Histone H2A Phosphorylation in Animal Cells: Functional Considerations†

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ABSTRACT: We have sought to determine the role of histone H2A phosphorylation in chromatin by examining the distribution of the phosphorylated and unphosphorylated forms of this core histone within the nuclei of mouse and human cells. At any time, only about 15% of the H2A of whole chromatin is in the phosphorylated form, and its phosphate is rapidly turned over, even in quiescent cells that contain a functional nucleus. The phosphorylations and dephosphorylations are not specifically related to progress through the cell cycle, nor to DNA synthesis or repair, and they are not selectively nucleolar. Euchromatin is substantially enriched with phosphorylated H2A but is not the exclusive repository of it. Possible roles of this modification of H2A are considered.

Of the histones in mammalian cells, only H1, H3, and H2A undergo abundant phosphorylation after their incorporation into chromatin (Gurley et al., 1975). The phosphorylations and dephosphorylations of H1 and H3 are cell cycle stage specific events, possibly related to the condensation and decondensation of chromatin (Gurley et al., 1975, 1978a; Hohmann et al., 1976; Bradbury et al., 1974). In contrast, the phosphorylation and dephosphorylation of H2A occur at relatively constant rates in both proliferating and quiescent cells (Gurley et al., 1975, 1978a; Prentice et al., 1978); at any given time, only a small fraction of the H2A complement is in the phosphorylated form (Balhorn et al., 1972; Dolby et al., 1979; D'Anna et al., 1980; Pantazis & Bonner, 1981; Joseph et al., 1981). The phosphate of H2A turns over continuously, independent of the replicative state of the cells, suggesting that the role of phosphorylated H2A in chromatin is unlike that of either phosphorylated H1 or H3. As yet, however, the physiological significance of H2A phosphorylation has not been established.

Using cells from three species of mice which differ in their heterochromatin content, Halleck and Gurley noted a correlation between the amount of a highly phosphorylated H2A variant and the abundance of heterochromatin and postulated that H2A phosphorylation plays a role in heterochromatin condensation (Gurley et al., 1978b; Halleck & Gurley, 1980). We have used nuclease digestion of whole chromatin to separate its euchromatic and heterochromatic subclasses in order to determine in a more direct fashion whether phosphorylated H2A is selectively localized in the transcriptionally active or inactive regions. Deoxyribonuclease I (DNase I, EC 3.1.4.5) begins it digestion of chromatin preferentially at transcriptionally active and potentially active regions of the genome and has been used to resolve the constituents of the active regions from those of the inactive (Garel & Axel, 1976; Weintraub & Groudine, 1976) and to locate various chromatin proteins with respect to the expressed regions of the genome (Nelson et al., 1978; Sealy & Chalkley, 1978; Mayfield et al., 1978). Our results show that phosphorylated H2A is enriched in the DNase I sensitive fraction of chromatin and that this fraction is indeed transcriptionally active. They also show that the phosphorylation of H2A is not exclusively associated with the processes of either DNA synthesis or repair nor with selective expression of the nucleolus.

Materials and Methods

Cell Culture. NCTC clone 929 mouse cells, strain L (Earle, 1943), were propagated and synchronized as described previously (Prentice et al., 1978). The radioactive labeling of asynchronous cells was done in half-gallon glass disposable roller bottles and that of G1 phase cells in plastic T-75 flasks.

Human fibroblast (HF) cultures were initiated from explants of newborn foreskin tissue and grown in Ham's F-12 medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere.

^{32}P Labeling. The L cell growth medium was replaced with low phosphate (0.18 mM, 5% of normal) medium (Prentice et al., 1978), and the cells were incubated in it at 37 °C for 30 min. The [^{32}P]PO_4 was added to a final concentration of 100 μCi/mL, and the cultures were incubated for another 1.5 h.

HF cells, between passages 6 and 12, were grown to confluence in T-75 flasks, washed twice with phosphate-buffered saline, and refed with serum-free Ham's F-12 medium containing 10 μg/mL ascorbate. After 2 days, the now quiescent cells were refed with Ham's F-12 medium containing 0.1 mM phosphate (10% of normal), 10 μg/mL ascorbate, 0.1% bovine serum albumin, and 100 μCi/mL [^{32}P]PO_4 and incubated at 37 °C for 2 h.

Quantitation of Phosphorylated H2A by Radiolabeling. L cells were labeled in medium containing 1 μCi/mL [^{14}C]lysine for 48 h (approximately two generations) and then labeled for 1.5 h with [^{32}P]PO_4 as described above. The nuclei were isolated, and the acid-extractable proteins were obtained and resolved by extended electrophoresis on Triton gels as described elsewhere under Materials and Methods. H2A was located by using purified marker H2A obtained by the method of Johns (1979).

DNase I Digestion and Protein Isolation. Unless stated otherwise, all procedures were carried out on ice. The cells were harvested by scraping with a rubber policeman, collected in ice-cold saline (0.85% NaCl), and pelleted by centrifugation at 800*g* for 2 min. The nuclei were isolated by the method of Penman (1966). The nucleoli were isolated from purified nuclei by the procedure of Bhorjee & Pederson (1973) and acid extracted as detailed below. In pilot studies using 50 mM sodium bisulfite, it was found that phosphatase inhibitors to prevent histone dephosphorylation during the initial isolation

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were unnecessary (Gurley et al., 1975; D'Anna et al., 1978). This may be due to the lack of a potent histone phosphatase in L cells.

The purified nuclei were suspended in 10 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 3 mM $MgCl_2$, and 1 mM $CaCl_2$ at a chromatin DNA concentration of 10–20 A_{260} units/mL and incubated with or without DNase I (Sigma type DN-CL, 100 units/mL) for 5–10 min at 37 °C. The digestion and subsequent handling were carried out in the presence of phosphatase inhibitors, 10 mM sodium arsenate and 10 mM fructose 1,6-bisphosphate. These inhibitors were used because they do not inhibit DNase I action. The reaction was stopped by adding ethylenediaminetetraacetic acid (EDTA) (pH 8.0) to 4 mM and placing the reaction vessel on ice. Digested samples were centrifuged at 10000*g* for 10 min to separate the solubilized chromatin (supernatant) from the unsolubilized (pellet).

The amount of acid-soluble A_{260}-absorbing material released by DNase I digestion was determined by the method of Garel & Axel (1976). The total amount of A_{260}-absorbing material released was determined by measuring the absorbance of the supernatant material before the acid-solubility assay. The total A_{260} content of the sample before digestion was determined by using the method of Saiga & Kinoshita (1976).

