Histone 4 Phosphotransferase Activities in Proliferating Lymphocytes

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ENZYME SPECIFIC FOR SER-47*

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RUTHANN A. MASARACCHIA,‡ BRUCE E. KEMP,§ AND DONAL A. WALSH

From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

Two histone H1 phosphotransferase activities are observed in DE52 chromatography or sucrose gradient ultracentrifugation of murine lymphosarcoma cell extracts. One of these enzymes (H4-Kinase-(I)) has been purified several thousandfold by ion exchange and Sephadex G-200 chromatography. H4 was the only phosphorylated product detected when an equimolar mixture of histones H1, H2A, H2B, H3, and H4 was incubated with H4-Kinase-(I) and [γ-32P]ATP. The phosphorylation rates of H2A, H2B, H1, or H3 was <20% of that observed with H4. The apparent K_m values for ATP and H4 were 69 and 16 μM, respectively. The molecular weight of H4-Kinase-(I), as estimated by gel filtration, is 102,000, consistent with an S_value of 5.2 obtained from sucrose gradient centrifugation. H4-Kinase-(I) catalyzed the formation of 1.02 mol of phosphoserine/mol of H4. Peptides corresponding to the two amino acid sequences containing serine in H4 have been synthesized. The peptide Ac-Ser-Gly-Arg-Gly-Gly-Lys-Gly-Gly (residues 1 to 7) was not a substrate for H4-Kinase-(I) when tested at concentration of up to 1 mM. In contrast, the second peptide containing residues 43 to 49 with an added COOH-terminal glycine (i.e. Val-Lys-Arg-Ile-Ser-Gly-Leu-Gly) was phosphorylated with an apparent K_m of 35 μM. It is concluded that H4-Kinase-(I) specifically phosphorylates Ser-47 in Histone H4. The second H4-phosphotransferase (H4-Kinase-(II)) was partially purified by ion exchange chromatography and ammonium sulfate precipitation. H4-Kinase-(II) phosphorylated the H4 peptide containing Ser-1 (residues 1 to 7) but not the H4 peptide containing Ser-47 (residues 43 to 49).

Phosphoryl groups, as esters of either seryl (1-5), threonyl (2, 3, 6), or histidyl moieties (7), are important substituents of histones. Extensive evidence indicates a dynamic role for post-translational phosphorylation or dephosphorylation of these proteins with such modifications potentially associated with DNA replication, gene expression, the orderly assembly of the υ body, and/or the separation of the genome at cell division. In the developing trout testis Dixon and his associates (8-11) have indicated that all five major histones (H1, H2A, H2B, H3, and H4) can be phosphorylated. Four of these five histones, with H2B excepted, have been shown to be phosphorylated in somatic and cultured mammalian cells in response to a variety of stimuli (13-24).

The phosphorylation of H4 in the developing spermatocyte (8, 9) and in dividing duck erythrocytes (25) and of H2A in developing spermatocytes (8-11) and in hepatic regeneration of the rat (23) occurs at the NH2-terminal acetyl serine in the identical sequence Ac-Ser-Gly-Arg-Gly-Lys-Gly. However, a consistent pattern of control of phosphorylation of H2A and H4 is not apparent. H4 phosphorylation in the developing spermatocyte does not occur until several hours after synthesis of the histone, whereas in the duck erythrocyte H4 phosphorylation is associated with cytoplasmic synthesis and rapid dephosphorylation occurs after H4 enters the nucleus. Similarly, H2A is phosphorylated shortly after synthesis in developing spermatocytes but, following partial hepatectomy, phosphorylation occurs on H2A present prior to the initiation of regeneration. Low levels of H4 phosphorylation and high, relatively constant levels of H2A phosphorylation occur throughout the cell cycle of rapidly proliferating Chinese hamster ovary cells (15, 16), HeLa cells (22), and HTC cells (21), but specific sites have not been identified. A specific H4 kinase has been identified (7) that catalyzes the formation of phosphohistidyl-H4 during liver regeneration (26).

In both rapidly proliferating mammalian cells (15, 16, 20, 21) and trout spermatocytes (8-11) H1 is phosphorylated at multiple sites. During sperm maturation Dixon and his associates have shown there is a complex sequential pattern in which 3 serine residues in the COOH-terminal portion of the protein are phosphorylated at identical sequences of Lys-Ser-Pro-Lys. In mammalian cells multiple phosphorylation of H1 occurs in late G1 and continues through M after which the histone is dephosphorylated (16). It is probable that a portion of this phosphorylation is catalyzed by a growth-associated histone kinase described by Langan and co-workers (27, 28).

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‡ Special Fellow of the National Institutes of Health.

§ Present address, Clinical Biochemistry, Flinders University of South Australia, Bedford Park 5042, South Australia.
by Hohmann et al. (29), by Lake and Salzman (13) and Lake
(30). This enzyme phosphorylates seryl residues 16 and 80
and threonyl residues 16, 153, and 180 of H1; the amino acid
sequences at positions 16, 153, and 180 are similar to that
observed in trout testis, i.e. Lys-[Ser-Thr-Pro-Lys-Langan (31,
32) has identified Ser-38 and Ser-106 of H1 as the sites
phosphorylated by the cAMP-dependent protein kinase and
an enzyme designated IIK2, respectively.

