Sedat, J., & Manuelides, L. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 331-350.
Stephens, R. E., Pan, C. J., Ajiro, K., Dolby, T. W., & Borun, T. W. (1977) *J. Biol. Chem. 252*, 166-172.
Terasima, T., & Tolmach, L. J. (1963) *Exp. Cell Res. 30*, 344-362.
Tsanev, R., & Russev, G. (1974) *Eur. J. Biochem. 43*, 257-263.
Weintraub, H., Palter, K., & Van Lente, F. (1975) *Cell 6*, 85-110.
Worcel, A. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 313-324.

Comparison of the Structures of Human Histones 1A and 1B and Their Intramolecular Phosphorylation Sites during the HeLa S-3 Cell Cycle†

Kozo Ajiro,‡ Thaddeus W. Borun, Seth D. Shulman, Gail M. McFadden, and Leonard H. Cohen*

ABSTRACT: A structural comparison of two subtypes of histone 1 (H1) in HeLa S-3 cells (H1A and H1B) shows that the cationic C-terminal half of H1A is smaller and more hydrophobic (containing ca. seven more valyl residues) than the comparable region of H1B, suggesting that the two proteins differ substantially in their interactions with DNA, proteins, or both. Differences between the N-terminal halves of H1A and H1B were found only in the short region between residues 16 and 30. H1B was found to have more phosphorylation sites than H1A. For location and comparison of phosphorylation sites within H1A and H1B during the S phase and mitosis, cells at these two stages were incubated with [^{32}P]orthophosphate, and tryptic peptide maps of the *N*-bromosuccinimide fragments were examined. In S-phase cells, most of the ^{32}P incorporated was at two places in the C-terminal cationic tail of each subtype. Experiments with hydroxyurea at the time of the G_1-S transition suggest that the onset of phosphorylation at one of these sites at that time does not require a substantial amount of DNA replication but that the onset of phosphorylation at the other site does. Phosphate groups at the former type of site were shown to be of shorter duration than those of the latter type of site. These observations show that the two principal S-phase phosphorylations are functionally distinct and suggest that one may precede, and the other follow, replication forks. Besides the two principal S-phase phosphorylation sites present in both subtypes, several additional sites in the H1B molecule were found to contain small but significant amounts of ^{32}P in labeled S-phase cell cultures. One of them was tentatively identified as a threonyl residue missing from H1A, at position ~17 in the N-terminal region. These sites were all sites of major phosphorylations in mitotic cells, suggesting that their phosphorylation during interphase may be related to the existence of compacted chromosomal regions in interphase cells. In H1A, in contrast, few of the principal mitotic phosphorylations were detectable in S-phase cell cultures. Such differences between H1 subtypes, in both the number and the regulation of their phosphorylations, suggest that the subtypes are nonrandomly arranged in the chromosomes and represent a means of modulation of chromatin organization through subtype-selective phosphorylations.

In the preceding paper (Ajiro et al., 1981), we showed that the HeLA histone 1 (H1)[1] fraction contains two principal subtypes. H1A and H1B, which differ in molecular weights and have different phosphorylation levels at all stages of the HeLa S-3 cell cycle. H1A is predominantly unphosphorylated in G_1, monophosphorylated in the S phase, and tetraphosphorylated during mitosis. In contrast, H1B is predominantly monophosphorylated in G_1 and diphosphorylated in the S phase and appears to have five to eight phosphate groups in mitosis. These striking and different changes in H1A and H1B phosphorylation levels during G_1, the S phase, and mitosis add to a body of evidence that H1 phosphorylation comprises a complex set of reactions, the effects of which depend upon the intramolecular locations of phosphorylation sites. These results also suggest that different H1 subtypes might have different effects on chromatin conformation, and that their coexistence within a single cell type might thus provide one of the levels of control over chromosome organization, provided they are not randomly dispersed through the chromatin.

In the present paper, we have extended our studies of the functions of H1 subtypes and their phosphorylation during the HeLa cell cycle in two ways. First, we have asked whether the structural differences between H1A and HB are sufficient to support the notion that the H1 subtypes differ functionally. Second, we have examined the intramolecular sites of phosphorylation of H1A and H1B labeled with ^{32}P during the S phase and mitosis. In both types of study, chromatographically resolved H1A and H1B were each cleaved to N- and C-terminal fragments with *N*-bromosuccinimide (NBS), and then tryptic digests of the NBS fragments were analyzed by two-

† From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104 (K.A. and T.W.B.), The Naval Research Laboratory, Space Science Division, Washington, D.C. 30375 (S.D.S.), and The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 (G.M.M. and L.H.C.). *Received January 3, 1979.* This investigation was supported by U.S. Public Health Servic Research Grants CA-11463, CA-12544, CA-21069, and CA-17856 from the National Cancer Institute and GM-24019 from the National Institute of General Medical Sciences and by an appropriation from the Commonwealth of Pennsylvania. T.W.B., a recipient of Research Career Development Award CA-00088 from the National Cancer Institute, died suddenly since submission of this paper.

‡ Present address: Aichi Cancer Center, Research Institute, Laboratory of Cell Biology, Chikusa-ku, Nagoya 464, Japan.

[1] Abbreviations used: H1, histone 1; NBS, *N*-bromosuccinimide; TLC, thin-layer chromatography; TEA, triethylamine; $NaDodSO_4$, sodium dodecyl sulfate; HMG, high mobility group of proteins; HU, hydroxyurea.

0006-2960/81/0420-1454$01.25/0

dimensional peptide mapping. Prior separation of the NBS fragments proved valuable for resolving tryptic phosphopeptides identical in map position but located in different halves of the H1 molecule. By use of these procedures, we have identified the approximate locations of phosphorylation sites in human H1. We have also established homologies and differences among the first 116–118 amino acid residues in the N-terminal halves of H1A and H1B. Finally, we have attempted to probe relationships between chromatin replication and phosphorylations occurring at different sites of the H1 molecule by asking which of the predominant S-phase phosphorylations depend on the presence of replicated chromatin.

Experimental Procedures

Materials

NBS, fluorescamine, triethylamine (TEA), and 0.2 M sodium citrate buffer, pH 2.2, were purchased from Pierce Chemical Co., Rockford, IL. Tyrosine was obtained from Sigma Chemical Co. Trypsin treated with TPCK [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] and chymotrypsin were purchased from Worthington Biochemical Corp., Freehold, NJ. Protease of *Staphylococcus aureus*, strain V8, was purchased from Miles Research Products, Elkhart, IN. Sephadex G-100 was obtained from Pharmacia Fine Chemicals. Thin-layer cellulose plates (20 × 20 cm), coated with a 250-μm layer of Avicel, were purchased from Analtech, Inc., Newark, DE. Varsol coolant for electrophoresis was purchased from Savant Chemical Co.

Methods

Cell Culture and Synchronization. HeLa S-3 cells were maintained in suspension culture in Joklik-modified Eagle's minimum essential spinner medium supplemented with 3.5% calf serum and 3.5% fetal calf serum (complete medium A) as described by Marks et al. (1973). Synchronization by selective detachment in mitosis was carried out at 37 °C as described by Ajiro et al. (1981). Synchronization by two 12-h cycles of blockade with 1 mM HU was as described by Stephens et al. (1977). In all experiments, progress of cells, from mitosis through G_1 and into the S phase, was monitored by labeling 2-mL cell culture samples containing (1.5–5) × 10^5 cells/mL with 0.2 μCi of [^{14}C]thymidine for 30 min at 37 °C at hourly intervals and determining levels of incorporation of [^{14}C]thymidine into acid-precipitable material as described by Marks et al. (1973).

^{32}P Labeling of H1 Subtypes in S-Phase and Mitotic Cells. To obtain ^{32}P-labeled H1A and H1B fractions from mid-S-phase cells, 2 × 10^8 HeLa S-3 cells, at a concentration of 1.7 × 10^6 cells/mL, were labeled with 100 μCi/mL [^{32}P]orthophosphate in medium C (Ajiro et al., 1981) from 8 to 11 h after synchronization by selective detachment in mitosis. To obtain ^{32}P-labeled H1 components from mitotic cells, 1.7 × 10^8 selectively detached mitotic cells, blocked with 0.05 μg/mL colcemid, were resuspended to a concentration of 1.66 × 10^6 cells/mL in medium C containing 0.05 μg/mL colcemid and 100 μCi/mL [^{32}P]orthophosphate and were incubated for 3 h at 37 °C. After labeling, H1A and H1B from S-phase and mitotic cells were extracted and purified as previously described (Ajiro et al., 1981).

^{32}P Labeling of H1 Subtypes at the Time of the G_1–S Transitions. During G_1, at 2.5 h after synchronization by selective detachment in mitosis, a culture containing 5 × 10^8 HeLa cells at a concentration of 5 × 10^5 cells/mL was divided into two parts, and one part was treated with 5 mM HU. At 6 h after mitosis, the cells of each culture were harvested and resuspended to a concentration of 2.5 × 10^6 cells/mL in 100 mL of medium C containing 56 μCi/mL [^{32}P]orthophosphate; the cells that had been pretreated with HU during G_1 received 5 mM HU as well. Both cultures were then incubated at 37 °C for 3 h.

For preparation of HU-synchronized early S-phase cells, HeLa cell cultures were subjected to two 12-h cycles of 1 mM HU blockade as previously described (Stephens et al., 1977). One hour after reversal of the second blockade, 9 × 10^8 cells were harvested, resuspended at 6 × 10^6 cells/mL in medium C containing 33 μCi/mL [^{32}P]orthophosphate, and incubated at 37 °C for 3 h.