The supernatant fraction (solubilized material) and the pellet (unsolubilized chromatin) were extracted separately with 0.4 N H_2SO_4 for 30 min and centrifuged at 17000*g* for 20 min to remove the DNA and associated "acidic" proteins. Additional extractions with acid removed no more histone, indicating that all of the histone proteins were solubilized by 30 min. The acid-soluble supernatant material was resolved electrophoretically as detailed below.

The nuclei were also fractionated into chromatin and "nucleoplasm" by soaking them in 0.14 M NaCl, 10 mM Tris (pH 8.0), and 1 mM $MgCl_2$ for 10 min, followed by centrifugation at 800*g* for 2 min. The supernatant liquid was saved, and the pelleted nuclei were lysed in 2 mM EDTA (pH 8.0). The chromatin was collected by centrifugation at 10000*g* for 10 min, while the supernatant fraction was combined with the previous supernatant liquid and designated the "nucleoplasmic" material. Both fractions were acid extracted as described above.

Whole nuclei were acid extracted for use as a control. Before acid extraction, isolated chromatin and the chromatin in whole nuclei were "sheared" with micrococcal nuclease (Sigma grade VI; 30 units/mL) according to the method of Noll et al. (1975) to open up the chromatin structure and prevent protein entrapment.

The acid-extracted proteins were concentrated, and the H_2SO_4 was removed by repeated cycles of diluting with water and concentrating with a Millipore CX-10 immersible molecular separator. The protein content was determined by either turbidity (Hnilica, 1975) or Coomassie assay (Bradford, 1976).

Electrophoresis and Measurement of Radioactivity. Electrophoresis of the basic chromatin proteins was performed by using three gel systems. The acid–urea (Panyim & Chalkley, 1969) and Triton (Zweidler & Cohen, 1972; Gurley & Walters, 1973) gels were cast as tubular gels 100 mm in length and 5 mm in diameter. They were routinely preelectrophoresed for 3 h at 2–3 mA/gel and then electrophoresed with 20–50 μg of the sample for 4–6 h at 1.5 mA/gel. The extended Triton gels were electrophoresed for 24 h. The sodium dodecyl sulfate ($NaDodSO_4$) gels were cast as 10–20% convex exponential acrylamide gradient slabs. Electrophoresis of samples was done according to Adamietz et al. (1979). The gels were stained with 0.5% amido black in 45% methanol and 10% acetic acid for 15 min and destained by diffusion in 45% methanol and 10% acetic acid.

The tubular gels were scanned at 600 nm with a Gilford Model 240 spectrophotometer equipped with a linear transporter and recorder or at 580 nm with an ISCO Model 1310 gel scanner in combination with an ISCO Model UA-5 absorbance monitor. The tubular gels were cut laterally into 1-mm slices and prepared for scintillation counting as described previously (Prentice et al., 1978). The slab gels were dried under vacuum onto a stiff paper backing for autoradiography.

In molar terms, the abundance of H2A is equal to that of any of the other three core histones, and the efficiencies of their extractions with dilute mineral acid are essentially equivalent. For comparative purposes, therefore, it would seem reasonable to relate the amount of radioactivity in the ^{32}P-labeled H2A gel band to the amount of protein in any one of the core histones as long as the same band is always used. One exception would be H3 which, because of its cysteine residue, is susceptible to dimerization. In the acid–urea gels, H4 resolves clearly from the other histones, and quantitative measurements of it are reliable and high reproducible. Thus, when the acid–urea gels were employed, numerical estimates of the relative specific activities of H2A in the euchromatic and heterochromatic fractions from any given sample were made in this way, i.e., counts per minute (cpm) in the phosphorylated H2A band/densitometric area of the H4 band of the same gel.

When $NaDodSO_4$ gels were used, the phosphorylated form of H2A comigrated with the nonphosphorylated. For comparison of the amounts of ^{32}P-labeled H2A between the euchromatic and heterochromatic fractions from a single source, euchromatic samples containing different amounts of protein were resolved electrophoretically, and the amido black stained profiles were compared with those of the heterochromatic samples. Electrophoretograms of the gel lanes with as nearly identical amounts of core histones as possible were autoradiographed and the autoradiograms compared. The evaluations of relative specific activity made in this way were subjective but not ambiguous.

Labeling of Nascent RNA and Resolution of Oligonucleosomes by Zone Velocity Sucrose Gradient Centrifugation. DNA synthesis was suppressed by 5-min pretreatment of L cells with 2.5 mM hydroxyurea, and then the cells were labeled with 40 μCi/mL [^3H]uridine for 15 min in the presence of hydroxyurea. The nuclei were isolated and digested at 37 °C with 100 units/mL DNase I, and the oligonucleosomal material was resolved by zone velocity centrifugation on a 5–20% convex exponential sucrose gradient in 2 mM EDTA, pH 7.0. The gradients were centrifuged in a Beckman SW27.1 rotor at 27000 rpm for 16 h at 4 °C and then pumped out and monitored for the absorbance at 254 nm and radioactivity.

Isolation of DNA and RNA and the Hybridization Methods. DNA was isolated from the DNase I solubilized and unsolubilized fractions of L cell nuclei by the method of Weintraub & Groudine (1976), with the modification that instead of the proteinase K digestion step, the crude DNA was treated with 0.3 N NaOH for 10 min at 37 °C. Before treatment with NaOH, the DNA in the unsolubilized fraction was digested to a size equivalent to that of the solubilized fraction by brief treatment with micrococcal nuclease. The solutions were neutralized and extracted with phenol/chloroform and chloroform/isoamyl alcohol, and the DNA was precipitated with ethanol. The amount of DNA was measured by the diphenylamine assay (Burton, 1956). The lengths of

