration using a 2.5 × 90 cm Sephadex G-25F column eluted with 1% acetic acid. Aliquots of each fraction were used for radioactivity measurements, adsorption at 230 nm, and the amino-group assay using fluorescamine (as described by Roche) with glycine (1–100 nmol) as standards and employing a Perkin-Elmer MPF-3L fluorimeter. Peaks were pooled and lyophilized (see below). The void-volume peak (A) was suspended in 10% acetic acid, 1% β-mercaptoethanol, and 5 M urea and fractionated on a 1.9 × 65 cm Sephadex G-75SF column with 1% acetic acid as the eluant. Peaks AI and AII from this column were dissolved in 50% Me_2SO–10% acetic acid and further fractionated on (carboxymethyl)cellulose by using a step gradient ranging from 1 mM acetic acid–2 mM pyridine, pH 5.5, to 1 M acetic acid–2 M pyridine, pH 5.5 (Hartley, 1972). For this and several other columns, approximately 10% of the effluent was diverted via a stream splitter through an amino acid analyzer equipped with an *o*-phthalaldehyde detection system. The void-volume material from this column was further fractionated under identical conditions of elution on a (diethylamino)ethylcellulose column. Soluble thermolysin and subtilisin digests were initially fractionated on a 2.4 × 40 cm Bio-Gel P-2 column in 1% acetic acid. Pools of labeled peaks were made and samples lyophilized.

Peptide Mapping. Pools from the protease digests fractionated by gel filtration were separated by two-dimensional mapping on Whatman 3MM paper ($18^1/_4 \times 22^1/_2$ in.). About 200–300 nmol of peptide was dissolved in 0.2 mL of 10% acetic acid and applied as a spot. Descending chromatography for the first dimension used butanol–water–acetic acid–pyridine (15:10:3:12) as the solvent (Sautiere et al., 1968) and was performed for 17 h with phenol red as the marker. The second dimension of fractionation used high-voltage electrophoresis at pH 3.5 in 5% acetic acid–0.5% pyridine at 200 V for 1.5–3 h, depending on the mobilities of the peptides to be separated. After drying, papers were autoradiographed by using Kodak X-ray film, usually for 48 h. All radioactive spots were cut out of the original map and sewn onto the center of a second sheet of paper and subjected to electrophoresis at pH 6.5 for 1–2 h in 10% pyridine–0.3% acetic acid for a third dimension of fractionation. Maps were lightly sprayed with 0.2 M *N*-ethylmorpholine, pH 8.5, until damp, and then peptides were detected by spraying with fluorescamine (10 mg/60 mL of acetone) and viewing under ultraviolet light. Highly fluorescent and radioactive spots were cut out and eluted with 1 mL of 10% acetic acid by using paper wicks.

Equal aliquots of each peptide isolated were taken for (1) radioactivity measurement, (2) determination of amino acid composition, and (3) amino-terminal analysis. Isotopic measurements were made by using Hydromix (York Chemicals) as the scintillant and by counting in a Beckman LS-250 scintillation counter. Samples for amino acid analysis were hydrolyzed in 6 N HCl, 0.1% phenol, and 1% β-mercaptoethanol for 20 h at 110 °C under nitrogen. Analyses were performed on a modifed JEOL 5AH analyzer with a

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; TNBS, trinitrobenzenesulfonic acid; Gdn·HCl, guanidine hydrochloride; Me_2SO, dimethyl sulfoxide.

single-column method using Durrum resin and *o*-phthalaldehyde detection; sensitivity was 200–500 pmol full scale. Amino-terminal analyses were performed by using microdansylation (R. W. Hartley, unpublished experiments). Samples were separated on polyamide sheets (Gallard Schlesinger) by using at least three internal standards, with approximately 1 nmol of peptide analyzed. Peptide identification was made from amino acid composition and confirmed in ambiguous cases by amino-terminal amino acid identification. Specific radioactivity of the peptides was computed by dividing the ^{14}C counts obtained by the total nanomoles of arginine in the peptide, determined by using norleucine as an internal standard to correct for sample losses during amino acid analysis. This specific activity was then compared to the input specific radioactivity of the acetic anhydride used for the particular labeling experiment.

Acetylation of Chromatin under Varying Conditions. Thymus chromatin was isolated by using the two procedures described above and, in addition, the saline citrate procedure described by Busch (1968). Hot–cold acetylation was carried out as described above, but varying pH (7.5, 8, and 9) and salt concentration (0 added, 0.15 M, and 2 M NaCl). Histones were digested with trypsin, and the soluble peptides were analyzed by two-dimensional analytic thin-layer peptide maps.

Thin-Layer Analytic Peptide Mapping. Tryptic digests of acetylated histones were fractionated in two dimensions on 20 × 20 cm Eastman thin-layer cellulose plates. About 2–5 nmol of sample dissolved in pH 3.5 pyridine–acetate buffer was spotted per plate. The first dimension was electrophoresis in 1% pyridine–10% acetic acid, pH 3.5, at 30 mA for 50 min with cold tap water cooling in Varsol. Plates were air-dried and run in ascending chromatography for 3–4 h by using the same solvent used for paper mapping. Peptides were detected by a cadmium acetate–ninhydrin dip (L. Robinson, personal communication) or by the fluorescamine spray as described above. Autoradiography was performed as described above.

Acetylation of Polylysine–DNA Complexes. Poly(D-lysine) hydrobromide (average degree of polymerization = 335) at 10 mg/mL in water was added slowly with stirring to calf thymus DNA at 1.2 mg/mL in 90 mM Tris–borate, pH 8.3, in 35 mM EDTA or in 0.05 M sodium borate, pH 9.0, in amounts sufficient to give final molar ratios of lysine to DNA phosphate of 1:2.5, 1:5, and 1:10. Acetic anhydride was then added at a 10- or 100-fold molar excess over lysine and allowed to react for 20 min at 4 °C. DNA concentration was measured before and after centrifugation at 10000*g* for 10 min. Supernatants were dialyzed, and the TNBS assay was performed on aliquots.

