Phosphorylation of High Mobility Group 14 Protein by Cyclic Nucleotide-dependent Protein Kinases*

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Chromosomal high mobility group (HMG) proteins have been examined as substrates for cGMP-dependent and cAMP-dependent protein kinases. Of the four HMG proteins only HMG 14 contained a major high affinity site which could be phosphorylated by both enzymes, preferentially by cGMP-dependent protein kinase. One mol of 32P was incorporated/mol of HMG 14. Kinetic analysis revealed apparent $K_m$ and $V_{max}$ of 40.5 $\mu$M and 14.7 $\mu$mol/min/mg, respectively, for cGMP-dependent protein kinase, and 123 $\mu$M and 11.1 $\mu$mol/min/mg, respectively, for cAMP-dependent protein kinase. Tryptic maps of 32P-labeled phosphopeptides of HMG 14 demonstrated phosphorylation of the same site by both enzymes. The tryptic fragment containing the major phosphorylation site was identified by amino acid composition and sequence as HMG 14 (residues 4-13): H-Lys-Val-Ser(P)-Ser-Ala-Glu-Gly-Ala-Ala-Lys-OH. HMG 14 and HMG 17 also contained minor sites which could be phosphorylated by cGMP-dependent protein kinase. Tryptic phosphopeptides mapping suggested that the same minor site was phosphorylated on both HMG 14 and 17. On the basis of amino acid composition, the tryptic peptides carrying the minor phosphorylation sites were identified as H-Leu-Ser(P)-Ala-Lys representing residues 23-26 and 27-30 of HMG 14 and HMG 17, respectively.

A group of chromatin-associated proteins characterized by a high content of acidic and basic residues was originally described by Goodwin et al. (1). Although a number of proteins were initially described in this high mobility group, subsequent studies indicated that several were proteolytic products of histone H1 (2). The four major HMG proteins designated HMG 1, 2, 14, and 17 have been well characterized as distinct entities. HMG 1 and 2 show considerable sequence homologies (3) as do HMG 14 and 17 (4, 5). Because these proteins are abundant in nuclei, a structural rather than an enzymatic function for HMG proteins was proposed by these authors.

Evidence derived from preferential sensitivity of defined genes in chromatin to nuclease attack has suggested that HMG 14 and 17 are associated with transcriptionally active genes. Genes which are actively transcribed exhibit increased sensitivity to DNase I when compared to transcriptionally inactive genes (6, 7). H6, the trout analog of HMG 17 (8), was selectively released from chromatin under conditions in which nucleae preferentially degraded transcriptionally active chromatin (9). HMG proteins were also preferentially released from duck erythrocyte chromatin during limited DNase 1 digestion (10). Reconstitution studies performed by Weisbrod et al. (11) indicated that either HMG 14 or HMG 17 restored DNase 1 sensitivity to chromatin depleted of these proteins. These authors indicated that 1 mol of HMG 14 or 17/10-20 nucleosomes restored full DNase 1 sensitivity as monitored by globin gene transcription. Evidence for HMG 14 and 17 binding to nucleosomes depleted of these proteins was presented (11). Two specific binding sites for HMG 14 and 17 on nucleosome cores were indicated by studies of Mardian et al. (12). Mechanisms through which HMG 14 and 17 specifically associated with transcriptionally active genes and the function of these proteins remains uncertain.

HMG proteins are subject to post-translational modification, including acetylation of ε-amine groups in lysine residues (13), methylation of arginine guanidino groups (14), and poly-ADP ribosylation (15). Recent studies indicate that HMG 14 and 17 are phosphorylated (16, 17). Levy-Wilson (17) noted that butyrate treatment of HeLa cells promoted hyperphosphorylation of HMG 14 and 17. Butyrate treatment is known to cause hyperacetylation of histones (18) and to enhance DNase 1 sensitivity (19). In butyrate-treated HeLa cells most HMG 14 and 17 was found in fraction MN1, which is reported to be enriched in transcribed DNA sequences (20).