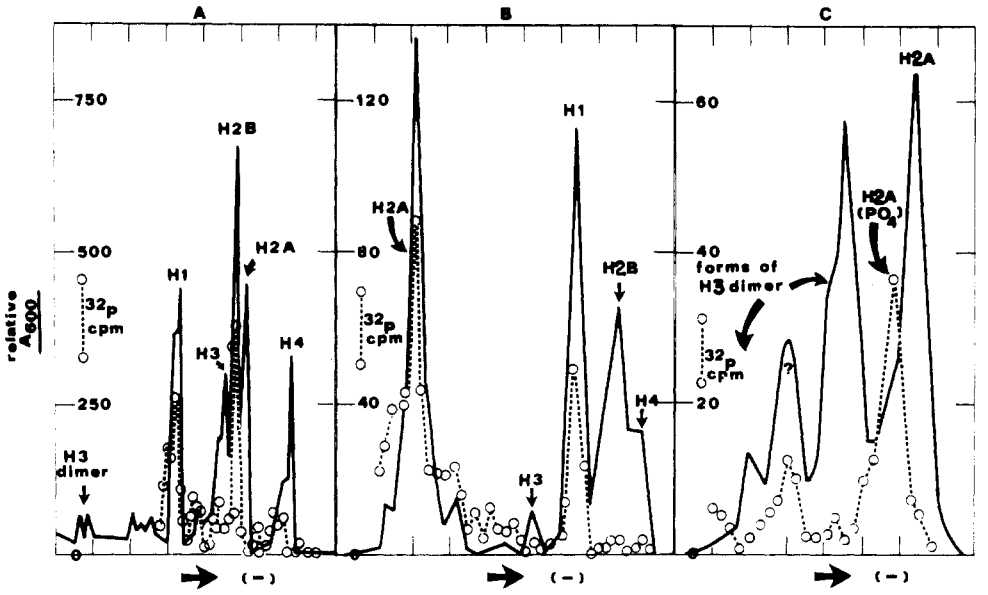

FIGURE 1: Electrophoretogram of the acid-soluble proteins from nuclei of [^{32}P]PO_4-labeled G1 phase L cells. Proteins were resolved on (A) an acid–urea gel, (B) a Triton gel, and (C) a Triton gel, extended electrophoresis. Only the H2A region is shown. Positions of migration of proteins were determined by using purified histone fractions obtained by the method of Johns (1979).

the DNA fragments from both the solubilized and unsolubilized fractions were determined by sucrose-gradient fractionation to range from 75 to 1500 nucleotides, with a median length of 400–500 nucleotides.

To obtain polysomal RNA, we labeled L cells for 4 h with 10 μCi/mL [^{3}H]adenosine, and the polysomes were isolated and RNA was extracted by the method of Palmiter (1974). The mRNA was isolated by chromatography on poly(U)–Sepharose (Boone & Moss, 1977; Cooper & Marzluff, 1978) and its concentration determined by the absorbance at 260 nm.

The RNA–DNA hybridizations were done on nitrocellulose filters in RNA excess according to the method of Gillespie (1968). DNA retention on the filters was monitored with [^{3}H]thymidine-labeled DNA, and proper corrections were made to achieve equivalent binding of DNA from both fractions.

Inhibition of Macromolecular Synthesis. L cells were treated with either 1 μg/mL actinomycin D, 2.5 mM hydroxyurea, or 10 μg/mL cycloheximide for 15 min to inhibit RNA, DNA, or protein synthesis, respectively. They were then incubated with [^{32}P]PO_4 in the continued presence of the inhibitors. The nuclei were isolated and proteins acid extracted and resolved electrophoretically.

Treatment of Cells To Damage the DNA. The DNA of the L cells was damaged either by treatment with 0.5 mg/mL ethyl methanesulfonate (EMS) for 2 h or by ultraviolet (UV) irradiation to produce total exposures from 24 to 190 ergs/mm^2. The cells were then labeled with [^{32}P]PO_4 as described above. The nuclei were isolated and proteins acid extracted and resolved electrophoretically.

Results

Characteristics of H2A Phosphorylation. A small but significant portion of the H2A of L cells can be phosphorylated regardless of the phase of the cell cycle; whether synchronized in G1 or S phase or unsynchronized, the cells incorporate [^{32}P]PO_4 into it. Exposing the cells to [^{32}P]PO_4 for as little as 30 min or as much as 150 min does not change the extent of ^{32}P labeling of H2A, a result that is consistent with rapid turnover of the phosphate on this histone. Certification that the phosphorylated histone is actually H2A was achieved by using three electrophoretic systems: the acid–urea gel system

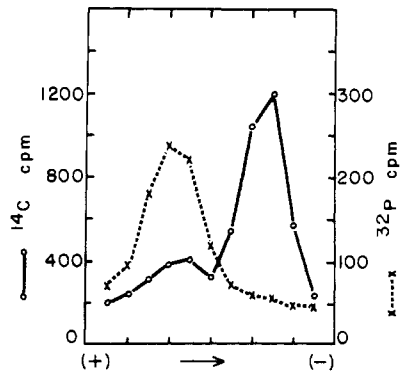

FIGURE 2: Electrophoretogram of the histone H2A region of a Triton gel after extended electrophoresis. The other histones have been electrophoresed off the gel, and H2A and its phosphorylated component are near the bottom of the gel. The sample was from the acid-soluble proteins from nuclei of asynchronous L cells labeled for approximately two generations with [^{14}C]lysine and then pulsed briefly with [^{32}P]PO_4.

of Panyim & Chalkey (1969) in which phosphorylated H2A migrates more slowly than unphosphorylated, its mobility corresponding closely to that of H2B (Figure 1A); a Triton gel system similar to that of Zweidler & Cohen (1972) (Gurley & Walters, 1973) in which the migration of both forms of H2A is greatly retarded, separating them clearly from H2B and the other histones (Figure 1B); and the $NaDodSO_4$ gel system of Adamietz et al. (1979) in which both the phosphorylated and unphosphorylated forms of H2A comigrate (Figure 5). Prolonged electrophoresis in the Triton system resolves the two forms well enough to show that the phosphorylated form is only a small fraction of the H2A complement (Figure 1C).

The distribution of H2A between its phosphorylated and unphosphorylated forms was determined by growing the cells for several generations in a medium containing [^{14}C]lysine so as to label the histones "uniformly", resolving the H2A species by prolonged electrophoresis on Triton gels, and determining the amount of carbon-14 in each of the two H2A bands. Shortly before the end of the incubation period, [^{32}P]PO_4 was added to the culture medium so that the phosphorylated form of H2A was doubly labeled with carbon-14 and phosphorus-32 (Figure 2). From the amount of carbon-14 in each of the two

