An H3 Histone-specific Kinase Isolated from Bovine Thymus Chromatin

Charles R. Shoemaker† and Roger Chalkley
From the Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52242

(Received for publication, August 1, 1977, and in revised form, November 30, 1977)

A substantial portion of the histone phosphorylating activity of bovine thymus chromatin can be isolated by extraction in 0.2 M NaCl. The specificity of this extract for either free histones or washed chromatin substrates was compared. The salt-extracted kinase enzyme favors H2b as the major acceptor when whole free histone is the substrate and H3 when the substrate is intact chromatin.

The H3 kinase activity of bovine thymus tissue has been purified free from other detectable histone kinase activities by ammonium sulfate fractionation and is highly specific for H3 histone when assayed either with chromatin or isolated whole histone. The activity is cAMP-independent. Tryptic peptide mapping of the labeled H3 histone reveals a single site of phosphorylation. This site appears to be identical with the major site of metaphase-associated H3 phosphorylation in hepatoma tissue culture cells. No corresponding H3 phosphorylation has been detected in thymus tissue in vivo.

A variety of in vivo histone phosphorylation events have been characterized in recent years. Langan (1) reported a cAMP-dependent phosphorylation of H1 histone in rat liver and suggested its role in the regulation of transcription. Phosphorylation of H1, H4, H2a, and H3 histones has been described in dividing cells. H1 phosphorylation has been observed in many cell types at specific times during the cell cycle (2-5) and has been implicated as functioning both in DNA synthesis and cell division. Specific phosphorylation of newly synthesized histones H1, H2a, and H4 has been reported which may be involved in the deposition of histone on DNA (6-8). H3 histone is phosphorylated immediately prior to and during mitosis and has been argued to have a role in chromatin condensation (9).

The study of in vivo histone phosphorylation has suffered from a lack of information concerning the specific enzymes involved. Histones in solution are excellent substrates for many protein kinases (10) and indeed have frequently been used to assay for the kinase activity of enzymes extracted from a lack of information concerning the specific enzymes involved. Histones in solution are excellent substrates for many protein kinases (10) and indeed have frequently been used to assay for the kinase activity of enzymes extracted from

Generally the preferred substrates. Workers utilizing nuclear incubation systems have obtained varying results. Thus Klein-smith et al. (11) incubated lymphocyte nuclei with [33P]orthophosphate and observed incorporation in several fractions with H1 the most extensively modified. In contrast, Rickwood et al. (12), utilizing mouse liver, brain, and kidney nuclei, concluded that H2a was the primary acceptor, although they observed H1 was the most highly modified when nuclei from dividing L 1210 cells were used. However, in this earlier work the specific phosphopeptides modified in vitro were not identified and compared with those observed in vivo. This comparison is all the more important since many of these systems were from nondividing tissues which show only very low levels of histone phosphorylation in vivo.

Both Langan (13) and Lake (14) have compared the sites of H1 modification in vivo to those obtained upon incubation of partially purified enzymes with a free H1 substrate as a means to identify the physiological H1 kinase enzymes.

In this paper we study the endogenous histone phosphorylating activity of bovine thymus chromatin, comparing histone kinase specificity for free histone and chromatin substrates. A new cAMP-independent protein kinase with distinct specificity for H3 has been partially purified and evidence is presented that the phosphorylation site is identical with that recurring during metaphase-associated H3 phosphorylation in HTC cells.

**MATERIALS AND METHODS**

**[γ-32P]ATP Synthesis**—The method employed is a modification of the method used by Post and Sen (15). Ten milliCuries of [32P]orthophosphate (Amerham/Searle) in 1.2 ml of dilute HCl were neutralized with 0.3 ml of 0.5 M Tris-HCl, pH 8.0. The final reaction mixture containing the [32P]orthophosphate in a solution (2.5 ml) of 1 mM ATP, 0.2 mM ADP, 1 mM phosphoglyceric acid, 0.04 mM NAD*, 2 mM MgCl2, 10 mM 2-mercaptoethanol, 7 units of 3-phosphoglyceric phosphokinase (Sigma), and 4 units of 3-phosphoglyceric phosphokinase (Sigma) was incubated at 35°C for 30 min and quenched by boiling for 5 min. After cooling, the mixture was centrifuged at 12,000 x g for 30 min, and the supernatant was stored at -20°C. The extent of the reaction was determined using ascending cellulose-polyethyleneimine (PEI) chromatography in 0.5 M potassium phosphate buffer, pH 3.45. [32P]orthophosphate and [γ-32P]ATP spots were located by autoradiography, cut out, and counted on a model 1042 Nuclear Chicago gas-flow counter. Yields of 80 to 90% [γ-32P]ATP were usually obtained.