Neither the enzymes responsible for phosphorylation of HMG proteins nor the sites of phosphorylation in these proteins have been identified. Sun et al. (21) reported that A-kinase catalyzed the thio-phosphorylation of HMG 1 when the ATP analog adenosine 5′-O-(3-thiotriophosphate) was used. In the present studies, we have examined the phosphorylation of purified HMG proteins as catalyzed by several purified protein kinase enzymes. HMG 14 was found to be specifically phosphorylated by cyclic nucleotide-dependent protein kinases, with preference for G-kinase. Sequence analysis of HMG 14 revealed a major phosphorylation site unique to HMG 14 and a minor phosphorylation site common to both HMG 14 and HMG 17.

**EXPERIMENTAL PROCEDURES**

Preparation of Enzymes and HMG Proteins—Purification of G-kinase from bovine lung and R6 from bovine heart has been described (22). The catalytic subunit of A-kinase was prepared according to Zoller et al. (23). The specific activities of G-kinase and A-kinase were 1.4 × 10⁶ and 2.2 × 10⁵ units/mg, respectively. One unit of activity equals 1 pmol of 32P from [γ-32P]ATP incorporated into histone type II A at 1 mg/ml/min.

HMG proteins were isolated by perchloric acid extraction from approximately 1 kg of calf thymus, without prior isolation of chro-
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matin, as described by Sanders (24). HMG proteins were separated by carboxymethyl-Sepharose chromatography (24). Fractions containing HMG 14 and HMG 17 were rechromatographed on carboxymethyl-Sephadex to remove contaminating histone fractions and the identity of HMG 14 and HMG 17 was confirmed by amino acid analysis. Further impurities were removed in each of the HMG protein preparations, no significant 32P incorporation into the contaminants was observed (see Fig. 1).

HMG proteins were analyzed on 15% polyacrylamide gels containing 2.5% urea in 0.9 M acetic acid (25) and 17.5% polyacrylamide gels containing sodium dodecyl sulfate (26).

Phosphorylation of Substrates—Reaction mixtures were run at 30 °C and contained 50 mM potassium phosphate, pH 6.9, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.1 mM [γ-32P]ATP (specific activity of 100-200 cpm/pmol), 2 mg/ml of bovine serum albumin, 0.4 μM cGMP, 0.1-0.5 mg/ml of enzyme, and protein substrate as indicated. Assays of initial reaction rates were run in a volume of 50 μl for 5-10 min and were determined to give a linear response of 32P incorporation as a function of time at enzyme levels indicated when histone H1 or HMG 14 were used as substrates. Control reactions were run in the absence of substrate and any self-phosphorylation of enzyme was subtracted from data. All assays were performed in duplicate and were initiated with the addition of [γ-32P]ATP and terminated with glacial acetic acid by adjusting the reaction to 30% (v/v). Determination of the stoichiometry of 32P incorporation into HMG 14 and HMG 17 was done by removal of an aliquot containing 1.5 nmol of substrate from the reaction at various times and combining with a volume of glacial acetic acid to make a 30% (v/v) solution. 32P incorporation was determined on phosphocellulose paper according to Glass et al. (28). Attempts to determine the stoichiometry of 32P incorporation into HMG 14 on paper discs with 25% trichloroacetic acid (29) or with the silicic acid-trichloroacetic acid reagent (30) gave consistently less than stoichiometric incorporation (0.2 and 0.6 mol of 32P/mol of substrate, respectively), suggesting that incomplete precipitation of substrate occurred with these reagents. This is consistent with the known solubility of the perchloric acid salts of basic HMG proteins.