Results

Acetylation of chromatin was used in this study to attempt to ascertain the chemical availability of various lysyl residues in the histones when complexed with DNA; by analogy with simple globular proteins, chemical modification might be expected to affect primarily those residues near the surface of the nucleoprotein complex and to modify with less facility those located in the internal areas of the particles. We chose to study thymus chromatin due to its relatively low content of nonhistone proteins and the known sequences of the four smaller calf histones. [^{14}C]Acetic anhydride was used to allow quantitation of the degree of substitution. Since our goal was peptide isolation and quantitation, we have used sequential modification of chromatin histones with labeled and unlabeled acetic anhydride (or the opposite). Thus, in a hot–cold modification, chromatin was acetylated with [^{14}C]acetic anhydride; then, after disruption of histone–DNA and histone–histone interactions in Gdn·HCl, any unmodified lysyl residues were fully substituted with cold acetic anhydride. This should lead to a homogeneous population of proteins in which all lysyl residues are substituted; the specific radioactivity of the acetyl substituent on any particular lysyl residue should be a measure of its availability in the native complex.

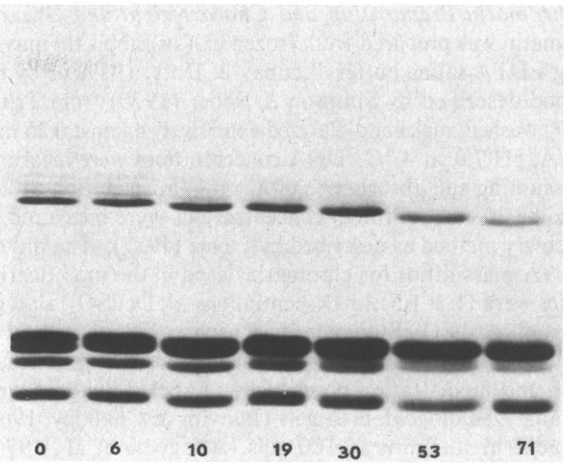

FIGURE 1: $NaDodSO_4$ gel electrophoresis of histones from acetylated chromatin. Chromatin was acetylated to varying degrees with acetic anhydride; the degree of modification assessed by TNBS assay is shown below each slot of the electrophoretic gel.

The converse experiment (cold–hot), acetylation of chromatin with cold acetic anhydride and acetylation in guanidine using labeled reagent, was used as a control. Here, the high specific activity of the acetyl substituent signifies a residue which was relatively inaccessible in the native complex. In either case, full acetylation of lysyl residues in the histones should lead to a new population of molecules which, on peptide analysis, will have tryptic cleavages only at arginyl residues.

Quantitation of the Modification. Modification of chromatin with acetic anhydride at varying molar excesses (acetic anhydride to DNA base pairs) at pH 9 leads to increasing incorporation of radioactive acetate until a plateau is reached at a molar excess of about four (Simpson, 1971). This ratio of reagent to nucleoprotein was used in all experiments to ensure a more homogeneous population of modified proteins, although modification at lower excesses of acetic anhydride or lower pH, both leading to lower levels of substitution, produced quantitative but not qualitative alterations in the results which are presented (see below). From the known specific activity of the radioactive reagent, it was calculated that 70% of the total lysyl residues of the histones was modified under the conditions of fourfold molar excess, pH 9.0. Measurement of the number of free amino groups in the chromatin proteins before and after acetylation using the TNBS method confirmed the degree of modification calculated by isotopic measurements, suggesting that little or no acetylation of tyrosyl residues occurs when calf thymus chromatin is modified in vitro under these conditions, in contrast to the conclusion previously drawn by Simpson (1971) using hydroxylamine reversal of acetylation as the criterion for tyrosyl acetylation. Whether this discrepancy arises from the presence of more nonhistone proteins in the rat liver chromatin previously employed, removal of acetyl groups from lysyl residues by neutral hydroxylamine, noted by others (Perlmann, 1966), or a combination of these remains open.

Figure 1 shows an electrophoretic analysis of acetylated histones using $NaDodSO_4$-containing polyacrylamide gels. The mobility of acetylated histones, particularly those richer in lysine, is increased when compared to unmodified proteins.

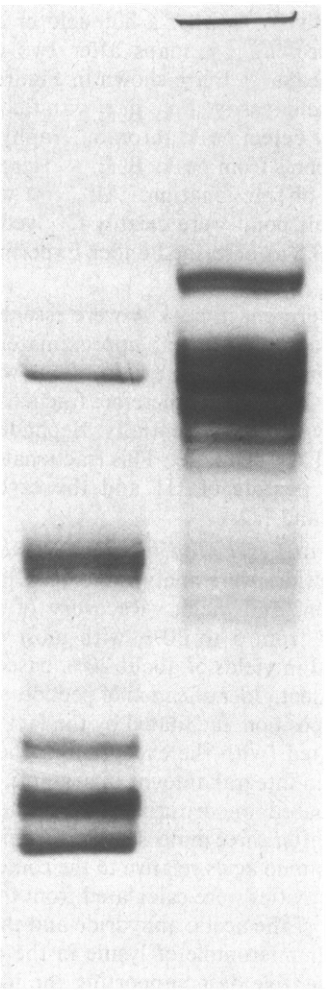

FIGURE 2: Electrophoresis of acetylated histones on Triton–acid–urea gel. Control (left) or acetylated (right) histones, modified to 70% of total lysyl residues substituted, were electrophoresed on acid–urea gels containing Triton X-100.