S-Phase Pulse–Chase Experiments. In the early S phase, at 6 h after synchronization by selective detachment in mitosis, 1200 mL of cells at 3 × 10^5 cells/mL was harvested by centrifugation at 6000*g* for 5 min at 37 °C. The cells were immediately resuspended to a concentration of 1.8 × 10^6 cells/mL in medium C containing 44 μCi/mL [^{32}P]orthophosphate and were incubated for 3 h at 37 °C. The labeled cells were harvested by centrifugation and incubated at 37 °C in 1200 mL of complete medium A. Samples (400 mL) were removed at 0, 2, and 4 h after resuspension. H1A and H1B isolated from these samples were digested with trypsin and the peptides resolved in the two-dimensional system described below. Quantitation of ^{32}P radioactivity in different tryptic phosphopeptides was carried out by scanning the TLC plates with a position-sensing proportional counter (PSPC) (Baird et al., 1979).

Bisection of H1 Subtypes with NBS. H1A and H1B were bisected with NBS (Bustin & Cole, 1969; Sherod et al., 1974). Samples of 1–5 mg of chromatographically purified H1A or H1B were incubated at a concentration of 500 μg/mL in a reaction mixture containing 10% acetic acid and 2 mM NBS for 60–70 min at room temperature, and then 0.4 volume of 25 mM tyrosine was added to terminate the reaction. The mixture was clarified by centrifugation at 10000*g* for 10 min at 3 °C and the supernatant dialyzed against 5% acetic acid at 3 °C to remove NBS and tyrosine. It was then lyophilized and the extent of H1 cleavage determined by electrophoresis on $NaDodSO_4$–polyacrylamide gels. N- and C-terminal fragments were separated from each other and from uncleaved molecules by chromatography on 1.5 × 90 cm columns of Sephadex G-100 in 10 mM HCl as described by Bustin & Cole (1970). ^{32}P radioactivity was estimated by Cherenkov counting in the ^{3}H channel of a Beckman LS-30 liquid scintillation counter set at a gain of 220. The N- and C-terminal fragments, detected by ultraviolet absorbtion at 230 nm, were pooled and lyophilized.

Resolution of Tryptic Peptides by Electrophoresis and Chromatography on Cellulose TLC Plates. Purified N- and C-terminal NBS fragments of H1A and H1B were dissolved at 2.5 mg/mL in 0.05 M sodium bicarbonate, pH 8.0, and were digested at 22 °C by three additions of trypsin (1 mg/mL in 1 mM acetic acid) at hourly intervals until the final trypsin/H1 ratio was 0.06. After the final addition of trypsin, the incubation was continued at 37 °C for 2 h. The mixture was lyophilized, resuspended in 100 μL of water, relyophilized, and dissolved in 3 μL of electrophoresis buffer (butanol–acetic acid–water–pyridine, 50:25:900:25; pH 4.7), and 2 μL was spotted near the corner of a 20 × 20 cm glass plate with a 250-μm coating of Avicel cellulose (Analtech, Inc.). Resolution in the first dimension was by electrophoresis at 600 V for 50 min in a chamber that permitted the simultaneous electrophoresis of two TLC plates under Varsol maintained at 17–19 °C by circulation of ice–water through cooling coils (M. Weigert and L. H. Cohen, unpublished design). After

Table I: Amino Acid Composition of NBS Fragments

	N terminus (mol %)		C terminus (mol %)	
amino acid	H1A	H1B	H1A	H1B
Asp	0.6	0.8	2.3	2.7
Thr	4.3	4.7	5.4	4.5
Ser	7.5	7.6	5.0	6.0
Glu	6.9	6.8	2.4	2.7
Pro	11.4	10.0	9.6	9.0
Gly	8.1	6.9	8.3	7.9
Ala	29.8	30.0	*19.7*[a]	*25.1*
Val	5.7	5.6	*7.6*	*3.1*
Met	0	0	0	0
Ile	1.6	1.6	0.7	0.9
Leu	5.3	6.2	3.4	3.6
Tyr	0	0	0	0
Phe	0	0	0.4	0.5
His	0.1	0.2	0	0.4
Lys	15.1	15.9	34.4	32.0
Arg	3.4	4.0	0.8	1.3

[a] Principal differences are italicized.

electrophoresis, the plates were dried, first in a warm air stream for 10–15 min, then overnight over NaOH in a vacuum desiccator. Resolution in the second dimension (within 18 h after electrophoresis) was by ascending chromatography in a solution (butanol–acetic acid–water–pyridine, 48.8:15.2:60.4:75.6) which was prepared the day before use.

Detection and Elution of Tryptic Peptides. The method used to stain thin-layer plates was a modification of previously described procedures (Felix & Jimenez, 1974; Mendez & Lai, 1975). After chromatography, the TLC plates were dried in a stream of warm air for about 5 min and then washed twice with acetone to remove primary amines which interfere with the fluorescamine staining reaction. The plates were sprayed lightly with 10% TEA in acetone, then with 0.01% fluorescamine in acetone, and again with 10% TEA. Fluorescent spots were scraped off the plates, and the peptides were eluted with 6 N HCl for 2 days.

Amino Acid Analysis. Purified N- and C-terminal fragments of H1A and H1B and tryptic peptides of these fragments were dissolved in 6 N HCl, sealed under N_2 in glass tubes, and hydrolyzed at 100 °C for 30 h (Moore & Stein, 1963). After hydrolysis, the samples were vacuum dried, dissolved in 50 μL of pH 2.2 sodium citrate sample dilution buffer, and analyzed in a Durrum D-500 amino acid analyzer. No correction was made for serine loss.

Autoradiography. For detection of ^{32}P-labeled phosphopeptides, TLC plates containing tryptic maps of the N- and C-terminal fragments of H1A and H1B from S-phase and mitotic cells labeled with ^{32}P were exposed to Du Pont Cronex 4 X-ray film, and the films were developed in a Kodak RP-OMAT automatic X-ray film developer.

Results

NBS Cleavage of HeLa Histones H1A and H1B. For facilitation of structural comparisons of subtypes of H1A and H1B, the chromatographically isolated protein subtypes were cleaved with NBS. The cleavage products are shown in Figure 1, resolved by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate ($NaDodSO_4$). It can be seen that the N-terminal fragments of H1A and H1B have nearly the same mobility whereas the C-terminal fragment of H1B appears to be larger than that of H1A, indicating that the previously described molecular weight difference between the two proteins (Ajiro et al., 1981) is due to differences in the region C terminal to the single tyrosyl residue present in these proteins.

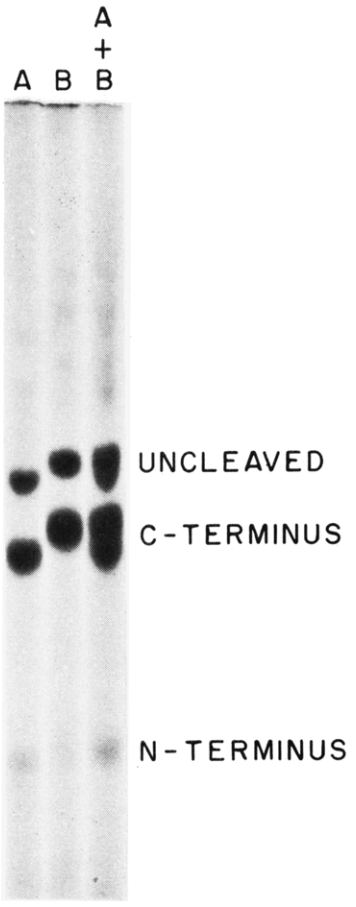

FIGURE 1: $NaDodSO_4$–acrylamide gel electrophoresis of NBS cleavage products of H1A and H1B. A 500-μg sample of chromatographically purified protein in 1 mL of 10% acetic acid and 2 mM NBS was incubated for 70 min at room temperature (22 °C). The reaction was terminated by adding 0.4 mL of 25 mM tyrosine solution. After 10 min at room temperature, the solutions were dialyzed overnight against 500 mL of 5% acetic acid and lyophilized. A mixture consisting of 15 μg of cleaved H1A (A), 15 μg of cleaved H1B (B), and 15 μg of a mixture of both (A + B) was run on 0.4 × 11 cm $NaDodSO_4$–acrylamide gels for 16 h at 30 V and was stained with 0.1% Coomassie blue, as previously described in Ajiro et al. (1980). Weak staining of the N-terminal NBS fragments of H1 histones has been previously reported (Sherod et al., 1974).

The cleavage products were separated by chromatography on Sephadex G-100. From the amino acid analyses of these products (Table I), it can be seen that the N-terminal fragments of H1A and H1B have very similar compositions. In contrast, the C-terminal fragments of these two H1 subtypes differ considerably in content of alanyl and valyl residues. The results in Figure 1 and Table I indicate the following: (a) H1A (M_r ~21 000) cannot be a cleavage product derived from H1B (M_r ~22 000) since it has more valyl residues than H1B. (b) The greatest differences in composition between H1A and H1B are in their C-terminal portions. (c) The C-terminal portion of H1A is considerably more hydrophobic than that of H1B.