Histone H3 is phosphorylated to a limited extent during
spermatocyte maturation (11) and during mitosis in mamma-
lian cells (15) although the phosphorylation site(s) is not
known. Histone 2B is the best histone substrate for the
phosphotransferases. The CAMP-dependent protein kinase has
phosphorylated by the CAMP-dependent protein kinase and
identified as phospho-H4 in vivo

Although the sites of histone phosphorylation and their
potential physiological significance is beginning to be docu-
mented, our current knowledge of the responsible protein
kinesases is still fragmentary. Pioneering studies of Lake and
Salzman (13) and Lake (30) and of Langan and co-workers (1,
6, 27, 28, 31, 32) have initiated the characterization of the H1
phosphotransferases. The cAMP-dependent protein kinase has
been extensively characterized, but this enzyme may be of
minimal significance in histone phosphorylation. This manu-
script reports a continuation of our previous studies (33) of
the characterization of protein kinase activities in proliferat-
ing lymphocytes. Two enzymes with activities toward H4 are
recognized, one apparently specific for Ser-1, the other for
Ser-47. The latter enzyme which accounts for the greater
proportion of activity has been partially purified and a prelimi-
nary characterization is described.

EXPERIMENTAL PROCEDURES

Tissue Source and Extract Preparation - Cortisol-sensitive (P1798/
5) lymphosarcomas were serially transplanted subcutaneously in 6-
to 8-week-old BALB/c mice. Tumors (20 to 50 g total) were removed
14 to 20 days after implantation and washed in cold 0.9% NaCl
solution. All subsequent steps were carried out at 4°C. Cells were
prepared in RPMI 1640 culture media obtained from Grand Island
Biological Co. by the procedure of Rosen et al. (34). Cell pellets
were homogenized with a Dounce homogenizer in 10 mM Tris/chloride
(pH 7.4) containing 2 mM EDTA, 0.1 mM phenylmethylsulfonyl
fluoride, and 15 mM β-mercaptoethanol (Buffer A). The homogenate
was centrifuged at 12,000 × g for 10 min. The pellet obtained
was rehomogenized in 0.2 M KCl in Buffer A, and this suspension
was centrifuged at 12,000 × g for 10 min. The supernatant
solution was combined with that obtained from the initial centrifu-
gation. The combined supernatant solutions were centrifuged at
270,000 × g for 90 min. The enzyme solution obtained by this
procedure was desalted by gel filtration on a column (1.4 × 24 cm)
of Sephadex G-25 equilibrated with Buffer A. This fraction, design-
ated as the "high speed supernatant extract," was utilized as the source of enzyme for preliminary characterization of
multiple histone kinase activities (Figs. 1 and 2) and as the starting
point for H4 kinase purification.

Assay of Protein or Peptide Phosphotransferase Activity - Phospho-
transferase assays were performed by adaptations of the methods of
Reimmann et al. (35) or Kemp et al. (36). The reaction mixture
contained 2 μmol of MES buffer (pH 6.8), 1 μmol of MgCl2, and 10
μmol of [γ-32P]ATP (100 to 200 dpm/μmol) in a total volume of 100
μl. Appropriate substrates and enzymes were added as noted in
individual experiments. All incubations were at 30°C for 10 min
unless specified otherwise. The amount of 32P-labeled product
was determined by one of three procedures. 32P-labeled proteins (H2A,
H2B, H3, H4, histone mix, etc.) were isolated by Assay II according
to the procedure of Reimmann et al. (35) with the following two
exceptions. In the experiment of Fig. 4A in which low concentrations
of H4 were used the ethanol and ether washes were replaced by an
acetic wash. In all experiments measuring incorporation into H1,
18% trichloroacetic acid was used. 32P-labeled proteins were isolated
by Assay II. A 50-μl aliquot of reaction mixture was pipetted onto
a 24 cm paper chromatography plate (Whatman-P3; 25 cm). The plate
was dried, and immersed in 30% acetic acid at 0°C. [γ-32P]ATP was removed by sequential washing with 30% acetic acid at 0°C,
15% acetic acid at 0°C (twice), 15% acetic acid at 25°C (twice), and acetone. Ten milliliters of each solvent was used per paper; all other conditions of the
washing procedure were as described by Reimmann et al. (35). Alter-
natively, 32P-labeled proteins or peptides were isolated by the procedure of Kemp et al. (36) utilizing Dowex 1-X8 (acetate) chromo-
matography in 30% acetic acid (Assay III). With H4 as substrate
identical results were obtained by all three assays. One unit of H4 kinase is defined as that amount of enzyme which
will catalyze the transfer of 1 pmol of phosphate from ATP/ min
at an assay concentration of 35 μM H4.

Preparation of Histones - Histone fractions H1, H2B, H3, and (H4 + H2A) were prepared from calf thymus by the procedure of Johns
et al. taking the precaution to inhibit proteolysis during tissue extrac-
tion by the addition of bisulfite (38). Subsequent separation of H4
and H2A was performed by gel filtration on Bio-Gel P-60 equili-
ibrated in 0.01 M HCl according to the procedure of Hnilica and Bess
(39). Purified histone fractions were stored as aqueous solutions of
30% ethanol and 20% (v/v) 1 M NaCl, the ethanol and 1 M NaCl
concentration of stock solutions was determined by quantitative
amino acid analysis and biuret protein analysis. The identity
and purity of the histone fractions was confirmed by amino acid analysis and by electrophoresis in 13% polyacrylamide, 2.5 M urea (pH 3.2)
according to the procedure of Panyim and Chalkley (36). Purified
H1 contained no detectable contaminants: H2A contained no H4 but a
small amount (0.5 to 2%) of H3; H2B contained approximately 6%
H2A and 5% H4. Purified H4 contained no contaminants detectable
by electrophoresis (Fig. 4) and the average mole composition of each
amino acid in comparison (expressed as percentage) to that deter-
mined from the sequence studies of DeLange et al. (40) and Ogawa
et al. (41) was 99.6 ± 1.01 (S. E.). Commercially available mixed
histone preparations were found to contain several unidentified
histones which migrated in the H2A/H4 region of 13% polyacry-
lamide, 2.5 M urea gels. For the purposes of this report (Figs. 2 and
4) we have prepared a histone mixture composed of approximately
equimolar H1, H2A, H2B, H3, and H4.

