Modulation by Exogenous Histones of Phosphorylation of Non-Histone Nuclear Proteins in Isolated Rat Liver Nuclei*

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SUMMARY

The phosphorylation of specific non-histone proteins in isolated rat liver nuclei is modified by the addition of exogenous histone fractions. The added histones are quantitatively adsorbed and their presence in the chromatin has been verified by electron microscope autoradiography. Histone fractions F1 and F2A1 stimulate phosphorylation of different nuclear phosphoproteins. F1 stimulates the phosphorylation of a protein fraction with a molecular weight of about 40,000, while F2A1 enhances phosphorylation of a protein fraction with a molecular weight of about 22,000. F1 and F2A1 each inhibit the phosphorylation of a specific low molecular weight nuclear protein fraction. Histones F2A2, F2B, and F3 have little effect on the phosphorylation of non-histone proteins under these conditions. The results suggest that individual histones may be involved in the control of phosphorylation of specific non-histone nuclear proteins, particularly at times when the concentrations of nuclear proteins are changing relative to one another.

Recent investigations suggest that histones may play a role in nuclear protein phosphorylation. For example, the addition of histones to partially purified calf thymus nuclear phosphoproteins stimulates their phosphorylation (9). Added histones have also been shown to effect a dissociation of the regulatory and catalytic subunits of a cyclic AMP-dependent protein kinase from bovine brain (10).

The present study is an examination of the effects of exogenous histone fractions upon the phosphorylation of non-histone proteins in isolated rat liver nuclei. The results indicate that individual histones differ in their effects on the phosphorylation of specific phosphoproteins of hepatic cell nuclei.

MATERIALS AND METHODS

Nuclei were isolated from livers of young male Sprague-Dawley rats by centrifugation through 2.2 M sucrose-3 mM MgCl2, as described previously (8). The nuclei were washed twice with 140 mM NaCl-3 mM MgCl2 and were resuspended in approximately 5 volumes of 5 mM Tris-HCl, pH 7.5, containing 20% glycerol (v/v) and 3 mM MgCl2.

Isolation and Isotopic Labeling of Histone Fractions—Calf thymus histone fraction F1 was obtained by perchloric acid extraction (11) of isolated thymocyte nuclei. Histone fractions F2A1, F2A2, F2B, and F3 were prepared by the method of Johns (12) and Phillips and Johns (13). The F2A1 histone was further purified by the method of Wangal et al. (14).

For studies of the extent of histone binding to isolated nuclei, electron microscope autoradiography, some of the tyrosyl residues of calf thymus histones were iodinated by treatment with 125I in the presence of an oxidizing agent as follows: 100 mg of total histone were dissolved in 2.0 ml of 0.05 M Tris-HCl, pH 7.4, containing 20% glycerol (v/v) and 3 mM MgCl2. Histones F2A1, F2A2, F2B, and F3 were prepared by the method of Johns (12) and Phillips and Johns (13). The F2A1 histone was further purified by the method of Wangal et al. (14).

For studies of histone intranuclear localization by electron microscope autoradiography, some of the tyrosyl residues of calf thymus histones were iodinated by treatment with 125I in the presence of an oxidizing agent as follows: 100 mg of total histone were dissolved in 2.0 ml of 0.05 M Tris-HCl buffer at pH 7.4. To the clear solution were added, in the order listed: 5 mCi (0.1 ml) of Na 125I (New England Nuclear, Inc., Boston, Mass.); 1 ml of 0.05 M phosphate buffer, pH 7.4; 100 μl of 0.01 M KI; and 78 mg of ammonium persulfate. After 4 hours at 25°C, the precipitated protein was dissolved by the addition of 20 μl of β-mercaptoethanol and by acidification with 300 μl of 6 N HCl. The resulting solution was dialyzed against 2-liter portions of 0.01 N HCl three times, and then dialyzed against water in the same way. The histones were precipitated in 10 volumes of acetone, washed with acetone and ether, and dried. The yield of 125I-labeled histone was 85 mg.

