Fulmer, A., & Fasman, G. (1979) *Biochemistry 18*, 659–668.
Griffith, J., & Christiansen, G. (1978) *Annu. Rev. Biophys. Bioeng. 7*, 19–35.
Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 195–199.
Houssier, C., & Fredericq, E. (1966) *Biochim. Biophys. Acta 120*, 113–130.
Houssier, C., Bontemps, J., Emonds-Alt, X., & Fredericq, E. (1977) *Ann. N.Y. Acad. Sci. 303*, 170–189.
Lee, K. S. (1980) Ph.D. Thesis, Yale University.
Marion, C., & Roux, B. (1978) *Nucleic Acids Res. 5*, 4431–4449.
McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119–146.
Newman, J., Swinney, H., & Day, L. A. (1977) *J. Mol. Biol. 116*, 593–606.
O'Konski, C. T., Yoshioka, K., & Orttung, W. H. (1959) *J. Phys. Chem. 63*, 1558–1565.
Olins, A. L. (1977) *Biophys. J. 17*, 115a.
Olins, A. L. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 325–329.
Olins, A. L., & Olins, D. E. (1979) *J. Cell Biol. 81*, 260–265.
Olins, A. L., Carlson, R. D., Wright, E. B., & Olins, D. E. (1976) *Nucleic Acids Res. 3*, 3271–3291.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879–1883.
Renz, M., Nehls, P., & Hozier, J. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 245–252.
Rill, R., & Van Holde, K. E. (1974) *J. Mol. Biol. 83*, 459–471.
Ris, H. (1973) *Ciba Found. Symp. 28*, 7–22.
Steinmetz, M., Streeck, R. Z., & Zachau, H. G. (1978) *Eur. J. Biochem. 83*, 615–628.
Suan, P., Bradbury, E. M., & Baldwin, J. P. (1979) *Eur. J. Biochem. 97*, 593–602.
Thoma, F., Koller, Th., & Klug, A. (1979) *J. Cell Biol. 83*, 403–427.
Tinoco, I. (1955) *J. Am. Chem. Soc. 77*, 4486–4489.
Tirado, M. M., & Garcia de la Torre, J. (1979) *J. Chem. Phys. 71*, 2581–2587.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Worcel, A., & Benyajati, C. (1977) *Cell 12*, 83–100.
Wu, H. M., Dattagupta, N., Hogan, M., & Crothers, D. M. (1979) *Biochemistry 18*, 3960–3965.
Zirkin, B. R., & Wolfe, S. L. (1972) *J. Ultrastruct. Res. 39*, 496–508.

Phosphorylation States of Different Histone 1 Subtypes and Their Relationship to Chromatin Functions during the HeLa S-3 Cell Cycle†

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ABSTRACT: The histone 1 (H1) fraction of HeLa S-3 cells contains two principal subtypes, H1A (M_r ~21 000) and H1B (M_r ~22 000). In G_1 cells, the H1 molecules are distributed among several phosphorylation states, most H1A molecules containing 0 or 1 phosphate groups and most H1B molecules containing 0, 1, 2, or 3 phosphate groups. Both subtypes undergo a general increase in phosphorylation levels of ~1 P/mol during the S phase and a further increase of 3–4 P/mol during mitosis. These two increases affect most of the H1 molecules and thus reflect phosphorylations occurring widely throughout the chromatin, presumably in association with replication and mitotic chromosome condensation. During all these periods, multiple phosphorylation levels of H1 molecules persist, as does the phosphorylation differential between H1A and H1B. Thus, there appear to be phosphorylation states that only some of the H1 molecules occupy, a fact that may be related to the conformational diversity in interphase and mitotic chromatin. The existence of differences between H1A and H1B phosphorylation states throughout the cell cycle, and within a single cell type, is in accord with the hypothesis that the H1 subtypes are functionally distinct, such that subtype-specific phosphorylations contribute to the control of chromatin organization.

The eukaryotic cell is thought to be able to exert control over fine details of the organization of its chromatin (Comings, 1972; Sedat & Manuelides, 1978). This degree of control undoubtedly reflects a number of functional needs: during interphase, active genes must be exempt from the high degree of compaction required to keep the large eukaryotic genome within reasonable physical bounds; there must be a precise schedule of unpackaging of genomic segments for purposes of replication; and after replication has been completed, the chromatin must be folded reproducibly into the variety of ordered structures of the mitotic chromosomes. A major element in the control of chromatin organization is histone 1 (H1),[1] which plays a role in the higher order coiling of the elemental chromatin fiber (Mirsky et al., 1968; Bradbury et al., 1973; Worcel, 1978; Cole et al., 1978; Gaubatz et al., 1978; Renz et al., 1978; Oudet et al., 1978).

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[1] Abbreviations used: H1, histone 1; PCA, perchloric acid; Cl_3CCO-OH, trichloroacetic acid; MeOH, methanol; HOAc, acetic acid; GdmCl, guanidinium chloride; CHO, Chinese hamster ovary.

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There are multiple types of the H1 molecule (Kinkade & Cole, 1966a,b). These vary in their proportions in different tissues (Kinkade, 1969; Panyim et al., 1971) and in different states of tissue differentiation (Hohmann & Cole, 1971), a fact which has led to the proposal that there are functional differences between H1 subtypes [reviewed by Cole (1975) and Hohmann (1978)]. recently, a series of switches in synthesis among several subtypes, not only of H1 but of H2A and H2B as well, was shown to occur during early embryogenesis in the sea urchin (Hill et al., 1971; Cohen et al., 1973, 1975; Seale & Aronson, 1973; Ruderman & Gross, 1974; Newrock et al., 1978). Different subtypes are synthesized at different stages of development; after cessation of their synthesis, the early subtypes are retained in the chromosomes of subsequent cell generations. In view of evidence that, during replication, preexisting and newly synthesized histone molecules do not become randomly mixed in the replicated chromatin (Tsanev & Russev, 1974; Leffak et al., 1977; Newrock et al., 1978; Russev & Tsanev, 1979), Newrock et al. (1978) proposed that the replications occurring during this period could result in patterns of distribution of histone subtypes in chromatin, differing from one cell to another, that might be important in the establishment of cell phenotypes. This proposal raises the question, dealt with in the present series of papers, of whether histone subtypes within any one cell type display structural or functional differences that might serve in the production of diversity in chromatin function and structure within a single cell.

The influence of H1 on chromatin structure and function is modulated by phosphorylations at multiple sites within the H1 molecule. At least one phosphorylation is considered to be related to gene activation (Langan, 1969a,b) since it was detected in quiescent cell populations (rat liver) in response to hormonal stimuli known to activate specific genes and involves only a very small proportion of H1 molecules. Phosphorylations at other intramolecular sites occur in a large proportion of the H1 of cells traversing the cell cycle, and it has been suggested that such phosphorylations are instrumental in producing changes in chromosome configuration as needed for DNA replication and for mitosis (Balhorn et al., 1972a,b; Lake et al., 1972; Marks et al., 1973; Langan & Hohmann, 1975; Gurley et al., 1975, 1978; Bradbury et al., 1973, 1974). Such correlations between H1 phosphorylations and chromatin conformation suggest how subtype distribution in chromatin might be important for the control of chromosome organization. If the diverse H1 subtypes in a cell were to differ with respect to the number or intramolecular locations of their phosphorylation sites, then kinases specific for different phosphorylation sites could be used for selective regional control over chromosome conformation, provided that the subtypes are nonrandomly distributed in the chromatin. That differences in phosphorylatability do exist among H1 subtypes was first indicated by the observation that one of the H1 subtypes of rabbit thymus lacks the seryl residue that is the target of a cAMP-dependent kinase prepared from rat liver (Langan et al., 1971). However, it is not known whether this subtype is contained in the same cells as the phosphorylatable subtypes.