This likely results from partial neutralization of these highly basic proteins, known to run with apparently higher molecular weights than more nearly neutral proteins of the same size on $NaDodSO_4$ gel electrophoresis (Weber, unpublished experiments; Panyim & Chalkley, 1971). In some preparations, histone bands decreased in staining intensity and new bands at lower molecular weights appeared after acetylation. H1 and H3 were most often found to be degraded. Only acetylated samples with little or no apparent proteolytic degradation were utilized for peptide analyses.

Electrophoresis of totally acetylated histone samples on acid–urea gels (Panyim & Chalkley, 1969) similarly resulted in five discrete protein bands, in this case migrating much slower than unmodified histones. This result is similar to that reported previously by Wong & Marushige (1976). Even under conditions of partial substitution (chromatin modified to 70% lysyl acetylation at a fourfold molar excess of acetic anhydride to DNA base pairs), the acetylated histone bands are only slightly more diffuse than the bands observed for totally modified proteins.

Samples electrophoresed on Triton–acid–urea gels are shown in Figure 2. This electrophoretic system detects slight differences in amino acid composition or chemical modification (Alfageme et al., 1974). The number of bands and their sharpness are quite comparable to those observed for totally acetylated histones. No unmodified histones are detected; the heterogeneity in accessibility to in vivo acetylation of histones

Table I: Cold–Hot Acetylation of Chromatin Histones[a]

	content		rel sp radioact. (cpm/pmol of Lys)	
histone	unmodified	acetylated	sample 1	sample 2
H3	0.94	0.64	5.5	5.0
H2B	0.94	1.29	1.0	1.0
H2A	0.99	0.94	1.7	1.3
H4	1.00	1.00	3.8	2.9

[a] Histones were modified by cold–hot acetylation as described under Experimental Section. Histone contents were determined by densitometric scanning of stained sodium dodecyl sulfate gels and are expressed relative to H4 = 1.0. The gels were sliced, and after overnight incubation at 37 °C in 0.1% $NaDodSO_4$, the slices were counted. Relative specific radioactivities were calculated from the counts, normalized to the content of the histone and the known amino acid composition of the proteins, and are expressed relative to the specific radioactivity of H2B = 1.0.

(Cousens et al., 1979) is not mirrored when chromatin is acetylated in vitro with acetic anhydride. Taken together, these data support the contention that totally acetylated histones comprise a derived population of five proteins equivalent in their homogeneity to the parent histone proteins.

Previous measurements of the extent of acetylation of the various histones in vitro have indicated that, among the four smaller proteins, H2A and H2B were more readily modified than the arginine-rich histones H3 and H4 (Simpson, 1971; Wong & Marushige, 1976). This observation was confirmed by using the cold–hot acetylation protocol described above for the current system. Histones acetylated with cold acetic anhydride and then reacetylated with [^{14}C]acetic anhydride after dissociation in guanidine hydrochloride were electrophoresed on $NaDodSO_4$-containing gels, and the gels were stained and scanned and then sliced for determination of incorporated radioactivity. The results in Table I demonstrate the reproducibility of the modification and clearly show that the specific activity (radioactive acetate per histone lysyl residue) in this cold–hot experiment is highest for H3 and H4. Thus, since these histones are most highly acetylated in guanidine hydrochloride, they are the most unreactive when complexed in chromatin.

Peptide Isolation. After acetylation in chromatin, the modified histones were totally water soluble; however, after total acetylation when dissociated from DNA, the proteins could be solubilized only in strongly denaturing solvents such as 5 M Gdn·HCl, 10% acetic acid, or 1% $NaDodSO_4$. Attempts were made to fractionate these fully acetylated histones using a wide variety of techniques including isoelectric focusing, hydroxyapatite and DEAE-Bio-Gel chromatography, and gel filtration in acid, Gdn·HCl, or $NaDodSO_4$. Due to aggregation of samples or insufficient resolution, all methods tried failed to resolve the five individual modified proteins; gel filtration in $NaDodSO_4$ or Gdn·HCl yielded the best results but resolved only H1 and H4 as pure entities. Preparative electrophoresis in acetic acid–urea–polyacrylamide gels did resolve individual proteins, but the method led to losses of such magnitude as to make it impractical for further peptide analysis.

Accordingly, we decided to attempt to purify the desired peptides from proteolytic digests of total acetylated histones. Trypsin digestions were monitored by using a pH stat; routinely, cleavage of 90–95% of the theoretical number of arginyl bonds was obtained. After digestion, about 25% of both the mass of protein and the radioactivity in a hot–cold acetylation was insoluble in 10% acetic acid. Analysis of this material on $NaDodSO_4$–polyacrylamide gels demonstrated that about

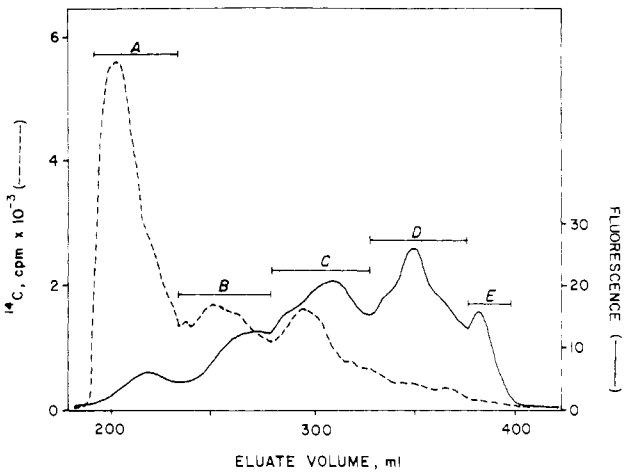

FIGURE 3: Gel filtration fractionation of peptides in a tryptic digest of acetylated histones. The digest was applied to a 2.5 × 90 cm column of Sephadex G-25F and eluted with 1% acetic acid. Aliquots of each fraction were counted to measure [^{14}C]acetate (---) and reacted with fluorescamine to determine peptide amino groups (—). Pools were made as indicated by the bars for further analysis.