Assays of cyclic nucleotide binding to G-kinase and RII were accomplished with a Wittmann-Liebold modified Beckman 890C spin column. The amino acid sequence analysis (31) of the aromatic amide of 32P-labeled HMG proteins was determined by paper electrophoresis on cellophane (0.7 × 2.0 cm) of AGI-X10 in 30% (v/v) acetic acid (31). The solution was lyophilized and the protein taken up in 0.1 M NH4HCO3 (pH 7.9) to 2 mg/ml and digested with trypsin. Digestion was in a room temperature water bath and was initiated with the addition of 20 μg of trypsin/mg of solution. Fresh trypsin, at 20 μg/ml of solution, was added every 48 h until digestion was complete. Eight days and a final trypsin concentration of 80 μg/ml were required at an enzyme to substrate ratio of 1:25. The reaction was monitored by removing aliquots of the trypsic digests containing 0.2-0.4 nmol of 32P and analyzing these by two-dimensional mapping on cellulose plates (20 × 20 cm). High voltage electrophoresis in the first dimension was carried out in a pH 4.7 buffer containing 1-butanol/pyridine/glacial acetic acid/water (2:1/1:36) for 30 min at 1000 V. Thin layer chromatography in the second dimension was carried out in 1-butanol/ pyridine/glacial acetic acid/water (15:10:3:2) for 4.5 h. Autoradiograms were exposed for 16 h. Visual detection of peptides was performed with fluorescamine spray (32).

Isolation of 32P-labeled Phosphopeptides from HMG 14 Phosphorylated by G-kinase—Tryptic digests of 32P-labeled HMG 14 were hydrolyzed in 6 N HCl in sealed tubes at 110 °C for 4 h. 32P-labeled protein hydrolysates were resolved by high voltage electrophoresis with pH 1.9 buffer (33), 32P-containing peak which eluted with an elution volume to void volume ratio of 1.65 was pooled and evaporated to dryness in vacuo at 40 °C. The residue was dissolved in 50% (v/v) aqueous methanol and applied as a band to a cellulose plate and then layer chromatography was performed for 7 h. The radioactive band was located by autoradiography and extracted with methanol/glacial acetic acid/water (50:15:35). The solvent was removed in vacuo at 40 °C, the residue was dissolved in 20 μg/ml of bovine serum albumin as a standard.

Phosphoamino Acid Determination—Phosphoserine and phosphothreonine detection was performed by partial acid hydrolysis in 6 N HCl in sealed tubes at 110 °C for 4 h. 32P-labeled protein hydrolysates were resolved by high voltage electrophoresis with pH 1.9 buffer (33) for 10 min at 14.2 V with pH 1100 V with pH 7.9 buffer. The radioactive bands were exposed to x-ray film for 3 days. The 32P-containing peak which eluted with an elution volume to void volume ratio of 1.65 was pooled and evaporated to dryness in vacuo at 40 °C. The residue was dissolved in 50% (v/v) aqueous methanol and applied as a band to a cellulose plate and thin layer chromatography was performed for 7 h. The radioactive band was located by autoradiography and extracted with methanol/glacial acetic acid/water (50:15:35). The solvent was removed in vacuo at 40 °C, the residue was dissolved in 20 μg/ml of bovine serum albumin as a standard.

RESULTS

Phosphorylation of HMG Proteins—HMG proteins isolated from calf thymus by perchloric acid extraction contain histone H1. G-kinase catalyzed phosphorylation of three prominent protein bands in this preparation (Fig. 1). The...
proteins were tentatively identified as histone H1, a proteolytic fragment of histone H1, and HMG 14 by their relative rates of migration compared to histones in two separate gel systems: 1) acetic acid/urea and 2) sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Isolation and purification of the HMG proteins was performed by carboxymethyl-Sephadex chromatography and provided histone-free protein preparations. Positive identification of HMG 14 and HMG 17 was confirmed by amino acid analysis of the purified proteins (4, 5).

Phosphorylation of the individual HMG proteins by G-kinase followed by gel electrophoresis and autoradiography substantiated the observation that besides histone H1, HMG 14 is readily phosphorylated (Fig. 1). No significant phosphate incorporation into HMG 1, HMG 2, or HMG 17 was observed. Phosphorylation of HMG 14 resulted in a slight reduction in mobility compared to the nonphosphorylated protein (Fig. 1A) which is consistent with stoichiometric phosphate incorporation to give an increase in weight and/or alteration in net charge.