In preliminary communications (Ajiro et al., 1975, 1976), we reported that the H1 fraction of HeLa cells is resolvable into two principal subtypes which are phosphorylated to different maximal levels at all phases of the cell cycle. A difference in the phosphorylation level between two proteins in the H1 fraction has also been detected in Chinese hamster ovary (CHO) cells (Gurley et al., 1978) and in tadpole liver (Morris & Cohen, 1979). In none of these reports was chemical confirmation provided that both proteins were actually H1. The two papers in the present series represent an analysis of differences between the HeLa cell H1 subtypes with regard to their primary structure, phosphorylation levels, and intramolecular phosphorylation sites. This paper describes the levels of phosphorylation of each H1 subtype during G_1, the S phase, and mitosis and discusses possible relationships of these levels to transcription, replication, and chromosome condensation.

Experimental Procedures

Joklik-modified Eagle's minimal essential spinner medium (medium A), Eagle's minimal essential medium (medium B), calf serum, fetal bovine serum, and Eagle's spinner salts were obtained from Grant Island Biological Co. (GIBCO), Grand Island, NY. Phosphate-free medium A was made up from components purchased from Sigma Chemical Co., St. Louis, MO and GIBCO. Medium C was phosphate-free medium A containing 3.5% calf serum and 3.5% fetal bovine serum and was used in all ^{32}P-labeling experiments. Analytical-grade Amberlite CG-50 was purchased from Mallinckrodt Chemical Works, St. Louis, MO. "Absolute" grade guanidinium chloride and urea were obtained from Research Plus Laboratories, Inc., Denville, NJ. Colcemid was purchased from CIBA Pharmaceutical Co., Summit, NJ. Hydroxyurea and thymidine were purchased from Sigma Chemical Co. All other chemicals were Baker-analyzed or equivalent reagent-grade materials. [2-^{14}C]Thymidine and carrier-free [^{32}P]orthophosphoric acid were obtained from New England Nuclear Corp., Boston, MA.

HeLa S-3 Cell Culture and Synchronization. Logarithmically growing HeLa S-3 cells were maintained in suspension culture in medium A supplemented with 3.5% each of calf serum and fetal calf serum (complete medium A) as previously described (Marks et al., 1973). synchronization was carried out at 37 °C either by selective detachment of mitotic cells (Terasima & Tolmach, 1973; Borun et al., 1975) or by two cycles of blockade with 1 mM hydroxyurea (Stephens et al., 1977). Two methods were employed to synchronize cells by selective detachment in mitosis. In most experiments reported here, a 6-L culture of log-phase cells at a concentration of 5×10^5 cells/mL was diluted with 1.2 L of warm complete medium A and was treated for 12 h with 2 mM thymidine. At the end of this thymidine block, the culture was harvested by centrifugation at 600*g* for 15 min, the cell pellets were suspended in 6 L of warm complete medium B, and 100 mL of this culture was plated in each of 60 2-L Blake bottles. After 8 h of incubation at 37 °C to allow attachment of most cells, the Blake bottles were given four vigorous shakes to remove nonadherent cells, and the remaining cell sheet in each bottle was washed with 40 mL of warm complete medium A which was then discarded. The cell sheets in the Blake bottles were then covered with 60 mL of warm complete medium A and incubated at 37 °C. After 2 h, mitotic cells were collected by gently rocking 200 mL of warm complete medium A over the cell sheet in each of four bottles and cycling this 200 mL through 15 bottles, such that all 60 bottles were subject to the collection. A second cycle of detachment was then carried out, employing 200 mL of warm complete medium A to remove the mitotic cells from 20 bottles. At the end of this process, which lasted 1 h, 1200 mL of detached cells at a concentration of about $(3–4) \times 10^5$ cells/mL was obtained. Routine microscopic examination of the cells showed over 95% of the cells to be in mitosis in every case except where noted. In some experiments, the preliminary thymidine block was omitted to

enable us to determine whether possible artifacts generated by such presynchronization affected H1 phosphorylation levels during the HeLa cell cycle (Figure 5, Table III). The zero time in these experiments was always the midpoint of the time it took to carry out the selective detachment of the mitotic cells. In experiments in which we wished to label mitotic cells with ^{32}P, complete medium A containing 0.05 μg/mL colcemid was used to wash and overlayer the adherent cells after the initial hard shake. Under these conditions, cells entered but did not leave the mitotic state unless the colcemid-containing medium was subsequently removed.

After selective detachment in mitosis, the rate of DNA replication in synchronized cultures was assayed at hourly intervals by pulse labeling 2-mL aliquots of the culture with [2-^{14}C]thymidine as previously described (Marks et al., 1973). In HeLa S-3 cells synchronized by either of these selective detachment methods, G_1 lasted for about 5–6 h after mitosis, followed by the S phase which lasted from about 5–6 to about 13–15 h after mitosis. Only if the rate of thymidine incorporation during G_1 was <10% of the peak rate in the S phase was the H1 phosphorylation status studied. On this basis, we estimate that approximately 90% of the cells in the G_1 cell cultures were in G_1 and that a similar fraction of the cells in the mid-S-phase cultures were in the S phase.

^{32}P-Labeling Experiments. Carrier-free [^{32}P]orthophosphoric acid was diluted with phosphate-free medium A, sterilized by filtration through a 0.22-μm Millipore filter, and then added to suspension cultures at10–100 μCi/mL as indicated in the figure legends. The cells to be labeled were harvested by centrifugation at 600*g* for 5 min at 37 °C, then were resuspended in medium C, at the concentrations indicated in the figure legends, and were incubated with ^{32}P for 3 h at 37 °C.

Cell Fractionation and Histone 1 Isolation. Histone 1 isolation from HeLa cells was carried out at 3 °C. Cell pellets were harvested by centrifugation at 600*g* for 5 min, washed 3 times with 50 mL of spinner salts, and lysed by washing 3 times with 20–50 mL of 80 mM NaCl, 20 mM EDTA, and 1% Triton X-100 containing 0.05 M sodium bisulfite (pH 7.2). The bisulfite was used to inhibit phosphatase activities (Gurley et al., 1975). The nuclei were collected by centrifugation at 1400*g* for 10 min. The nuclear pellet was washed twice with 0.14 M NaCl containing 0.05 M sodium bisulfite to remove saline-soluble nuclear proteins. Histones were obtained by three extractions of the nuclei in 0.4 N H_2SO_4, precipitated with 4 volumes of cold ethanol, washed once with 80% ethanol, and stored overnight at −70 °C. The histone pellets were suspended in water (approximately 4 mg/mL) and centrifuged to remove undissolved material. The supernatant was mixed with an equal volume of 10% perchloric acid (PCA) to precipitate nucleosomal core histones (H2A, H2B, H3, and H4), and after being chilled for 10 min at 3 °C, this suspension was centrifuged at 10000*g* for 10 min at 3 °C and washed once with 5% PCA. The two PCA supernatants were combined, and H1 was precipitated by the addition of 0.2 volume of cold 120% trichloroacetic acid (Cl_3CCOOH). After 30 min at 3 °C, the suspension was centrigfuged for 30 min at 10000*g*. The pellet was washed once with acetone containing 0.3 HCl and 3 times with acetone and dried with a stream of anhydrous nitrogen.