For studies of the extent of histone binding to isolated nuclei, histone fractions F2A1, F2A2, and F3 were enzymatically labeled...
with $^3$H-acetyl groups (15). Isolated calf thymus nuclei were suspended in 0.25 M sucrose-3 mM CaCl$_2$ at a concentration of 40 mg of nuclei (dry weight) per ml. The suspension (200 ml) was added to an incubation medium consisting of: 100 ml of 0.1 M sodium phosphate buffer, pH 6.8, in 0.25 M sucrose; 80 ml of 0.1 M glucose containing 3.75 mg of NaCl + 4.2 mg of MgCl$_2$-4H$_2$O per ml; and 20 ml of H$_2$O containing 1 mg of sodium [methyl-$^3$H]acetate with a specific activity of 930 mCi per mmole (New England Nuclear, Inc., Boston, Mass.). The suspension was transferred to a 1-liter low form culture flask in a water bath at 37$^\circ$ and shaker for 15 min. Following incubation, the suspension was quickly chilled to 4$^\circ$, and the individual histone fractions were isolated (11-14).

Histone Binding to Isolated Nuclei—The extent of histone binding to isolated rat liver nuclei was measured at pH values 6.0, 7.0, and 7.5. In all cases, individual histone fractions labeled with [3H]acetate were present at concentrations ranging from 1.0 to 6.0 mg/2.0 ml. In most experiments, aliquots of the nuclear suspension containing 15 mg of total nuclear protein were incubated for 15 min at 0$^\circ$ in 2.0 ml of medium containing 100 $\mu$moles of sodium acetate and 20 $\mu$moles of magnesium acetate. The final pH of the suspension was 6.0. Similar incubations were carried out in 50 mM Tris-HCl buffers at pH 7.0 and pH 7.5. After incubation, the nuclei were sedimented by centrifugation at 1000 x g for 10 min. The pellets were dissolved in 0.5 ml of Soluhene (New England Nuclear, Inc.); 15 ml of Bray's solution (16) were added and the $^3$H activity measured by scintillation spectrometry. Aliquots of the supernatant were counted under identical conditions to determine the fraction of radioactive histone remaining in solution.

Assay for Phosphorylation of Acidic Nuclear Proteins—The transfer of phosphoryl groups from [$\gamma$-$^32$P]-ATP to nuclear acidic proteins was measured under the conditions used for the study of histone binding. The nuclear suspension, after 15 min of exposure to unlabeled histones at 0$^\circ$, was supplemented with 60 $\mu$l of H$_2$O containing 10 $\mu$moles of [$\gamma$-$^32$P]-ATP of specific activity $3 \times 10^9$ cpm per nmole (New England Nuclear, Inc.). After 5 min at 37$^\circ$, the reaction was stopped by the addition of 2.0 ml of 0.5 N HCl. The nuclei were centrifuged and the supernatant containing the histones was decanted. After an additional extraction in 0.25 N HCl, the nuclei were washed successively with chloroform-methanol (1:1, v/v) containing 0.25 N HCl, chloroform-methanol (2:1, v/v) containing 0.25 N HCl, and ether. The nuclear phosphoprotein fraction was extracted in phenol, following the procedure of Teng et al. (8). The nuclear residue, suspended in 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol, was extracted with an equal volume of phenol. The phenol phase was dialyzed against a series of buffers selected to restore the phenol-soluble proteins to an aqueous phase comprising 0.01 M sodium phosphate, pH 7.1, 0.14 M 2-mercaptoethanol, and 0.1% SDS. The isolated phosphoprotein fraction was then further characterized by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS at pH 7.1 (8), applying 150 $\mu$g of protein to each 0.5 cm x 6 mm gel. The gels were stained with Amido black 10B and destained in methanol-acetic acid as described previously (8). Densitometric patterns of the stained protein bands were obtained using a Joyce-Loebl microdensitometer to scan positive transparent photographs of the gel. The distribution of radioactive phosphorus in the gel was determined in two ways. In most experiments, the gels were swollen by immersion in 7% acetic acid and then sliced longitudinally into four slices with a device that held the gels in position while cutting wires traversed their length. Each flat center slice of 1.5-mm thickness was dried under vacuum to form a thin film on Whatman No. 50 paper. The dried slices were then placed in contact with Kodak Royal X-Omat film for 1 to 7 days, and the film was developed. The resulting autoradiographs were scanned with the Joyce-Loebl microdensitometer to indicate the relative activities of the individual protein bands. In the autoradiographic procedure, resolution and intensity of the labeled bands vary with the specific activity of the phosphate incorporated and with the length of time of exposure of the film to the isotope. Since $^{32}$P has a short half-life (11.3 days), autoradiographs prepared on different days using the same lot of [$\gamma$-$^32$P]-ATP would be expected to differ in intensity and resolution. Therefore comparisons have been made only of autoradiographs prepared at the same time. In some experiments, $^{32}$P distribution in the gel was determined by transverse sectioning and counting individual gel slices (7).