**Chromatin and Histone Preparation**—Chromatin and histone preparations were prepared as described by Panyim et al. (16). Chromatin was solubilized by shearing to top speed in a Vir-Tis "45" homogenizer for 1 min.

**Histone Fractionation**—Individual histone fractions were obtained by the modified procedure of John (17) as described by Oliver et al. (18).

**Polyacrylamide Gel Electrophoresis**—All gel electrophoresis was performed by the method described by Panyim and Chalkley (19). The 9-cm gels contained 2.5 M urea and were run at room temperature. The 24-cm gels contained 1 M urea and were run at 4°C in an atmosphere of a 2-5% dimethyl sulfoxide/methanol solvent system.
electrophoresis buffer containing 1 M urea. Electrophoresis was terminated when the blue component of a methyl green tracking dye reached the bottom of the gel.

**Endogenous Histone Kinase Assay**—Nuclear suspensions or chromatin solutions were adjusted to a final concentration of 2 mg of DNA/ml (A260 = 40) in a 1-ml volume of 0.4 M MgCl₂ and 10 mM Tris-HCl, pH 7.4 (60 mM salt-washed chromatin is sonication to suspension). Fifty microliters of [γ-³²P]ATP (1 μM ATP, 2 × 10⁶ cpm/nmol) solution were added and the mixture incubated for 5 min at 37°C. The reaction was quenched by addition of 5 ml of a cold solution containing 12.5 mM EDTA and 100 mM Tris-HCl, pH 8.0, centrifuged at 7000 × g for 5 min, and the pellet washed once in the same buffer. The pellet was extracted by 1 ml of 6 M H₃O₄ overnight at 4°C and spun at 12,000 × g for 10 min. The supernatant was dialyzed against H₂O (1000 volumes) for at least 4 h and adjusted to 20% sucrose, 0.9 M acetic acid, and 0.5 M 2-mercaptoethanol. The final volume was 1.6 ml. An aliquot (100 μl) was then applied to a 24-cm polyacrylamide gel for electrophoresis. The histone region of the stained gel was sliced into 2-mm sections, dried at 60°C, and counted on a Nuclear Chicago gas-flow counter.

**Histone Kinase Assay using Chromatin Substrate**—Chromatin containing 2 mg of DNA (A₂₆₀ = 40) was adjusted to 0.2 M NaCl, 10 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4. After an hour at 4°C the precipitated salt-washed chromatin was pelleted by centrifugation at 7000 × g for 5 min and resuspended by sonication in 10 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4 (1 ml) containing the histone kinase to be assayed. The assay was then carried out in the same way as for the endogenous histone kinase.

**Histone Kinase Assay using Histone Substrate**—Bovine thymus histone was dissolved in a solution of 10 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4, and dialyzed against 100 volumes of the same buffer. A 1-ml solution was prepared containing 2 mg of histone (A₂₆₀ = 5.6), histone kinase, 10 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4. Fifty microliters of [γ-³²P]ATP (1 μM ATP, 2 × 10⁶ cpm/nmol) were added and the mixture incubated at 37°C for 6 min before quenching by addition of 10 μl of 36 M H₃O₄. Following dialysis against H₂O (1000 volumes), histone was analyzed for phosphorylation in the same way as for the endogenous histone kinase assay.

**ATPase Assay**—To monitor for the presence of ATPase activity during histone kinase assays an aliquot (approximately 1 μl) was removed from the assay reactions immediately prior to quenching, placed on cellulose-polyethyleneimine paper, and dried. Ascending chromatography was performed as previously described and analyzed for any significant changes in the percentage of [γ-³²P]ATP.