80% was large peptides, 10% was undigested histone, and the remainder was small peptides. On the basis of known sequences some of the large peptides would be expected to derive from most of the H1 and the carboxyl-terminal regions of the inner histones; this supposition was confirmed by further digestion of the insoluble fraction with thermolysin and β-subtilisin and peptide fractionation by gel filtration on Bio-Gel P-2 columns and two-dimensional mapping.

The soluble peptides in the tryptic digest were initially fractionated by gel filtration on Sephadex G-25; a typical elution profile of both radioactivity and peptide fluorescence with fluorescamine is shown in Figure 3. About one-third of the incorporated acetate elutes in the void volume in a typical hot–cold acetylation digest. About 10–20% of this peak (A) is undigested histone on $NaDodSO_4$ gel electrophoresis. The large peptides expected to migrate in peak A or in its tailing area are the carboxyl terminus of H1 (142 AA), the carboxyl terminus of H2A (40 AA), the cysteine-containing peptide of H3 (33 AA), the amino terminus of H1 (34 AA), and the carboxyl terminus of H2B (25 AA). Eluting after this excluded peak are two defined regions of smaller peptides, peaks B and C, which together contain the bulk of the remaining radioactive label in a hot–cold acetylation. The elution profiles of radioactivity and peptide amount reverse in peak C. The final peaks pooled from the column, D and E, are defined by peptide elution peaks: radioactivity tails off through this region of the profile. Recovery of radioactivity from gel filtration columns was usually 90% or greater.

After this initial size fractionation, we attempted subfractionation of peaks A–E by ion-exchange chromatography with pyridine–acetate gradients on columns containing (carboxymethyl)cellulose, phosphocellulose, or sulfoethyl-Sephadex. In no case was satisfactory resolution of the peptide mixture achieved; fractions tended to contain several peptides, due to the similarity in composition of many of the peptides. We therefore elected to further fractionate the peptide mixtures by three-dimensional mapping on paper. Descending chromatography in butanol–water–acetic acid–pyridine was used for the first dimension, followed by electrophoresis at two different pH values to achieve purification. These methods allowed purification of 22 of the 31 predicted peptides containing lysine and/or tyrosine from the 4 smaller histones in sufficient yield for identification and determination of acetate specific radioactivity in either a hot–cold or a cold–hot experiment. Representative maps after two dimensions of separation for peaks A–E are shown in Figure 4; the figure indicates both the intensity of staining with fluorescamine and the radioactivity detected by autoradiography. Most of the peptides in the pools from peaks B–E are cleanly resolved by two dimensions of fractionation. All those which partially overlapped at this point were cleanly resolved after electrophoresis at pH 6.5 as described under Experimental Section (Figure 4).

The peptides present in peak A were essentially insoluble in the chromatography buffer; approximately 50% of the radioactivity remained at the origin. This was in part undigested histone. Peak A was therefore fractionated by column chromatography using, sequentially, Sephadex G-75, CM-cellulose, and DEAE-cellulose. This fractionation yielded the amino-terminal peptide of H1 and the carboxyl-terminal peptides of H1 and H2A.

Quantitation of Lysyl Modification in Isolated Peptides. The isolated peptides were analyzed for [^{14}C]acetate, amino acid composition, and yield. Recovery of peptides after mapping varied from 5 to 30%, with most of the smaller peptides isolated in yields of about 20%, based on the lysine and arginine content. Identification of peptides was made from amino acid composition, facilitated by the fact that all tryptic peptides generated (with the exception of the C terminals) should contain an integral amount of arginine. The purity of peptides was based on criteria of a single, radioactive, fluorescent spot after three dimensions of mapping and integral values of other amino acids relative to the content of arginine. Specific radioactivities were calculated from the known input specific activity of the acetic anhydride and the incorporated radioactivity per micromole of lysine in the peptide. Containing representative data supporting the identification of these peptides, Table II presents the experimentally determined and theoretical compositions of peptides we assign to H3 in the acetylated chromatin. In all cases, agreement is quite good, although glycine and serine contents tend to be high, probably due to background from the paper used for peptide isolation.

In addition to the tryptic peptides isolated, several lysyl or tyrosyl sites were identified from peptides obtained after thermolysin digestion. A number of other peptides obtained from thermolysin or subtilisin digestions gave identifiable compositions; the peptides were too small to give unambiguous sequence localizations, however. Regions of the four smaller histones for which no tryptic peptides were obtained are (1) the large hydrophobic cysteine-containing peptide of H3 (1 Lys), (2) three peptides in the carboxyl-terminal regions of H4 (3 Lys and 1 Tyr; 1 Lys localized in a thermolysin peptide), (3) a large centrally located peptide in H2B (2 Tyr and 4 Lys; 2 Lys and 2 Tyr localized by thermolysin peptides), and (4) a large centrtal H2A peptide (2 Tyr). The carboxyl-terminal peptide of H2A (7 Lys) was obtained in a cold–hot experiment only.

Averaged results from five distinct hot–cold experiments are depicted for the partial sequence of H1 and the total sequences of the four smaller histones in Figure 5. The figure depicts the positions of lysyl and arginyl residues for each protein on the abscissa, with sites of tryptic cleavage at arginyl residues shown by the arrows. The histogram indicates the specific activity of identified acetyl lysyl residues relative to the input specific activity of acetic anhydride. A "U" over a lysyl residue denotes that that particular residue was not identified in the isolated peptides. Except where individual residues within an arginyl peptide have been further identified