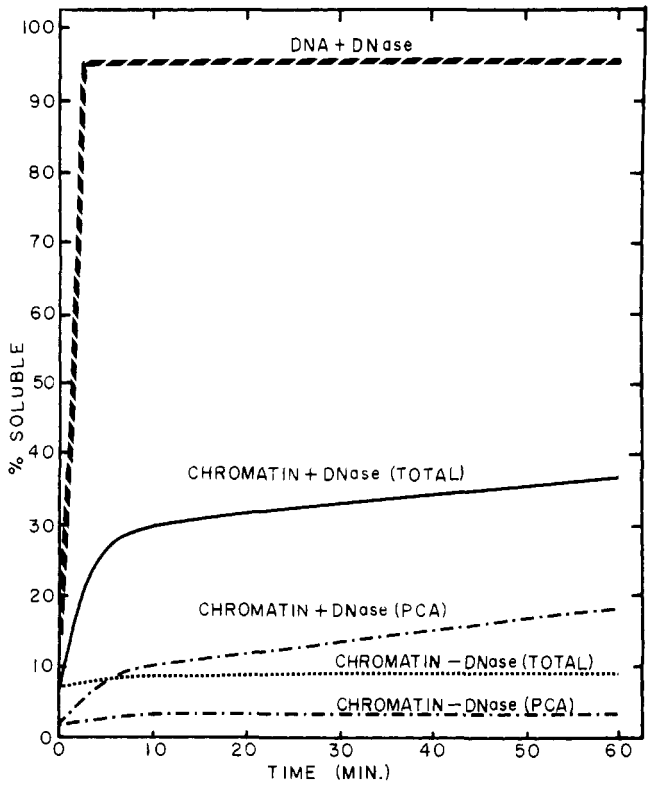

FIGURE 3: Digestion kinetics of DNase I on protein-free DNA or chromatin (nuclei). Total indicates the entire amount of A_{260}-absorbing material released, while PCA indicates the entire amount of A_{260}-absorbing material released that was soluble in perchloric acid. The amount of material released by incubation of nuclei without DNase I is also shown.

bands, the phosphorylated form of H2A was calculated to range from 10 to 20% of the total, with a mean value of 15.4%. This is very close to the amount seen by Dolby et al. (1979) in HeLa cells.

The pattern of histone phosphorylation of early-passage human foreskin fibroblasts (HF) is similar to that of the L cells. During exponential growth, cultures of unsynchronized cells incorporate phosphate into H1, H3, and H2A, but during quiescence, H2A is phosphorylated virtually exclusively. As in the L cells, the phosphorylated form of H2A is but a small fraction of the total H2A of chromatin. These findings suggest that some, if not all, of the phosphorylation of H2A is unrelated to the progress of the cells through the mitotic cycle and raise the following question: "What is the functional significance of H2A phosphorylation and dephosphorylation in chromatin?" Among the possibilities are an involvement in transcription, DNA repair, or nucleolar function, each of which shall be considered here.

Distribution of Phosphorylated H2A between Euchromatin and Heterochromatin. The extent to which H2A is phosphorylated in transcriptionally active and inactive portions of chromatin was examined. The nuclei of [^{32}P]PO_4-labeled L cells were isolated and digested briefly with DNase I, and estimates of the specific activities of the H2A in both the solubilized and unsolubilized chromatin fractions were made. The kinetics of DNase I action on the chromatin of L cell nuclei and on purified DNA are shown in Figure 3. The purified DNA was solubilized almost completely within 2 min whereas the chromatin was solubilized in a biphasic manner—rapidly for the first 5–10 min and slowly thereafter. Without exogenous nuclease, a small amount of 260-nm-absorbing material was released, but only at the outset. Thus, the release of most of the 260-nm-absorbing material is attributable to the action of the DNase I.

Nuclei from [^{32}P]PO_4-labeled L cells were digested briefly with DNase I, and the solubilized material was separated from the unsolubilized by centrifugation. The basic proteins of each of the two fractions were obtained by extraction with dilute mineral acid, resolved electrophoretically on both acid–urea and Triton gels, and stained with amido black. The gels were sliced laterally, and the radioactivity of each slice was determined.

Mock digestions of nuclei isolated from [^{32}P]PO_4-labeled G1 phase cells, from which the DNase I was omitted, did release some radioactive basic proteins, but virtually none that migrated in the core histone region of the acid–urea gels. In addition, nuclei that had not been digested were fractionated into "nucleoplasm" and chromatin, and the basic proteins of each fraction were resolved electrophoretically. The major ^{32}P-labeled histone of the chromatin fraction was H2A; there was little if any H2A, phosphorylated or otherwise, in the nucleoplasmic fraction. Several phosphorylated nonhistone proteins were present in the nucleoplasmic fraction, but they were clearly separated from the nucleosomal core histone region of the gels. This result and that of the mock digestions indicate that any H2A, phosphorylated or not, which is released during the digestion of nuclei with DNase I both is of nucleosomal origin and released specifically by the action of the nuclease.

Nuclei from [^{32}P]PO_4-labeled unsynchronized L cells were digested briefly with DNase I, the solubilized and unsolubilized fractions obtained and acid extracted, and their basic proteins resolved electrophoretically. From the A_{600} and radioactivity profiles (Figure 4A), it is evident that most of the ^{32}P of the unsolubilized fraction is associated with the H1 and H2B regions of the acid–urea gels. As already mentioned, the H2B-associated radioactivity is actually phosphorylated H2A. These results are confirmed by the Triton gel separations of the same material (Figure 4C). In contrast, there is very little stainable protein in the core histone region of the acid–urea gels of the DNase I solubilized fraction, but there is a major peak of radioactivity at the expected position of migration of phosphorylated H2A (Figure 4B). These findings are confirmed by the corresponding Triton gels (Figure 4D). By inspection, the amount of radioactivity in the H2A band relative to the amount of H2A protein (an estimate of its specific activity) is much greater in the DNase I solubilized (Figure 4B,D) than in the DNase I unsolubilized fraction (Figure 4A,C) or than in the extract of chromatin that had not been nuclease treated. The solubilized fraction is also enriched with phosphorylated H1 and a plethora of phosphorylated nonhistone protein (Figure 4B,D).

The basic proteins from the DNase I solubilized and unsolubilized fractions of chromatin were also resolved by unidimensional $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5A) and the ^{32}P-labeled constituents located by autoradiography (Figure 5B). Figure 5B shows that phosphorylated H2A is present in the DNase I solubilized fraction at much higher specific activity than in the unsolubilized fraction (compare lanes 2 and 3). Lane 1 is from undigested nuclei, and lanes 4 and 5 are from the solubilized and unsolubilized fractions, respectively, of the mock digest. Phosphorylated H2A is conspicuously absent from the solubilized fraction (lane 4) of the mock digest, again indicating the need for DNase in its release.