**Tryptic Peptide Mapping and Autoradiography**—All protein solutions were extensively dialyzed against H₂O and adjusted to 10 mM ammonium acetate, pH 8.0, prior to tryptic mapping. Digestion was initiated by addition of 10 μl of a 1 mg/ml solution of trypsin (twice crystallized, Worthington). After stirring for 1 h at room temperature and freeze drying, the trypsin solution was added and stirred overnight. The solution was then lyophilized and the trypic peptides dissolved in H₂O. An aliquot was spotted on Whatman No. 3MM chromatography paper and dried. Descending chromatography was performed overnight in a buffer containing 1-butanol: acetic acid: water: 2-mercaptoethanol. The final pattern of the H3 histone kinase shown in Fig. 1 represents the phosphorylation profile into the histone region of a gel. A substantial amount of label has become incorporated into the H3 histone region with significant incorporation into H1 and lesser amounts into the other three histone regions (phosphohistones move slightly slower than parental histones due to the negatively charged phosphates). Although the extent of phosphate incorporation into H3 represents about one phosphate group per 10⁵ H3 molecules, these levels were obtained after short incubations and under conditions which were not fully optimized. More recently, techniques have been developed which substantially increase the phosphate incorporation into H3 (see below). The pattern of ³²P incorporation into histone shown in Fig. 1 has proven to be very reproducible although minor variations between preparations do occur, particularly involving the H1 histone region.

**EVIDENCE OF H3 PHOSPHORYLATION**—Several observations have confirmed that H3 histone has indeed been modified under our incubation conditions.

1. H3 histone isolated from bovine thymus is found in parental, mono-, and diacetylated forms. Each of these forms can be resolved on polyacrylamide gels as seen in Fig. 1. Phosphate incorporation into the H3 histone region of the gel follows the distribution profile that would be expected if H3 and its acetylated products were to be phosphorylated.

2. Histone H3 was labeled with ³²P as in Fig. 1 and H3 was purified to homogeneity by chemical fractionation (18). A polyacrylamide gel of the purified H3 was shown to have ³²P associated strictly with the H3 region. In addition a tryptic digest of the H3 fraction was found to contain a phosphopeptide with identical electrophoretic and chromatographic mobility to the phosphate peptide found during in vivo mitotic labeling of H3 (Fig. 3).

C. B. Shoemaker and R. Chalkley, manuscript in preparation.
5804 H3 Histone Kinase

Experimental systems were employed in an effort to determine histone labeled in vitro by the endogenous kinase action of purified H3 histone (Fig. 3c) demonstrates that the in vivo tryptic mapping as the H3 phosphopeptide obtained from the histone fraction. Following such a reaction, a distinct shift in H3 protein to the slower moving modified forms can been shown to contain phosphate by the presence of [2-35]P. The histones were isolated, separated on polyacrylamide gels, and examined for phosphorylation. The [35]P incorporation into H3 increased approximately 400% over that of cells not exposed to colcemid, consistent with the results of Gurley et al. (9). An autoradiogram of a tryptic map of the purified H3 histone (Fig. 3c) demonstrates that the in vivo phosphorylation of H3 histone occurs primarily on a single tryptic peptide. This peptide has the same mobility upon tryptic mapping as the H3 phosphopeptide obtained from histone labeled in vitro by the endogenous kinase action of bovine thymus chromatin.

In Vivo Histone Phosphorylation in Thymus—Two experimental systems were employed in an effort to determine...
Histone Kinase 5805

Electrophoresis pH 6.5

+ --- Electrophoresis pH 6.5 --- +

Fig. 3. Tryptic mapping and autoradiography of purified 32P-labeled H3 histone. (a) is a tryptic map of bovine thymus H3 stained with “color dip” (23). Autoradiography was performed on tryptic maps of purified H3 following (b) endogenous histone kinase assay of bovine thymus chromatin or (c) in vivo labeling of colcemid-blocked HTC cells with 32PO4. Histone fractionation, tryptic mapping, autoradiography, and 32P-labeling procedures are described under “Materials and Methods.”

Fig. 4. The 32P incorporation profiles of the histone region of 24-cm polyacrylamide gels. 32P-Histones were obtained following the in vivo labeling of rat thymus (●—●) or in vitro labeling of isolated rat thymus nuclei (○—○). In vivo and in vitro 32P labeling was performed on 3-month-old male Sprague-Dawley rats as described under “Materials and Methods.” The dotted line is a scan of the histone region of a gel of rat thymus histone at 600 nm (arbitrary units).

Extraction and Analysis of Histone Kinases of Bovine Thymus Chromatin

Extraction—A buffered solution containing 0.2 M NaCl effectively solubilizes much of the endogenous histone phosphorylating activity of bovine thymus chromatin (Table I). A salt wash of this type will consistently extract greater than 60% of the enzymatic activity. When the endogenous histone kinase assay is performed in the presence of 0.2 M NaCl, H1 phosphorylating activity is reduced to about 40% of that in chromatin at low ionic strength while H3 phosphorylating activity remains the same. Thus solubilization appears to have no effect on the ability of endogenous kinase to phosphorylate H3 in chromatin.