H1A and H1B Phosphorylation Sites during the S Phase and Mitosis. Autoradiograms of the tryptic peptide maps of H1A and H1B from HeLa cells incubated with ^{32}P, seen in Figures 2 and 3, reveal that the pattern of phosphorylation changes considerably between the S phase and mitosis.

The phosphopeptide map of H1A labeled with ^{32}P during the mid-S phase (Figure 2a) contained two predominant spots, AC-1 and AC-4 (named according to rules described in the legend), both of which were found in the C-terminal NBS fragment (Figure 2c). When labeled with ^{32}P during mitosis,

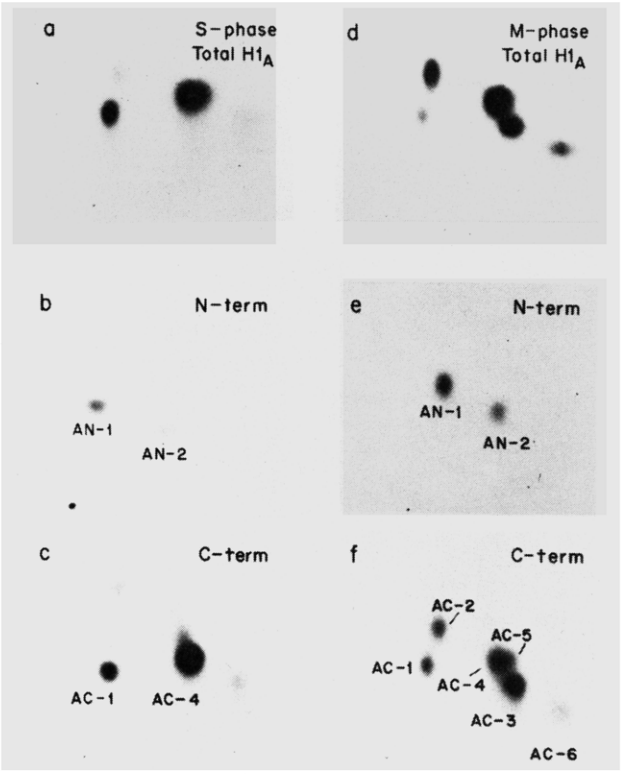

FIGURE 2: Autoradiographs of ^{32}P-labeled tryptic peptide maps of H1A and its NBS fragments. Top panels (a and d): A 30-μg sample of H1A (~30 000 cpm) from ^{32}P-labeled S-phase cells and 20 μg of H1A (~40 000 cpm) from ^{32}P-labeled mitotic cells were each mixed with 300 μg of unlabeled H1A carrier from log-phase cells, digested with trypsin, and then resolved by electrophoresis and chromatography on TLC plates as described under Methods. These tryptic fingerprints were then autoradiographed for 2 days. To obtain the autoradiographs in the middle and bottom panels, 32 μg of ^{32}P-labeled S-phase H1A (~32 000 cpm) and 17 μg of ^{32}P-labeled mitotic H1A (~34 000 cpm) were each mixed with 700 μg of unlabeled H1A carrier and cleaved with NBS. After cleavage, N- and C-terminal NBS fragments of these two types of ^{32}P-labeled H1A were purified by chromatography on Sephadex G-100, digested with trypsin, and fingerprinted as described under Methods. Middle panels (b and e): Tryptic maps of the N-terminal NBS fragments of H1A from ^{32}P-labeled S-phase (~1400 cpm) and mitotic (~2800 cpm) HeLa S-3 cells prepared as described under Methods. Bottom panels (c and f): Tryptic maps of the C-terminal NBS fragments of H1A from ^{32}P-labeled S-phase (~12 300 cpm) and mitotic (~12 000 cpm) cells. The autoradiographs in the middle and bottom panels represent 6-week exposures. The phosphopeptides in this and in subsequent figures were named in the following way: A or B indicates the H1 subtype; N or C indicates the NBS fragment that contains the peptide; the number indicates the relative proximity of the spot to the origin. Where it is not known which of two overlapping spots is present, both numbers are used.

this fragment yielded five principal labeled peptides: AC-1, AC-4, and three essentially mitosis-specific phosphopeptides, AC-2, AC-3, and AC-6 (Figure 2f). The N-terminal NBS fragment contained less than 10% of the ^{32}P radioactivity incorporated into H1A during the S phase. It can be seen in Figure 2b that most of this was found in two peptides, AN-1 and AN-2. In mitotic cells (Figure 2d–f), where the ^{32}P incorporation into H1 was twice that in S-phase cells (Ajiro et al., 1981), the relative labeling of these two N-terminal peptides was increased, accounting for over 20% off the total ^{32}P incorporated into H1A.

The patterns of H1B phosphorylation (Figure 3) were more complex than those of H1A phosphorylation, in accord with the observation (Ajiro et al., 1981) that H1B contains more phosphate groups per molecule than H1A at all phases of the cell cycle. It is nevertheless apparent that, as in the case of H1A, H1B from ^{32}P-labeled S-phase cells yielded two pre-

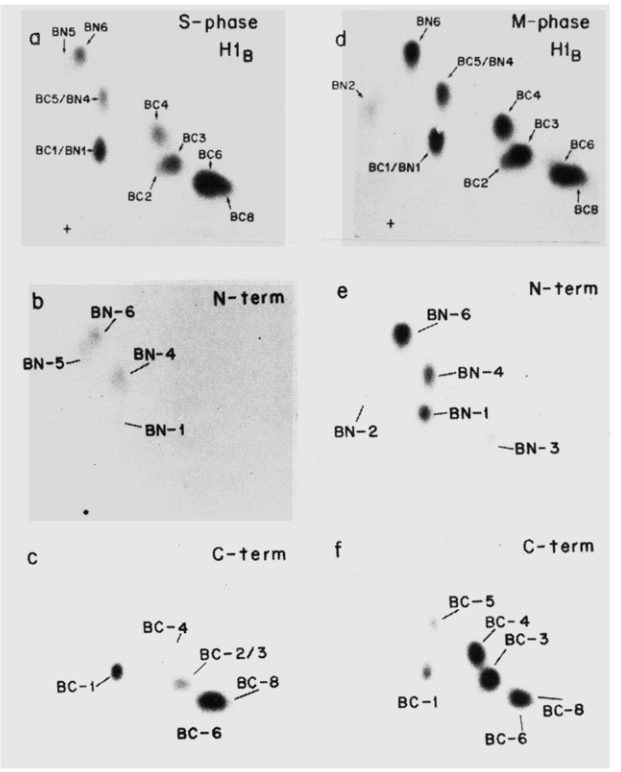

FIGURE 3: Autoradiographs of ^{32}P-labeled tryptic peptide maps of H1B and its NBS fragments. Top panels (a and d): A 70-μg sample of H1B (~140 000 cpm) from ^{32}P-labeled S-phase cells and 50 μg of H1B (~200 000 cpm) from labeled mitotic cells were each mixed with 300 μg of unlabeled H1B carrier, digested with trypsin, and fingerprinted as described under Methods. The tryptic maps were then autoradiographed for 2 days. To obtain the autoradiographs in the middle and bottom panels, 30 μg of ^{32}P-labeled S-phase H1B (~60 000 cpm) and 19 μg of ^{32}P-labeled mitotic H1B (~76 000 cpm) were each mixed with 1 mg of unlabeled H1B carrier and cleaved with NBS. After cleavage, the N- and C-terminal NBS fragments of these two types of ^{32}P-labeled H1B were purified by chromatography, digested with trypsin, and fingerprinted as described under Methods. Middle panels (b and e): Tryptic maps of the N-terminal NBS fragments of H1B from ^{32}P-labeled S-phase (388 cpm) and mitotic (2800 cpm) HeLa S-3 cells prepared as described under Methods and autoradiographed for 6 weeks. Bottom panels (c and f): Tryptic maps of C-terminal NBS fragments of H1B from ^{32}P-labeled S-phase (2496 cpm) and mitotic (7500 cpm) HeLa S-3 cells, autoradiographed for 6 weeks.

dominant radioactive spots, BC-1 and BC-6 (Figure 3a,c). The map position of BC-1 is the same as that of AC-1, but that of BC-6 is not the same as that of AC-4. As in H1A, both of these spots arose from the C-terminal NBS fragment. In addition to these two phosphopeptides, the C-terminal NBS fragment of H1B from cells labeled with ^{32}P during the S phase yielded three less heavily labeled peptides, BC-2, BC-3, and BC-4 (Figure 3c). In mitotic cells (Figure 3d–f), the ^{32}P-labeling of BC-2, BC-3, and BC-4 as well as that of BC-5 was greatly increased relative to that of BC-6. Thus, whereas the intensity of BC-1 and BC-6 labeling during the S phase was considerably greater than that of other phosphopeptides, the differences were reversed during mitosis.