H2A Phosphorylation and DNA Synthesis. DNase I acts preferentially upon decondensed chromatin, including regions that are transcriptionally active (Garet & Axel, 1976;

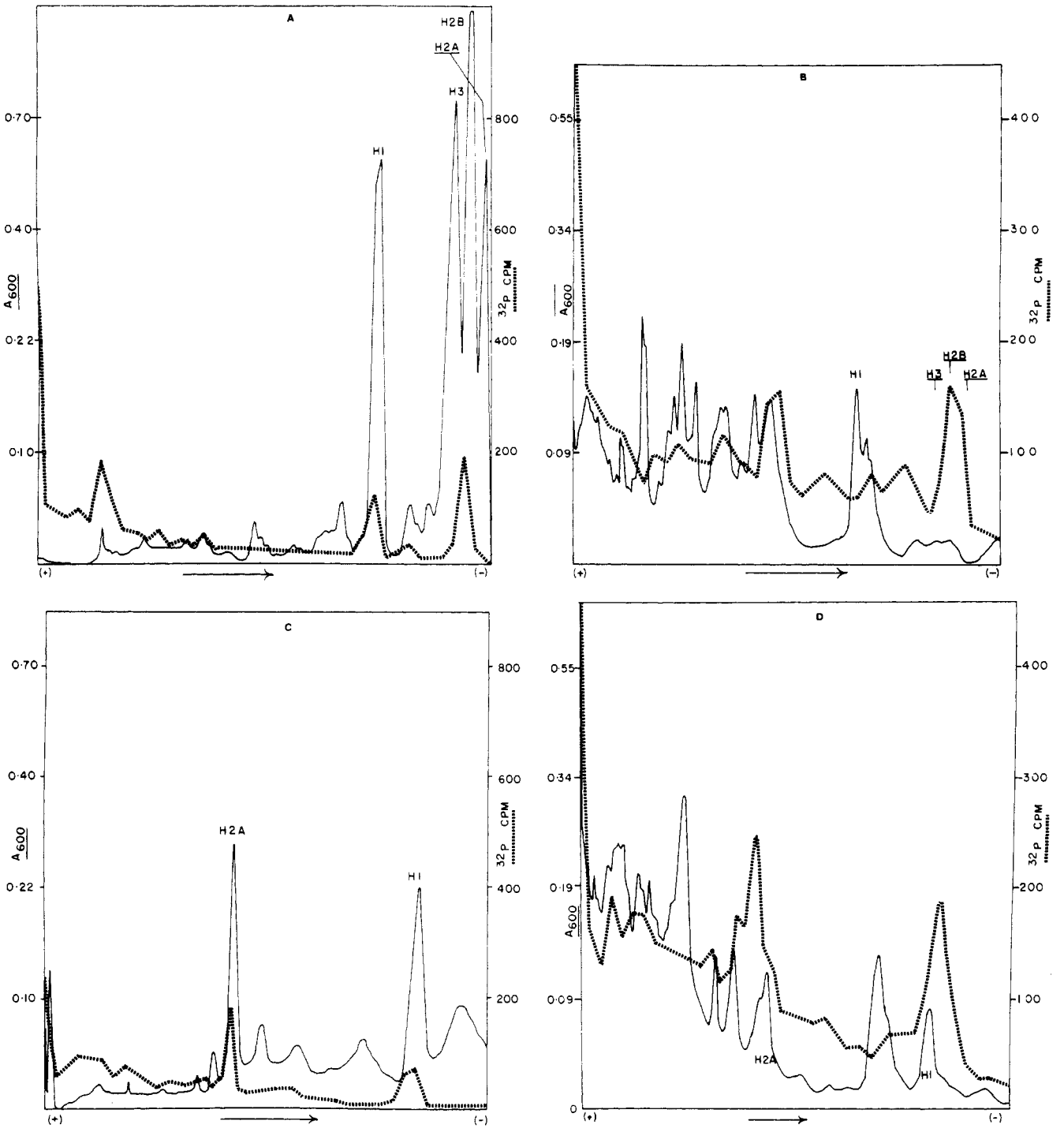

FIGURE 4: Electrophoretograms of the acid-soluble proteins obtained from a DNase I digestion of nuclei from [^{32}P]PO_4-labeled asynchronous L cells. The proteins were resolved electrophoretically. (A) Unsolubilized material, acid–urea gel; (B) DNase I solubilized material, acid–urea gel; (C) unsolubilized material, Triton gel; (D) DNase I solubilized material, Triton gel.

Weintraub & Groudine, 1976) and possibly replicative fork regions. Considering the possibility that the phosphorylation of H2A, like the poly(ADP-ribosylation) of the histones (Jump et al., 1979), could be a replicative function, we exposed G1 phase L cells to [^{32}P]PO_4 and after brief DNase I digestion separated their chromatin into solubilized and unsolubilized fractions. The acid-extractable proteins of these fractions were resolved electrophoretically on acid–urea gels, the protein and radioactivity profiles of which are shown in Figure 6A,B. Because the cells were in G1 phase, there was not much phosphorylation of H1. It is notable that ^{32}P-labeled H2A is present in both the solubilized and unsolubilized fractions, but by inspection, its specific activity is much greater in the former. This distribution indicates an enrichment of the phosphorylated form of H2A in the DNase I sensitive region of chromatin of G1 phase cells similar to that seen in asynchronous cells, approximately 40% of which are in S phase (data not shown). Thus, H2A phosphorylation must not be an activity that is peculiar to the replicative fork.

Transcriptional Characteristics of the DNase I Solubilized Chromatin. It is well established that DNase I acts preferentially at the transcriptionally active and potentially active regions of the genome. For the studies reported here, we verified the DNase I specificity in two ways. In the first method, hydroxyurea-treated L cells were pulsed briefly with high specific activity [^{3}H]uridine to label the nascent RNA. The nuclei were isolated and digested with DNase I, and the oligonucleosomes thus produced were resolved by zone velocity

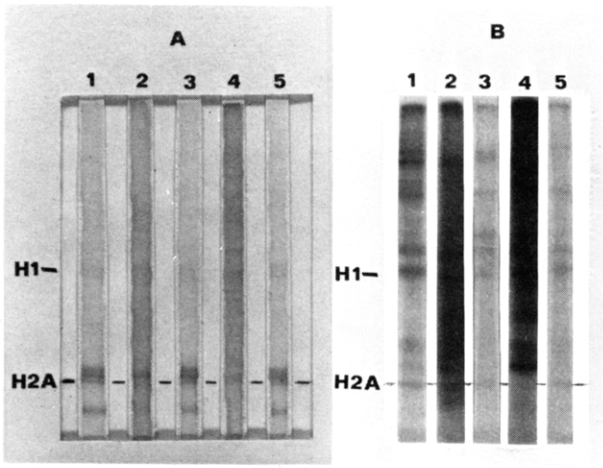

FIGURE 5: Electrophoretogram of the acid-soluble proteins obtained by incubation with or without DNase I of nuclei from [^{32}P]PO_4-labeled asynchronous L cells. The proteins were resolved on a $NaDodSO_4$ gel (A) and the ^{32}P-labeled proteins detected by autoradiography (B). Lane 1, material from unfractionated nuclei; lanes 2 and 3, DNase I solubilized and unsolubilized material, respectively, from a DNase I digestion; lanes 4 and 5, the solubilized and unsolubilized material, respectively, from nuclei incubated in digestion buffer without DNase I. Amounts of protein applied to the gel to get approximately equal straining intensities were the following: lanes 1, 3, and 5, 10 μg; lanes 2 and 4, 80 μg.

centrifugation through a sucrose gradient. The distribution of radioactivity across the oligonucleosomal profile following digestion with DNase I is shown in Figure 7. The results indicate the presence of nascent RNA in association with the DNase I released oligonucleosomes.