The rates of phosphorylation of the HMG proteins and histone H1 were compared as substrates for both G-kinase and A-kinase (Table I). HMG 14 phosphorylation was faster with G-kinase than with A-kinase and greater than that of histone H1 with both enzymes. Histone H1 phosphorylation was faster with A-kinase than with G-kinase and is a reflection of the $V_{\text{max}}$ for each enzyme at the saturation concentrations of substrate used. As shown by Zeilig et al. (38), the affinity of G-kinase is greater than the affinity of A-kinase for histone H1, though the $V_{\text{max}}$ is approximately 4-fold higher for the latter enzyme.

Stoichiometry of Phosphorylation of HMG 14 and HMG 17—A time course of $^{32}$P incorporation into HMG 14 with G-kinase at 0.12 µg/ml and A-kinase at 0.19 µg/ml was performed. A stoichiometry of 1 mol of phosphate/mol of HMG 14 was observed with each enzyme individually or together in the reaction (Fig. 2). Therefore, a single identical site was likely phosphorylated by both kinases. When substantially higher amounts of G-kinase (0.38 µg/ml) were used, phosphorylation to a stoichiometry of greater than one but less than 2 mol of phosphate/mol of HMG 14 occurred (Fig. 3). Also, phosphate incorporation into HMG 17 could be achieved with G-kinase during longer reaction times at these higher enzyme concentrations, although less than 0.2 mol of phosphate/mol of HMG 17 was observed. This finding was substantiated by gel electrophoresis and autoradiography of $^{32}$P-labeled HMG 17 with proportionate amounts of HMG 17 and $^{32}$P incorporation demonstrating a slight decrease in migration rate (data not shown). The change in migration of only a
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Phosphorylation site kinetics were observed. Under the experimental conditions the major site of phosphorylation was defined; phosphorylation of the minor site was not readily observed under initial rate conditions with the enzyme and substrate concentrations used. With G-kinase the apparent $K_m$ was 40.5 $\mu$M and the $V_{max}$ was 14.7 mmol/min/mg. With A-kinase the apparent $K_m$ was 123 $\mu$M and the $V_{max}$ was 11.1 mmol/min/mg. G-kinase thus has a preference for HMG 14 with respect both to affinity and to rate of phosphorylation.

Preparation and Isolation of $^{32}$P-labeled Phosphopeptides from $^{32}$P-Labeled HMG 14—To identify the site(s) of phosphorylation, HMG 14 was phosphorylated with G-kinase under conditions which label a single or multiple number of sites. Excess ATP was removed by anion exchange chromatography and $^{32}$P-labeled HMG 14 was digested with trypsin. Autoradiograms of trypsin digests are shown in Fig. 5. Single site phosphorylation reveals a single prominent $^{32}$P-labeled phosphopeptide while multiple site phosphorylation conditions demonstrate two prominent $^{32}$P-labeled phosphopeptides, one of which migrates identically with the single site phosphopeptide.

Tryptic digests were fractionated by cation exchange chromatography (Fig. 6). Digests from single site reaction conditions, representing the major phosphorylation site on HMG 14, revealed a single radioactive peak eluting at a pH of 3.6. No additional major radioactive peaks were observed. Digests from multiple site reaction conditions, representing the major and minor phosphorylation sites, were fractionated into two radioactive peaks. One eluted in the same position as the

![Image](4664_Fig_3.png)

**Fig. 3** Stoichiometry of HMG 14 and HMG 17 phosphorylation with G-kinase. Reactions were run for the times indicated with 0.36 $\mu$g/ml of G-kinase and 31 $\mu$M of HMG 14 (---) and 36 $\mu$M HMG 17 (---). Molecular weights used were 10,400 (4) and 9,247 (5) for HMG 14 and HMG 17, respectively.

fraction of HMG 17 after phosphorylation contrasts with the change in migration of all of HMG 14 after phosphorylation (Fig. 1). No phosphorylated impurities were observed. A-kinase at concentrations up to 1 $\mu$g/ml was not able to phosphorylate HMG 17 nor to phosphorylate HMG 14 in excess of 1 mol of $^{32}$P/mol of HMG 14. These data suggested that, in addition to a major site readily phosphorylated by G-kinase, HMG 14 contains a minor site capable of being phosphorylated specifically by G-kinase. A minor site specific for G-kinase is also present on HMG 17.