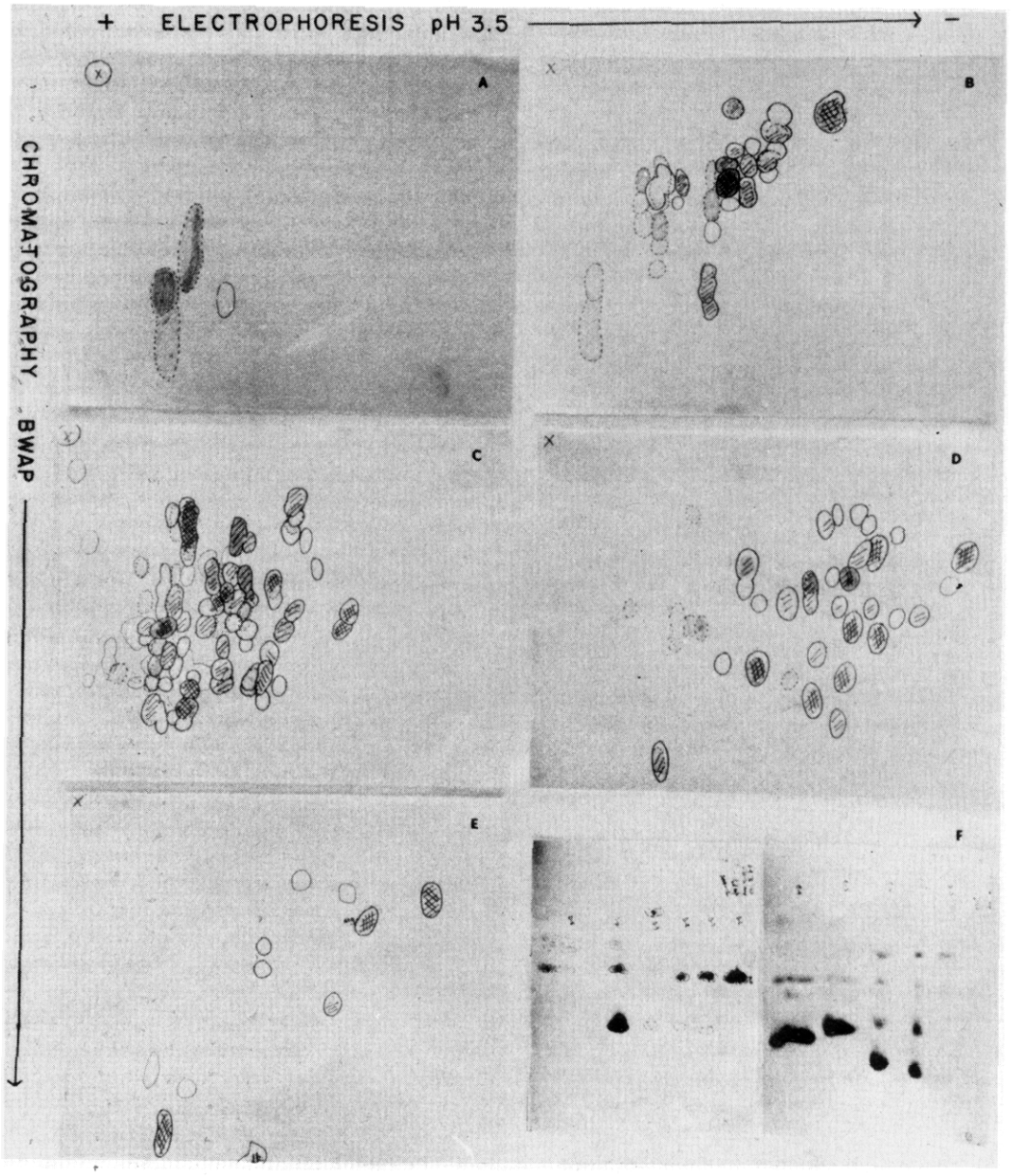

FIGURE 4: Fingerprints of peptides in a tryptic digest of acetylated histones. Peaks pooled from gel filtration as shown in Figure 3 were analyzed by two-dimensional paper peptide mapping using descending chromatography as the first dimension and electrophoresis for the second. Peptide spots detected by fluorescence are outlined. The crosshatching indicates fluorescence and the shading indicates the relative intensities of the spots detected upon autoradiography of the papers. Section F shows representative maps obtained in a third dimension of fractionation (electrophoresis at pH 6.5). This figure is a photograph of the autoradiogram, with the positions of nonradioactive, fluorescamine positive spots indicated by outlining.

Table II: Composition of Peptides from Histone H3[a]

peptide (no.)	Lys	His	Arg	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe
1–2 (2)			1.0 (1)					0.2	1.1 (1)						
3–8 (3)	1.0 (1)		1.0 (1)		1.6 (2)		1.1 (1)		1.1 (1)						
9–17 (2)	1.5 (2)		1.0 (1)		1.1 (1)	1.0 (1)		3.2 (2)	1.3 (1)						
18–26 (5)	1.8 (2)		1.0 (1)		1.2 (1)	0.6	1.2 (1)		2.9 (3)						
27–40 (2)	2.8 (3)	0.9 (1)	1.0 (1)		1.0 (1)	0.9 (1)		2.2 (2)	2.1 (2)	1.0 (1)			0.9 (1)		
41–49 (3)			2.0 (2)		0.9 (1)			2.1 (1)	1.4 (1)	1.0 (1)			1.1 (1)	0.7 (1)	
50–53 (1)			2.0 (2)				1.1 (1)					1.1 (1)			
54–63 (1)	1.0 (1)		1.0 (1)		0.8 (1)	1.1 (1)	1.9 (2)					1.0 (1)	1.7 (2)	0.5 (1)	
64–69 (5)	0.8 (1)		1.0 (1)			0.2	1.4 (1)	0.2					1.0 (1)		0.9 (1)
70–72 (3)			1.0 (1)							1.0 (1)			1.0 (1)		
73–83 (3)	0.9 (1)		1.0 (1)	1.9 (2)	1.1 (1)	0.2	1.9 (2)		1.3 (1)			0.8 (1)	1.0 (1)		0.7 (1)
117–128 (3)	1.2 (1)		1.0 (1)	1.1 (1)	1.1 (1)		1.3 (1)	0.6	1.1 (1)	0.8 (1)	0.4 (1)	1.6 (2)	1.0 (1)		
130–131 (1)			1.0 (1)									0.9 (1)			
132–134 (1)			1.0 (1)				1.2 (1)	1.8 (1)							

[a] The peptides are indicated by sequence position. The number after each peptide is the number of independent isolates analyzed. Numbers after the amino acid contents are the theoretical composition.

