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Phosphorylation of the DNA-binding domain of nonhistone high-mobility group I protein by cdc2 kinase: Reduction of binding affinity

(growth-associated histone H1 kinase/DNA–protein interaction)

RAYMOND REEVES[†‡], THOMAS A. LANGAN[§], AND MARK S. NISSEN[†]

[†]Department of Biochemistry/Biophysics and [‡]Program in Genetics and Cell Biology, Washington State University, Pullman, WA 99164; and [§]Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262

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ABSTRACT **Mammalian high-mobility group I nonhistone protein (HMG-I) is a DNA-binding chromatin protein that has been demonstrated both *in vitro* and *in vivo* to be localized to the A+T-rich sequences of DNA. Recently an unusual binding domain peptide, "the A·T-hook" motif, that mediates specific interaction of HMG-I with the minor groove of DNA *in vitro* has been described. Inspection of the A·T-hook region of the binding domain showed that it matches the consensus sequence for phosphorylation by cdc2 kinase. Here we demonstrate that HMG-I is a substrate for phosphorylation by purified mammalian cdc2 kinase *in vitro*. The site of phosphorylation by this enzyme is a threonine residue at the amino-terminal end of the principal binding-domain region of the protein. Labeling of mitotically blocked mouse cells with [^{32}P]phosphate demonstrates that this same threonine residue in HMG-I is also preferentially phosphorylated *in vivo*. Competition binding studies show that cdc2 phosphorylation of a synthetic binding-domain peptide significantly weakens its interaction with A+T-rich DNA *in vitro*, and a similar weakening of DNA binding has been observed for intact murine HMG-I protein phosphorylated by the kinase *in vitro*. These findings indicate that cdc2 phosphorylation may significantly alter the DNA-binding properties of the HMG-I proteins. Because many cdc2 substrates are DNA-binding proteins, these results further suggest that alteration of the DNA-binding affinity of a variety of proteins is an important general component of the mechanism by which cdc2 kinase regulates cell cycle progression.**

The mammalian nonhistone high-mobility group proteins (HMGs) are a relatively abundant class of nuclear proteins thought to be important structural components affecting the conformation and function of chromatin (1). HMG-I and HMG-Y are isoform members of a subgroup of these nonhistone proteins (collectively called the HMG-I family) that are most likely produced by translation of mRNAs derived from a common precursor by alternative splicing (2, 3). The HMG-I proteins (not to be confused with the unrelated HMG-1 chromatin protein; ref. 1) are DNA-binding proteins that *in vitro* preferentially bind to stretches of A+T-rich DNA sequence (4–6). *In vivo* the HMG-I proteins have been immunolocalized to the A+T-rich G/Q and C bands of mammalian metaphase chromosomes (7). Members of the HMG-I family have been shown to specifically bind in the narrow minor groove of DNA by a specific 11-amino acid-binding-domain peptide (BD) (in single-letter code, TPKRPRGRPKK) which, because of its predicted structure, is called the "A·T-hook" motif (8).

HMG-I proteins are of considerable interest because they are expressed at elevated levels in proliferating cells (9, 10) and seem preferentially associated with an undifferentiated or neoplastically transformed cellular phenotype (2, 3, 11–13). This relationship suggests that whatever other functions these proteins might have in the cell, members of the HMG-I family may also play an important *in vivo* role in division and/or in maintaining the undifferentiated state of cells.

Along with histone H1, the HMG-I proteins appear to be among the most highly phosphorylated protein species in the nucleus (10, 14–16). The extensive phosphorylation of histone H1 that occurs in proliferating cells (17, 18) is catalyzed by an enzyme, formerly termed growth-associated H1 kinase, the activity of which is sharply elevated at mitosis (19–21) and which has recently been shown to be a mammalian homolog of yeast cdc2 kinase (22). In yeast, cdc2 kinase is required at two points in the cell cycle: (*i*) at a point called "start" in late G_1, which commits the cell to a round of cell division, and (*ii*) at a point just before entry into mitosis (23–26). A homolog of *cdc2* is also present in the human genome that can complement mutations in the yeast *cdc2* gene (27). The protein kinase component of *Xenopus* maturation-promoting factor and protein kinases present in starfish and clam oocytes, each of which is associated with the initiation of meiotic or mitotic divisions, are also encoded by homologs of the *cdc2* gene (28–32). Thus, it seems likely that the kinase is present in all eukaryotic cells. These findings provide evidence that this type of protein kinase participates in universal mechanisms for the control of cell-cycle progression. Elucidation of these mechanisms clearly depends on identification of the physiological substrates for cdc2 kinase.