Electron Microscope Autoradiography—Samples of rat liver nuclei incubated in the presence of [$\gamma$-$^32$P]-labeled histones were pelleted by centrifugation and fixed in 2.5% glutaraldehyde-0.05 M sodium cacodylate buffer, pH 6.8, for 4 hours at 0$^\circ$. After washing in 0.2 M sucrose at pH 6.8, the nuclei were postfixed in buffered osmium tetroxide, pH 6.8, for 90 minutes at 0$^\circ$. The nuclear pellets were dehydrated through an ethanol series and embedded in Epon. Autoradiographs were prepared by the method of Caro and van Tubergen (17).

Tests for Contamination of Nuclear Acidic Proteins by Histones—The nuclear acidic protein fraction was prepared in phenol (8) from nuclei which had been previously exposed to [3H]labeled histone fractions. ($^3$H activity in the acidic protein fraction would indicate contamination by histones.) The proteins were dissolved in 0.5 ml of Soluhene and counted in Bray's solution as described, using a Packard model 3375 scintillation spectrometer.

RESULTS AND DISCUSSION

Introduction of Histones into Isolated Nuclei—Rat liver nuclei were isolated and exposed to increasing concentrations of individual histone fractions which had been labeled enzymatically with [methyl-$^3$H]acetate (15). After 15 min at 0$^\circ$, the amounts of radioactive histone bound to the nuclei and remaining in the supernatant were determined (Fig. 1). The binding of exogenous histones F2A1, F2A2, and F3 is nearly quantitative under these conditions. More than 95% of the added histone was recovered in the nuclei. Studies of the time course of histone uptake indicated that binding was complete within 5 min. The extent of binding of histone fraction F1 could not be determined in this way because F1 is not a substrate for the histone acetylases and could not be labeled with [3H]acetate. However, it is known that histone fraction F1 is readily adsorbed by nuclei isolated from calf thymus lymphocytes (18, 19) and it has been observed to enter the nuclei of intact cells of the salivary glands of dipteran larvae (20).

When similar binding studies were carried out with histones which had been labeled with $^{131}$I, it became possible to determine the sites of histone adsorption by electron microscope autoradiography. The nuclei were exposed to $^{131}$I-labeled total calf thymus histone for 5 min at 0$^\circ$. Electron microscope autoradiographs show that the isotopic histone enters the isolated rat liver nuclei (Fig. 2). The intranuclear distribution of the added histone was studied by counting and scoring the positions of the grains in more than 50 autoradiographs. The great majority of the grains (74 ± 5%) were found associated with the chromatin inside the nucleus and more than 1 grain length away from the inner nuclear.
Fig. 1. Uptake of exogenous histone fractions by isolated rat liver nuclei. Increasing amounts of histone labeled with $^3$H-acetyl groups were incubated for 15 minutes at 0°C with 2.0-ml aliquots of a nuclear suspension containing 15 mg of total nuclear protein. The distribution of radioactive histone in nuclei, supernatant, and phenol-soluble nuclear proteins is plotted against the amount of histone added. O—O, histone bound to nuclei; •---•, histone remaining in supernatant; Δ—Δ, histone in phenol extract. A, uptake of histone fraction F2A1; B, uptake of histone fraction F2A2; C, uptake of histone fraction F3.

membrane. This provides a conservative estimate of intranuclear binding because approximately 10% of the nuclear cross-sectional area is within 1 grain length of the nuclear membrane. Most of the remaining grains were associated with chromatin located near the nuclear envelope. Very few grains were located directly on the membrane or outside of the nuclear envelope. Control experiments were carried out to test whether the fixation procedures employed for electron microscopy could have extracted labeled histone from the nuclei. Nuclei were exposed to [3H]acetate-labeled F2A1, suspended in 2.5% glutaraldehyde 0.05 M sodium cacodylate buffer, pH 6.8, for 4 hours at 0°C, and then centrifuged to pellet the fixed nuclei. Less than 2% of the labeled histone was removed from the nuclei by this fixation procedure.

FIG. 2. Evidence for intranuclear localization of histone added to rat liver nuclei. Total calf thymus histone, labeled with $^3$H, was incubated with isolated rat liver nuclei for 5 min at 0°C. After washing, the nuclei were fixed, sectioned, stained, and prepared for electron microscope autoradiography (17). Note that histone binding, as revealed by grain distribution, occurs throughout the nucleoplasm. × 15,000.