Ion-Exchange Column Chromatography. H1 histones were purified and resolved into component subfractions by chromatography on Amberlite CG-50 resin (200–400 mesh) by the method of Kinkade & Cole (1966a). The columns (0.5-cm diameter and 40–100 cm long) were washed prior to each run with 20 mL of 20% guanidinium chloride in 0.1 M sodium phosphate buffer, pH 6.8, and then equilibrated with 6% guanidinium chloride in the same buffer. The H1 samples were dissolved in 0.5 mL of water, and 5–10% of each sample was reserved for analysis by gel electrophoresis. The rest was made 6% with respect to GdmCl in 0.1 M phosphate buffer, pH 6.8, and loaded on the column. After the column was washed for 2–12 h with 6% GdmCl dissolved in 0.1 M phosphate buffer, a 150- or 200-mL 7–13% GdmCl gradient in phosphate buffer was run through the columns. Multiple columns for H1 preparations from different stages of the cell cycle (G_1, S, and M) were usually run at the same time from a single gradient source and pumped from the bottom of each column with a Desage multichannel peristaltic pump. Usually, 1-mL fractions were collected every 48 min. The absorbance of the fractions was measured at 218 nm, and ^{32}P radioactivity was measured by Cherenkov counting (^3H channel, gain at 220) with a Beckman LS-230 liquid scintillation counter. The H1A and H1B subfractions were pooled separately and recovered by precipitation with 7 volumes of acetone containing 0.3% HCl.

Polyacrylamide Gel Electrophoresis. Acrylamide gel electrophoresis in long, small-diameter gels to resolve phosphorylation levels of H1 was performed at room temperature by a modification of the method of Panyim & Chalkey (1969). The gels contained 14% acrylamide, 0.08% methylenebis-(acrylamide), 2.5 M urea, and 5% acetic acid. Gels 1.8 mm in diameter and 270 mm long were cast in 300-mm-long Microtrol tubes obtained from Drummond Scientific Co., Broomall, PA, and run in an electrophoresis apparatus designed by Alfageme & Cohen (C. R. Alfageme and L. H. Cohen, unpublished experiments). After preelectrophoresis overnight at 200 V, the gels were loaded with 20 μL of 1 M cysteamine and run at 150 V to scavenge free radicals (Alfageme et al., 1974). After 1 h of scavenging, any remaining cysteamine solution was removed. The protein samples (0.5–5.0 μg/gel) were loaded in a solution containing 2 M urea, 0.1 M mercaptoacetic acid, 2% mercaptoethanol, and 0.01% pyronine Y tracking dye. Constant voltage (500 V) was then applied for 16 h. After extrusion from the glass tubes with a long stainless-steel rod, the gels were stained for 3 h with 0.1% Coomassie blue dissolved in 5 parts of methanol (MeOH), 5 parts of water, and 1 part of acetic acid (HOAc) and destained with 5 parts of MeOH, 16 parts of H_2O, and 1 part of HOAc. The gels were scanned at 550 nm with a Gilford spectrophotometer with a 0.5 × 1.2 mm slit. For the autoradiographic analysis, gels were dried on Whatman 3 MM paper in a vacuum chamber and covered with two sheets of X-ray film (Cronex 4, Du Pont). After sufficient exposure, the autoradiographs were developed by using a Kodak RP-OMAT processor. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was carried out on 0.4 cm in diameter, 13-cm-long 12% acrylamide gels for 18 h at 40 V as previously described (Alfageme et al., 1974).

Results

Resolution and Properties of H1A and H1B. The chromatographic properties of ^{32}P-labeled H1 histones extracted from HeLa cells incubated with [^{32}P]orthophosphate at three stages of the cell cycle, G_1, mid-S, and mitosis, are shown in Figure 1. In each case, two well-resolved peaks were obtained, designated A and B. Over 90% of the H1 was found in these two peaks, the remainder being eluted as three very minor components subsequent to peak B.

It can be seen in the upper panel of Figure 1 that peak B is heterogeneous. However, phosphorylated proteins are eluted

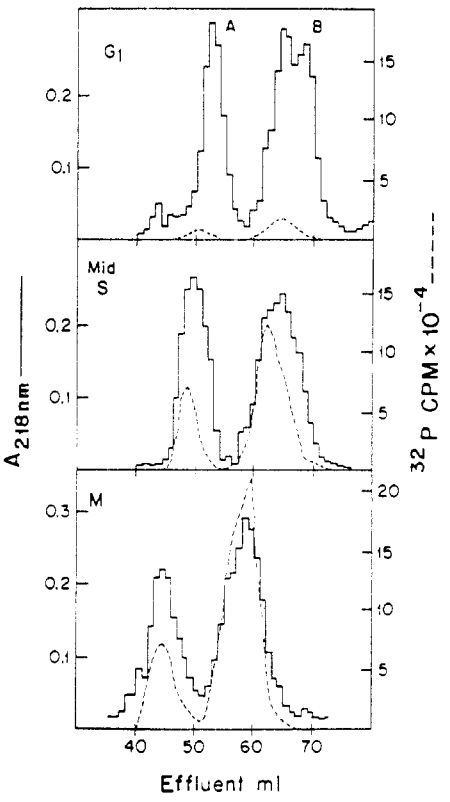

FIGURE 1: Chromatographic resolution of ^{32}P-labeled H1 components extracted from HeLa S-3 cells at various stages of the cell cycle. Upper panel: At 2 h after selective detachment in mitosis, G_1 cells were harvested and incubated at 1.6×10^6 cells/mL in medium C containing 100 μCi/mL [^{32}P]orthophosphate for 3 h at 37 °C as described under Experimental Procedures. Middle panel: At 8 h after selective detachment in mitosis, S-phase cells were incubated as described above with 100 μCi/mL [^{32}P]orthophosphate. Lower panel: Mitotic cells were collected by selective detachment in medium A containing 0.05 μg/mL colcemid as described under Experimental Procedures and then were incubated as described above with 0.05 μg/mL colcemid and 100 μCi/mL [^{32}P]orthophosphate. After incubation, the ^{32}P-labeled H1 fractions were prepared and chromatographed on Amberlite CG-50 columns as described under Experimental Procedures. The results in these panels represent the A_{218} and ^{32}P content of H1 components recovered from 2.5×10^8 G_1, S-, and M-phase cells.

earlier than their unphosphorylated counterparts in this chromatographic system (Gurley et al., 1975). We have concluded that phosphorylation is the principal source of the chromatographic heterogeneity of peak B because of several facts: (1) Only the more rapidly eluted of the B components is labeled with ^{32}P. (2) During the S phase (Figure 1, middle panel), the second component of the H1B peak is diminished in amount compared with H1B from G_1 cells, and there is a large increase in ^{32}P incorporation, indicating conversion of the second component into the first by phosphorylation. (3) In long acid–urea gels, the proteins in the nonradioactive portions of the chromatographic H1A and H1B peaks are electrophoretically homogeneous (data not shown) whereas the ^{32}P-containing portions of these peaks contain components with mobilities expected for phosphorylated forms of H1A and H1B (see below). (4) $NaDodSO_4$ gel electrophoresis of successive fractions from a chromatogram of HeLa cell H1 shows the A and B components to be monodisperse (Figure 2). (5) The amino acid compositions of the two B components are indistinguishable from each other but quite distinct from that of the A component (Table I).

Thus, the A peak and the B peak each contains either a single protein or a distinctive group of closely related proteins. During $NaDodSO_4$ electrophoresis H1A and H1B have the mobilities of two H1 bands previously described for other

Table I: Amino Acid Composition of HeLa H1 Components[a]

amino acid	H1A (residues/100 residues)	H1B (residues/100 residues) peak 1	H1B (residues/100 residues) peak 2
Asp	2.1	2.2	2.0
Thr	5.0	4.5	4.3
Ser	6.0	5.6	6.3
Glu	3.7	3.6	3.4
Pro	9.7	9.1	9.2
Gly	7.7	6.7	6.7
Ala	*22.4*	*27.0*	*27.2*
Val	*7.0*	*3.8*	*3.5*
Ile	0.9	0.9	0.9
Met	0	0	0
Leu	4.4	4.9	4.9
Tyr	0.3	0.5	0.5
Phe	0.6	0.6	0.6
His	0.1	0.1	0.1
Lys	28.4	28.8	28.6
Arg	1.7	1.7	1.8

[a] Samples (50 μg) of chromatographically purified H1A and H1B fractions from log-phase HeLa S-3 cells were sealed in glass tubes which were previously flushed with nitrogen, and the samples were hydrolyzed in 6 N HCl for 30 h at 110 °C. The hydrolyzed samples were lyophilized and dissolved in 50 μL of sodium citrate buffer, pH 2.2. Portions (5 and 20 μL) of the samples were analyzed by using a Durrum amino acid analyzer. No corrections were made for serine loss during hydrolysis. H1B peaks 1 and 2 correspond in chromatographic position to the two H1B peaks seen in the top panel in Figure 1. The principal differences between H1A and H1B are italicized.