cdc2 kinase recognizes substrates that contain the consensus sequence (Zaa)-Ser/Thr-Pro-(Xaa)-Zaa, where Xaa is frequently a polar residue, Zaa is generally lysine or arginine, and parentheses indicate residues present at some but not all sites (33). The consensus sequence is based on characterization of the sites in histone H1 phosphorylated by mammalian cdc2 kinase. These sites in H1 have also been shown to be phosphorylated by yeast and *Xenopus* oocyte cdc2 kinases (22), and most cdc2 phosphorylation sites characterized follow this consensus (34). Inspection of the three potential DNA-binding domains present in all mammalian HMG-I proteins (cf. Fig. 1, ref. 8) showed that either one (murine) or two (human) of these domains in individual proteins contain amino acid residues matching this cdc2 consensus sequence. We, therefore, proceeded to examine phosphorylation of these sites in murine HMG-I *in vivo* and *in vitro*.

MATERIALS AND METHODS

Purification of Proteins and Peptides. Crude HMG protein samples were obtained by extracting murine R1.1 (American

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Abbreviations: BD, DNA-binding domain peptide; HMG-I, high-mobility group I nonhistone protein; RP-HPLC, reversed-phase HPLC.

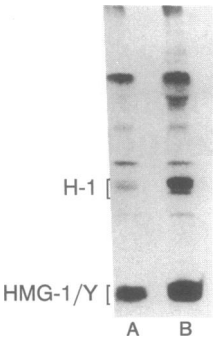

FIG. 1. Autoradiogram of SDS/PAGE separated *in vivo* ^{32}P-labeled total nuclear proteins isolated from nonproliferating (*A*) and mitotically blocked, exponentially growing (*B*) murine NIH 3T3 cells by extraction with 5% perchloric acid. Radiolabeled protein samples were isolated as described and electrophoretically separated on 14% SDS/PAGE as described (10) and exposed for autoradiography for 12 hr.

Type Culture Collection, No. TIB42) ascites cells with 5% perchloric acid followed by precipitation of the acid-soluble proteins by addition of an equal volume of 50% trichloroacetic acid (8). The precipitated proteins were collected by centrifugation, washed with acetone, and dried. Pure HMG-I and HMG-Y proteins were isolated from the crude extract by reversed-phase HPLC (RP-HPLC) as described (10). Peptides were synthesized on an Applied Biosystems model 431A peptide synthesizer and purified by C_{18} RP-HPLC as reported (8). Concentrations of protein and peptides were determined by standard methods (8). Autoradiography and electrophoretic separation of proteins by SDS/PAGE followed published procedures (10).

Cell Culture and *in Vivo* Labeling. NIH 3T3 murine cells obtained from the American Type Culture Collection were maintained as attached monolayers in Dulbecco's modified Eagle's medium/5% calf serum (complete medium). Nonproliferating and partially synchronous populations of proliferating cells were obtained as described (35). Nonproliferating cells were labeled with inorganic [^{32}P]phosphate (New England Nuclear) for 4 hr (50 μCi/ml; 1 Ci = 37 GBq) in complete medium/0.5 mM adenosine (Sigma). Mitotically blocked cells in partially synchronous populations were similarly labeled for 4 hr (between 18 and 22 hr after seeding) in the presence of nocodazole (Sigma) at 0.4 μg/ml. After ^{32}P-labeling, cells were harvested, washed once with phosphate-buffered saline solution, and resuspended in 500 μl of 5% perchloric acid. Cells were lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation. Carrier protein (bovine serum albumin, 50 μg) was added to the supernatant, and proteins were extracted and purified by RP-HPLC as described above.

***In Vitro* Phosphorylation of Proteins and Peptides by cdc2 Kinase.** Mammalian cdc2 kinase was purified from washed chromatin of logarithmic-phase Novikoff rat hepatoma cells and quantified as described (22, 36, 37). *In vitro* radiolabeling of pure proteins with phosphate was done with [γ-^{32}P]ATP (ICN) under the conditions for assay of growth-associated (cdc2) kinase activity described (36, 37) at a substrate concentration of 0.2 mg/ml (5-min incubation at 37°C). Quantitations of the extent and stoichiometry of *in vitro* phosphorylation of pure HMG-I proteins and synthetic peptides by cdc2 kinase were done as reported (22, 36, 37) at an enzyme concentration of 0.07 unit/ml and substrate concentrations of 0.5 mg/ml for incubation times of up to 4 hr at 37°C. Determinations of the time course of the reactions showed that phosphorylation was complete by this time. Phosphorylated HMG-I protein and peptides were isolated from the kinase reaction mixtures by RP-HPLC.