The N-terminal NBS fragment of H1B (Figure 3b) contained about 13% of the ^{32}P incorporated into H1B during the S phase, principally in four phosphopeptides, BN-1, BN-4, BN-5, and BN-6. When HeLa cells were labeled with ^{32}P during mitosis, the relative labeling of the N-terminal NBS fragment of H1B was increased 2–3-fold, such that about 30% of the total mitotic H1B radioactivity was found in N-terminal peptides BN-1, BN-2, BN-3, BN-4, and BN-6. Peptide BN-5 was not significantly labeled during mitosis [compare (b) and

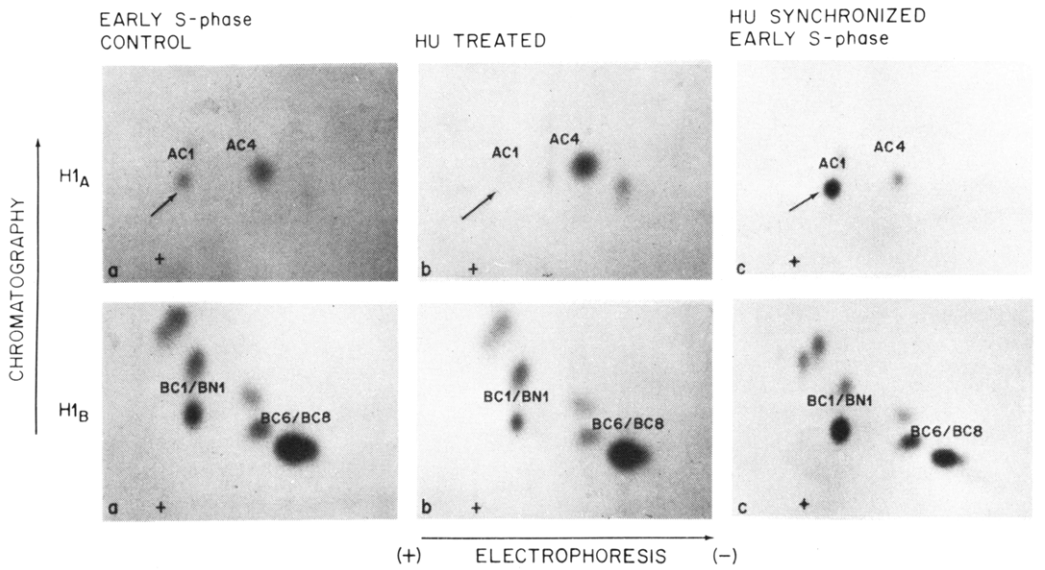

FIGURE 4: Effects of HU on the ^{32}P labeling of HeLa H1A and H1B phosphopeptides in cells at the G_1–S transition. (a) Autoradiographs of tryptic maps of H1A (upper panel) and H1B (lower panel) from control cells that were synchronized by mitotic selective detachment and incubated with [^{32}P]orthophosphate during the interval from 6 to 9 h after mitosis (early S phase), as described under Experimental Procedures. (b) Autoradiographs of peptide maps of H1A and H1B from cells blocked at the G_1/S boundary by 5 mM HU as described under Experimental Procedures and incubated with [^{32}P]orthophosphate in the presence of HU during the same interval as in (a). The tryptic map of H1A from the HU-treated cells was autoradiographed for 2 weeks. All other maps in this figure were autoradiographed for 1 week. (c) Autoradiographs of the tryptic maps of H1A and H1B from HU-synchronized cells that were labeled with ^{32}P during the interval from 1 to 4 h after reversal of the second cycle of HU blockade as described under Experimental Procedures.

(e) in Figure 3]. In contrast, peptide BN-2, barely labeled at all during the S phase, was fairly well labeled during mitosis.

Relationships between Replication Onset and S-Phase Phosphorylations. In the mammalian cell cycle, H1 phosphorylation is activated at or slightly preceding the onset of DNA replication (Stevely & Stocken, 1968; Marks et al., 1973; Gurley et al., 1974). The S-phase phosphorylations occur at two main intramolecular sites, as indicated by the foregoing data and by results obtained with other types of cells (Hohmann et al., 1976; Balhorn et al., 1975). We would expect that if these reactions are prerequisites of DNA replication they would be activated even if the onset of replication were largely prevented by an inhibitor while any that are consequences of replication would be blocked. We have therefore studied the effects of inhibiting the onset of replication, with respect to the ^{32}P labeling of specific sites in H1A and H1B at the time of the G_1–S transition.

In Figure 4, the (a) panels are autoradiographs of peptide maps of H1A and H1B from control cells that were synchronized by selective detachment and labeled with ^{32}P during the initial 3 h of the S phase, i.e., 6–9 h after mitosis; the (b) panels show the results obtained with cells from the same synchronized culture as the control cells but treated with 5 mM HU both prior to and during the ^{32}P-labeling period. Measurements of [^{14}C]thymidine incorporation (Figure 5, left panel) indicate that DNA replication began to accelerate at about 6 h after mitosis in the control cells and was completely inhibited in the HU-blocked cells. The autoradiograms in Figure 4a,b indicate that this inhibition of replication onset affected the onset of ^{32}P labeling of different sites in H1 to differing degrees. This is more clearly seen in the case of H1A. In the HU-blocked cells, peptide AC-1 was not detectably labeled with ^{32}P. The failure of the blocked cells to begin AC-1 phosphorylation was not due to a direct inhibition of AC-1 phosphorylation by HU, since HU did not block ^{32}P labeling at this site in mid-S-phase cells (not shown). These results suggest that phosphorylation at site AC-1 occurs only in replicated chromatin. In contrast, the onset of labeling at site AC-4 did not require appreciable DNA replication (Figure 4b), suggesting that the activation of phosphorylation at site AC-4 can precede the onset of replication and can affect the phosphorylation of H1A molecules located in unreplicated chromatin.

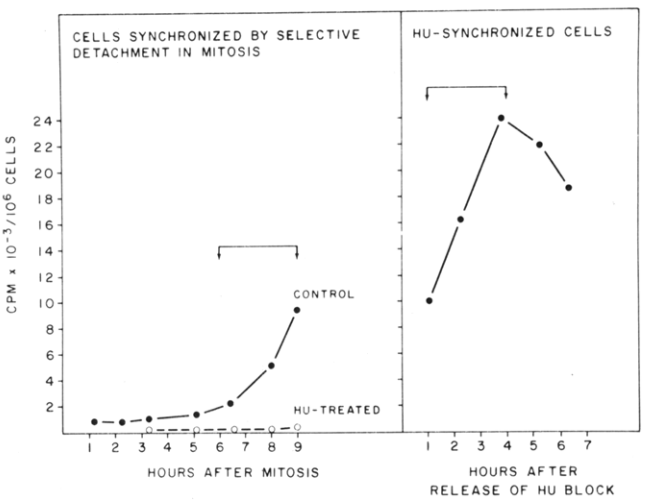

FIGURE 5: Effects of HU on the incorporation of [^{14}C]thymidine into HeLA cells during G_1 and the S phase. In the experiments described in Figure 4, parallel cultures which did not receive ^{32}P were sampled at the indicated times and incubated with [^{14}C]thymidine for 30 min as described under Experimental Procedures. Acid-precipitable radioactivity incorporated into 1×10^6 cells was then determined as previously described (Marks et al., 1973). The brackets indicate the times when ^{32}P was present in the control, HU-blocked, and HU-synchronized cultures that were used for H1 ^{32}P-labeling experiments.

In the case of H1B, inhibition of the onset of replication did not completely abolish the ^{32}P labeling of any spot in the peptide map. However, it did result in reduced intensity of labeling of spot BC-1/BN-1, which contains two phosphopeptides, relative to that of BC-6. This reduction in intensity must be principally in peptide BC-1 because peptide BN-1 was only slightly labeled in S-phase cells (Figure 3). These results suggest that, as in the case of site AC-1 in H1A, the activation of phosphorylation at site BC-1 during the G_1–S transition

Table II: ^{32}P Radioactivity[a] in Principal Tryptic Phosphopeptides of H1A after Labeling and Chasing during the S Phase

phosphopeptide	before chase	after 4-h chase
AC-4	882	395
AC-1	115	161

[a] Counts per minute per peptide.

requires the onset of DNA replication whereas phosphorylation of site BC-6, and at a number of the sites of minor phosphorylation, does not.

The preceding data suggested that phosphorylation at site AC-4 in H1A and at site BC-6 in H1B can occur in unreplicated regions of the chromatin whereas phosphorylation at site AC-1 in H1A and at site BC-1 in H1B occurs only in replicated regions. As a futher test of this interpretation, we examined H1 phosphorylation in cells in the early S phase, shortly after release from the HU blockade. Cells were synchronized by two cycles of HU blockade (Stephens et al., 1977) and labeled with [^{32}P]orthophosphate during the period from 1 to 4 h after the release of the second HU block. The c panels of Figure 4 are autoradiographs of the phosphopeptides of H1A and H1B from these cells. It is evident that the ^{32}P labeling of H1A is much less intense at site AC-4 than at site AC-1, a result opposite to that obtained when cells synchronized solely by selective detachment were used, whether labeled in early S (Figure 4, panel a) or in mid-s (Figure 2). Similarly, in H1B, the ^{32}P labeling at site BC-6, relative to the labeling of spot BC-1/BN-1, is significantly less intense in the HU-synchronized cells than in cells synchronized by selective detachment. The diminished labeling of AC-4 and BC-6 under these conditions is readily explained since, during the HU treatment, these sites should have become occupied in those H1 molecules that normally become phosphorylated at the time of the G_1–S transition. It is noteworthy, therefore, that the rate of DNA replication almost immediately after removal of the HU was much higher than that in the early S-phase cells that had been synchronized by selective detachment (Figure 5). Thus, some process important for replication occurred during the HU blockade. The correlation of the replication rate with the degree of occupancy of sites AC-4 and BC-6 suggests that it may have been the phosphorylation at this type of site that prepared the chromatin for replication during the blockade. The increased replication rate following the removal of HU is accompanied also by an increase in ^{32}P labeling at site AC-1 in H1A and at an analogous site in H1B (probably BC-1), supporting the conclusion reached above that phosphorylation at sites AC-1 and BC-1 depends on the presence of replicated chromatin.

