Articles

Four Distinct Cyclin-Dependent Kinases Phosphorylate Histone H1 at All of Its Growth-Related Phosphorylation Sites†

Richard A. Swank,*,‡ John P. H. Th'ng,‡ Xiao-Wen Guo,‡ Joe Valdez,§ E. Morton Bradbury,‡,§ and Lawrence R. Gurley§

School of Medicine, Department of Biological Chemistry, University of California—Davis, Davis, California 95616, and Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Received June 16, 1997; Revised Manuscript Received September 8, 1997⊗

ABSTRACT: In mammalian cells, up to six serines and threonines in histone H1 are phosphorylated *in vivo* in a cell cycle dependent manner that has long been linked with chromatin condensation. Growth-associated H1 kinases, now known as cyclin-dependent kinases (CDKs), are thought to be the enzymes responsible for this process. This paper describes the phosphorylation of histone H1 by four different purified CDKs. The four CDKs phosphorylate only the cell cycle specific phosphorylation sites of H1, indicating that they belong to the kinase class responsible for growth-related H1 phosphorylation *in vivo*. All four CDKs phosphorylate all of the interphase and mitotic-specific H1 sites. In addition to the (S/T)PXK consensus phosphorylation sites, these four CDKs also phosphorylate a mitotic-specific *in vivo* H1 phosphorylation site that lacks this sequence. There is no site selectivity among the growth-related phosphorylation sites by any of the four CDKs because all four CDKs phosphorylate all relevant sites. The results imply that the cell cycle dependent H1 phosphorylations observed *in vivo* must involve differential accessibility of H1 sites at different stages of the cell cycle.

How a cell controls its proliferation is a question of fundamental importance to both biological and medical sciences. Work for the last 30 years has implicated the activity of growth-associated H1 kinase and the phosphorylation of histone H1 as important biochemical processes in cell proliferation control (Ord & Stocken, 1968; Gurley & Walters, 1971; Balhorn et al., 1972; Bradbury et al., 1973, 1974a,b; Gurley et al., 1973, 1974, 1975, 1978a,b, 1981, 1995; Hardie et al., 1976; Inglis et al., 1976). Bradbury et al. (1973, 1974a) found a correlation between H1 phosphorylation, H1 kinase activity, and chromosome condensation and observed (Bradbury et al., 1974b) that the addition of heterologous H1 kinase to the plasmodium of the slime mold *Physarum polycephalum* 3 h before metaphase caused an advancement of mitosis of up to 1 h. This lead to the proposals that H1 kinase activity controls the cell cycle and through H1 phosphorylation initiates chromosome condensation. Work in mammalian systems, including Chinese hamster CHO[1] (Gurley et al., 1974, 1975, 1978a,b, 1981; Hohman et al., 1975, 1976), Chinese hamster V-49 (Lake & Salzman, 1972; Lake et al., 1972; Lake, 1973a,b), HeLa cells (Lake, 1973b; Marks et al., 1973; Ajro et al., 1975, 1981), rat nephroma cells (Lake et al, 1972), hepatoma HTC cells (Balhorn et al., 1975), and rat hepatoma (Langan et al., 1980) also suggested that H1 phosphorylation was linked to mitotic chromosome condensation.

Recently, evidence linking histone H1 phosphorylation levels with chromosome condensation has been reported using the kinase inhibitor staurosporine. Th'ng et al. (1994) demonstrated that treatment of *murine* cells with staurosporine prevented histone phosphorylation and entry into mitosis. When these cells are blocked in mitosis with nocadozole, the addition of staurosporine caused rapid histone dephosphorylation and rapid chromosome decondensation. Also, Gurley et al. (1995) showed that staurosporine and Hoechst 33342 blocked both interphase and mitotic H1 phosphorylation sites in CHO cells and prevented the condensation of G2 phase chromatin into mitotic chromosomes. These data are the most recent direct evidence linking histone H1 phosphorylation levels to chromosome condensation.

The role of protein phosphorylation as a primary control mechanism for cell cycle progression has been demonstrated by recent genetic studies that have identified the family of cyclin-dependent kinases (CDKs) that are necessary for cell cycle progression. CDKs are composed of catalytic and regulatory subunits and their activity is controlled through

† This work was supported by NIH Grant GM 45890-03 to E.M.B. and by the United States Department of Energy.