Tryptic Peptide Analysis of ^{32}P-Labeled HMG-I Proteins. RP-HPLC-purified murine HMG-I proteins phosphorylated either *in vitro* by cdc2 kinase or *in vivo* as a result of metabolic labeling with inorganic [^{32}P]phosphate were dissolved in 100 mM NH_4HCO_3, pH 8.0/0.1 mM $CaCl_2$ containing trypsin added at 1:10 (wt/wt) ratio. After overnight incubation at room temperature, the pH of the reaction mixture was adjusted to 2 with trifluoroacetic acid, and peptides were resolved by C_{18} RP-HPLC. Chromatographic fractions were collected and counted for radioactivity, and the ^{32}P-labeled peptides in the indicated fractions (see text) were collected and sequenced (Applied Biosystems 470A sequencer).

Fluorescence Competition DNA-Binding Assays. Quantitative fluorescence competition DNA-binding assays followed described procedures (8). Briefly, A+T-rich substrate DNA (the 3′ untranslated tail region of bovine interleukin 2 cDNA; ref. 8) was titrated with the fluorescent dye Hoechst–Roussel 33258 with or without phosphorylated and nonphosphorylated peptide competitor. The Michaelis–Menten equation was fit to the data (fluorescence vs. [dye]) using the program ENZFITTER (38), permitting the direct determination of the dissociation constant K_d of the dye (in the absence of competitor) or the apparent dissociation constant K_{app} (in the presence of competitor). Dissociation constants of the peptides K_{pep} were obtained by using the relation $K_{app} = K_d (1 + [\text{peptide}]/K_{pep})$.

RESULTS

As shown in Fig. 1, *in vivo* ^{32}P-labeling of mitotically blocked murine cells revealed extensive phosphorylation of HMG-I/Y proteins as well as of histone H1. Compared with nonproliferating cells, the increase in phosphorylation is 2- to 3-fold for HMG-I/Y and >6-fold for histone H1. This smaller relative increase in phosphorylation of HMG-I/Y (compared with H1) is due to the more extensive *in vivo* phosphorylation of the murine protein in nonproliferating cells at additional sites that are probably not substrates for cdc2 kinase (see below). *In vitro* experiments with purified mammalian cdc2 kinase (36, 37) demonstrated that pure murine HMG-I and HMG-Y proteins are efficient substrates for this enzyme; the rates of phosphorylation were ≈40% that of histone H1 (Fig. 2). On longer incubation with excess cdc2 kinase, the stoichiometry of phosphorylation of HMG-I was found to be 0.7 mol per mol of protein, close to the value predicted for murine proteins from inspection of their amino acid sequences for consensus cdc2 sites (2). Failure to reach the predicted stoichiometry of one phosphate per molecule of protein may indicate that the HMG-I substrate protein, which is isolated

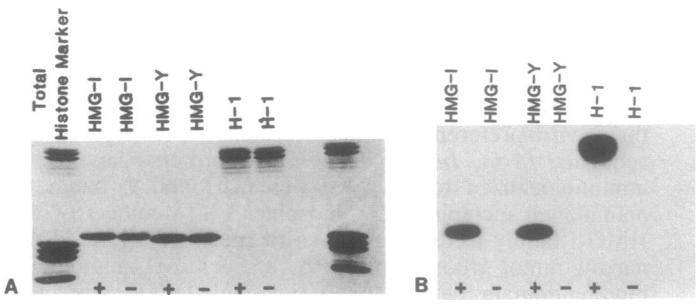

FIG. 2. *In vitro* phosphorylation of HMG-I, HMG-Y, and histone H1 by cdc2 kinase. Purified rat cdc2 kinase was used to phosphorylate the indicated purified proteins with (+) or without (−) the addition of the kinase. (*A*) Coomassie-stained SDS/PAGE gel. (*B*) Autoradiogram of *A*. Radioactive spots were cut out and counted by Cerenkov radiation. The rates of phosphorylation of HMG-I and HMG-Y were 37% and 46%, respectively, of the rate of H1 phosphorylation.