Peptide Synthesis - The peptides Leu-Ser-Gly-Arg-Gly-Lys-Gly,
corresponding to amino acids 1 to 6 in H4, and Val-Lys-Arg-Ile-Ser-
Gly-Leu-Gly, corresponding to amino acids 43 to 49 of H4 plus an
edited COOH-terminal glutamic acid, were synthesized by the pepti-
de synthesis method of Gutte and Merrifield (42) as previously
described (36). In this text these peptides are designated the Ser-1
peptide and the Ser-47 peptide, respectively. Purified peptides were
phosphorylated by chromatography on Sephadex G-25 and sulfoacryl
(SP)-Sepharose (30). The products from Merrifield syntheses were
applied to a column (2.5 × 80 cm) of Sephadex G-25 equilibrated with 0.04 M
acetic acid. The eluted fractions containing the peptide were pooled
and lyophilized, and the residue was dissolved in 2 ml of 0.2 M
pyridine/aic acid (pH 2.8). The sample was applied to a column (1.9 × 80 cm) of SP-Sephadex equilibrated with the same buffer at
50°C. The peptide was eluted with a concave gradient of 0.2 M
pyridine/aic acid, pH 2.8, to 2 M pyridine/aic acid, pH 5.1, in a
total volume of 500 ml. Fractions containing the peptide were pooled
and lyophilized. The respective amino acid composition of peptides
Ser-1 and Ser-47 were: Val, 1.00; Lys, 1.17; Arg, 0.97; Ile, 0.98; Ser,
0.98; Gly, 2.01; Leu, 1.00; and Ser, 0.67; Gly, 3.21; Arg, 0.92; Lys,
1.08. For these amino acid analyses, hydrolysis in 5.7 N HCl was
performed for 30 h and no correction has been made for the concom-
itant hydrolysis of the NH2-terminal acetyl group and the destruc-
tion of serine. The peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, which
corresponds to the amino acid sequence of pyruvate kinase that is
determined as phospho-H4 in vivo

2 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid.
of 20 ml of acetone. The precipitate was collected by centrifugation, suspended in 2 ml of distilled H2O, and dialyzed against four changes of 1000 volumes of H2O for 1 h at 4°C. To the dialyzed solution was added 2 ml of 0.2 M NH4HCO3 (pH 7.8) containing 0.2 mg of thermolysin. After the solution had been incubated at 40°C for 3 h, additional thermolysin (0.1 mg dissolved in 100 µl of 0.1 M NH4HCO3) was added, and the digestion was continued for 1 h at 40°C. The proteolytic digestion was terminated by addition of acetic acid to a final concentration of 30%. The products were applied to a column (2.5 x 80 cm) of Sephadex G-25 equilibrated with 30% acetic acid. One major 32P-peptide, comprising >80% of the total radioactivity, was eluted as a symmetrical peak between 102 and 113 ml. The fractions were pooled, concentrated by lyophilization, and rechromatographed on Sephadex G-25 under identical conditions. The peptide fraction obtained was radiochemically pure on the basis of the symmetrical elution profile from Sephadex G-25 and by paper electrophoresis at pH 1.9 but contained several nonradioactive peptides.

Other Methods - Electrophoresis of phosphorylated histone products was performed according to the procedure of Panyim and Chalkley (38) in pre-electrophoresed 13% polyacrylamide gels (8 cm) containing 2.5% urea, 0.9% acetic acid, pH 3.2, using a methylene green tracking dye. All gels were stained with Coomassie blue in 45% methanol, 7.5% acetic acid, 47.5% water and destained overnight in the same buffer. Gels containing phosphorylated products were sliced horizontally into 2-mm sections. Each section was placed on a piece (1 x 1.5 cm) of Whatman No. 31 ET chromatography paper, dried for 1 h at 115°C, and counted in 5 ml of toluene-based scintillation fluid containing 4 g/liter of Omnifluor (New England Nuclear).

Paper electrophoresis of 32P-labeled peptides was performed at pH 1.9 on strips (57 cm) of Whatman No. 3MM chromatography paper at 3000 V for 60 min at 25°C using a solvent of 14 M urea, 0.6 M formic acid, pH 1.9.

Samples (10 to 300 nmol) for amino acid analysis were hydrolyzed in 2 ml of triple-distilled 5.7 N HCl in vacuo for 30 h at 105°C. Amino acid analysis was performed on a Durrum D-500 amino acid analyzer as previously described (36). Protein concentrations were determined by biuret or fluorescamine assays (45). Peptide concentrations were determined by quantitative amino acid analysis. During purification of the peptide concentrations were determined by fluorescamine assays (45).