Table II: Incorporation of ^{32}P into H1A and H1B[a]

	G_1	S	M
H1A			
cpm $\times 10^{-3}$	38	251	374
protein (μg)	203	213	190
cpm $\times 10^{-3}/\mu$g	*0.2*	*1.2*	*2.0*
H1B			
cpm $\times 10^{-3}$	109	720	1363
protein (μg)	333	294	299
cpm $\times 10^{-3}/\mu$g	*0.3*	*2.4*	*4.6*
H1B sp act./H1A sp act.	1.5	2.0	2.3

[a] Incorporation of ^{32}P and amount of protein found in H1A and H1B extracted from 2.5×10^8 G_1, S-phase, and mitotic HeLa S-3 cells labeled with ^{32}P at a concentration of 100 μCi/mL as described in the legend of Figure 1.

vertebrates (Panyim & Chalkley, 1971; Weintraub et al., 1975), corresponding to molecular weights of ~21 000 and ~22 000. The most distinctive compositional differences between H1A and H1B are in alanine and valine content (Table I); H1A is substantially more hydrophobic than H1B, containing ~7 more valyl residues.

It is evident in Figure 1 and Table II that HeLa cells contain approximately 1.5 times more H1B than H1A, indicating that these two H1 types cannot be uniformly paired with each other throughout the HeLa genome.

H1 Phosphorylation Levels during the HeLa Cell Cycle. The data of Figure 1 indicate a progressive increase in phosphorylation of H1A and H1B during the HeLa cell cycle, judged from both the rate of ^{32}P incorporation and the chromatographic positions of the protein peaks relative to the radioactive peaks. In G_1 cells, a small proportion of H1A and a major proportion of H1B appear to contain phosphate groups; the low rate of ^{32}P incorporation in such cells suggests that most of these phosphate groups have persisted from the preceding cell cycle. In the lower two panels of Figure 1, it can be seen that the S phase and mitosis are periods of much more active ^{32}P incorporation than G_1. In S-phase cells, a

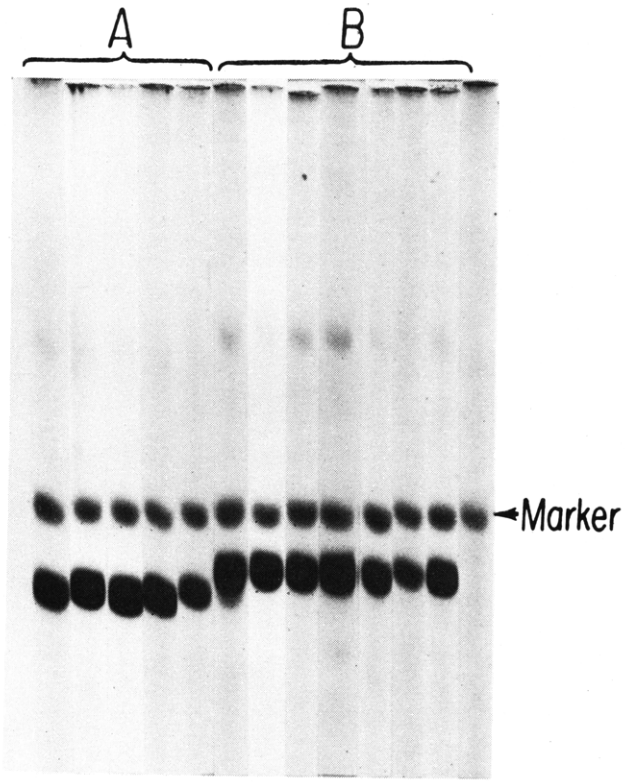

FIGURE 2: $NaDodSO_4$ electrophoresis of chromatographic components of HeLa H1. Approximately 4.5 mg of H1 from 3×10^8 log-phase HeLa S-3 cells was resolved on an Amberlite CG-50 column as described under Experimental Procedures. Fractions throughout the A and B regions of the chromatogram were precipitated for 30–60 min with 7 volumes of acetone containing 0.1 volume of 1 N HCl at room temperature. After centrifugation at 8000*g* for 30 min at 22 °C, the precipitates were resuspended in $NaDodSO_4$ sample buffer, and 10-μg samples were subjected to electrophoresis as described under Experimental Procedures. After 2 h of electrophoresis, a sample of a chromatographic fraction containing 2 μg of unphosphorylated H1B was applied to each gel as a marker, and electrophoresis was continued for 16 h. The gels were stained with Coomassie blue as described under Experimental Procedures.

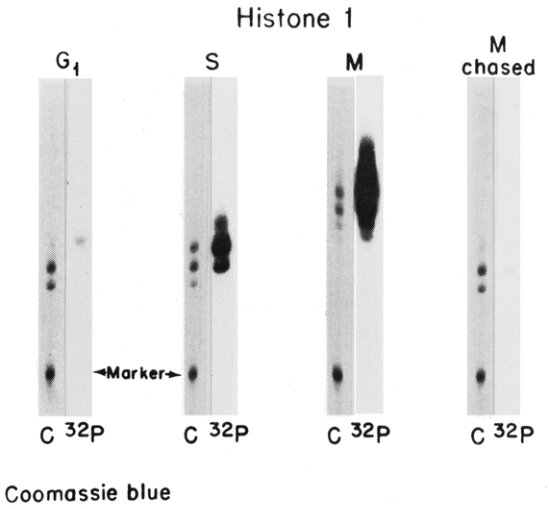

FIGURE 3. Acid–urea gel electrophoresis and autoradiography of ^{32}P-labeled total H1 extracted from HeLa H-3 cells at various stages of the cell cycle. HeLa S-3 cells (3×10^8) were synchronized by selective detachment in mitosis as described under Experimental Procedures and incubated in 850 mL of complete medium A at a concentration of 3.5×10^5 cells/mL at 37 °C. At 3 h after selective detachment, 3×10^7 G_1 cells were harvested and incubated at 1.6×10^6 cells/mL in medium C containing 100 μCi/mL [^{32}P]orthophosphate for 3 h at 37 °C. At 8 h after selective detachment, 3×10^7 S-phase cells were similarly incubated with ^{32}P. After labeling, H1 fractions were prepared from these G_1 and S-phase cells as described under Experimental Procedures. During the same experiment, 2.1×10^8 mitotic HeLa cells were collected by selective detachment in 520 mL of Medium A containing 0.05 μg/mL colcemid, harvested at 600*g* at 37 °C, and incubated with 0.05 μg/mL colcemid and 100 μCi/mL [^{32}P]orthophosphate as described above. After incubation, 1.7×10^8 mitotic ^{32}P-labeled cells were harvested at 3 °C, and the H1 fraction was immediately prepared. The remaining 4×10^7 cells were also harvested, but at 37 °C, resuspended in complete medium A, and then incubated in the absence of ^{32}P at 37 °C. At 3 h after resuspension, 2×10^7 cells were harvested from this culture by centrifugation at 3 °C, and the H1 fractions were prepared. All of these ^{32}P-labeled H1 fractions (0.5 μg of H1 from cells labeled during G_1 or the S phase, 0.75 μg of H1 from cells labeled during colcemid blockade in mitosis, and 1.5 μg of H1 from cells which had been ^{32}P labeled during mitosis and chased for 3 h into G_1) were electrophoresed on long acid–urea gels as described under Experimental Procedures. The gels were stained, photographed, and autoradiographed as described under Experimental Procedures. The marker protein, 0.2 μg of sea urchin (*Strongylocentrotus purpuratus* blastula) H1, was mixed with the HeLa H1 fractions prior to electrophoresis.

majority of the H1 molecules are phosphorylated; in mitotic cells, almost all of them are phosphorylated. Moreover, the early elution positions of the mitotic H1A and H1B suggest that the molecules contain more phosphate groups during mitosis than they do in the S phase.