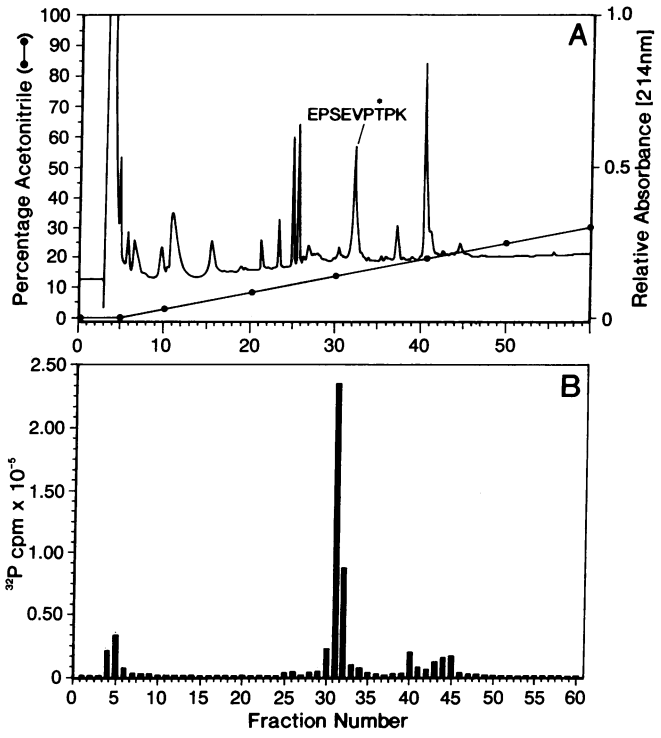

FIG. 3. Tryptic peptide analysis of *in vitro* cdc2-labeled murine HMG-I protein. Tryptic peptides were separated by RP-HPLC (*A*) and counted for radioactivity (*B*). The indicated ^{32}P-labeled peptide was sequenced, and the site of *in vitro* cdc2 phosphorylation was determined (*) (*A*). This phosphorylation site corresponds to threonine residue 53 at the amino-terminal hook end of the principal DNA-binding BD peptide (*TPKRPRGRPKK) of the murine HMG-I protein (8).

from proliferating murine ascites cells, is already partially phosphorylated. Tryptic peptide analysis of murine HMG-I protein phosphorylated to this level by cdc2 kinase (Fig. 3) revealed that a single peptide (EPSEVPTPK) contained most of the radioactive phosphate; the threonine residue was the site of phosphorylation as determined by sequence analysis (Fig. 4*A*). These sequencing results were confirmed by phosphoamino acid analysis of HMG-I protein labeled *in vitro* by cdc2 kinase, which showed phosphothreonine as the sole phosphoamino acid present (Fig. 4*B*). It is to be noted that this *in vitro* cdc2 kinase-phosphorylated peptide contains the amino-terminal A·T-hook amino acids of the internal HMG-I BD (residues 53–62) that most strongly binds to DNA (8) and that the threonine residue conforms to the consensus sequence of cdc2 kinase phosphorylation sites (22, 34).

Similar experiments involving ^{32}P-labeling of exponentially growing mammalian cells blocked in mitosis by treatment with nocodazole indicated that this same threonine residue in the DNA-binding domain was phosphorylated *in vivo* (Fig. 5, *). In addition to the indicated cdc2 site (*; at residue 53), two other major sites of *in vivo* phosphorylation (Fig. 5, +) occur in murine HMG-I, both of which are also located in potential DNA-binding domains (at residues 21 and 77) of the mouse protein (8). The kinases responsible for the *in vivo* modification of these additional phosphorylation sites (+) remain to be determined. Nevertheless, additional experiments involving *in vivo* ^{32}P labeling of HMG-I proteins in either nondividing, randomly proliferating, or mitotic-phase NIH 3T3 cells indicate that *in vivo* the cdc2 site (*) is much more highly phosphorylated in both mitotic phase and randomly growing cells than in nondividing cells; the highest level of phosphor-