Although there are two principal sites of phosphorylation in each H1 subtype during the S phase, few molecules of H1A acquire more than one phosphate group during this phase (Ajiro et al., 1981). To help determine how phosphorylations at sites AC-1 and AC-4 produce a phosphorylation level of one phosphate group per H1A molecule during the course of chromosome replication, we examined the relative stabilities of the phosphate groups at these two sites. HeLa cells synchronized by selective detachment were labeled with [^{32}P]-orthophosphate for 3 h during the early S phase as described under Experimental Procedures. The cells were then washed and incubated without ^{32}P for 4 h. H1A was isolated from cell samples withdrawn before and after the chase period, and tryptic peptide maps were prepared. The radioactivities in phosphopeptides AC-1 and AC-4, determined with the PSPC (Baird et al., 1979), are shown in Table II. We cannot

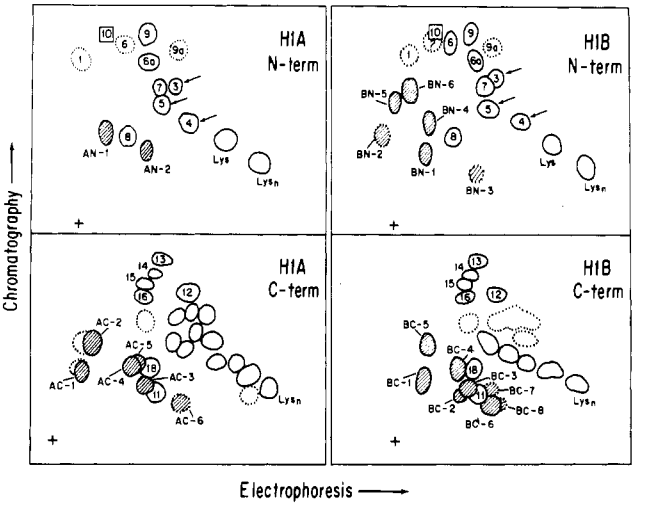

FIGURE 6: Tryptic peptide maps of NBS fragments of HeLa H1A and H1B. After autoradiography to detect ^{32}P-labeled phosphopeptide, the TLC plates containing the tryptic peptide maps of the N- and C-terminal NBS fragments of HeLa H1A and H1B, shown in part in Figures 3 and 4, were stained with fluorescamine as described under Methods, and observed under UV light. Phosphopeptides labeled with ^{32}P are indicated in this figure by shaded spots, named as described in the legend of Figure 2. The numbered, fluorescamine-stained peptides were cut out and their amino acid compositions determined as described under Methods. These compositions are compared with partial rabbit and calf H1 sequences in Table II. The location of peptide 10 is shown by a box in the upper panels of this figure because it contains the tyrosine residue which is cleaved with NBS. Thus, peptide 10 was found in uncleaved H1A and H1B but was absent from NBS fragments of these H1 subfractions. Faintly or indistinctly stained and lightly labeled peptides are indicated by dotted perimeters. Peptide 6a has one more Lys residue than 6 but otherwise has the same composition. Peptide 9a has one more Lys residue than 9 but otherwise has the same amino acid composition. Arrows indicate the three tryptic peptides, 3, 4, and 5, which are different in the N-terminal NBS fragments of H1A and H1B.

determine the kinetic parameters of dephosphorylation at these sites because, judging from the fact that the amount of ^{32}P in AC-1 increased during the chase, there appear to have been substantial residual sources of ^{32}P in the cells during the chase period. However, it is evident that during the S phase the phosphate group at site AC-1 is much less transient than that at site AC-4 and that dephosphorylation of site AC-4, if first order, occurs with a half-life considerably less than 4 h.

Partial Sequence Comparison of HeLa H1A and H1B. Although the N-terminal NBS fragments of HeLa histones H1A and H1B have similar compositions (Table I) and molecular weights (Figure 1), they yield different radioactive phosphopeptides, whether labeled during the S phase or mitosis (compare Figures 2b,e with 3b,e). To explore how the primary structures of H1A and H1B might explain these differences, we determined the amino acid compositions of the fluorescamine-stained tryptic peptides of the N-terminal NBS fragments of H1A and H1B (Figure 6) and aligned these peptides with peptides within known sequences of H1 subtypes of rabbit thymus (RTL-2, RTL-3, and RTL-4) and calf thymus (CT-L-3) (Rall & Cole, 1971; Jones et al., 1974; Hsiang & Cole, 1977) as indicated in Table III, It can be seen in Figure 6 and Table III that the unphosphorylated peptides derived from this region of H1A are identical with those from H1B in map position and composition, except for peptides 3, 4, and 5. These three peptides show a fair degree of homology with residues 20–34 in CTL-1 and thus appear to include residues 17–31 in HeLa H1A and H1B. Peptide 2 of CTL-1 (Ser-Pro-Ala-Lys) was not found among the peptides from HeLa H1A and H1B. The absence of this peptide is not unique to HeLa cell

Table III: Amino Acid Compositions of Tryptic Peptides from Human (HeLa) H1A and H1B and Alignment by Homology with Known Mammalian H1 Sequences

peptide[a] no.	H1[b]	residues per mol	peptide[a] no.	H1[b]	residues per mol
1	CTL-1	Thr Ser Glu_2 Pro_4 Ala_6 Lys^{15}	9	CTL-1	Ser_2 Gly Ala_2 Val Leu_2 Lys^{64} $(Lys)^i$
	H1A, H1B	Thr Ser Glu_2 Pro_4 Ala_7 Lys		H1A, H1B	Ser_2 Gly Ala_2 Val Leu_2 Lys $(Lys)^i$
2	CTL-1	Ser Pro Ala Lys^{19}	10	RTL-3	Asp Glu Gly_2 Ala_3 Val Leu Tyr Lys^{76}
	RTL-2	– – – –		RTL-2	Asp Glu Gly Ala_4 Val Leu Tyr Lys
	H1A, H1B	– – – –		H1A, H1B	Asp Glu Gly Ala_4 Val Leu Tyr Lys
3[c]	CTL-1	Thr Pro Val Lys^{23}	11	RTL-3	Asn_2 Ser Arg^{80}
	H1A	Ala Pro Val Lys		H1A, H1B	Asn_2 Ser Arg
	H1B	Thr Pro Val Lys			
4	CTL-1	Ala_2 Lys^{26} $(Lys_3)^d$	12	RTL-3	Ile Lys^{82}
	RTL-4	Ala Arg (Lys_3)		H1A, H1B	Ile Lys
	H1A	Ala_2 Lys $(Lys_3)^e$	13	RTL-3	Gly Leu_2 Lys^{86}
	H1B	Ala Arg $(Lys_3)^e$		H1A, H1B	Gly Leu_2 Lys
5[c]	CTL-1	– Pro Gly Ala_2 Arg^{34} (Arg) (Lys)	14	RTL-3	Ser_2 Val Leu Lys^{91}
	RTL-4	Ser – Gly Ala_3 Lys (Arg) (Lys)		H1A, H1B	Ser_2 Val Leu Lys
	H1A	Thr Pro Gly_2 Ala Arg $-^g$ $(Lys)^h$	15	RTL-3	Thr_2 Glu Gly Val Leu Lys^{98}
	H1B	Ser^f – Gly Ala_3 Lys $-^g$ $(Lys)^h$		H1A, H1B	Thr_2 Glu Gly Val Leu Lys
6	CTL-1	Thr Ser_2 Glu Pro_2 Gly Ala Val Ile Leu Lys^{48}	16	RTL-3	Thr Ser_2 Gly_3 Ala Phe Lys^{107}
	H1A, H1B	Thr Ser_2 Glu Pro_2 Gly Ala Val Ile Leu Lys		H1A, H1B	Thr Ser_2 Gly_3 Ala Phe Lys
7	CTL-1	Ser Ala_3 Val Lys^{54}	17	RTL-3	Asn Leu Lys^{110}
	H1A, H1B	Ser Ala_3 Val Lys			
8	CTL-1	Glu Arg^{56}	18	RTL-3	Ser Glu Pro Gly Ala_3 Lys_2^{119}
	H1A, H1B	Glu Arg		H1A, H1B	Ser Glu Pro Gly Ala_3 Lys_2

[a] The order of the peptides and the positions of the C-terminal residue of each peptide are those in the composite of the H1 sequence cited from the literature. [b] Abbreviations for the various H1 proteins are as follows: CTL-1, calf thymus H1 subfraction 1; RTL-2, rabbit thymus H1 subfraction 2; RTL-3, rabbit thymus H1 subfraction 3; RTL-4, rabbit thymus H1 subfraction 4; H1A, human (HeLa cell) H1A; H1B, human (HeLa cell) H1B. [c] In the case of H1A, the assignment of peptides 3 and 5 to these positions in the order of peptides is tentative, since these peptides do not bear a close homology to known H1 sequences. Thus, the order of these two peptides may be the reverse of that shown here. [d] Residues that would be expected to yield amino acids or homopeptides during tryptic cleavage are shown in parentheses. [e] The existence of Lys_3 is indicated by the amino acid composition of the N-terminal NBS fragment, which shows three Lys residues in excess of those found in heteropeptides, the existence of lysine homopeptides in the tryptic map (Figure 6), and the presence of a Lys-Lys-Lys sequence following lysyl residue 26 in CTL-1. [f] The serine/lysine ratio of peptide 5 of H1B was 0.4–0.6 in two independent peptide maps, indicating that some H1B molecules may lack a seryl residue in this region of the molecule. [g] The absence of an arginyl residue following peptide 5 is indicated by the amino acid composition of the N-terminal NBS fragment and also by the failure to detect free arginine in the tryptic map. [h] The presence of a lysyl residue between peptide 5 and peptide 6 is indicated by the detection of peptide 6a, identical with peptide 6, except for having two lysyl residues. [i] The presence of a lysyl residue between peptide 9 and peptide 10 is indicated by the detection of peptide 9a, identical with peptide 9, except for having two lysyl residues.