It follows that the fixation media employed in electron microscope autoradiography do not solubilize histones, and they are not likely to preferentially extract histones from the outside of the nuclei, leaving only those inside the nuclei to be visualized by autoradiography. The fact that binding of exogenous histones occurs throughout the nucleoplasm and is not restricted to the nuclear surface is important in considering their effects on nuclear phosphoprotein metabolism.

The results indicate that the concentrations of particular histones within nuclei can be altered by varying the amount of histone added to the medium. The addition of 10 mg of purified F2A1 to a nuclear suspension containing 15 mg of total nuclear protein raises the intranuclear concentration of that particular histone about 7-fold.

Recent studies of histone binding to calf thymus chromatin show that extra histone has only minor effects on free DNA-phosphate content and template activity (21). This is consistent with the view that the binding of exogenous histones by chromatin fractions involves regions of the DNA which already have some histone (and non-histone) proteins bound to them. There are strong indications that histones interact with nuclear acidic proteins (15, 22-27). The formation of complexes with nuclear phosphoproteins is clearly demonstrable in vitro (15, 18) and is sometimes highly selective (15). Such interactions are likely to occur when individual histone fractions are added to isolated hepatocyte nuclei.

Effects of Histones on Nuclear Phosphoprotein Phosphorylation —The phosphorylation of nuclear acidic proteins has been followed by incubating isolated rat liver nuclei in the presence of [γ-$^3$P]ATP, and subsequently measuring the radioactivity of the phosphoprotein bands separated by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS at pH 7.1 (8). The distribution of $^3$P activity in the proteins of unsupplemented nuclei (controls) is shown in the uppermost curve of Fig. 3. The other curves in Fig. 3 show the distribution of $^3$P activity in
FIG. 3. Effects of exogenous histone F2A1 on [32P]phosphate incorporation into acidic nuclear proteins of isolated rat liver nuclei. Nuclei containing a total of 15 mg of protein were exposed to the indicated amounts of histone F2A1 for 15 min at 0° and subsequently were incubated for 5 min at 37° with [γ-32P]ATP. The nuclear phosphoproteins were extracted and separated by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS (8). The distribution of radioactivity, as determined by autoradiography of longitudinal gel slices, is plotted against the molecular weight of the acidic nuclear protein bands. The autoradiographs were prepared and developed simultaneously.

Regions in which exogenous histones exert notable effects are indicated by ↓. A, control, nuclei with no added histone; B, nuclei + 2 mg of F2A1; C, nuclei + 4 mg of F2A1; D, nuclei + 8 mg of F2A1.

Different histone fractions differ in their effects on nuclear acidic protein phosphorylation. Fig. 4 illustrates a comparison of the effects of exogenous F1 and F2A1 histones on 32P uptake into the phosphoproteins of isolated rat liver nuclei. (All the autoradiographs upon which the data in Fig. 4 are based were prepared and developed simultaneously, but not at the same time as the autoradiographs shown in Fig. 3.) While both histones inhibit the phosphorylation of a low molecular weight protein fraction (Region II, Fig. 4), each histone stimulates phosphorylation of a different set of nuclear proteins. Histone F2A1 augments phosphate uptake into a protein fraction of approximate molecular weight 22,000 (Region II, Fig. 4), while the most striking effect of histone F1 is a stimulation of phosphorylation of proteins in the molecular weight range 30,000 to 60,000 (Region I, Fig. 4).

Histones F2A2, F2B and F3 were also tested for their effects on the phosphorylation of different non-histone nuclear proteins. These histone fractions had only a slight influence on 32P incorporation into the nuclear phosphoproteins.