Histone Kinase Specificity for Chromatin or Free Histone Substrates—The salt extract was assayed for its histone kinase activity on both salt-washed chromatin and soluble histone (Table II). The salt extract retains the same histone kinase activity and specificity for chromatin as seen before extraction. Keeping the assay conditions as similar as possible, the salt extract has significantly more activity for soluble histone than for salt-washed chromatin. In addition, substantial 32P is now detected in the H2b region of the gel. This “H2b” phosphorylating activity is cAMP-dependent and is particularly evident in the cytoplasm and in impure nuclei.

Partial Purification of H3-Kinase—Further study was initiated in an effort to distinguish the H3 phosphorylating activity from other histone kinases that are present in chromatin. The 0.2 M NaCl extract of bovine thymus chromatin was fractionated on the basis of solubility in ammonium

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts per min in phosphohistone region of gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>1910 3030 460 350 230 170</td>
</tr>
<tr>
<td>H3</td>
<td>450 1230 160 100 90 90</td>
</tr>
<tr>
<td>H2b</td>
<td>820 3140 660 460 200 190</td>
</tr>
<tr>
<td>H2a</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td></td>
</tr>
<tr>
<td>Back-</td>
<td></td>
</tr>
<tr>
<td>ground</td>
<td></td>
</tr>
</tbody>
</table>

### Table II

Histone kinase activity of 0.2 M NaCl extract of bovine thymus chromatin employing chromatin or soluble histone substrates

A solution (1 ml) consisting of bovine thymus chromatin (A260 = 50), 0.2 M NaCl, 10 mM MgCl2, and 10 mM Tris-HCl, pH 7.4, was prepared. After an hour at 0°C the chromatin was pelleted at 12,000 X g for 10 min. Two hundred microliters of the supernatant (0.2 NaCl extract) was used in each assay. Assays were performed as described under “Materials and Methods,” except that 0.2 M NaCl was present. Background was calculated as for Table I. Phosphorylation due to the endogenous histone kinase activity remaining in the salt-washed chromatin has been subtracted. Soluble thymus histone has no endogenous kinase activity.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Counts per min in phosphohistone region of gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>300 770 370 130 150 100</td>
</tr>
<tr>
<td>H3</td>
<td>600 2500 3080 180 150 90</td>
</tr>
<tr>
<td>H2b</td>
<td></td>
</tr>
<tr>
<td>H2a</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td></td>
</tr>
<tr>
<td>Back-</td>
<td></td>
</tr>
<tr>
<td>ground</td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on October 31, 2017
sulfate of increasing concentration. Fig. 5 demonstrates that the H3 kinase activity precipitates in the 50 to 70% saturation range and, in this way, can be separated from a major portion of the protein in the salt extract (the ammonium sulfate fractionation is significantly more effective when performed in separate 10% cuts than when a 50% saturation cut is immediately employed). From the results in Table III and Fig. 6 it can be seen that the H3 kinase activity present in the

50 to 70% ammonium sulfate cut has also been purified from all other detectable histone kinases present in the salt extract and as such is extremely specific for H3 histone. No qualitative difference in either the histone specificity or the phosphorylation site can be observed whether the substrate is free histone or chromatin. Physiologically active concentrations of cAMP do not activate the H3 kinase activity for free histone and may actually provide a slight inhibition (Table III). The heat-stable inhibitor of cAMP-dependent protein kinase did not inhibit the H3-kinase.

**Table III**

Histone kinase activity of 50 to 70% ammonium sulfate fraction of 0.2 M NaCl extract of bovine thymus chromatin

Ammonium sulfate fractionation of 0.2 M NaCl extract of bovine thymus chromatin was performed, on a larger scale, similar to that described in Fig. 5. Fifty microliters of the 50 to 70% ammonium sulfate cut were used in each assay. Assays were performed as described under "Materials and Methods" except that 0.2 M NaCl was present. Fifty microliters of 10^{-4}M cAMP was added, where indicated, prior to the final volume adjustment. Background was calculated as for Table I. Phosphorylation due to the endogenous histone kinase activity remaining in the salt-washed chromatin has been subtracted.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>At 4.75 x 10^{-4} M cAMP</th>
<th>Counts per min in phosphohistone region of gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt-washed thymus</td>
<td>-</td>
<td>180, 1440, 150, 230, 130, 180</td>
</tr>
<tr>
<td>Histone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble thymus</td>
<td>-</td>
<td>90, 3650, 160, 100, 90, 60</td>
</tr>
<tr>
<td>Histone</td>
<td>+</td>
<td>70, 3060, 160, 80, 70, 60</td>
</tr>
<tr>
<td>Soluble thymus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