H1 subtypes since the residues corresponding to peptide 2 of CTL-1 are missing also from RTL-2, a rabbit thymus H1 subtype.

Peptide 10 was isolated from typtic peptide maps of uncleaved H1A and H1B. It contains the sole tyrosyl residue present in each of these proteins, which is the NBS cleavage site. Since the molecular weights of the N-terminal NBS fragments of H1A and H1B are quite similar (Figure 1), this tyrosyl residue must be similarly located in both molecules (~residue 70) (Table III). We have also isolated and analyzed peptides 11–16 and 18 from the C-terminal NBS fragments of H1A and H1B. There are indications that peptide 17 (Asn-Leu-Lys) is also present in the tryptic maps of these two HeLa H1 subtypes (in a map position between peptides 12 and 18) but is contaminated by other overlapping peptides. Thus, these data in Table III provide information about the extent of homology between HeLa H1A and H1B for over half of the lengths of the molecules, i.e., to Lys_{118} in H1A and Lys_{116} in H1B (which corresponds to Lys_{120} in RTL-3).

It is evident in Table III that peptide 16 contains the only phenylalanyl (Phe) residue in H1A and H1B. A Phe residue in this region of trout H1 has been shown to be a primary cleavage site during digestion with chymotrypsin under conditions described by MacLeod et al. (1977). The C-terminal chymotryptic fragment of each H1 subtype was isolated and its length determined from its composition (Table IV). From these analyses, it was calculated that this C-terminal fragment has 105 residues in H1A and 116 residues in H1B. When added to the number of residues found between the N terminus and the Phe residue (i.e., in peptides 1, 3–15, and part of 16

Table IV: Amino Acid Compositions of Chymotryptic C-Terminal Fragments of Human (HeLa) H1A and H1B

amino acid	H1A (mol %)	H1B (mol %)	H1A (residues/mol)[a]	H1B (residues/mol)
Asp	1.1	1.0	1.1	1.2
Thr	4.7	3.5	4.9	4.1
Ser	3.2	2.9	3.4[b]	3.4[b]
Glu	1.2	1.1	1.2	1.3
Pro	13.0	12.8	13.6	14.8
Gly	5.7	4.3	6.0	5.0
Ala	24.0	31.2	25.1	36.2
Val	7.5	0.9	7.9	1.0
Ile	0.07	0	<0.1	0
Leu	1.0	0.9	1.0	1.0
Tyr	0	0	0	0
Phe	0	0	0	0
Lys	38.4	41.4	40.3	48.2
Arg	0.1	0	0.1	0
total			105[b]	116[b]

[a] On the basis of the assumption that there are integral numbers of leucyl and glycyl residues. [b] The value for serine is nonintegral, as expected from the fact that 10–15% is destroyed during acid hydrolysis. Therefore, we estimate that the number of seryl residues is four per fragment.

in Table III), these values indicate that H1A contains a total of 209 amino acid residues, and H1B contains 218 residues.

Discussion

Structural Differences between HeLa H1A and H1B. There have been no previous studies comparing structural aspects of different H1 subtypes in a single cell type, nor has structural information been available about human H1 histones. The present data suggest that HeLa H1A and H1B have identical structures between residues 1–17 and 30–118 but have differences in the region between residues ~17 and 30 and pronounced differences in the highly basic C-terminal halves of these H1 subtypes. These observations confirm previous indications (Bustin & Cole, 1970; Hsiang & Cole, 1977; MacLeod et al., 1977) that H1 molecules generally contain a relatively invariant hydrophobic central globular region (Hartman et al., 1977) which is flanked by a N-terminal domain that contains a variable region and a highly basic C-terminal domain.

While previously available evidence has revealed that the sequence of the C-terminal domain is variable from one organism to another, it has suggested that this domain does not vary much among H1 subtypes from a single organism (Hsiang & Cole, 1977). This is clearly not the case in human HeLa S-3 cells, however, where the C-terminal domains of H1A and H1B differ in size, by ~9 residues (H1B > H1A), and in hydrophobicity, H1A containing eight valyl residues in this region of the molecule and H1B containing only one. It can be seen in Table IV that all of these extra valyl residues are located in the final 102 residues of the cationic C-terminal tail of H1A, where they represent the principal source of hydrophobicity other than proline and alanine. It would appear likely that this striking difference between H1A and H1B, in the most cationic portion of the H1 molecule, produces a signficiant difference in the manner in which these subtypes bind to DNA and in their effects on chromatin conformation. It may also be relevant to the reported subtype specificity of association between H1 and HMG proteins (Smerdon & Isenberg, 1976).

Intramolecular Locations of H1A and H1B Phosphorylation Sites. In both H1A and H1B, the two principal sites labeled during the S phase are C terminal to the NBS cleavage site (Figures 2 and 3). Other experiments, in which the labeled C-terminal NBS fragments were cleaved with a protease from *Staphylococcus aureus*, V8 strain, revealed that all of the ^{32}P in these fragments was C terminal to the glutamyl residue at position ~112, and therefore in the cationic tail that is thought to bind to DNA.

During mitosis, phosphorylation continues to occur at these two sites in each subtype, particularly at sites AC-4 in H1A and BC-6 in H1B. In addition, a number of other sites are very actively phosphorylated at this stage. Of these, some are in the C-terminal tail, and others are in the N-terminal regions. There are more such sites in H1B than in H1A, a fact that accounts for the higher maximal phosphorylation state attained by H1B (Ajiro et al., 1981). Several of these sites incorporate ^{32}P to a small extent in S-phase cells. The number of such sites labeled during the S phase, as well as their labeling intensities, was greater in H1B than in H1A, thus accounting for the previous observation that H1B molecules span a wider range of phosphorylation states than do H1A molecules during the S phase (Ajiro et al., 1981).

Differences between subtypes may help to explain differences among results obtained with various cell types in previous studies of H1 phosphorylation in interphase cells. Lake (1973) detected substantial interphase phosphorylation within the N-terminal domain by examining the fragments obtained from the total H1 fraction of Chinese hamster V79 cells. In Chinese hamster ovary (CHO) cells, however, phosphate incorporation in this region of H1 has been reported to be restricted to mitosis (Hohmann et al., 1976; Gurley et al., 1978). However, the H1 fraction of CHO cells contains a relatively minor protein component that resemble HeLa H1B in having more phosphate groups than the principal H1 protein during mitosis (Hohmann et al., 1976), and the presence of a moderate amount of radioactivity in the N-terminal region of such a protein during interphase might not be readily detectable by examination of fragments prepared from the total H1 fraction. Sherod et al. (1975), working with unsynchronized hepatoma tissue culture cells, could not detect ^{32}P incorporation into the N-terminal NBS fragments prepared from the total H1 fraction, but in similar experiments with Ehrlich ascites tumor cells, they found evidence of two sizes of N-terminal NBS fragment, presumably arising from different H1 subtypes, the larger of which was much more prominently labeled than the smaller. Thus, the existence, within a single cell type, of diverse H1 subtypes differing in their phosphorylation properties may be a fairly general occurrence, and some cells may not have easily detectable amounts of a subtype that undergoes significant N-terminal phosphorylation during interphase.

There are more than enough ^{32}P-labeled phosphopeptides in the tryptic maps of both H1A and H1B to account for the highest phosphorylation levels attained by these H1 subtypes during the HeLa cell cycle. One possible explanation might be that no molecules ever have all of their phosphorylation sites occupied simultaneously. Another is that extra labeled phosphopeptides may have been generated by cleavage at alternative sites in the vicinity of adjacent basic residues. Peptides 6a and 9a are unphophosphorylated examples of such extra peptides (Figure 6 and Table III). In addition, Dixon et al. (1975) and Langan (1976) have reported that peptide bonds between lysine and phosphoserine or phosphothreonine are poorly cleaved and may generate multiple tryptic phosphopeptides containing the same phosphorylation site.

By taking into account cleavage artifacts of these types and by examining differences between the peptide maps of the N-terminal NBS fragments of H1A and H1B shown in Figure 6, we have tentatively identified the intramolecular locations of the principal phosphorylation sites in the N-terminal regions of HeLa H1A and H1B as indicated in Table V. These identifications are based on the following considerations:

(1) The principal phosphopeptides in the tryptic map of the N-terminal fragment of H1A (AN-1 and AN-2) are different

Table V: Tentative Identification of the Intramolecular Locations of Phosphopeptides from the N-Terminal Regions of (HeLa) H1A and H1B

H1A		H1B	
peptide[a]	^{32}P-labeled phosphopeptide[b]	peptide[a]	^{32}P-labeled phosphopeptide[b]
5 (mono P)	AN-1	1 (mono P)	BN-5
(5 + Lys) (mono P)	AN-2	3 (mono P)	BN-4
		(1 + 3) (mono P)	BN-6
		(1 + 3) (di P)	BN-2
		5 (mono p)	BN-1
		(5 + Lys) (mono P)	BN-3

[a] See Table III for composition of peptides and Figure 6 for tryptic map locations. [b] See Figure 6 for tryptic map location of phosphopeptides.

from any of those in the corresponding map of H1B. Examination of Table III shows that the only hydroxy amino acid containing peptides in the N-terminal halves of H1A and H1B that are not common to both H1 subtypes are peptide 5 of H1A and peptides 3 and 5 of H1B, suggesting that the differences in the labeled phosphopeptide maps are due to phosphorylation of residues in these three peptides.