The magnitude of the effects produced by different histones on the phosphorylation of different non-histone proteins of rat liver nuclei are summarized in Table I. The specific 32P activities are presented for proteins in three regions of the polyacrylamide gel in which the effects of histones were notable, as well as for the total non-histone protein fraction. For example, proteins in Region I incorporate about 35 pmol of phosphate per mg of protein in the absence of added histone F1 and about
FIG. 4. Comparison of effects of exogenous histones Fl and F2Al on phosphorylation of rat liver nuclear acidic proteins. Isolated liver nuclei containing a total of 15 mg of protein were exposed to the individual histone fractions for 15 min at 0° and subsequently were incubated with [γ-32P]ATP as described under “Materials and Methods.” The distribution of 32P activity is plotted against the molecular weight of the acidic nuclear protein bands. The autoradiographs were prepared and developed simultaneously. Regions in which exogenous histones exert notable effects are indicated by ↓. Note the differences in effects of Fl and F2Al on stimulation of nuclear protein phosphorylation in Regions I and II. Both histones inhibit phosphorylation of a low molecular weight protein fraction in Region III. A, control, nuclei with no added histone; B, nuclei + 12 mg of histone F2Al; C, nuclei + 12 mg of histone Fl. 50 pmoles phosphate per mg of protein when Fl is added. Proteins in Region II incorporate about 39 pmoles of phosphate per mg of protein in the absence of histone F2Al and about 68 pmoles per mg in its presence. Phosphorylation in Region III is very active; proteins in this size category incorporate about 285 pmoles of phosphate per mg of protein in the absence of added histone. Histones Fl and F2Al inhibit phosphorylation in this region by 60 and 85%, respectively. Because of the contrasting effects of histones on phosphorylation of different nuclear acidic proteins, the over-all level of incorporation shows little change (Table I, Column 5).

Addition of exogenous histones to isolated nuclei has no effect upon the final recovery of total non-histone protein and, according to densitometric analysis of the polyacrylamide gel patterns, no effect on the final recoveries of individual non-histone protein fractions. However, the percentage of protein comprising phosphorylated species in a particular region of the gel cannot be determined, and it is possible that changing amounts of minor phosphorylated protein species could contribute to the observed changes in specific activity.

Control experiments have established that none of the observed shifts in radioactivity patterns represent contamination by phosphorylated histones. The acidic protein fractions isolated as described (8) do not show the presence of histone bands when subjected to analytical electrophoresis by the Panyim and Chalkley procedure (29), nor can the presence of radioactive histone bands be detected. In addition, treatment of nuclei with [H-]acetyl-labeled histones does not lead to contamination of the nuclear phosphoprotein fraction (Fig. 1). Under the conditions of these experiments, endogenous histone F2Al shows negligible 32P uptake. Histone Fl is phosphorylated in isolated rat liver nuclei but endogenous Fl does not contaminate the acidic protein fraction, and exogenous Fl induces changes in the phosphorylation of proteins of molecular weights very different from that of Fl itself.

Similar responses are observed when individual histone fractions are added to suspensions of isolated calf thymus nuclei. The histones have differential effects on the phosphorylation of specific acidic proteins, but the magnitude of the stimulation or inhibition is less than that observed with rat liver nuclei. In
both nuclear types the inhibition of phosphorylation of a low molecular weight fraction is pronounced. Comparisons have also been made of calf thymus nuclei isolated in isotonic sucrose solutions (30) with those prepared in nonaqueous media (31). In both cases exogenous histones modify the phosphorylation of nuclear acidic proteins in a similar fashion.

The interaction between histones and acidic chromosomal proteins is evident in two ways. The effects on phosphorylation show that different histones may modify the structure and metabolic activity of individual nuclear phosphoproteins. Conversely, it is known that an acidic protein fraction which binds selectively to histones F2A1 and F3 functions as a histone deacetylase (15). This mutual interdependence of enzyme systems regulating acetylation of histones and phosphorylation of nuclear acidic proteins may represent an important aspect of interlocking controls in the regulation of genetic activity.

The relative concentrations of histones and nuclear acidic proteins would be expected to vary during the cell cycle since it is known that histone synthesis occurs during the S phase (32, 33), whereas the synthesis of individual non-histone proteins proceeds throughout the cell cycle (34, 35). The possibility that alterations in intranuclear histone concentration during S might differentially affect the phosphorylation of acidic proteins involved in genetic control mechanisms should be mentioned because it is known that the induction of some enzymes (e.g., alkaline phosphatase induction in HeLa S3 cells treated with hydrocortisone (36)) is limited to the synthetic phase of the cell cycle. This response is known to require new messenger RNA synthesis (39) and the rate of RNA synthesis doubles shortly after the HeLa S3 cell enters the S phase (40).

We have recently observed that the rate of phosphorylation of HeLa nuclear acidic proteins varies during the cell cycle, with high rates occurring in S and low rates in the period, late G2 to M, when RNA synthesis is suppressed (41). Whether such changes in nuclear protein phosphorylation are causally related to altered patterns of transcription remains to be determined, but the temporal correlations are clear.

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