Figure 1 also reveals that differences between H1A and H1B phosphorylation are striking at all three phases of the cell cycle examined. In H1 from cells labeled with ^{32}P during G_1, a much larger fraction of H1B than H1A is eluted together with the ^{32}P peak. At all stages, moreover, the specific radioactivity of H1B was over twice that of H1A (Table II).

An analysis of the successive increases in H1 phosphorylation levels during the HeLa cell cycle from G_1 through mitosis was carried out by electrophoresis of the total H1 fraction in 27-cm-long acid–urea gels. In such gels, the decrease in H1 mobility produced by a single phosphate group is discernible (Balhorn et al., 1972b). The gels, stained for protein, are shown together with their autoradiograms in Figure 3. In S-phase cells, much of the H1 has a lower mobility than that found in G_1 cells. In mitotic cells, there is a large further decrease in mobility of most of the H1. The ^{32}P radioactivity relative to the protein content in the gel patterns increases progressively with the decline in mobility, consistent with the view that the mobility differences are manifestations of phosphorylation differences. It can also be seen (Figure 3, M chased) that when colcemid-blocked cells were allowed to proceed into G_1 subsequent to removal of the colcemid, the H1 regained essentially its original spectrum of higher mobilities, and 95% of the ^{32}P radioactivity acquired during mitosis was lost. These results indicate that H1 gains phosphate groups in S-phase cells and is raised to considerably higher phosphorylation levels in mitotic cells. The phosphate groups added during mitosis are radidly removed following mitosis, in accord with earlier observations (Marks et al., 1973; Gurley et al., 1974; Balhorn et al., 1975).

The electrophoretic procedure was applied to chromatographically resolved H1A and H1B from cells at various stages of the cell cycle, with the results shown in Figures 4 and 5. In the cases of the G_1 and S-phase cells (Figure 4), evenly spaced bands of H1A and H1B are obtained. Three facts suggest that each H1 band differs from its neighboring band by a single phosphate group: (1) the fastest band (marked 0) is not labeled with ^{32}P at any stage of the cycle (or in nonsynchronized log-phase cells, data not shown), indicating that the protein in this band is unphosphorylated; (2) the radioactivity relative to the amount of protein in each band increases with decreasing mobility, consistent with a stepped increase in the number of phosphate groups per molecule; (3)

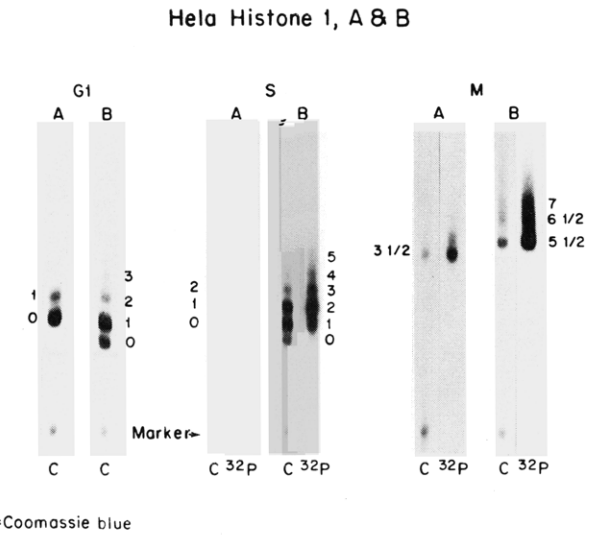

FIGURE 4: Acid–urea gel electrophoresis and autoradiography H1A and H1B from G_1, S-phase, and mitotic cells. H1A and H1B from unlabeled G_1 and ^{32}P-labeled S-phase and mitotic HeLa S-3 cells were resolved by chromatography on 40 × 0.6 cm Amberlite GC-50 columns as described under Experimental Procedures. Optimal amounts for electrophoretic resolution, 0.50 μg of H1A and 0.25 μg of H1B from G_1 cells, 2 μg of H1A or H1B from S-phase cells, and approximately 0.3 μg of H1A and 0.5 μg of H1B from mitotic cells, were resolved by electrophoresis on 27-cm-long acid–urea gels for 16 h at 500 V as described under Experimental Procedures. The marker protein, 0.2 μg of sea urchin (*S. purpuratus* blastula) H1, was mixed with the HeLa H1 fractions prior to electrophoresis. The gels were stained, photographed, and autoradiographed as described under Experimental Procedures. For preparation of H1 from G_1 cells, 4.7×10^8 cells were synchronized by selective detachment in mitosis and incubated at 37 °C for 2 h as described under Experimental Procedures. For preparation of the ^{32}P-labeled S-phase cells, a large culture of cells was synchronized by two cycles of 1 mM hydroxyurea blockade as previously described (Stephens et al., 1977). At 6 h after the reversal of the second hydroxyurea block, 8.7×10^8 S-phase cells were harvested and incubated at 3.9×10^6 cells/mL in medium C with 33 μCi/mL [^{32}P]orthophosphate at 37 °C for 3 h. ^{32}P-Labeled late S-phase cells synchronized by mitotic selective detachment gave similar results (data not shown). For preparation of ^{32}P-labeled mitotic cells, 4×10^8 cells were synchronized by selective detachment in mitosis in complete medium A containing 0.05 μg/mL colcemid as described under Experimental Procedures. The cells were harvested by centrifugation at 37 °C and incubated at 2.7×10^6 cells/mL in medium C containing 0.05 μg/mL colcemid and 33 μCi/mL [^{32}P]orthophosphate for 3 h at 37 °C.

the interband distance is quite uniform, and its value (1% of the migration of the fastest component) is consistent with a single phosphate difference between adjacent bands.[2]

It is also evident that unphosphorylated H1A migrates more slowly than unphosphorylated H1B, with its mobility essentially equal to that of monophosphorylated H1B. Thus, prior resolution of these components of the HeLa H1 fraction is essential for determining even the average levels of phosphorylation of H1.

In the case of H1 from mitotic cells, the displacements of the bands from the position of the parent molecule are not exact multiples of the spacing found in S-phase preparations. No such deviation was observed in the H1 of CHO cells (Gurley et al., 1975). The anomalous mobilities of mitotic H1 in HeLa cells were confirmed by electrophoresis of mixtures of H1B from S-phase and mitotic cells (data not shown).

[2] A single charge alteration would represent about 1.5% of the total cationic charge (~65+ at the pH of the gel). A phosphate group would be expected to bear slightly less than one anionic charge, assuming the pK_a is ~2. Thus, the 1% mobility difference between bands is best reconciled with a difference of one phosphate group.