Materials - Homogeneous bovine skeletal muscle cAMP-dependent protein kinase catalytic subunit (46) and regulatory subunit (47), and bovine heart cAMP-dependent protein kinase regulatory subunit (46) were generously provided by Dr. R. Krebs and his associates. The preparation of glycogen synthetase, phosphorylase kinase, phosphorylase b, casein, the heat-stable inhibitor protein of the cAMP-dependent protein kinase, tropomyosin subunit, reduced carboxymethylated maleylated lysozyme, and [γ-32P]ATP was as previously described (35, 48-50). The intact 30 S and 50 S Escherichia coli ribosomes, purified L7L12 from E. coli, and purified myosin light chains from human skeletal muscle and platelets were generous gifts of J. W. B. Hershey, R. R. Traut, both of University of California at Davis, and R. S. Adelstein, National Institutes of Health, respectively. Pyridine and HCl for peptide purification and hydrolysis, respectively, were redistilled before use. Other materials were from commercial sources identified previously (33, 35, 36, 48) or from the following vendors: aminopeptidase M, chymotrypsin, phosphatase, Sigma Chemical Co.; SP-Sephadex, CM-Sephadex, Sephadex G-200, Pharmacia Fine Chemicals Inc., urea, Schwarz/Mann; thermolysin, Calbiochem; Fluram, Roche Diagnostics Corp.

RESULTS

Multiple Histone Kinases in Soluble Extracts of Lymphosarcoma Cells

Sucreose density gradient ultracentrifugation of soluble extracts from lymphosarcoma cells indicates the presence of multiple histone kinases (Fig. 1). The cAMP-dependent protein kinase is the predominant activity and is readily detected with H2B as substrate (Fig. 1A). The s_{20w} of this enzyme (6.8 S) is characteristic of both the type I and II isozymes from most tissues (52-54). In the absence of cAMP a low level of H2B phosphorylation, probably due to spurious dissociation of the cAMP-dependent holoenzyme, is detected in the 6.8 S region of the gradient (Fig. 1, A and B), but the predominant cAMP-independent H2B phosphotransferase activity sediments at 5.8 S. A major histone phosphotransferase, sedimenting in the same region of the gradient, is also detected with H2A as substrate (Fig. 1C). As anticipated from the results of Langan and co-workers (25, 31, 32), multiple H1 phosphotransferase activities are detectable (Fig. 1B). The assay of fractions for H1 protein kinase(s) was performed in the presence of the heat-stable inhibitor protein of the cAMP-dependent protein kinase thus eliminating activity due to either free catalytic subunit of the protein kinase (48) or to histone-promoted dissociation of the cAMP-dependent holoenzyme (54). Two H1 phosphotransferases different from the cAMP-dependent enzyme are recognized. The major fraction which has a sedimentation coefficient of 6.5 S appears to be a distinct entity from the H2A-H2B phosphotransferase which sediments at 5.8 S. A second H1 protein kinase, comprising approximately 25% of the total activity, sedimented as a broad peak in the 8.0 S region of the gradient. At least two H4 phosphotransferases with sedimentation coefficients of 7.2 S and 7.5 S are detected by this experimental design (Fig. 1C). Neither activity can be attributed to either cAMP-dependent protein kinase or the catalytic subunit thereof since the assays were performed in the presence of the heat-stable inhibitor protein of the cAMP-dependent protein kinase.

Multiple histone kinase activities in lymphosarcoma extracts are also indicated by DE52 chromatography, a procedure.
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FIG. 2. Fractionation of histone phosphotransferase activities of lymphosarcoma P1798/S on DEAE-cellulose. The high speed supernatant extract from 35 g of lymphosarcoma tumors (see "Experimental Procedures") containing 350 mg of protein was applied to a column (1.9 x 19 cm) of DE52 equilibrated with Buffer A. The column was washed with Buffer A until the absorbance at 280 nm decreased to less than 0.2 and subsequently eluted with a 0 to 400 mM linear gradient of KCl in Buffer A (total volume, 180 ml). Each fraction contained 2.45 ml. Protein phosphotransferase activity was determined by Assay I with 40 μg of histone H4 (Panel A), histone mix in the presence (●●●●) or absence (○○○○) of 2 μM CAMP (Panel B), or histone H1 (Panel C).

FIG. 3. Fractionation of H4-Kinase-(I) on CM-Sephadex. Partially purified H4-Kinase-(I), Fraction III (57 mg of protein), obtained by the purification procedure detailed in the text and equilibrated in Buffer B by dialysis, was applied to a column (0.6 x 6 cm) of CM-Sephadex equilibrated in Buffer B. The column was washed with Buffer B until the absorbance at 280 nm decreased to less than 0.2. The column was eluted with a 0 to 300 mM linear gradient of KCl in Buffer B (total volume, 200 ml). H4 phosphotransferase activity of each fraction (1.5 ml) was measured by Assay I in the presence of 68 μM (γ-32P)ATP and 40 μg of H4.

dialed against 250 volumes of Buffer B, the dialyzed solution was centrifuged at 22,000 g for 20 min, and the precipitate was discarded. The supernatant solution (Fraction III) was applied to a column (0.6 x 6 cm) of CM-Sephadex equilibrated with Buffer B. H4 phosphotransferase was eluted as a single broad peak by a linear salt gradient (Fig. 3). The fractions containing the enzyme were pooled (Fraction IV) and dialyzed against 30 volumes of Buffer B for 4 h. The dialyzed protein was applied to a column (0.6 x 6 cm) of DE52 equilibrated with Buffer B. The column was washed with at least 5 volumes of Buffer B and the enzyme was eluted as a single narrow peak with 100 mM KCl in Buffer B (Fraction V). This fraction, containing approximately 150 μg of protein, was applied to a column (1.5 x 72 cm) of Sephadex G-200 equilibrated with 100 mM KCl in Buffer B. Approximately 90% of the applied enzyme was eluted as a single symmetrical peak of activity in the region corresponding to a molecular weight of 102,000. This enzyme (Fraction VI) or the enzyme at the previous purification stage (Fraction V) could be stored at -70° for several months with less than 5% loss of enzyme activity.