(2) In studies of calf thymus H1 phosphorylation in vitro by a protein kinase isolated from growing cells (Langan, 1976), the principal ^{32}P-labeled tryptic phosphopeptide obtained from the N-terminal NBS fragments of unfractionated calf thymus H1 was (ThrSerGlx$_2$Pro$_4$Lys)ThrPO$_4$–ProValLys, which was not easily cleaved at the lysyl–phosphothreonine bond. It is evident in Table III that the composition of this phosphopeptide corresponds closely to that of the first 20 residues of HeLa H1B, composinig peptides 1 and 3. BN-6, the principal ^{32}P-labeled tryptic peptide in the N-terminal region of H1B, has an electrophoretic mobility consistent with the charge/mass ratio calculated for peptide 1 uncleaved from monophosphorylated peptide 3. Some degree of cleavage of this peptide would be expected to occur, and BN-4 has map coordinates consistent with its being monophosphorylated peptide 3. These tentative identifications of BN-4 and BN-6 are supported by the failure to obtain corresponding phosphopeptides from the N-terminal NBS fragment of H1A, in which peptide 3 lacks a phosphorylatable amino acid residue (Table III). Thus, several lines of evidence indicate that the principal phosphorylation site in the N-terminal region of H1B is the threonyl residue at position 17, a residue not present in H1A (Table III).

(3) We infer that AN-1 and BN-1 are monophosphorylated peptide 5 of H1A and H1B, respectively, from the fact that peptide 5 of H1A has a slightly higher map position than peptide 5 of H1B, a relationship also true of phosphorylated peptides AN-1 and BN-1. Further, the electrophoretic mobilities of AN-1 and BN-1 are consistent with charge/mass ratios calculated for these putative phosphopeptides.

(4) These assignments of AN-1 and BN-1 also provide a plausible explanation for the existence of AN-2 and BN-3. Since, in the H1 molecule, peptide 5 appears to be preceded and followed by lysyl residues, one should expect to find a phosphorylated form of peptide 5 containing an extra lysyl residue, particularly in the event that the phosphoseryl or phosphothreonyl residue is next to a lysyl residue as is the case in the rabbit thymus H1, RTL-4 (Hsiang & Cole, 1977). An extra lysyl group would increase the electrophoretic mobility and decrease the chromatographic mobility of the phosphopeptide, resulting in a displacement of the map position like that observed.

(5) The only peptide in the N-terminal region of H1B that could, when phosphorylated, have the map position of BN-5 is peptide 1, comprising residues 1–16. Furthermore, the existence of such a phosphorylation site within peptide 1 would provide a simple explanation for the presence of BN-2 in the H1B map since any molecules phosphorylated at that site as well as at residue 17 would give rise to a diphosphorylated tryptic peptide containing residues 1–20.

(6) These assignments of phosphopeptides explain also the observed replacement of BN-5 by BN-2 in the tryptic map of H1B as the cells proceed from the S phase to mitosis. Thus, if all of the H1B molecules become phosphorylated at residue 17 during mitosis, cleavage at that residue would be inhbited, with the result that residues 1–16 would be found in BN-2 (diphosphorylated peptide 1 plus 3) instead of BN-5 (monophosphorylated peptide 1). That extensive phosphorylation at residue 17 does occur during mitosis is indicated by the marked increase in the relative intensities of BN-6 and BN-4 in Figure 3 (panel d).

None of the above assignments includes the seryl residue at position ~37 that has been implicated in glucagon activation of transcription in liver (Langan, 1969). The peptide maps of the N-terminal fragments did, however, contain a few labeled spots too faint to be reproduced in photographs, and, thus, there appear to be a number of phosphorylations, in addition to those reported here, that are confined to a very small proportion of the H1 molecules or are extremely transient.

As yet, we do not know the locations of the phosphorylation sites in the cationic tails of H1A and H1B, other than that they are all C terminal from position ~112. In trout testis during spermatogenesis, the principal site of H1 phosphorylation was shown by Dixon et al. (1975) to be a seryl residue at position 156, almost exactly in the middle of the cationic tail. Their data also indicated that one or two other seryl residues in trout H1, closer to the C terminus, are also phosphorylated. Langan (1978) has reported the locations of three sites of in vitro enzymatic phosphorylation of H1 preparations from a variety of mammals to be residues ~136, ~153, and ~180, in addition to the sites in the N-terminal region, at residues ~16 and ~37.

Functional Differences between the Two Major S-Phase Phosphorylations. During the S phase, phosphate incorporation occurs principally at two sites in each H1 subtype. Since the predominance of ^{32}P labeling at these sites was shown not to be due to especially rapid turnover, we conclude that during the S phase more H1 molecules have phosphate at these two sites than have phosphate at their other phosphorylation sites. [An alternative possibility, that the other sites have unlabeled phosphate, acquired at another period of the cell cycle, is unlikely since (a) there is little phosphate incorporated into H1 during G_1 and (b) almost all of the phosphate incorporated during mitosis is removed by early G_1 (Ajiro et al., 1981).] The two sites in H1A (AC-1 and AC-4) resemble those in H1B (BC-1 and BC-6, respectively) in that they are located in the cationic tail, as well as in their relative labeling intensities (in both the S phase and mitosis). During and after HU synchronization of the cells, moreover, both subtypes exhibit similar alterations in the relative ^{32}P labeling at these sites. Furthermore, AC-1 and BC-1 are congruent in peptide map position, and probably are identical peptides. While AC-4 and BC-6 are not identical peptides, the close similarities in their functional properties suggest that they too may come from similar positions in their respective molecules. These results indicate that the roles of these two phosphorylations are the same for both H1 subtypes. Since they are the predominant H1 phosphorylations during the S phase, one or both of them must be responsible for the general increase in the phosphorylation states of H1A and H1B, by ~1 P/mol, that occurs during the course of the S phase (Ajiro et al., 1981).

Our results show also that there are distinct differences between these two phosphorylations. The phosphate group at site AC-4, although not extremely transient, has a lifetime that is a relatively small fraction of the S phase whereas that at AC-1 is much more stable. The results of the experiments with HU show that there are also major differences between the two types of phosphorylation in their relationships to the process of chromatin replication. They suggest that the onset of one of them, at AC-4 and BC-6 in H1A and H1B, respectively, is activated at the time of the G_1–S transition, preceding any substantial extent of DNA replication. Fur-

thermore, when we allowed phosphate to accumulate at AC-4 and BC-6 by inhibiting the onset of DNA replication, release of the block was followed by replication at enhanced rates. These characteristics suggest that this type of H1 phosphorylation can occur in advance of replication forks and is required for replication to procede. Phosphorylation at AC-1 and BC-1, on the other hand, appears to depend on the presence of replicated chromatin, suggesting that this type of phosphorylation may be confined to chromatin that has been replicated.

This interpretation of the data, that one of the two principal S-phase phosphorylations occurs in H1 molecules ahead of replication forks and is relatively short-lived while the other occurs in H1 molecules behind replication forks and is quite stable, provides an explanation for our observation (Ajiro et al., 1981) that few H1A molecules contain more than one phosphate group during the S phase even though H1A undergoes both of these phosphorylations during this phase. Furthermore, it is consistent with the observed net increase of ~ 1 in the number of phosphate groups in H1A and H1B during the S phase, described in the same paper.

The relative transience of the phosphate group at site AC-4 agrees with the proposed function of this phosphorylation, producing a chromatin conformation that permits replication, since persistence of such a group after the replication fork passes might allow overreplication to occur. Mere removal of this phosphate group would not, however, permit a distinction between replicated and unreplicated chromatin and would thus not assure against overreplication as long as the kinase for this site were active. In contrast, the addition of a stable phosphate group in H1 molecules behind the replication fork, as at site AC-1, could provide replicated chromatin with distinctive properties. For example, it might promote dephosphorylation at the putative prereplication phosphorylation site, or in some other way lock the chromatin into a conformation incompatible with replication.

The hypothesis that the phosphorylation at sites AC-1 and BC-1 is postreplicative makes no assumptions as to whether it is confined to previously existing or to newly synthesized H1 molecules. Also, we do not wish to imply that phosphorylation at sites AC-4 and BC-6 is exclusively prereplicative. It is quite conceivable that phosphorylation at this type of site might be involved in preparing chromatin for transcription as well as for replication. It is evident also that active phosphorylation at sites AC-4 and BC-6 occurs not only during the S phase but also during mitosis as well (Figures 2 and 3), when neither replication not transcription occur. Thus, assignment of a single functional role for this phosphorylation on the basis of our data is not straightforward.