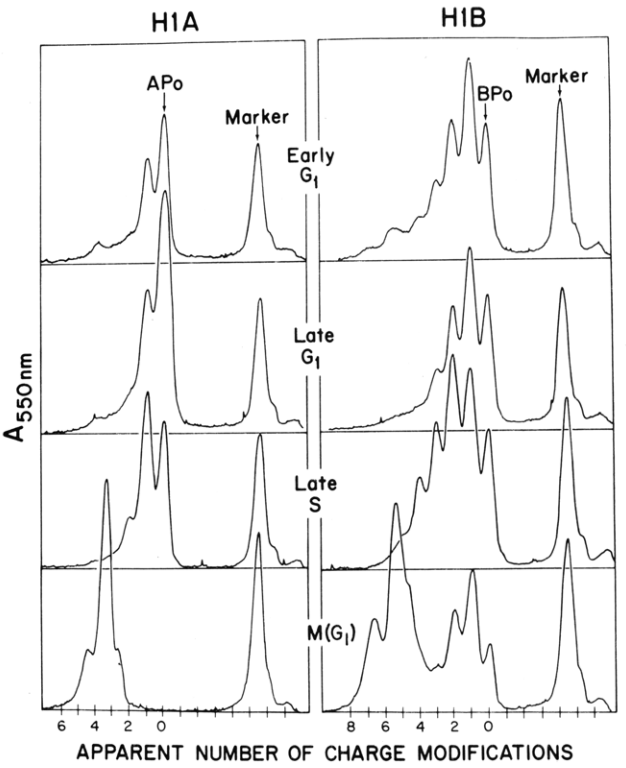

FIGURE 5: Densitometric scans of stained acid–urea gel electrophoretograms of chromatographically resolved H1A and H1B from G_1, S-phase, and mitotic cells. The cells were synchronized by selective detachment in mitosis without pretreatment with thymidine as described under Experimental Procedures. H1 fractions were resolved by electrophoresis at 600 V for 13.3 h on 27-cm-long polyacrylamide gels. The gels, stained with Coomassie blue, were scanned at 550 nm as described under Experimental Procedures. The 0 positions in these tracings correspond to the positions that are not labeled when cells are give to ^{32}P (see Figures 3 and 4). In this experiment, 2.9×10^8 cells were collected from 90 Blake bottles containing monolayers of log-phase HeLa S-3 cells by using 1000 mL of medium A as described under Experimental Procedures (culture 1). After this selection procedure, colcemid was added to each Blake bottle culture to a concentration of 0.05 μg/mL, and the bottles were incubated at 37 °C for 2 h to accumulate mitotic cells, which were then harvested as described under the Experimental Procedures (culture 2). Samples of 7×10^7 cells were harvested from culture 1 at 2 h (early G_1), 4 h (late G_1), and 12 h (mid-S-phase) after mitosis and processed as described under Experimental Procedures. In this experiment, the mitotic cells in culture 2 were contaminated with a small number of G_1 cells because the cell cultures were not given a hard shake prior to detachment of the motitic population. Because of the omission of the hard shake, some very early G_1 cells, past the colcemid blocking point but not yet firmly adherent to the glass bottles, were inadvertently shaken off with the mitotic population which was blocked with colcemid. As a result, substantial amounts of H1A and H1B at low phosphorylation levels were present, all of which were pooled in the H1B fraction because of the chromatographic similarity of highly phosphorylated H1B to unphosphorylated and monophosphorylated H1 (cf. Figure 1).

Either there is one or more nonintegral charge modifications of H1 at this stage in HeLa cells or there are phosphorylations that affect mobility through effects on protein conformation as well as on ionic charge. Therefore, while it is evident from the autoradiographs in Figure 4 that H1A and H1B are highly phosphorylated in mitotic cells, the positions of the bands provide only an approximate estimate of the number of phosphate groups per mole, and this is indicated in Figure 4 by nonintegral values.

For measurement of the amounts of different phosphorylated H1A and H1B species present in G_1, the S phase, and mitosis, the experiment shown in Figure 5 was performed. In this experiment, cells were synchronized by selective detachment

Table III: Levels of H1A and H1B Phosphorylation during the HeLa S-3 Cell Cycle[a]

apparent P/mol	H1A (% of total)			H1B (% of total)		
	M	G_1	S	M	G_1	S
0	[b]	57	34	[b]	22	14
1	[b]	27	48	[b]	35	25
2	[b] (11)[b]	8	13	[b] [b]	23	28
3	(69)[b]	4	3	[b]	11	17
4	(19)[b]	3	2	(24)[b]	5	11
5	(~1)[b]			(47)[b]	3	4
6				(26)[b]	1	1
7				(~2)[b]		

[a] Apparent levels of HeLa H1A and H1B phosphorylation during G_1, the S phase, and mitosis. G_1 and S-phase cells were harvested at 4 and 12 h, respectively, after selective detachment in mitosis as described in the legend of Figure 5. After the gels shown in Figure 5 were scanned, the areas under the various peaks in these gels were integrated and divided by the total area of the H1A or H1B subfraction. Thus, the various levels of phosphorylation are indicated in this table as the percent of total H1A or H1B. Because of difficulties in accurately quantitating the exact percentage of the various highly phosphorylated mitotic H1 species, only relative quantities of these phosphorylated species are indicated. [b] In calculations of the percentages of H1A and H1B at each phosphorylation level in M-phase cells, the lower levels of phosphorylation were ignored because they represent principally H1 molecules from G_1 cells in the mitotic cell preparation (see legend of Figure 5). The percentages of the various phosphorylated forms in mitotic cells are therefore shown in parentheses. In addition, they are placed between integral phosphorylation levels because the electrophoretic mobilities of these forms do not correspond precisely to the mobilities expected from integral numbers of charge modifications.

in mitosis *without* prior synchronization with thymidine, to avoid the possibility of drug-induced artifacts. After chromatography and electrophoresis of the H1 fraction, the gels were scanned (Figure 5), and the amounts of each of the H1A and H1B parent and phosphorylated species were determined from these profiles. Results of these determinations are summarized in Table III. The data show that during G_1 and the S phase, a large fraction of H1B molecules contain more phosphate groups than do most H1A molecules. By the end of the S phase, most molecules of both H1 types contain at least one more phosphate group over the numbers found during G_1. During M, the levels increase again,[3] in the case of most H1A molecules by two to four phosphate groups and in the case of most H1B molecules by three to five groups. At their highest phosphorylation levels during mitosis, most H1B molecules contain, on the average, two more phosphate groups than H1A. Ancillary experiments (data not shown) revealed that the majority of ^{32}P associated with HeLa H1 histones was converted into inorganic orthophosphate by treatment with alkaline phosphatase and therefore represents monoesterified orthophosphate. Less than 10% of the ^{32}P, the nature of which is unknown, was resistant to alkaline phosphatase under the optimal conditions described by Mellgren et al. (1977). None of the ^{32}P was released as AMP- or iso-ADP-ribose by treatment with snake venom diesterase, showing that our H1 histone preparations contained no poly(ADP)-ribose. However, since poly(ADP)-ribose is usually degraded by a glycohydrolase during the isolation of nuclei (Kidwell & Mage, 1976), our data do not reveal whether ADP ribosylation occurs in addition to the modifications that we have observed.

[3] Because the mitotic cell population used in this particular experiment contained some G_1 cells and because unphosphorylated H1A is eluted together with the most highly phosphorylated species of M-phase H1B, the M(G_1)-phase H1B shown in the lower right-hand panel of Figure 5 contains some unphosphorylated H1A and some H1B at low levels of phosphorylation.

Discussion

There is evidence that H1 phosphorylation in mammalian cells is a complex of several phenomena that are intimately related to modulations of chromosome structure (Dolby et al., 1979) and reflect processes relating to at least three kinds of chromosomal function: gene transcription (Langan & Hohmann, 1975), DNA replication (Balhorn et al., 1972b; Marks et al., 1973; Gurley et al., 1975), and mitosis (Lake et al., 1972; Marks et al., 1973; Gurley et al., 1975; Bradbury et al., 1973, 1974). In this discussion, we shall consider how these chromosomal functions might be related to the observed phosphorylation levels of the two principal H1 subtypes in HeLa S-3 cells at various stages of the cell cycle.