Prolonged incubation of each of the HMG proteins with agarose-immobilized alkaline phosphatase did not enhance their total phosphorylation by G-kinase.

Phosphorylation of HMG 14 by G-kinase was completely dependent on cGMP. In contrast to arginine-rich nucleosomal core histones which not only serve as substrates but also activate cyclic nucleotide-dependent protein kinase (26), HMG proteins had no effect on the activity of G-kinase assayed either without or with cGMP. Because arginine-rich histones are noncompetitive inhibitors of cyclic nucleotide binding to both G-kinase and the regulatory subunits of A-kinase (22), the HMG proteins were also tested as potential inactivators of cyclic nucleotide binding to G-kinase and to $R_{II}$. No significant inactivation of binding was observed (data not shown). This is consistent with the low arginine but high lysine content of HMG proteins and is analogous to the lack of inactivation of binding observed with lysine-rich histone H1 (22).

Two nuclear kinases from rat liver and two casein kinases from reticulocytes were unable to significantly phosphorylate HMG 14 (Table II).

Partial acid hydrolysis of $^{32}$P-labeled HMG 14 and $^{32}$P-labeled HMG 17 and subsequent analysis by high voltage electrophoresis at pH 1.9 were performed. Autoradiograms revealed that substantially all the radioactivity comigrated with authentic phosphoserine and inorganic phosphate though a trace estimated at 1-2% of phosphothreonine could be detected in the hydrolysates of both substrates.

Kinetics of HMG 14 Phosphorylation—The rate of HMG 14 phosphorylation by G-kinase and A-kinase as a function of substrate concentration is shown in Fig. 4. Typical saturation curves were converted to Eadie-Hofstee plots and single phosphorylation site kinetics were observed. Under the experimental conditions the major site of phosphorylation was defined; phosphorylation of the minor site was not readily observed under initial rate conditions with the enzyme and substrate concentrations used. With G-kinase the apparent $K_m$ was 40.5 $\mu$M and the $V_{max}$ was 14.7 mmol/min/mg. With A-kinase the apparent $K_m$ was 123 $\mu$M and the $V_{max}$ was 11.1 mmol/min/mg. G-kinase thus has a preference for HMG 14 with respect both to affinity and to rate of phosphorylation.

![Image](4664_Fig_4.png)

**Fig. 4** Kinetics of HMG 14 phosphorylation by G-kinase and A-kinase. Reactions were run for 10 min with 0.25 $\mu$g/ml of G-kinase (C) or 0.40 $\mu$g/ml of A-kinase ( ). HMG 14 concentrations ranged from 6.35-127 $\mu$M. Data represent the average of two experiments.

| Enzyme          | Ratio of $^{32}$P incorporated to HMG 14 |
|-----------------|-----------------------------------------|
|                 | units/ml | mol/mol   |
| G-kinase        | 420      | 1.09      |
| A-kinase        | 420      | 0.84      |
| Nuclear kinase I| 200      | <0.01     |
| Nuclear kinase II| 172      | 0.02      |
| Casein kinase 1 | 302      | 0.04      |
| Casein kinase 2 | 2320     | 0.11      |
Panels kinase.

Two-dimensional mapping of each phosphopeptide was performed. Peptide detection with fluorescamine, coupled with autoradiographic analysis showed a single fluorescent peptide on plates which was superimposed on a single radioactive peptide on the autoradiogram (Fig. 5, C and D). The NH2-terminal amino acid of the major site phosphopeptide was found to be lysine and the NH2-terminal amino acid of the minor site phosphopeptide was found to be leucine. High voltage electrophoresis at pH 1.9 of the dansylated hydrolysates confirmed the existence of 32P-labeled phosphoserine in each of the phosphopeptides.