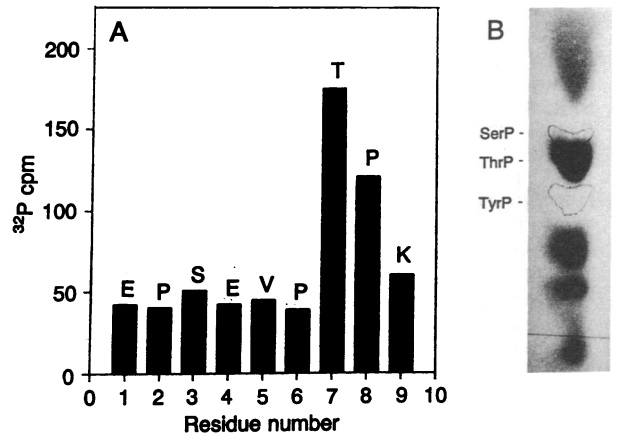

FIG. 4. Phosphoamino acid analysis of murine HMG-I phosphorylated by cdc2 kinase *in vitro*. (*A*) Histogram of ^{32}P-labeled amino acids (in one-letter code) released during automated sequencing of the *in vitro*-labeled peptide shown in Fig. 3. (*B*) HMG-I was phosphorylated for 4 hr at 37°C as described, and an aliquot of the reaction mixture was spotted on Whatman p81 phosphocellulose paper. After removal of [^{32}P]ATP by washing with 75 mM phosphoric acid, the phosphorylated HMG-I was eluted from the paper with 6 M HCl, and partial acid hydrolysis and phosphoamino acid determination were done as described (22). [^{32}P]Phosphoamino acids were visualized by autoradiography. Dashed lines show the position of carrier phosphoamino acids stained with ninhydrin. Other radioactive spots represent phosphopeptides and free inorganic [^{32}P]phosphate present in the partial acid hydrolysate.

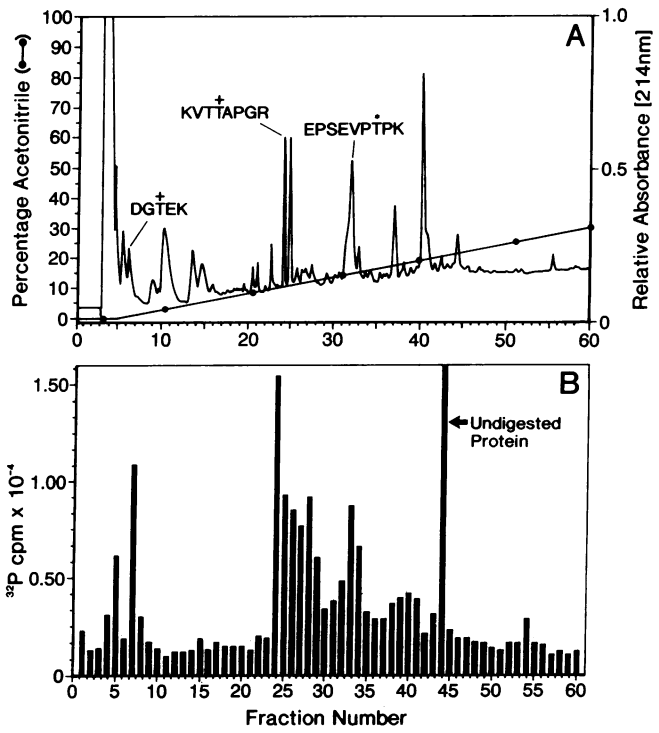

FIG. 5. Tryptic peptide analysis of *in vivo* ^{32}P-labeled murine HMG-I protein. Mitotically blocked NIH 3T3 cells were *in vivo* ^{32}P-labeled, and HMG-I protein was isolated and RP-HPLC-purified as described earlier (except that 100 μg of purified murine ascites HMG-I was substituted for carrier bovine serum albumin). After digestion, tryptic peptides were separated by RP-HPLC (*A*) and counted for radioactivity (*B*). The indicated ^{32}P-labeled peptides were sequenced, and the sites of *in vivo* phosphorylation were determined (*A*). Note that one *in vivo*-phosphorylated peptide is the same as the single peptide labeled *in vitro* by cdc2 kinase (*) (Fig. 3). In addition, as noted in text, two other sites of *in vivo* phosphorylation, denoted by + above the indicated amino acid sequences, are both located in potential DNA-binding domains (at residues 21 and 77) of the murine HMG-I protein (8).