Table I: Hybridization of L Cell mRNA with DNA from DNase I Solubilized and Nonsolubilized Fractions of Chromatin[a]

	^{3}H-labeled mRNA hybridized with L cell DNA (cpm)[b]		
R_0t (M s)	DNase I solubilized	DNase I unsolubilized	no DNA
5×10^{-1}	125 ± 25.0	16 ± 1.0	6 ± 3.6
5×10^{0}	320 ± 51.0	27 ± 5.6	8 ± 3.6
5×10^{1}	7325 ± 645.0	110 ± 49.0	

[a] L cell DNA fragments (57–1500 nucleotides in length; median length, 400–500 nucleotides) from the solubilized and nonsolubilized fraction obtained after brief DNase I digestion were bound to nitrocellulose filters. High specific activity [^{3}H]adenosine-labeled poly(A+) mRNA from rapidly growing L cells was hybridized to the membrane-bound DNA at each of the indicated R_0t values by the method of Gillespie (1968). [b] Mean value ± SD of the mean.

The second method of evaluating the transcriptional relevance of the DNase I solubilized fraction of chromatin was to hybridize its DNA with the cytoplasmic RNA of the cells. Polysomal RNA labeled with [^{3}H]uridine was isolated from L cells and its mRNA obtained by poly(U)–Sepharose chromatography. DNA was purified from DNase I solubilized and unsolubilized chromatin fractions, bound to nitrocellulose filters, and hybridized with the [^{3}H]mRNA. Table I shows that the DNA obtained from the DNase I solubilized fraction is enriched in transcribed sequences approximately 66-fold over that in the unsolubilized fraction. This RNA–DNA hybridization and the nascent RNA study demonstrate for the L cell system that in the early stages of DNase I digestion there is preferential release of transcriptionally active chromatin.

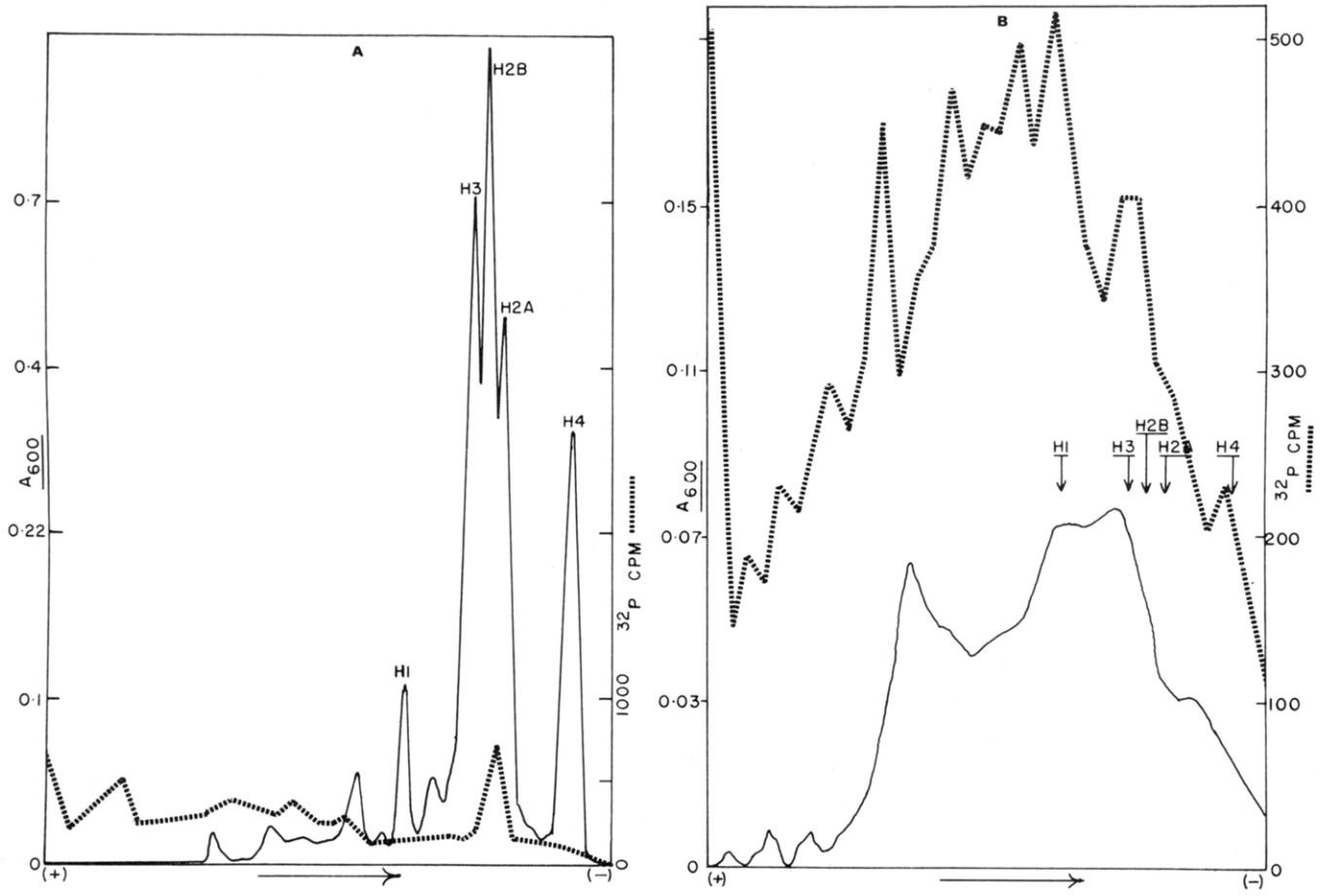

FIGURE 6: Electrophoretograms of the acid-soluble proteins obtained from a DNase I digestion of nuclei from [^{32}P]PO_4-labeled G1 phase L cells. The proteins were resolved on acid–urea gels. (A) Unsolubilized material; (B) DNase I solubilized material.