A summary of the purification procedure and of the phosphotransferase activities with various histones is presented in Table I. For Fractions I, II, and III, the phosphorylation of
endogenous substrates was determined from assays performed in the absence of added histones and this value was subtracted from the activity expressed in the complete system. For initial fractions this correction for endogenous phosphorylation was substantial, and, in consequence, the accuracy of these determinations was compromised. The initial fractionation may also have resulted in either the activation of the enzyme, the removal of histone kinase inhibitors, or the removal of acidic or other proteins that would complex or modify the histone substrates. It appears that the efficacy of the purification scheme can also be evaluated by assays performed with the Ser-47 peptides (see below). Extensive enrichment of H4-Kinase-(I) is apparent, but a full interpretation of the selective removal of other histone kinases is complex. The cAMP-dependent protein kinase, which has an isoelectric point below pH 5.5 (55) is removed by CM-Sephadex chromatography (Fig. 1C) and with the molecular weight of 102,000 calculated from Sephadex G-200 chromatography (see above). The relative distribution of H4-Kinase activities between the two fractions identified by both sucrose gradient ultracentrifugation (Fig. 1C) and DEAE-cellulose chromatography (Fig. 2C) would suggest that H4-Kinase-(II) has an $s_{20,w}$ of 7.2.

Properties of H4-Kinase-(I)

**Molecular Size**—In 5 to 15% sucrose density gradients purified H4-Kinase-(I) sedimented with a $s_{20,w}$ of 5.2 (Fig. 1D), a value that is compatible with the major species of H4 kinase detected in crude extracts (Fig. 1C) and with the molecular weight of 102,000 calculated from Sephadex G-200 chromatography (see above). The relative distribution of H4-Kinase activities between the two fractions identified by both sucrose gradient ultracentrifugation (Fig. 1C) and DEAE-cellulose chromatography (Fig. 2C) would suggest that H4-Kinase-(II) has an $s_{20,w}$ of 7.2.

**Substrate Specificity of H4-Kinase-(I)—**Urea-polyacrylamide gel electrophoresis of the $^{32}$P-labeled product obtained from the incubation of pure H4 with purified H4-Kinase-(I) indicated that all the radioactivity co-migrated with the H4 histone (Fig. 4A). Similarity, essentially all (>95%) of the $^{32}$P-labeled protein produced from the incubation of purified H4-Kinase-(I) with an equimolar mixture of five histone fractions (H1, H2A, H2B, H3, and H4) was H4; a low level of $^{32}$P labeled protein (<5%) migrated in the H2A-H2B and H1 regions (Fig. 4B). In a similar experiment using a crude tissue extract many phosphorylated histones were readily detected (Fig. 4C). Time course studies of purified histone phosphorylation by H4-Kinase-(I) confirmed that H4 was the optimum histone substrate (Fig. 5A). $[^3P]Phosphate was incorporated into the H2B preparation at 20% of the rate of H4 phosphorylation; however, urea-polyacrylamide gel analyses of the phosphorylated H2B preparation indicated that 40% of the total $^{32}$P-labeled protein was H4, a contaminant in the H2B preparation. Low rates of incorporation were observed with H1 or H2A. No activity was detected with H3 (not shown). None of the following phosphoproteins could serve as substrates for purified H4-Kinase-(I) in the standard assay conditions at the protein substrate concentration indicated: phosphorylase b, 1 mg/ml; phosphorylase b kinase, 0.15 mg/ml; glycogen synthetase, 0.5 mg/ml; myosin light chain, 0.6 mg/ml; troponin I, 0.3 mg/ml; troponin T, 0.5 mg/ml; 30 S ribosomes, 1.5 mg of protein/ml; 50 S ribosomes, 1.6 mg of protein/ml; ribosomal protein L7/L12 from Escherichia coli, 0.3 mg/ml; bovine cAMP-dependent protein kinase regulatory subunit type II, 0.65 mg/ml; casein, 1 mg/ml; and reduced carboxymethylated maleylated lysozyme, 0.86 mg/ml. A low level of phosphorylation was observed with protamine and phosvitin.

**Kinetic Constants and Metal Ion Requirements of H4-Kinase-(I)—**Kinetic constants for H4-Kinase-(I) were evaluated by standard Michaelis-Menten kinetics (Fig. 6). The high
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FIG. 4. Gel electrophoresis profiles of phosphorylated histones. Purified histone H4 (112 μg; A) or histone mixture (200 μg; B and C) was incubated with either purified H4-Kinase-(I), Fraction V (0.8 μg protein; A and B), or high speed supernatant extract, Fraction I (60 μg protein; C) and [γ-32P]ATP with other components of the standard assay reaction mixture (see "Experimental Procedures") for 10 min at 30°. At the termination of the reaction a fraction of each incubation mixture (A, 4.48 μg of histone; B, 17 μg of histone; C, 17 μg of histone) was applied to 13% polyacrylamide, 2.5 M urea gels, pH 3.25, and electrophoresed according to the procedure of Panyim and Chalkley (38). The radioactive content of gel slices was determined as indicated under "Experimental Procedures." Panel A inset indicates the electrophoresis profile of purified H4 as detected by staining with Coomassie blue.