Levels of H1A and H1B Phosphorylation during Mitosis. That the highest levels of chromatin compaction during mitosis are correlated with the highest levels of H1 phosphorylation has been indicated previously (Marks et al., 1973; Ajiro et al., 1976; Hohmann et al., 1976) and most clearly demonstrated in studies with CHO cells by Gurley et al. (1978). The data in the present paper show that in mitotic HeLa cells both H1A and H1B contain more phosphate groups per molecule and incorporate more ^{32}P than in other periods of the cell cycle. A majority of H1 molecules appear to have acquired three to five phosphate groups in addition to those present in the late S phase, and few, if any, are unphosphorylated.

The present data show also that H1B is considerably more phosphorylated that H1A, as judged both by rate of ^{32}P incorporation and by electrophoretic mobility. Table III shows that H1B, when maximally phosphorylated, contains two more phosphate groups than does H1A. Thus, H1B is phosphorylated at more sites in the molecule than is H1A, or more of its sites are occupied simultaneously on any one molecule, or both. That H1B does indeed have more phosphorylation sites than does H1A is shown in the accompanying paper (Ajiro et al., 1981).

Levels of H1A and H1B Phosphorylation during G_1. In late G_1, H1 phosphorylation states are at their lowest levels. Nevertheless, a number of H1 molecules bear at least one phosphate group. As judged from the low rate of ^{32}P incorporation at this stage, most of these phosphate groups cannot be very short-lived, which suggests that their influence on chromatin at this time is more to maintain conformational states than to elicit changes. Approximately 43% of H1A and 78% of H1B molecules are phosphorylated (see Table III), most H1A molecules bearing zero to one phosphate groups and most H1B molecules bearing zero to three phosphates. To a small extent, these may include mitosis-related phosphate groups still to be removed, as well as the initial phases of replication-related phosphorylation, particularly since cell synchrony is not absolute. However, since over 95% of the ^{32}P incorporated into H1 in mitotic cells is lost in early G_1, and since little ^{32}P is incorporated during G_1 compared with the S phase or mitosis, it appears likely that most of the G_1 phosphorylation states, particularly in H_1B, were established in the preceding cell cycle or cycles, at the time of chromatin replication or in G_2, and serve functions not immediately related to mitosis or replication. Most of the phosphorylated H1 molecules in G_1 cells must be associated with transcriptionally inactive chromatin because transcription in higher eukaryotes is generally restricted to a small fraction of the DNA sequences. We suggest, therefore, that phosphate groups at some sites of the H1 molecule might be serving to maintain

much of the chromatin in conformations incompatible with transcription. This interpretation is in accord with evidence that in *Drosophila* there is at least one H1 phosphorylation related to heterochromatin maintenance (Blumenfeld et al., 1978; Blumenfeld, 1979; Billings et al., 1979), and with the observation that phosphorylation of H1 at certain sites increases its ability to cross-link DNA in vitro (Matthews & Bradbury, 1978).

Levels of H1A and H1B Phosphorylation during the S Phase. S-phase cells incorporated almost 7 times more ^{32}P into H1A and H1B than did G_1 cells, a ratio that is probably an underestimate because some of the labeled H1 in the G_1 samples was undoubtedly from cells that had entered the S phase toward the end of the labeling period. H1B is more phosphorylated than H1A during the S phase, as indicated both by its considerably higher ^{32}P specific activity and by the electrophoretic analysis of the numbers of phosphate groups per molecule.

A number of facts indicate that the ^{32}P uptake by H1 during the S phase represents a variety of kinds of phosphorylation, some involving all or most of the H1 molecules during the course of the S phase and others being confined to only a fraction of the H1 molecules. The H1 molecules of S-phase cells were found to be in a variety of phosphorylation states, ranging from zero to five phosphate groups per molecule. This multiplicity cannot have been due to contamination by mitotic cells, since no H1 at mitotic phosphorylation levels was detectable, although contamination with G_2 cells could conceivably have accounted for the amount of H1B having five or more phosphate groups per molecule and the amount of H1A having three or more phosphate groups per molecule ($\sim$5% in each case). It is likely, moreover, that stepped phosphorylation levels from zero to four phosphate groups per molecule exist within each cell throughout most of the S phase, since (a) these phosphorylation levels were found in both early and late S-phase cells (data not shown) and (b) a study of ^{32}P incorporation into various sites within these molecules (Ajiro et al., 1981) has shown that most of the phosphorylatable sites in H1B undergo phosphorylation in early S-phase cells as well as in late S-phase cells, showing that there is no unique temporal sequence in which the sites are phosphorylated during the course of the S phase. The fact that the higher phosphorylation states are represented by moderate numbers of molecules, and some states almost exclusively by H1B molecules, shows that in S-phase cells some of the phosphorylations are confined to H1 molecules in a limited proportion of the chromatin. Since H1 plays a role in the packing of supranucleosomal structures that must be unpacked in preparation for replication and repacked afterward, we suggest that these phosphorylations of H1 are involved in packing or unpacking such higher order structures and that H1B may play a greater role than H1A in the production of such structures.

On the other hand, it would appear that at least one phosphorylation occurs rather generally throughout the chromatin during the course of the S phase, involving H1 molecules of both subtypes. Thus, examination of Figure 5 reveals that there is a general increase in the amount of phosphate in both H1 subtypes by the late S phase over that seen in G1 cells, by approximately one phosphate group per molecule. At this stage, only 20% of the H1 molecules lack phosphate groups, as calculated from the data in Table III. Furthermore, the actual number of H1 molecules acquiring phosphate groups during the S phase must be substantially greater than that apparent at any given moment, since there is evidence that during the S phase preexisting H1 molecules undergo very active phosphorylation at an intramolecular site at which the phosphate group has a lifetime considerably shorter than the S phase (Ajiro et al., 1981). It is likely, therefore, that almost every H1 molecule in chromatin, regardless of its subtype, is elevated in phosphorylation level during some period of the S phase. Since the only process obviously affecting all of the chromatin during the S phase is replication, this general increase of about one phosphate group per H1 molecule may be related to replication throughout all regions of chromatin, whether packed in higher order supranucleosomal structures during the S phase or not. In view of recent evidence that H1, in addition to maintaining supranucleosomal packing, plays a role in the superhelical coiling of DNA in the individual nucleosome (Boulikas et al., 1980; McCleary & Fasman, 1980), such a phosphorylation might be involved in alterations in nucleosomal DNA conformation, which obviously must occur before and after replication.

The notion that during the S phase some kinds of H1 phosphorylation occur throughout the chromatin and affect both H1 subtypes, while others are subtype specific and limited to selected regions of the chromatin, is supported by studies of the intramolecular phosphorylation sites (Ajiro et al., 1981), showing that in the S phase H1A and H1B are both subject to very active phosphorylations at two intramolecular sites and that H1B undergoes in addition several minor phosphorylations. Furthermore, all of these minor phosphorylations become major phosphorylations during mitosis, supporting the notion that their roles in the S phase are principally in the production or maintenance of supranucleosomal packing.

Our data reveal also that, as in other mammalian cell lines (Jackson et al., 1976; Gurley et al., 1978), many of the new H1 molecules introduced into chromatin during replication become phosphorylated before the end of the S phase; otherwise, there would not have been the relative decrease in unphosphorylated H1 seen in Table III. In the case of H1B, even the monophosphorylated form decreases in relative amount during the S phase, indicating that some newly acquired H1B molecules receive more than one phosphate group. It seems doubtful that new H1 molecules need these phosphate groups simply in order to be deposited in chromatin, since there is a known case, the sea urchin gastrula, in which the new H1 molecules deposited in the nucleus during replication are not O-phosphorylated at all (Cohen et al., 1979). In that particular case, there is evidence that cells receiving these molecules do not enter another cell cycle for at least several days (unpublished experiments). In cultured mammalian cells, then, the observed phosphorylations of newly deposited H1 molecules may establish conditions required for the next cell cycle to occur. For example, some phosphorylation states existing in particular genomic regions prior to replication may have to be reproduced in replicated chromatin, and then retained through mitosis into the next cell cycle. This idea fits well with the evidence described above, indicating that a considerable number of H1 molecules in G_1 cells have phosphate groups that they acquired prior to the preceding mitosis. It is also in accord with evidence that in *Physarum* some of the phosphate incorporated into H1 prior to mitotic chromosome condensation is retained into the subsequent cell cycle (Fischer & Laemmli, 1980).