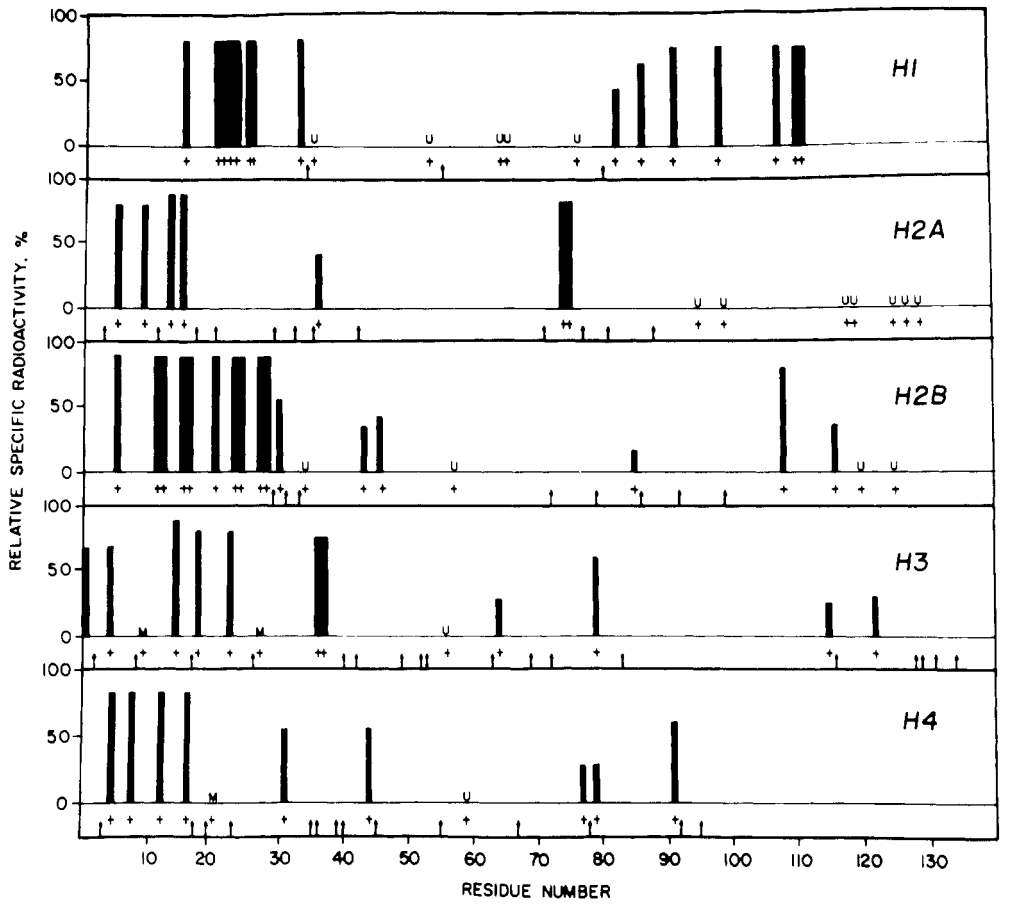

FIGURE 5: Reactivities of lysyl residues in the histones. For the amino-terminal portion of H1 and the total sequence of the four smaller histones, the figure indicates the positions of lysyl residues by (+) and the positions of arginyl residues, sites of tryptic cleavage of the acetylated proteins, by arrows. The relative specific radioactivity for each detected lysyl residue is shown by the vertical bars; this is the specific radioactivity of the acetyl lysyl residue relative to the specific radioactivity of the input acetic anhydride. M above a lysyl residue indicates a site of methylation in vivo. U above a lysyl residue indicates that the peptide containing that residue was not identified in the fractionation of hot–cold labeled histones. All lysyl residues within a given tryptic peptide are assigned the same relative specific radioactivity except in cases where further identification was possible in peptides isolated from thermolysin digests. The carboxyl-terminal peptide of H2A was labeled to a low specific activity in a cold–hot experiment. Lys-56 in H3 was identified as highly labeled in a cold–hot experiment.

by thermolysin peptides, the specific activity of the acetyl substituent is assumed to be the same for all lysyl residues within a given tryptic peptide.

The amino-terminal peptide of H1 is highly available for acetylation, suggesting a surface environment in chromatin. Peptides from the central, presumably globular region of the lysine-rich histone were not obtained. The carboxyl-terminal peptide of H1, 81–212, is highly accessible for acetylation. This peptide contains 49 lysyl residues; we assume that most of these are modified readily. Two residues in the region of this peptide which is not very highly basic (Lys-82 and -86) were identified in thermolysin peptides, and their acetyl substituents have lower specific activities than the carboxyl-terminal lysyl residues. These data are consistent with both amino- and carboxyl-terminal ends of H1 binding to DNA in a relatively exposed position in chromatin since, in contrast to the previous expectation (Simpson, 1971), lysyl residues interacting with DNA are quite capable of being acetylated by acetic anhydride (see below). Since the binding of H1 to DNA is obviously weakened by this degree of modification of basic residues, this result could also arise from a partial dissociation of the protein during the chemical modification.

The results for the four smaller histones are quite consistent with current ideas of organization of the core particle [for reviews see Kornberg (1977) and Felsenfeld (1978)]. Lysyl residues in the highly basic amino-terminal third of each molecule are highly reactive toward the acylating agent in chromatin. In general, lysyl residues in the central, less basic portion of the proteins are less available for modification, suggesting their being located in the inner nucleus of the core particle. There are exceptions to this generalization, however. Lysyl residues at positions 79 in H3, 44 and 91 in H4, 108 in H2B, and 74 and 75 in H2A are modified to nearly the same extent as those in the N-terminal regions. It seems plausible that these residues are on the surface of the globular histone core of the nucleosome, perhaps providing binding sites which aid in the definition of the path taken by DNA when folded around the protein nucleus.