Fig. 6. Characterization of the kinetic properties of H4-Kinase-(I). A, double reciprocal plot of initial velocity versus H4 concentration. [32P]Phosphate incorporation was measured by Assay I in the presence of 160 μM [γ-32P]ATP, 30 mM MgCl₂, and 1.6 μg of H4-Kinase-(I), Fraction V. B, double reciprocal plot of initial velocity versus ATP concentration. [32P]Phosphate incorporation was measured by Assay I in the presence of 44 ELM H4, 30 mM MgCl₂, and 0.38 μg of H4-Kinase-(I), Fraction V. C, double reciprocal plot of initial velocity versus Mg⁺⁺ concentration. [32P]Phosphate incorporation was measured by Assay I in the presence of 35 PM H4, 64 μM [γ-32P]ATP and 0.8 μg of H4-Kinase-(I), Fraction V. D, double reciprocal plot of initial velocity versus Ser-47 peptide concentration. [32P]Phosphate incorporation was determined by Assay II in the presence of 82 μM [γ-32P]ATP, 1.5 mM MgCl₂, and 1.8 μg of H4-Kinase-(I), Fraction V. Calculated Kₐ values were: H4, 16 μM; Ser-47 peptide, 43 μM; ATP, 69 μM. The apparent Kₐ for Mg⁺⁺ was 55 mM.

Identification of Site of Phosphorylation in H4 Catalyzed by H4-Kinase-(I)

Characterization of Modified Amino Acid—Prolonged incubation (2 h) of H4 with purified H4-Kinase-(I) and excess [γ-32P]ATP resulted in the incorporation of 0.94 mol of phosphate/mol of H4 (Fig. 5B). Additional of more enzyme and (γ-

affinity for H4 (Kₐ = 16.4 μM; Fig. 6A) is consistent with its assignment as the physiological substrate. The Kₐ for ATP (69 μM; Fig. 6B) is within the concentration range observed for other protein kinases (51). No phosphorylation of H4 was detected if 42 μM [γ-32P]GTP was substituted for [γ-32P]ATP in the standard assay. A double reciprocal plot of velocity versus magnesium chloride concentration was linear within the concentration range 3 to 8 mM and extrapolated to an apparent Kₐ of 55 mM (Fig. 6C). This value exceeds the Mg⁺⁺ concentration required to form the ATP-Mg⁺⁺ complex, the established substrate for other kinase-catalyzed reactions, and suggests that Mg⁺⁺ also activates the reaction by interaction with either the protein substrate or the enzyme. Under the described assay conditions, maximum activity was observed in the presence of 10 mM Mg⁺⁺; Mg⁺⁺ in excess of this concentration was inhibitory (Table II). Mn⁺⁺ can substitute for Mg⁺⁺ in the metal ion-ATP complex and is equally effective as an apparent activator of H4-Kinase-(I) (Table II). Mn⁺⁺, as well as KCl, K₂HPO₄, and CaCl₂, was inhibitory at high concentrations (Table II), implying a nonspecific inhibition of the enzyme by high ionic strength.

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Characterization of Modified Amino Acid—Prolonged incubation (2 h) of H4 with purified H4-Kinase-(I) and excess [γ-32P]ATP resulted in the incorporation of 0.94 mol of phosphate/mol of H4 (Fig. 5B). Additional of more enzyme and (γ-

affinity for H4 (Kₐ = 16.4 μM; Fig. 6A) is consistent with its assignment as the physiological substrate. The Kₐ for ATP (69 μM; Fig. 6B) is within the concentration range observed for other protein kinases (51). No phosphorylation of H4 was detected if 42 μM [γ-32P]GTP was substituted for [γ-32P]ATP in the standard assay. A double reciprocal plot of velocity versus magnesium chloride concentration was linear within the concentration range 3 to 8 mM and extrapolated to an apparent Kₐ of 55 mM (Fig. 6C). This value exceeds the Mg⁺⁺ concentration required to form the ATP-Mg⁺⁺ complex, the established substrate for other kinase-catalyzed reactions, and suggests that Mg⁺⁺ also activates the reaction by interaction with either the protein substrate or the enzyme. Under the described assay conditions, maximum activity was observed in the presence of 10 mM Mg⁺⁺; Mg⁺⁺ in excess of this concentration was inhibitory (Table II). Mn⁺⁺ can substitute for Mg⁺⁺ in the metal ion-ATP complex and is equally effective as an apparent activator of H4-Kinase-(I) (Table II). Mn⁺⁺, as well as KCl, K₂HPO₄, and CaCl₂, was inhibitory at high concentrations (Table II), implying a nonspecific inhibition of the enzyme by high ionic strength.