**Table III**

| Amino acid | Major site | Minor site |
|------------|------------|------------|
| Asx        | 0.18 (0)   | 0.26 (0)   | 0.14 (0)   |
| Ser        | 2.12 (2)   | 1.91 (2)   | 1.00 (1)   |
| Glx        | 1.19 (1)   | 1.19 (1)   | 0.15 (0)   |
| Pro        | 0.14 (0)   |            |            |
| Gly        | 1.25 (1)   | 1.46 (1)   | 0.31 (0)   |
| Ala        | 3.14 (3)   | 3.00 (3)   | 1.03 (1)   |
| Val        | 1.08 (1)   | 0.83 (1)   |            |
| Met        |            | 0.05 (0)   |            |
| Ile        | 0.03 (0)   | 0.05 (0)   |            |
| Leu        | 0.16 (0)   | 0.18 (0)   | 0.97 (1)   |
| Tyr        | 0.04 (0)   | 0.07 (0)   | 0.03 (0)   |
| Phe        | 0.02 (0)   | 0.03 (0)   | 0.03 (0)   |
| Lys        | 2.00 (2)   | 1.66 (2)   | 1.20 (1)   |
| His        | 0.02 (0)   | 0.03 (0)   | 0.05 (0)   |
| Arg        | 0.06 (0)   | 0.07 (0)   | 0.10 (0)   |

**Table IV**

| Cycle | PTH-amino acid | Yield* | 32P* |
|-------|----------------|--------|------|
| 1     | Lys            | 1.84   | 8    |
| 2     | Val            | 2.16   | 10   |
| 3     | Ser            | 0.54   | 447  |
| 4     | Ser            | 0.61   | 324  |
| 5     | Ala            | 1.57   | 187  |
| 6     | Glu            | 0.65   | 159  |
| 7     | Gly            | 0.57   | 105  |
| 8     | Ala            | 1.09   |      |
| 9     | Ala            | 0.68   | 79   |
| 10    | Lys            | 1.34   |      |
| 11    | None           |        |      |

*32P-labeled peptide (2.0 nmol) was added to the cup containing polybrene (approximately 4 mg) and subjected to automatic Edman degradation. Values represent total yields per cycle.

Aliquots (2/3) of each PTH-amino acid fraction were subjected to scintillation counting. The values shown represent the total radioactivity corrected for background.
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The amino acid sequence of the 32P-labeled phosphopeptide containing the major phosphorylation site was determined by Edman degradation in the spinning cup sequenator as H-Lys-Val-Ser-Ser-Ala-Glu-Gly-Ala-Ala-Lys-OH (Table IV). The yields of the PTH derivatives of serine (cycles 3 and 4) were lower than the yields of stable hydrophilic PTH amino acids such as PTH-lysine or PTH-alanine. However, these lower yields of the serine derivatives were expected on the basis of experimental data obtained from sequence analysis of serine containing peptides under identical conditions (35, 36). The established amino acid sequence corresponded to the fragment HMG 14 (residues 4–13). The PTH amino acid fractions collected from the converting flask showed that radioactivity did not appear before cycle 3 and was maximal in cycle 3. The radioactivity found in cycle 4 (Table IV) could be explained as the result of carryover of the highly polar phosphoserine amidinohexitolizoline of cycle 3.

This explanation is supported by the data of manual Edman degradation of phosphorylated HMG 14 (residues 4–13) in which the peptide was subjected to two-dimensional mapping after each degradation cycle.