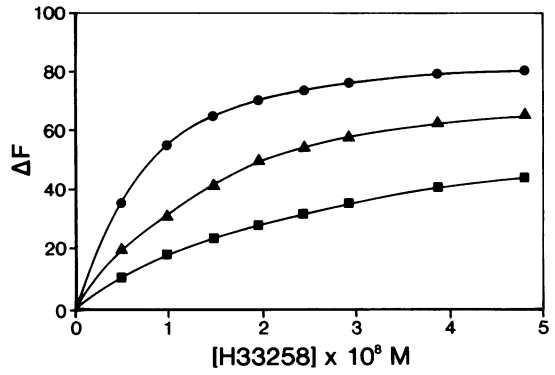

FIG. 6. Fluorescence competition assay of binding of a synthetic HMG-I-binding-domain peptide phosphorylated *in vitro* by cdc2 kinase to A+T-rich substrate DNA. The binding constant of an HMG-I 13-mer synthetic consensus BD (VPTPKRPRGRPKK) was determined by using a fluorescence competition assay as described in text (8). ●, Change in fluorescence (ΔF) seen when DNA was titrated with dye without competing ligands or with unphosphorylated (■), or cdc2 kinase-phosphorylated (▲), synthetic BDs. DNA concentration was fixed at 100 nM, the two BDs were at 5.8 μM each, and the dye concentration varied from 0 to 50 nM. K_d values derived for unphosphorylated and phosphorylated BDs are 1.9 μM and 4.2 μM, respectively, indicating ≈65% decrease in DNA-binding affinity of the BD after *in vitro* phosphorylation.

ylation of this threonine residue is found in the mitotic cells (data not shown). Together, these results demonstrate that the threonine residue in the principal DNA-binding domain of mammalian HMG-I proteins is phosphorylated by cdc2 kinase *in vitro* and that this same site is also preferentially phosphorylated *in vivo* in mitotically blocked cells.

A 13-residue synthetic peptide containing the "consensus" DNA-binding domain of mammalian HMG-I (8) (plus two additional amino-terminal amino acids), VPTPKRPRGRPKK, was prepared. This peptide was also found to be active as a substrate for cdc2 kinase, being phosphorylated at 57% of the rate of histone H1, which is an excellent substrate for mammalian and yeast cdc2 kinases (22, 34, 37). By using fluorescence competition assays (8), the stoichiometrically phosphorylated form of this peptide was found to bind significantly less tightly to substrate DNA than unphosphorylated peptide. As illustrated in Fig. 6, phosphorylation of the peptide by cdc2 kinase reduces its binding affinity to substrate DNA by ≈55–65% compared with unphosphorylated peptide. Phosphorylation does not, however, completely inhibit binding to A+T-rich DNA. This observation is consistent with the prediction that there are multiple intermolecular interactions involved in BD binding to the minor groove of A+T-rich DNA (8), and the interpretation that cdc2 kinase phosphorylation, although considerably weakening these interactions, does not necessarily completely eliminate all of them. Preliminary data show a similar weakening of DNA binding for the intact murine HMG-I protein phosphorylated *in vitro* (data not shown). Taken together, these data suggest that the phosphorylation of threonine residues in the hook region of the DNA-binding domains of the HMG-I proteins may have biologically functional significance, particularly with respect to the ability of these proteins to specifically and tightly bind to A+T-rich DNA.

DISCUSSION

cdc2 kinase has been shown to phosphorylate a considerable number of proteins, many of which are DNA-binding proteins (for review, see ref. 34). Most of these proteins are phosphorylated at sites that match the consensus sequence for cdc2 kinase recognition discussed above. Sea urchin sperm-specific histones H1 and H2B contain multiple repeats of such a sequence, Ser-Pro-basic amino acid-basic amino acid (39), which have been shown to be DNA-binding sites (40–43). Suzuki has proposed a structure for binding of these multiple repeats to A+T-rich DNA (40–42), which, although quite different in most respects, has some features in common with the A+T-hook motif predicted for HMG-I binding sites (8). These sperm histone sites are also phosphorylated *in vivo* (43, 44), and such modifications have been suggested to affect the DNA-binding characteristics of the amino-terminal ends of these proteins (40–44). In addition to mammalian somatic histone H1 (33), trout testis histone H1 and chicken erythrocyte histone H5 also contain sequences of this type, which are known to be phosphorylated *in vivo* (45, 46), and in several cases phosphorylation has been shown to affect the compactness of chromatin or histone H1-DNA complexes (44, 47, 48). The present demonstration that *in vitro* cdc2 kinase phosphorylation of an HMG-I DNA-binding domain reduces its affinity for DNA suggests that similar *in vivo* phosphorylations of intact HMG-I proteins may likewise alter their DNA-binding properties and thereby, perhaps, participate in cell cycle-regulated modulations of chromatin structure. Phosphorylation of recognition sites in other DNA-binding cdc2 kinase substrates may also diminish their DNA affinity. Such alterations in the affinity of DNA-binding proteins may be an important general component of the mechanism by which cdc2 kinase regulates cell cycle progression.

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