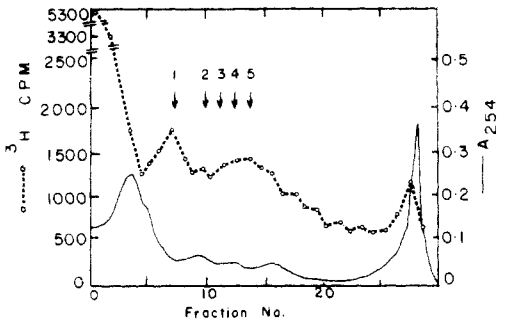

FIGURE 7: Sucrose-gradient profiles of the DNase I solubilized material obtained by the digestion of nuclei from [^{3}H]uridine-labeled L cells. The numbered arrows indicate the approximate sedimentation position of mononucleosomes, dinucleosomes, etc.

H2A Phosphorylation during the Repair of DNA Damage. A possible involvement of H2A phosphorylation in the repair of damaged DNA was investigated by exposing L cells to either ethyl methanesulfonate (EMS) or ultraviolet (UV) irradiation at doses which were determined to cause significant DNA damage and loss of division potential in these cells. Cells thus treated and untreated cells were then labeled with [^{32}P]PO_4, their acid-soluble nuclear proteins were isolated, and the radioactivity in the histones was compared after electrophoretic resolution. In response to either of these insults, there were no significant changes in the levels of the phosphorylated forms of H2A or H1 (Figure 8A,B; only the results from the EMS treatment are shown). If H2A phosphorylation were involved in the repair process, an increase in the level of phosphorylation in response to these treatments would be expected. The results suggest that participation in the process of DNA repair is not the primary role of H2A phosphorylation.

H2A Phosphorylation in the Nucleolus. The nucleoli of proliferating L cells were examined for possible enrichment of phosphorylated H2A for two reasons: (1) the nucleolus is the site of a significant fraction of the transcriptional activity of rapidly growing cells; (2) H2A phosphorylation within the nucleus could be associated partly or totally with nucleolar function. The histones of whole nuclei and of nucleoli were isolated from [^{32}P]PO_4-labeled L cells and resolved on acid–urea gels. From the electrophoretic profiles for protein and radioactivity (not shown), there did not appear to be any significant difference in the amount of phosphorylated H2A per unit histone protein between these two organelles. Thus, H2A phosphorylation must not be strictly or even principally nucleolar.

Is the Process of H2A Phosphorylation Dependent upon Simultaneous Macromolecular Synthesis? Asynchronous cultures of L cells were treated for 15 min with actinomycin D, hydroxyurea, or cycloheximide to inhibit RNA, DNA, or protein synthesis, respectively, and then the cultures were exposed to [^{32}P]PO_4. The nuclei of these cells were isolated and their acid-extractable proteins obtained and resolved on unidimensional $NaDodSO_4$–polyacrylamide gels (Figure 9A). The ^{32}P-labeled proteins were detected by autoradiography (Figure 9B).

The actinomycin D treatment caused no qualitative or obvious quantitative changes in the phosphorylation of the basic proteins. The only detectable change caused by the hydroxyurea treatment was to decrease the extent of H1 phosphorylation, not suprising because it is tied to progress from S through M phase of the cell cycle. The cycloheximide treatment caused notable decreases in the phosphorylation of all the phosphoproteins. This may be due to an increase in phosphate turnover (Balhorn et al., 1973). These results

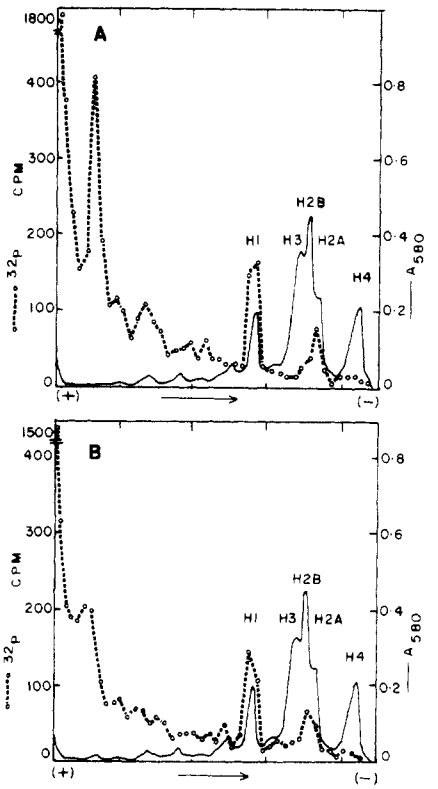

FIGURE 8: Electrophoretograms of the acid-soluble proteins obtained from nuclei of [^{32}P]PO_4-labeled asynchronous L cells. (A) Control (untreated) cells; (B) EMS-treated cells.

suggest that neither transcription nor DNA replication are simultaneously required for H2A phosphorylation in vivo.

Discussion

The patterns of phosphorylation and dephosphorylation of the histones of animal cells can be divided into two distinct groups—those related to proliferation and those not. Among the former are the phosphorylations of H1 and H3 of Chinese hamster ovary cells (Gurley et al., 1975), HeLa cells (Marks et al., 1973), and differentiating erythroblasts (Ruiz-Carrillo et al., 1976). They are cell cycle phase specific. In contrast, H2A phosphorylation, although not necessarily constant in rate throughout the cell cycle, is continuously active (Gurley et al., 1973, 1975, 1978a; Prentice et al., 1978) and is the only histone phosphorylation that is evident in quiescent cells, viz., human fibroblasts. These attributes suggest that the phosphorylation of H2A is independent of proliferation and not associated exclusively with any phase-specific event of the cell cycle.

Several lines of study have provided information about possible roles of H2A phosphorylation in animal cells. Ruiz-Carrillo et al. (1976) showed that in the differentiation of avian erythroid cells the phosphorylation of most histones is limited to the erythroblast stage, a time when proliferation is of paramount importance. The phosphorylation of H2A, however, continues much later in the maturation process, declining as the cells approach the ultimate erythrocyte state. The rate of decline approximately parallels that of transcription (Ruiz-Carrillo et al., 1974). Neumann et al. (1978) noted that the only nuclear protein to become more highly phosphorylated during dimethyl sulfoxide induced differentiation of erythroleukemia cells to the erythroid state was H2A. Likewise, Prentice and co-workers (Prentice et al., 1978) observed an increase in the extent of H2A phosphorylation of strain L mouse cells during an induction response to a glucocorticoid hormone. In these instances, the phosphorylation of H2A appears consistent with its involvement in the regulation of