The results for cold–hot acetylation (not shown) support the conclusions drawn in the hot–cold studies. That is, residues near the amino termini of the proteins are more available for modification when the histones are in chromatin and, hence, are modified to a lower specific activity in this type of experiment. The carboxyl-terminal peptide of H2A was labeled only slightly, indicating ready access of the acylating agent to those lysyl residues in chromatin. Most of the tyrosyl residues were found highly labeled in cold–hot experiments.

Acetylation of Chromatin under Different Conditions. Table III shows the results of acetylation of chromatin at different pH values. As expected, when chromatin is acetylated at pH values less than 9, always in 0.05 M sodium borate, the specific activity (cpm of acetate/mg of protein) of the incorporated acetate decreases with decreasing pH. Increasing ionic strength, at the constant pH of modification,

Table III: Acetylation of Chromatin Histones at Different pH Values[a]

pH	% incorporated	sp radioact.
9	10	4.1×10^5
8	7	3.5×10^5
7.5	5.3	2.4×10^5

[a] All modifications were in 0.05 M sodium borate. Specific radioactivity is cpm/mg of histone.

also decreases the specific activity of the modified proteins, consistent with expansion of the structure at lower ionic strengths, exposing lysyl residues for more ready modification.

Both these results in variability of modification could arise either from altered reactivity of the same lysyl residues or, alternatively, from qualitative changes in the lysyl residues which are and are not modified under the various conditions. This question was addressed by tryptic digestion, two-dimensional thin-layer peptide mapping, and autoradiography of samples modified under various conditions with [^{14}C]acetic anhydride. The results indicate that with the exception of four peptides, the peptide maps were qualitatively identical at all three pH values used for the modification. Thus, at lower pH values, the same peptides were labeled with acetate but to a lesser extent. This suggests that the major sites of acetylation are the same regardless of the pH at which the modification is performed. Similarly, varying salt conditions for the modification led only to quantitative variations in the amount of acetate in the modified peptides. No selective effects on particular peptides were noted except when the modification was performed in 2 M NaCl, where H1 was more highly acetylated relative to peptides from the inner histones than when the modification was done in 0.15 M NaCl.

Acetylation of Polylysine–DNA Complexes. Since the results obtained above indicated that the amino-terminal lysyl residues of the smaller histones are most available for acylation in vitro, and these regions of the histones have been thought to comprise the primary sites for protein interaction with DNA, we studied whether formation of an electrostatic complex between the ϵ-amino group of a lysyl residue in a polypeptide and DNA phosphate would preclude acetylation of the amino moiety.

Complexes of polylysine and calf thymus DNA were formed by direct mixing as described under Experimental Section. At this intermediate salt concentration, binding will likely be neither completely cooperative nor completely random (Leng & Felsenfeld, 1966). At a polylysine to DNA ratio of 1:2.5 (mol/mol), the complex mixture was turbid and about 25% of the DNA could be pelleted by a low-speed centrifugation. Complexes formed at lower ratios, 1:5 and 1:10, were visibly clear solutions. All complexes, as well as free polylysine, were acetylated at a 10- or 100-fold molar excess of acetic anhydride to lysine. TNBS assays were carried out on the initial mixtures and after acetylation. Essentially all the lysyl residues of the polylysine are modified under these conditions, irrespective of whether the poly(amino acid) is initially bound to DNA or free in solution. Thus, interactions of lysyl amino groups with nucleic acid phosphates apparently do not block modification by acetic anhydride, although we can not exclude the possibility that, after a certain level of acetylation, the complex dissociates and the remaining acetylation occurs on a polylysine molecule free in solution.

Discussion

The data obtained clearly indicate that (1) numerous histone lysyl residues in chromatin in addition to those known to be modified in vivo are acetylated when the nucleoprotein is treated with acetic anhydride in vitro and (2) there are certain identifiable residues whose reactivity when in chromatin is either high, on the one hand, or low, on the other. Could the modification of more lysyl residues in vitro be due to an unzippering of histones from DNA after initial modification of a few residues? This seems unlikely since (1) these levels of acetylation do not lead to histone dissociation from DNA for the four smaller basic proteins (Simpson, 1971; Wong & Marushige, 1976), (2) differential reactivity of certain residues is observed, (3) similar relative distributions of modified peptides are observed at different overall levels of modification, and (4) preliminary results using initial modification with ethyl acetimidate, followed by labeling with [^{14}C]acetic anhydride in Gdn·HCl, give data similar to those obtained in the cold–hot acetic anhydride experiments (L. O. Tack, unpublished experiments). Note that modification with ethyl acetimidate leads to minimal if any effects on chromatin structure (Tack & Simpson, 1977).

If one accepts the premise that in vitro acetylation is detecting those lysyl residues that are near the surface of the nucleoprotein complex, the observation that in vivo acetylation is so selective in the specific residues which are modified becomes even more striking. The activity of histone deacetylase "correcting" inappropriate acetylations is likely not the basis for the specificity, since inhibition of deacetylase activity by sodium *n*-butyrate (Boffa et al., 1978; Candido et al., 1978; Sealy & Chalkley, 1978) does not lead to acetylation in excess of the number of substituents per histone observable in the absence of this inhibitor (DeLange & Smith, 1975; Dixon et al., 1975; Ruiz-Carillo et al., 1975; Schauffhausen & Benjamin, 1976). Some of the selection in sites likely then derives from selective interactions of the acetyltransferase with local features of the polypeptide chain. Other aspects of specificity may relate to higher order chromatin structure or nucleosome heterogeneity, since there is a fraction of H4 molecules which is apparently never acetylated in HTC cells (Cousens et al., 1979).