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$^{32}$P-ATP at 2 h slightly increased H4 phosphorylation to 1.02 mol/mol. $^{32}$P-labeled H4 (phosphorylated to a level of 0.81 mol of $^{32}$P phosphate/mol of protein) was separated from $\gamma$-$^{32}$P-ATP using Dowex 1-X8-OH, pH 7, and incubated at 100° for 20 min in the presence of 0.1 N NaOH, 0.1 N HCl, or 0.1 N NaOH plus 0.1 N HCl. As determined by a second Dowex 1-X8-OH chromatography, no loss of protein-bound $^{32}$P phosphate was observed in the acid-treated sample as compared to the control. In contrast, the protein-bound $^{32}$P phosphate was entirely (>99.5%) alkali-labile. Fully phosphorylated H4, isolated from the Dowex 1-X8 acetate chromatography, was digested for 1 h at 105° with 5.7 N HCl. The partial acid hydrolysate was analyzed by paper electrophoresis at pH 1.9. The recovery of $^{32}$P as unhydrolyzed material, $[^{32}$P]phosphate, and $[^{32}$P]phosphoserine was 13, 73, and 13%, respectively. No phosphothreonine was detected.

The above data support the conclusion that H4-Kinase-(I) catalyzes the phosphorylation of a single serine residue in H4. Two serine moieties are present in H4 at positions 1 and 47 (40, 41); the NH2-terminal serine is acetylated. Thermolysin digestion of $^{32}$P-labeled H4 produced by H4-Kinase-(I) catalysis yields a single $^{32}$P-labeled peptide ("Experimental Procedures"). This peptide was tested for sensitivity to aminopeptidase M. Since the latter enzyme requires a free NH2-terminal amino acid, peptides such as would be derived from the NH2-terminus of H4 containing the acetylated serine are insensitive to digestion by this enzyme (56). The incubation of 100 nmol of radiochemically pure $^{32}$P-labeled peptide of H4 obtained by thermolysin digestion with 500 units of aminopeptidase M in 100 mM ammonium bicarbonate, pH 7.8, for 24 h at 25° completely (>99%) degraded the thermolysin peptide and formed a $^{32}$P-labeled product that was more electrophoretically as determined by paper electrophoresis at pH 1.9. (The mobilities of the unreacted peptide and the $^{32}$P product derived from aminopeptidase M digestion were 7.33 and 2.67 cm/Vh, respectively. Conditions of electrophoresis are given under "Experimental Procedures.")

Phosphorylation of Synthetic Peptides—Peptides corresponding to H4 amino acid sequences containing Ser-1 (residues 1 to 6) and Ser-47 (residues 43 to 49) were tested as substrates for purified H4-Kinase-(I) and with the fractions obtained during enzyme purification. The Ser-47 peptide was phosphorylated by an enzyme(s) in the high-speed supernatant. The Ser-1 peptide was not a substrate for H4-Kinase-(I), (Fig. 2; Table I) and the enzyme obtained by a subsequent ammonium sulfate fractionation catalyzed the phosphorylation of the Ser-1 peptide but not the H4 Ser-47 peptide. Phosphorylation of H2A, a histone with an NH2-terminal pentapeptide identical to that of H4, was also catalyzed by this fraction.

Nonidentity of H4-Kinase-(I) with cAMP-dependent Protein Kinase or Catalytic Subunit Thereof—Commercial "arginine-rich histone" (presumably H2A + H4) is one of the substrates for the cAMP-dependent protein kinase that Donnelly et al. (57) have implied permit an enhanced activity to be expressed in the presence of a heat-stable modulator. The elution of H4-Kinase-(I) from DEAE-cellulose partially overlaps that of the type I CAMP-dependent protein kinase; however, on the basis of several properties of the purified enzymes a clear distinction between the two enzymes can be made. H4-Kinase-(I) activity was neither stimulated by 1 mM cAMP or 1 mM cGMP nor inhibited by either the heat-stable inhibitor protein of the cAMP-dependent protein kinase (Table III) or by the regulatory subunit thereof (data not shown). In addition, H4-Kinase-(I) and the catalytic subunit of the cAMP-dependent protein kinase exhibited different substrate specificity (Table III). H4 was the preferred substrate for H4-Kinase-(I) (Table III; Fig. 5) and was a poor substrate for the catalytic subunit of the

### Table I

| Substrate                  | H4-Kinase-(I) | Catalytic subunit of cAMP-dependent protein kinase |
|----------------------------|---------------|--------------------------------------------------|
|                            |              | -Inhibitor Protein - | +Inhibitor Protein + |
| Histones                   |              | Protein              | Protein              |
| H4 (70 μM)                 | 24           | 28                   | 1.5                  | 1.5                  |
| H2A (50 μM)                | 2            | 1.7                  | 93                   | 10                   |
| Synthetic peptides         |              |                      |                      |
| Ser-47 (70 μM)             | 20           | 21                   | 0.84                 | 0.92                 |
| (Val-Lys-Arg-Ile-Ser-Gly-Leu-Gly) |            |                      |                      |
| Ser-1 (700 μM)             | <0.1         | <0.1                 |                      |                      |
| (Ac-Ser-Gly-Ary-Gly-Lys-Gly) |              |                      |                      |
| PK-1 (88 μM)               | 5.1          | 6                    | 160                  | 16                   |
| (Leu-Arg-Arg-Ala-Ser-Leu-Gly) |            |                      |                      |

* Measured in the presence of 10 mM MgCl2.