Taken together with results of studies of the intramolecular sites phosphorylated during the S phase and mitosis, reported in the accompanying paper (Ajiro et al., 1981), our results suggest that of the many phosphorylations that H1 molecules undergo during the S phase, one or two occur on all H1

molecules and are related to replication throughout the chromatin while others are subtype specific and occur only in some regions of the chromatin, where, depending on their intramolecular sites, they may serve to produce, maintain, or unfold higher order conformations in the chromatin. If H1 subtypes are arranged nonrandomly in the chromatin, such subtype-specific phosphorylations could furnish a means for fine control over chromosome organization as required for gene regulation, orderly chromosome replication, and orderly chromosome condensation at mitosis.

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References

Ajiro, K., Borun, T. W., & Cohen, L. H. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 581.

Ajiro, K., Borun, T. W., & Cohen, L. H. (1976) *Fed. Proc., Fed Am. Soc. Exp. Biol. 35*, 1623.

Ajiro, K., Borun, T. W., Shulman, S. D., McFadden, G. M., & Cohen, L. H. (1981) *Biochemistry*, following paper in this issue.

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

Balhorn, R., Balhorn, M., Morris, H. P., & Chalkley, R. (1972a) *Cancer Res. 32*, 1775–1784.

Balhorn, R., Chalkley, R., & Granner, D. (1972b) *Biochemistry 11*, 1094–1098.

Balhorn, R., Jackson, V., Granner, D., & Chalkley, R. (1975) *Biochemistry 14*, 2504–2511.

Billings, P. C., Orf, J. W., Palmer, D. K., Talmadge, D. A., Pan, C. G., & Blumenfeld, M. (1979) *Nucleic Acids Res. 6*, 2151–2164.

Blumenfeld, M. (1979) *Biochem. Genet. 17*, 163–166.

Blumenfeld, M., Orf, J. W., Sina, B. J., Kreber, R. A., Callahan, M. W., Mullins, J. I., & Snyder, L. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 866–870.

Borun, T. W., Gabrielli, F., Ajiro, K., Zweidler, A., & Baglioni, C. (1975) *Cell 4*, 59–67.

Boulikas, T., Wiseman, J. W., & Garrard, W. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 127–131.

Bradbury, E. M., Inglis, R. J., Matthews, H. R., & Sarner, N. (1973) *Eur. J. Biochem. 33*, 131–139.

Bradbury, E. M., Inglis, R. J., & Matthews H. R. (1974) *Nature (London) 247*, 257–261.

Cohen, L. H., McFadden, G., & Nardi, R. (1979) *J. Cell Biol. 83*, 171a.

Cohen, L. H., Mahowald, A. P., Chalkley, R., & Zweidler, A. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 588.

Cohen, L. H., Newrock, K. M., & Zweidler, A. (1975) *Science (Washington, D.C) 190*, 994–997.

Cole, R. D. (1975) *Mol. Biol. Mamm. Genet. Appar., Proc. Int. Symp. 1*, 93–103.

Cole, R. D., Lawson, G. M., & Hsiang, M. W. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 253–263.

Comings, D. E. (1972) *Adv. Hum. Genet. 3*, 237–431.

Dolby, T. W., Ajiro, K., Borun, T. W., Gilmour, R. S., Zweidler, A., Cohen, L. H., Miller, P., & Nicolini, C. (1979) *Biochemistry 18*, 1333–1344.

Fischer, S. G., & Laemmli, U. K. (1980) *Biochemistry 19*, 2240–2246.

Gaubatz, J., Hardison, R., Murphy, J., Eichner, M. E., & Chalkley, R. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 265–271.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1974) *J. Cell Biol. 60*, 356–364.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1975) *J. Biol. Chem. 250*, 3936–3944.

Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., & Tobey, R. A. (1978) *Eur. J. Biochem. 84*, 1–15.

Hill, R. J., Poccia, D. L., & Doty, P. (1971) *J. Mol. Biol. 61*, 445–462.

Hohmann, P. (1978) *Subcell. Biochem. 5*, 87–127.

Hohmann, P., & Cole, R. D. (1971) *J. Mol. Biol. 58*, 533–540.

Hohmann, P., Tobey, R. A., & Gurley, L. R. (1976) *J. Biol. Chem. 251*, 3685–3692.

Jackson, V., Shires, A., Tanphaichitr, N., & Chalkley, R. (1976) *J. Mol. Biol. 104*, 471–483.

Kidwell, W. R., & Mage, M. G. (1976) *Biochemistry 15*, 1213–1217.

Kinkade, J. M., Jr. (1969) *J. Biol. Chem. 244*, 3375–3386.

Kinkade, J. M., Jr., & Cole, R. D. (1966a) *J. Biol. Chem. 241*, 5790–5797.

Kinkade, J. M., Jr., & Cole, R. D. (1966b) *J. Biol. Chem. 241*, 5797–5805.

Lake, R. S., Goidl, J. A., & Salzman, N. P. (1972) *Exp. Cell Res. 73*, 113–121.

Langan, T. A. (1969a) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1276–1283.

Langan, T. A. (1969b) *J. Biol. Chem. 244*, 5763–5765.

Langan, T. A., & Hohmann, P. (1975) in *Chromosomal Proteins and Their Role in Regulation of Gene Expression* (Stein, G. S., & Kleinsmith, L. J., Eds.) pp 113–125, Academic Press, London and New York.

Langan, T. A., Rall, S. C., & Cole, R. D. (1971) *J. Biol. Chem. 246*, 1942–1948.

Leffak, I. M., Grainer, R., & Weintraub, H. (1977) *Cell 12*, 837–845.

Marks, D. B., Paik, W. K., & Borun, T. W. (1973) *J. Biol. Chem. 248*, 5660–5667.

Matthews, H. R., & Bradbury, E. M. (1978) *Exp. Cell Res. 111*, 343–351.

McCleary, A. R., & Fasman, G. D. (1980) *Arch. Biochem. Biophys. 201*, 603–614.

Mellgren, R. L., Slaughter, G. R., & Thomas, J. A. (1977) *J. Biol. Chem. 252*, 6082–6089.

Mirksy, A. E., Burdick, C. J., Davidson, E. H., & Littau, V. C. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 592–597.

Morris, S. M., Jr., & Cohen, P. P. (1979) *Biochem. Biophys. Res. Commun. 89*, 162–168.

Newrock, K. M., Alfageme, C. R., Nardi, R. V., & Cohen, L. H. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 421–431.

Oudet, P., Spadafora, C., & Chambon, P. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 301–312.

Panyim, S., & Chalkley, R. (1969) *Biochemistry 8*, 3972–3979.

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

Panyim, S., Bilek, D., & Chalkley, R. (1971) *J. Biol. Chem. 246*, 4206–4215.

Renz, M., Nehls, P., & Hozier, J. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 245–252.

Ruderman, J. V., & Gross, P. R. (1974) *Dev. Biol. 36*, 286–298.

Russev, G., & Tsanev, R. (1979) *Eur. J. Biochem. 93*, 123–128.

Seale, R. L., & Aronson, A. I. (1973) *J. Mol. Biol. 75*, 633–645.

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†

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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-

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[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.

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