Whatever the basis for specificity in vivo, it is apparent from these studies that in vitro acetylation with acetic anhydride does not provide a valid model for investigation of the effects of the in vivo chemical modification on chromatin structure and function. Histones which are not acetylated in vivo are highly modified in vitro, and those histones which are modified in both cases are modified at a number of additional sites when the acetylation is performed with acetic anhydride. It is also clear that the use of acetylation at high levels in vitro to measure the fraction of histone lysyl residues which are not interacting with DNA (Simpson, 1971) is not a valid approach, since synthetic complexes of polylysine with DNA are modified as readily as the free poly(amino acid). Results using a competitive method for judging acetylation rates at low excesses of reagent, which indicated that about 20% of the histone lysyl residues was available for modification at pH 8–10 (Malchy & Kaplan, 1976), may not be affected by our results. Determination of the sites of modification under these conditions might help in the interpretation of that data.

Finally, the relation of the current studies to chromatin structure is of interest. Those lysyl residues most readily modified in vitro, the inner histone amino-terminal regions and all of H1, are those most readily digested by trypsin (Weintraub & Van Lente, 1974), consistent with current notions which place H1 and the histone amino termini near the surface of the nucleosome in a position accessible to solution components. Additionally, however, certain internal

lysyl residues in the four smaller histones appear to be modified by acetic anhydride nearly as readily as those lysyl residues near the amino termini. This observation suggests that they may be at the surface of the protein core of the nucleosome. In this position, such residues may be of critical importance in providing DNA-binding sites which determine the path taken by the nucleic acid as it folds around the protein nucleus of the core particle. They may also provide the stabilization of binding which allows the core particle to largely remain folded after removal of the amino-terminal regions of H2A, H2B, H3, and H4 by trypsin (Whitlock & Simpson, 1977) and the template for the folding of 140 base-pair DNA by these same trypsin-created histones (Whitlock & Stein, 1978).

Acknowledgments

We thank Dr. Robert W. Hartley for use of his amino acid analyzer, advice on the analyses, and the microdansylation procedure for amino-terminal analysis. Dr. E. Appella provided us with the procedure for fingerprint peptide detection with fluorescamine. Dr. Brian Tack was of great help in the peptide analyses. We also thank Linda Propst for photographic assistance and D. Reggia for preparation of the manuscript.

References

Alfageme, C. R., Zweidler, A., Mahowald, A., & Cohen, L. H. (1974) *J. Biol. Chem. 249*, 3729–3736.

Allfrey, V. G., Faulkner, R., & Mirsky, A. E. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 786–794.

Boffa, L. C., Vidali, G., Mann, R. S., & Allfrey, V. G. (1978) *J. Biol. Chem. 253*, 3364–3366.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Busch, H. (1968) *Methods Enzymol. 12B*, 65–84.

Candido, E. P. M., Reeves, R., & Davie, J. R. (1978) *Cell 14*, 105–113.

Cousens, D., Gallwitz, D., & Alberts, B. M. (1979) *J. Biol. Chem. 254*, 1716–1723.

DeLange, R. J., & Smith, E. L. (1975) *Ciba Found. Symp. 28*, 59–70.

Dixon, G. H., Candido, E. P. M., Honda, B. M., Lowe, A. J., MacLeod, A. R., & Sung, M. T. (1975) *Ciba Found. Symp. 28*, 229–258.

Felsenfeld, G. (1978) *Nature (London) 271*, 115–122.

Garel, A., & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.

Gottesfeld, J. M., Murphy, R. F., & Bonner, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4404–4408.

Hartley, R. W. (1972) *Anal. Biochem. 46*, 676–680.

Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46*, 931–954.

Layne, E. (1957) *Methods Enzymol. 3*, 477–454.

Leng, M., & Felsenfeld, G. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1325–1332.

LeStourgeon, W. M., & Rusch, H. P. (1973) *Arch. Biochem. Biophys. 155*, 144–158.

Malchy, B. L., & Kaplan, H. (1976) *Biochem. J. 159*, 173–175.

Marushige, K. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3937–3941.

Marushige, K., Marushige, Y., & Wong, T. K. (1976) *Biochemistry 15*, 2047–2053.

Pages, M., & Alonso, C. (1978) *Nucleic Acid Res. 5*, 549–562.

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Panyim, S., & Chalkley, R. (1971) *J. Biol. Chem. 246*, 7557–7560.

Perlmann, G. E. (1966) *J. Biol. Chem. 241*, 153–157.

Ruiz-Carillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science 190*, 117–128.

Sautiere, P., Starbuck, W. C., Roth, C., & Busch, H. (1968) *J. Biol. Chem. 243*, 5899–5905.

Schaffhausen, B. S., & Benjamin, T. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1092–1096.

Sealy, L., & Chalkley, R. (1978) *Cell 14*, 115–121.

Simpson, R. T. (1971) *Biochemistry 10*, 4466–4470.

Simpson, R. T., & Sober, H. A. (1970) *Biochemistry 9*, 3103–3109.

Tack, L. O., & Simpson, R. T. (1977) *Biochemistry 16*, 3746–3753.

Wallace, R. B., Sargent, T. D., Murphy, R. F., & Bonner, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3244–3248.

Weintraub, H., & Van Lente, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4249–4253.

Weintraub, H., & Groudine, M. (1976) *Science 193*, 848–856.

Whitlock, J. P., Jr., & Simpson, R. T. (1977) *J. Biol. Chem. 252*, 6516–6520.

Whitlock, J. P., Jr., & Stein, A. (1978) *J. Biol. Chem. 253*, 3857–3861.

Wong, T. K., & Marushige, K. (1976) *Biochemistry 15*, 2041–2046.

Zubay, G., & Doty, P. (1959) *J. Mol. Biol. 1*, 1–20.