Based on the amino acid composition data, it is suggested that the peptides containing the minor phosphorylation sites are HMG 14 (residues 23–26) and HMG 17 (residues 27–30) (4, 5). In agreement with this suggestion, leucine was found to be the NH2-terminal amino acid and the peptide contained P-serine. A study was performed to demonstrate that the minor phosphorylation site on HMG 14 is the same as that phosphorylated on HMG 17. HMG 17 was phosphorylated by G-kinase and digested with trypsin. The digest of 32P-labeled HMG 17 was compared to the purified minor site phosphopeptide from 32P-labeled HMG 14 by two-dimensional mapping. A single major 32P-labeled phosphopeptide from the trypsin digests of phosphorylated HMG 17 was observed. The phosphopeptide from HMG 17 migrated with the minor site phosphopeptide from HMG 14 whether mapped separately or together (Fig. 7), indicating that the same site is present on both substrates. The proposed sequence is thus Leu-Ser(P)-Ala-Lys.

To determine whether the same major site as that phosphorylated by G-kinase was similarly labeled with A-kinase, the latter enzyme was used to prepare 32P-labeled HMG 14. Two-dimensional maps of the tryptic digest showed a single prominent phosphopeptide that migrated identically with the major site phosphopeptide prepared with G-kinase (data not shown).

**DISCUSSION**

Of the four HMG proteins, only HMG 14 was readily phosphorylated by cyclic nucleotide-dependent protein kinases. The primary sequences of the HMG proteins from calf thymus have been determined (4, 5). HMG 14 and HMG 17 have considerable sequence homology with three conserved regions which occur at the NH2-terminus involving residues 1–4, at a 27 amino acid residue region which contains the principal DNA binding segment on HMG 17 (41), and at a 7 amino acid residue region in the COOH-terminal end of the proteins. The major site of phosphorylation is in a sequence unique to HMG 14 in comparison with other HMG proteins. The site is specific for both A-kinase and G-kinase with preferences for the latter enzyme as indicated by kinetic evidence. The phosphorylation site is located outside the DNA binding segment at the NH2-terminal region with the principal DNA binding segment at the NH2-terminal region.

Amino Acid and Sequence Analysis—The two major site phosphoprotein preparations from single and multiple site reaction conditions, although isolated separately, behaved identically through each stage of purification. Their NH2-terminal amino acid and amino acid compositions did not differ significantly. The stoichiometry of the major site phosphopeptide was calculated to be 0.93 and 0.73 mol of 32P/mol of phosphopeptide from single and multiple site reaction conditions, respectively. Amino acid analysis of the major site and minor site phosphopeptides are shown in Table III. The major site phosphopeptide contains 10 amino acids including 2 serine residues. The minimal molecular weight was calculated to be 1026. The minor site phosphopeptide has four amino acids with a single serine and a minimal Mr = 498.
The residue sequence of Pro-Lys-Arg-Lys-Val-Ser(P)-Ser-Ala-Glu-Gly-Ala-Ala-Lys, and with the exception of residues 1-4, contains a sequence not found in HMG 17. The sequence contains two adjacent serines at positions 6 and 7. Phosphorylation appears to occur predominantly in residue 6, though the absence of $^{32}$P in serine 7 has not been completely established. Peptide mapping of the purified phosphopeptide revealed a single phosphorylated species containing 1 mol of $^{32}$P/mol of decapeptide, suggesting that phosphorylation of both serines does not occur on the same molecule. Although distribution of phosphates between the two serines on different molecules cannot be excluded, sequencing indicated predominant location of phosphoserine in position 6 of HMG 14.

Lysine 4 was not significantly removed by trypsin, presumably due to the influence of the acidic phosphoserine residue on the COOH-terminal side of the basic amino acid and/or because of the repetitive sequence of basic amino acids which tend to reduce the rate of hydrolysis by trypsin (42). An arginine residue which is removed during tryptic digestion is present on the NH$_2$-terminal side of lysine 4.