### Table II

Effect of inorganic salts on activity of H4-Kinase-(I)

Incorporation of $[^{32}$P]phosphate was measured by Assay I in the presence of 50 μM H4, 105 μM $\gamma$-$[^{32}$P]ATP, and 0.57 μg of H4-Kinase-(I), Fraction V.

| Molarity | H4 phosphotransferase activity |
|----------|--------------------------------|
|          | MgCl2 | MnCl2 | KCl | $K_HPO_4$ | CaCl2 |
| mM       |       |       |     |          |       |
| 0        | <1    | <1    | 36  | 36        | 36    |
| 2.5      | 22    | 27    | 31  | 36        | 30    |
| 5        | 34    | 32    | 32  | 34        | 34    |
| 10       | 36    | 34    | 32  | 34        | 15    |
| 20       | 32    | 30    | 29  | 30        | 6.4   |
| 50       | 21    | 18    | 25  | 21        | 4     |
| 100      | 12    | 6.4   | 20  | 12        | 3     |

* Measured in the presence of 10 mM MgCl2.
cAMP-dependent protein kinase. Similarly, the Ser-47 peptide was a poor substrate for the catalytic subunit of the cAMP-dependent protein kinase. H2A was actively phosphorylated by the latter enzyme but was a poor substrate for the H4-Kinase-(I) (Table III). The synthetic peptide PK-1, which is a good substrate for the cAMP-dependent enzyme (43, 44), is not phosphorylated by H4-Kinase-(I). The Ser-1 peptide is not significantly phosphorylated by either of these enzymes.

**DISCUSSION**

As documented by a recent review by Dixon et al. (8) and delineated briefly in the introduction of this report a wide spectrum of histone kinases may be presumed to exist. This concept is supported not only from the extensive documentation of specific histone phosphorylation that occurs under a gamut of biological circumstances (13-29) but also from the identification of different classes of phosphorylation sites as defined by their primary amino acid sequence (8). It is possible that a sequence of five or six amino acids that contain the phosphorylation site is insufficient to delineate the recognition parameters for a specific histone kinase, but the recent evidence obtained for the cAMP-dependent protein kinase (43, 44) has provided strong support for the concept that, at least with the latter enzyme, the primary structure of the protein substrate is a major determinant of substrate specificity. Similarly, a high degree of selectivity and specific interaction with phosphorylase kinase has been demonstrated for small peptides containing the phosphorylation site of phosphorylase (43, 58). Nevertheless, with the exception of the work of Langan and co-workers (4, 6, 28, 31, 32), Lake and Salzman (13) and Lake (30) who have initiated a characterization of multiple H1 kinases, little is known of histone-specific kinases.

As evidenced by the data of Figs. 1 and 2 multiple histone kinases are present in the proliferating lymphocyte: in this manuscript we have defined the properties of a specific H4-Kinase. The purified enzyme H4-Kinase-(I), which has been enriched several thousandfold, exhibits little activity towards other histones or phosphoproteins. The high affinity of the enzyme for both H4 and a seryl peptide derived from H4 (Fig. 6) provides the best evidence so far accumulated that H4 is the physiological substrate. H4-Kinase-(I) catalyzes the phosphorylation of one of the two seryl residues of H4. A phosphopeptide derived from H4 has not yet been obtained in a homogeneous form; however, the data which strongly supports the conclusion that Ser-47 and not Ser-1 is the H4 site phosphorylated as detected by sucrose gradient centrifugation (Fig. 10) has an sp0.1 of 7.2 S and is presumably the enzyme designated H4-Kinase-(I). The latter elutes from DEAE-cellulose at a higher salt concentration than the type I enzyme (Fig. 2A). This enzyme has only been minimally purified; however, the purest fraction obtained exhibits little activity towards the Ser-47 peptide but actively catalyzes the phosphorylation of the Ser-1 peptide, H4, and H2A. The latter two histones have an identical pentapeptide at the N1I-terminal. Presumably, H4-Kinase-(II) catalyzes the phosphorylation of H2A or H4, or both, as has been shown to occur during spermatocyte maturation (H2A and H4; Refs. 8-11), erythroblast proliferation (H4; Ref. 25), and/or hepatic regeneration (H2A; Ref. 23). Whether a single enzyme catalyzes each of these phosphorylations is unknown and, in view of the apparent differential control of each, is doubtful.

The phosphorylation of serine residue 47 of H4 has not been demonstrated with intact cell studies, but for several circumstances for which H4 phosphorylation has been shown to occur, the site of phosphorylation has yet to be determined. Typical examples include the phosphorylation of H4 that occurs throughout the cell cycle in Chinese hamster ovary cells (15, 16), HeLa cells (22), or HTC cells (21). The function of H4 phosphorylation by H4-Kinase-(I) remains speculative but it is of note that Ser-47 is a residue at the edge of the hydrophilic COOH-terminal region (i.e. residues 46 to 102) which also includes the α helical region and is associated with histone-histone binding. Conceivably, the phosphorylation of Ser-47 may be essential in determining the H4 conformation required for histone-histone interaction during cell division and chromatin assembly. A similar postulate has been expressed for the function of H1 phosphorylation at residues 37 and 105 which are also at the demarcation of the ternary structure of the protein. Bradbury and co-workers (59) have reported that as a consequence of in vitro phosphorylation of these 2 residues a large section of the NH2-terminus of H1 no longer binds to DNA. Finally, the identification of H4 as a protein with multiple phosphorylation sites permits it to be added to an evergrowing list of proteins for which multisite phosphorylation has become apparent.

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