Relationships between Mitotic and S-Phase Phosphorylations of H1B. There are two main differences between HeLa H1 subtypes with regard to phosphorylation. First, the total number of sites phosphorylated in H1B is greater than in H1A, a conclusion consistent with our estimates of the numbers of phosphate groups per molecule (Ajiro et al., 1976, 1981). Second, there are differences between the two proteins in the use of some of their phosphorylation sites during the S phase: although the two H1 subtypes incorporated ^{32}P to similar extents at the two principal phosphorylation sites described above, the extent to which additional sites were labeled during this phase was greater in the case of H1B than in the case of H1A. Indeed, most of the H1B phosphorylations that occur in mitotic cells were found to occur to a significant extent in S-phase cells as well. This observation cannot have been due to contamination by mitotic cells since the H1A obtained from the same S-phase cells did not contain ^{32}P in some of the sites phosphorylated in mitotic cells (e.g., AC-3 and AC-2 in Figure 2). These results agree with the observed presence in S-phase cells of substantial numbers of molecules of H1B, but not of H1A, having two, three, and four phosphate groups (Ajiro et al., 1981).

We conclude that several of the H1B phosphorylations that occur extensively throughout the chromatin during mitosis occur also during the S phase, but at that time they are confined to limited regions of the genome or are relatively transient, or both. Assuming that the roles of the major phosphorylations in mitotic cells are in the formation, maintenance, and possibly unfolding of the condensed mitotic chromosomes, we suggest that their occurrence in S-phase cells is related to various degrees of compaction present in certain chromosomal regions during interphase. The incorporation of ^{32}P into these sites of H1B during the S phase would then be explained by the need for the chromatin structures in these regions to be unfolded ahead of replication forks and reestablished in both sister chromatids following replication, in the S phase and G_2. We believe that some of the phosphate groups incorporated at this time are retained through the rest of the cell cycle and into the next cell generation. Thus, as cells proceed into G_1, phosphate groups added in mitosis are removed almost quantitatively (Balhorn et al., 1975; Ajiro et al., 1981), but a majority of H1B molecules retain one to three phosphate groups, most of which must have been acquired prior to mitosis (Ajiro et al., 1981). We have suggested that these phosphate groups may serve in the maintenance of certain chromatin conformations from one cell generation to the next, until such time as these conformations have to be unfolded again in preparation for replication of their DNA.

We do not suggest that H1 phosphorylations alone are responsible for the conformational diversity of interphase chromatin or the compactness of mitotic chromosomes; indeed, a number of other chromosomal components have been implicated, including H3 (Gurley et al., 1974), A24 (Matsui et al., 1979; Goldknopf et al., 1980), and nonhistone proteins (Adolph et al., 1977; Paulson & Laemmli, 1977).

The results of this paper and the preceding paper support the view that the various phosphorylations of H1 in mammalian cells serve diverse roles, including both the production and the disruption of chromatin conformations of various levels of complexity. Given the existence, within a single cell, of two or more H1 subtypes that differ substantially in primary structure as well as in the numbers, regulation, and intramolecular locations of their phosphorylations, a considerable degree of control over chromatin conformation through the agency of subtype-specific H1 phosphorylation can be imagined, provided there are mechanisms for situating histone subtypes in specific locations in the chromatin, as previous work has suggested (Newrock et al., 1978; Weintraub et al., 1978). Moreover, if the locations of histone subtypes were to differ from one cell type to another, H1 phosphorylation could play a role in cell-specific control of chromosomal function.

Acknowledgments

We thank Dr. Martin Weigert for advice regarding tryptic peptide mapping and Lynn Gatmaitan for help with the amino acid analyses.

References

Adolph, K. W., Cheng, S. M., & Laemmli, U. K. (1977) *Cell 12*, 805–816.

Ajiro, K., Borun, T. W., & Cohen, L. H. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1623.
Ajiro, K., Borun, T. W., & Cohen, L. H. (1981) *Biochemistry*, preceding paper in this issue.
Baird, W. M., Diamond, L., Borun, T. W., & Shulman, S. (1979) *Anal. Biochem. 99*, 165–169.
Balhorn, R., Jackson, V., Granner, D., & Chalkley, R. (1975) *Biochemistry 14*, 2504–2511.
Bustin, M., & Cole, R. D. (1969) *J. Biol. Chem. 244*, 5291–5294.
Bustin, M., & Cole, R. D. (1970) *J. Biol. Chem. 245*, 1458–1466.
Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., MacLeod, A. R., & Sung, M. T. (1975) *Ciba Found. Symp. 28*, 229–258.
Felix, A. M., & Jimenez, M. H. (1974) *J. Chromatogr. 89*, 361–364.
Goldknopf, I. L., Sudhakar, S., Rosenbaum, F., & Busch, H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1253–1260.
Gurley, L. R., Walters, R. A., & Tobey, R. A. (1974) *J. Cell Biol. 60*, 356–364.
Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., & Tobey, R. A. (1978) *Eur. J. Biochem. 84*, 1–15.
Hartman, P. G., Chapman, G. E., Moss, T., & Bradbury, E. M. (1977) *Eur. J. Biochem. 77*, 44–51.
Hohmann, P., Tobey, R. A., & Gurley, L. R. (1976) *J. Biol. Chem. 251*, 3685–3692.
Hsiang, M., & Cole, R. D. (1977) in *The Molecular Biology of the Mammalian Genetic Apparatus* (T'so, P., Ed.) pp 93–103, Elsevier/North-Holland Publishing Co., Amsterdam.
Jones, G. M., Rall, S. C., & Cole, R. D. (1974) *J. Biol. Chem. 249*, 2548–2553.
Lake, R. S. (1973) *J. Cell Biol. 58*, 317–331.
Langan, T. A. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1276–1283.
Langan, T. A. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1623.
Langan, T. A. (1978) *Methods Cell Biol. 19*, 127–142.
MacLeod, A. R., Wong, N. C. W., & Dixon, G. H. (1977) *Eur. J. Biochem. 78*, 281–291.
Marks, D. B., Paik, W. K., & Borun, T. W. (1973) *J. Biol. Chem. 248*, 5660–5667.
Matsui, S., Seon, B. K., & Sandberg, A. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6386–6390.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 65*, 281–292.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.
Newrock, K. M., Alfageme, C. R., Nardi, R. V., & Cohen, L. H. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 421–431.
Paulson, J. R., & Laemmli, U. K. (1977) *Cell 12*, 817–828.
Rall, S. C., & Cole, R. D. (1971) *J. Biol. Chem. 246*, 7175–7190.
Sherod, D., Johnson, G., & Chalkley, R. (1974) *J. Biol. Chem. 249*, 3923–3931.
Sherod, D., Johnson, G., Balhorn, R., Jackson, V., Chalkley, R., & Granner, D. (1975) *Biochim. Biophys. Acta 381*, 337–347.
Smerdon, M. J., & Isenberg, I. (1976) *Biochemistry 15*, 4242–4247.
Stephens, R. E., Pan, C. J., Ajiro, K., Dolby, T. W., & Borun, T. W. (1977) *J. Biol. Chem. 252*, 166–172.
Stevely, W. S., & Stocken, L. A. (1968) *Biochem. J. 110*, 187–191.
Weintraub, H., Flint, S. J., Leffak, I. M., Groudine, M., & Grainger, R. M. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 401–407.

Substrate and Product Inhibition Initial Rate Kinetics of Histone Acetyltransferase†

John E. Wiktorowicz,* Kenneth L. Campos, and James Bonner

ABSTRACT: Initial velocity and product inhibition kinetics of the histone acetyltransferase (EC 2.3.1.48) reaction indicate that the rat liver nuclear enzyme operates under a rapid equilibrium ordered bireactant mechanism. Histone adds first to the enzyme, and under the conditions of the experiment $K_a = 0$ as acetyl coenzyme A (CoA) concentration approaches saturating conditions. The K_m for acetyl-CoA was 2.10 ± 0.48 μM. Inhibition with acetyllysine resulted in a K_{iq} for the enzyme·acetyllysine complex of 1.96 ± 0.30 mM. Inhibition with CoA yielded K_{ip} for the ternary complex of 3.19 ± 0.48 μM. These results indicate that the enzyme activity is comparatively independent of histone concentration, and, since the enzyme is sensitive only to acetyl-CoA and CoA concentrations, the enzyme will tend to maintain histones in the acetylated state.

In chromatin, histones are organized in a repeating structure consisting of approximately 200 base pairs of DNA associated with one molecule of histone H1 and two molecules each of histones H2a, H2b, H3, and H4 (Kornberg & Thomas, 1974; Thomas & Kornberg, 1975; Simpson & Bustin, 1976; Weintraub et al., 1975; Shaw et al., 1976; Simpson & Whitlock, 1976). Since a distinctive feature of histones is their large complement of basic residues, particularly in the N-terminal regions, it is generally accepted that the histones interact with DNA through ionic interactions. Histones may undergo a series of postsynthetic modifications, including acetylation and methylation of lysyl residues and phosphorylation of seryl residues. The modifications of lysyl residues in particular can lead to altered positive charge densities in the critical regions

† From the Division of Biology, California Institute of Technology, Pasadena, California 91125. *Received September 9, 1980.* This work was supported in part by a fellowship grant from Damon Runyon–Walter Winchell Cancer Fund (DRG-240-FT) and U.S. Public Health Service Grant GM 137-62.

* Address correspondence to this author. He is a Fellow in Cancer Research supported by Grant DRG-240-FT of the Damon Runyon–Walter Winchell Cancer Fund.

0006-2960/81/0420-1464$01.25/0