The sequence Lys-Arg-Lys-Val-Ser(P)-Ser-Ala of residues 2-8 of HMG 14 resembles that of many protein substrates for cyclic nucleotide-dependent protein kinases where the general sequence Arg-Arg-X-Ser(P) has been found (31, 43-46). In most studies of protein substrates, A-kinase catalyzed phosphorylation with lower $K_m$ and/or higher $V_{max}$ values than G-kinase (46-50). There are, however, three reported substrate sites with kinetic preference for G-kinase: 1) histone H2B with the Lys-Arg-Lys-Val-Ser(P)-Ser-Ala sequence Lys-Arg-Lys-Val-Ser(P)-Ser-Ala (51, 52). As discussed by Glass et al. (47), the presence of arginine on the COOH-terminal side of Ser 32 in H2B is a negative determinant for A-kinase while a lysine in one of the required dibasic positions on the amino terminal side of the phosphorylatable serine (or threonine) may favor G-kinase. The site of HMG 14 has lysine in one of the dibasic positions as do other G-kinase sites, but comparison of amino acids immediately adjacent to Ser- or Thr(P) on either amino or carboxyl sides reveals no obvious unique features. Assuming that all phosphate is present in Ser 6 of HMG 14, a preference for only one amino acid residue between the dibasic region and the phosphorylatable serine is indicated for both G-kinase and A-kinase. Secondary structural features in proteins may also contribute to substrate specificities for G-kinase and A-kinase (53).

The minor site specifically phosphorylated by G-kinase occurs in a conserved region found associated with the DNA binding segment of both HMG 14 and HMG 17 containing Ser 24 and Ser 28, respectively (4, 5). The single leucine residue which occurs in each protein is adjacent to the NH$_2$-terminal side of serine. Phosphorylation of this serine did not affect the cleavage of the Arg-Leu bond at the NH$_2$-terminal side of the phosphopeptide. The sequence in HMG 14 is Ala-Arg-Leu-Ser(P)-Ala-Lys-Pro (4). The single basic residue on the amino terminal side of this domain may explain the slow rate of phosphorylation associated with this site. Ser 20 in HMG 14 appears to be a potential phosphorylation site because it contains the basic site determinants in the sequence Pro-Lys-Arg-Arg-Ser-Ala-Arg. However, no phosphopeptide containing this serine was identified, perhaps due to lack of an amino acid between the dibasic region and phosphorylatable serine or due to the secondary structure of the protein.

Saffer and Glazer reported phosphorylation of HMG 14 and 17 in $^{32}$P-labeled Erlich ascites and L1210 cells (16) and Levy-Wilson reported that phosphorylation of HMG 14 and 17 was enhanced by butyrate treatment of HeLa cells (17). The enzyme(s) responsible for in vivo phosphorylation of HMG proteins have not been identified. The site of HMG 14 which is phosphorylated in vitro by cyclic nucleotide-dependent protein kinases appears specific for these enzymes because it was not phosphorylated by either of two nuclear or casein kinases tested. However, it is not known whether this site is phosphorylated in vivo or is it known whether A-kinase or G-kinase catalyze such in vivo phosphorylation. Immunofluorescent studies have indicated that G-kinase may be located in nuclei (54) and that cGMP-dependent phosphorylation of non-histone nuclear proteins occurs (55, 56).

In contrast to studies of Sun et al. (21) where thiposphorylation of HMG 1 by A-kinase was observed, the present studies detected only minimal A- or G-kinase catalyzed phosphorylation of either HMG 1 or 2. Alkaline phosphatase treatment failed to increase subsequent phosphorylation, suggesting that endogenous phosphate was not obscuring possible phosphorylation sites. Additional studies will be required to clarify possible phosphorylation sites on HMG 1 and 2.

In vitro phosphorylation may not accurately reflect phosphorylation in vivo. Although all five histones are phosphorylated by both cyclic nucleotide-dependent protein kinases in vitro, only a fraction of histone H1 has been clearly shown to be a phosphorylation substrate in vivo (57). Although the four arginine-rich nucleosomal core histones are good substrates when mixed individually with the enzymes, these histones are not phosphorylated when organized in nucleosome structures (58).

Because HMG 14 and 17 are reported to be associated with actively transcribed genes, posttranslational modification of these proteins is of potential importance in exploring their function in vivo. Identification of a phosphorylation site which is specific for cyclic nucleotide-dependent protein kinases offers one approach for such studies.

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