Neutron scattering of chromatin (24) has shown that the DNA is wrapped around a core of non-H1 histones. As a consequence, chromosomal histones might be expected to be largely protected from the more nonspecific actions of protein kinases and thereby provide a substrate more capable of unambiguously identifying those histone kinases whose function is to phosphorylate non-H1 histone in vivo. Since protein kinase activity requires the presence of Mg^{2+} at a concentration which causes the precipitation of chromatin, we have employed sheared chromatin which forms a fine suspension under these conditions. Noll et al. (25) have reported that chromatin formed by extension at low ionic strength and shearing has lost the characteristic nuclease digestion pattern observed for nuclei. This observation suggests that sheared chromatin may have lost some of its native structure. We have found that despite any structural changes that may have

...
occurred, the endogenous histone kinase activities of sheared chromatin prepared from a variety of different tissue sources, retain the same histone specificity as that found in the nuclei from which it was prepared.4

The finding of substantial H3 phosphorylating activity in bovine thymus chromatin was unexpected and led us to analyze it further for possible physiological relevance. Very little bovine thymus chromatin was prepared when cell division, bovine thymus was thought to have a moderately high mitotic index (27, 28). With this rate of cell division, bovine thymus would be expected to have detectable in vivo H1 and H2a phosphorylation (2-5). Thus we cannot confidently ascribe our observation of H3 kinase activity to the presence of an enzyme which modifies this histone. When the barriers of compartmentalization are destroyed during homogenization, H3 becomes available to modification with high specificity.

Most of the endogenous histone kinase activity of bovine thymus chromatin can be solubilized in 0.2 M NaCl while retaining its activity and specificity. The salt extract so obtained can restore histone kinase activity to salt-washed chromatin or can phosphorylate soluble histone. A distinct difference exists in the specificity of the various histone kinases in the salt extract toward soluble histone or histone in its chromatin state. For instance, phosphorylation of H2b occurs much more efficiently in its free form while H3 histone is a good substrate in either form. In a similar experiment Chae et al. (29) have compared the histone specificity of a cAMP-dependent protein kinase isolated from rat liver for free histone or chromatin. They found H2b to be heavily phosphorylated when assayed in its free state yet no phosphorylation of H2b in chromatin was detected. In contrast H1 was effectively phosphorylated in both forms by the enzyme. These observations suggest that the structure of chromatin provides significantly more protection for some histone sites than for others and may indeed be a better substrate for the detection of true histone kinases than soluble histone. In addition, these results reveal the potential use of protein kinases as a probe of chromatin structure once the phosphorylation sites have been determined for the individual histones.

Ammonium sulfate fractionation of the salt extract makes possible the isolation of the H3 phosphorylating activity from other histone kinase activities. It reveals the presence of a cAMP-independent chromatin kinase with remarkable specificity for H3 histone. The enzyme phosphorylates a single site on H3 whether supplied chromatin or isolated whole histone as substrate. The high level of specificity and the ability to phosphorylate H3 histone in chromatin lends support to the idea that H3 is a physiological substrate of the enzyme. It is particularly intriguing that the phosphorylation site appears to be the same as occurs in vivo in HTC cells. Whatever the true function of the H3 kinase in thymus, the enzyme can be exploited to obtain substantial yields of phospho-H3 for efforts such as physical studies on the effects of phosphorylation on histone and chromatin structure or the determination of the site of phosphorylation.

Acknowledgments—We wish to thank Ms. Tracy Hart for her valuable technical assistance. We are also indebted to Doctors R. Roskoski and D. K. Granner as well as the members of our laboratory for their helpful discussion and critical reading of the manuscript.

REFERENCES

An H3 histone-specific kinase isolated from bovine thymus chromatin.
C B Shoemaker and R Chalkley


Access the most updated version of this article at [http://www.jbc.org/content/253/16/5802](http://www.jbc.org/content/253/16/5802)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/253/16/5802.full.html#ref-list-1](http://www.jbc.org/content/253/16/5802.full.html#ref-list-1)