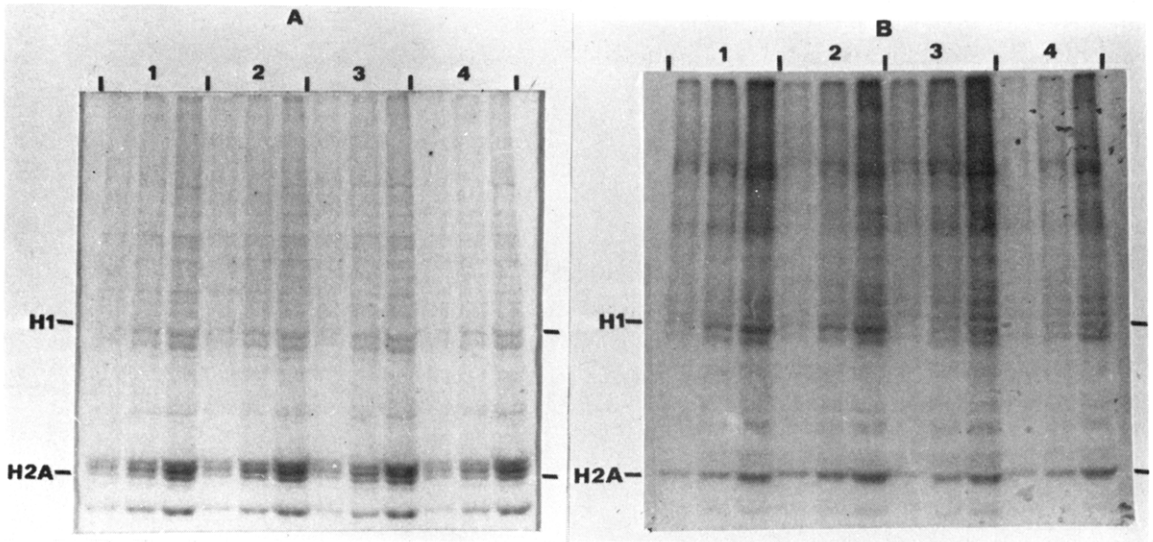

FIGURE 9: Electrophoretograms of the acid-soluble proteins obtained from nuclei of [^{32}P]PO_4-labeled asynchronous L cells treated with inhibitors of macromolecular synthesis. The samples were resolved on a $NaDodSO_4$ gel (A) and the ^{32}P-labeled proteins detected by autoradiography (B). Sample 1, control cells; sample 2, actinomycin D treated cells; sample 3, hydroxyurea-treated cells; sample 4, cycloheximide-treated cells. The three lanes for each sample contained (from left to right) 10, 20, and 40 μg of protein, respectively.

genetic expression, possibly transcription or a close correlate. This possibility had been suggested earlier by Gurley et al. (1973) and has support in the recent findings of Allis & Gorovsky (1981), who showed that in *Tetrahymena* the phosphorylation of H2A occurred almost exclusively in the transcriptionally active macronuclei and not in the transcriptionally inactive micronuclei.

Another view of the function of phosphorylated H2A is would Halleck & Gurley (1980; Gurley et al., 1978b). They examined the histones of two lines of *Peromyscus* (deer mouse) cells and observed a correlation between the amount of constitutive heterochromatin and a highly phosphorylated H2A variant (only one of the two major variant forms of H2A was highly phosphorylated). They proposed that the phosphorylation is causally linked to the condensation of constitutive heterochromatin.

If phosphorylated H2A were involved in genetic expression, it would probably be localized in euchromatin and if involved in the condensation of heterochromatin, probably be indigenous to that fraction. To evaluate these alternatives, we fractionated [^{32}P]PO_4-labeled chromatin into its euchromatic and heterochromatic constitutents with DNase I, electrophoretically resolved the acid-soluble proteins from each fraction, and obtained estimates of the H2A specific activities in them. The results show that there is an enrichment, that is, higher specific activity, of phosphorylated H2A in the solubilized fraction but that not all of the phosphorylated H2A is in the DNase I solubilized fraction; most, in fact, remains unsolubilized.

Under the conditions of our studies, a 10-min digestion of whole nuclei with DNase I releases a substantial amount of chromatin-associated material, only a small portion of which is DNA (Figure 3). Approximately 8% of the 260-nm-absorbing material of the nuclei, mainly nucleoplasmic constituents, is released by incubating them without DNase. An additional 22%, which may be considered chromatin associated, is released if DNase is included. Most of it, however, is nonhistone protein. The perchloric acid soluble fraction liberted by DNase I, mainly DNA and core histones, is 7% or less of the total. Thus, when whole nuclei are used in either the Garel & Axel (1976) or the Weintraub & Groudine (1976) procedures for chromatin fractionation, the euchromatic portion is endowed with an overwhelming abundance of nonhistone proteins, some of which are nucleoplasmic. For comparison of the ^{32}P-labeled H2A contents of the two fractions, equivalent amounts of core histones or, more specifically, of H2A must be used. The great abundance of nonhistone basic proteins in the euchromatic fraction complicates any prior assessment of its histone content. Furthermore, quantitation of the amount of H2A in the sample by amido black staining and densitometric scanning is difficult with 10-cm acid–urea gels because of the superimposition of the phosphorylated H2A band on H2B. The Triton gels, which clearly resolve H2A from the other histones, provide a better system for estimating the relative amounts of the two forms. As shown in Figure 2, the phosphorylated form is only about 15% of the H2A of the cell. Unfortunately, this method is not as easily applied to the euchromatic fraction because some of the basic, nonhistone proteins that make up a large part of this fraction are phosphate rich and may overlap the H2A region of the gel. Nevertheless, estimates of the distribution of H2A between the phosphorylated and unphosphorylated forms in the euchromatin fraction have been made and indicate that the phosphorylated form ranges from 46 to 48% of it. The phosphorylated form in both the heterochromatin fraction and whole chromatin accounts for no more than 20% of the total H2A.

Our results preclude an exclusively nucleolar role of phosphorylated H2A or one limited to DNA synthesis or repair. Moreover, they show that an inhibition of RNA synthesis does not suppress the phosphorylation of H2A, eliminating the possibility that transcriptional progress of RNA polymerase is needed for it to occur. The enrichment of the euchromatin with phosphorylated H2A suggests that a principal involvement in heterochromatin condensation is unlikely.

One other possible function of H2A phosphorylation, as yet untested but consistent with the results of the erythroid differentiation studies of Ruiz-Carrillo et al. (1976) and of Neumann et al. (1978), is that phosphorylated H2A is contained in all transcriptionally active and potentially active regions of the genome, the latter being facultative heterochromatin, currently in a condensed state. The rapid turnover of phosphate in these materials could be an expression of metabolic surveillance of those regions of the genome, possibly directed by nonhistone chromatin proteins, to distinguish active and potentially active chromatin from that which is tran-

scriptionally proscribed. Alternatively, H2A phosphorylation may be no more than an expression of the accessibility of the nucleosomes to protein kinases, the decondensed regions of the genome being affected most.

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