* Author to whom correspondence should be addresed. Present address: Department of Medical Genetics, University of Washington, Seattle, WA 98195-7720.

‡ University of California—Davis.

§ Los Alamos National Laboratory.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1997.

[1] Abbreviations: CDK, cyclin-dependent kinase; CHO, a fibroblast cell line originally derived from the ovary of a Chinese hamster; (S/T)PXK, the consensus amino acid sequence (serine or threonine), proline, any amino acid, lysine; Ac-SETAPAAPAAAPPAEK, an N-terminal tryptic peptide of histone H1 containing an N-acetylated serine (Ac-S), glutamic acid (E), threonine (T), alanine (A), proline (P), and lysine (K); HMG, high mobility group; HPLC, high-performance liquid chromatography; AU-PAGE, acid-urea polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; FM3A, a wild-type mouse mammary tumor cell line; FPLC, fast protein liquid chromatography.

S0006-2960(97)01436-0 CCC: $14.00

post-translational modification of these subunits and by association with inhibitor proteins [see reviews in Hunter and Pines (1994) and Lees (1995)]. Whereas understanding the regulation of these kinases continues to make progress, positive identifications of their *in vivo* substrate proteins, their *in vivo* phosphorylation sites and the substrate's function is largely unknown. For example, the CDK having maximum activity at mitosis ($p34^{CDC2}$/cyclin B) was thought to phosphorylate only consensus sites (S/T)PXK [see review by Moreno and Nurse (1990)]. However, our laboratory recently identified an *in vivo* mitotic-specific phosphorylation site on histone H1 contained in the N-terminal peptide Ac-SETAPAAPAAAPPAEK that has no such consensus sequence (Gurley et al., 1995). This raised the question as to whether this particular site is phosphorylated not by a CDK but by an unknown kinase.

Histone H1 contains six cell-cycle-specific phosphorylation sites (Hohmann et al., 1975, 1976; Gurley et al., 1995). However, attempts to examine these sites using purified CDKs have been limited to only one CDK ($p34^{CDC2}$/cyclin B). The resulting *in vitro* phosphorylation matched some, but not all, of the *in vivo* phosphorylation sites (Jerzmanowski & Cole, 1992). Therefore, it is clear that the examination of H1 phosphorylation sites with the other purified CDKs is necessary to determine if histone H1 is a natural substrate for the CDK class of kinases.

This paper describes the *in vitro* phosphorylation of histone H1 by four different CDKs and demonstrates that H1 is the natural substrate for all these kinases by comparing the *in vitro* phosphorylation sites to those phosphorylated *in vivo*. The data yield the surprising results that all four CDKs phosphorylate all the *in vivo* cell-cycle-dependant phosphorylation sites in H1, indicating that H1 cell-cycle-dependent phosphorylation is not a function of individual CDK site specificity. The data also demonstrates that the consensus sequence (S/T)PXK is not the sole requirement for the CDK phosphorylation of histone H1 *in vivo* as previously thought. These results indicate a need for a new paradigm in viewing H1 phosphorylation in the cell cycle. Our data suggests a model in which cell-cycle-dependent chromosomal rearrangements expose H1 sites that can be phosphorylated by any CDK available in that phase of the cell cycle. For example, in this paradigm, mitotic-specific H1 phosphorylation does not occur because $p34^{CDC2}$/cyclin B is active only in mitosis, but rather because the N-terminal tail of H1 is released from chromatin and exposed to this CDK at that time. The major question is whether chromosomal rearrangements during each phase of the cell cycle are initiated by the phosphorylation of phase specific sites on histone H1.

EXPERIMENTAL PROCEDURES

Purification of CDKs. The purification of CDKs from *murine* FM3A cells is described in Hamaguchi et al. (1992) with some modifications. Following the preparation of a whole cell extract and polyethyleneimine precipitation, samples are subjected to FPLC ion exchange chromatography (Mono S followed by Mono Q). Hydrophobic interaction chromatography (Phenyl Superose) was used to achieve highly purified kinase preparations (Swank, 1995). The four CDKs isolated are $p34^{CDC2}$/cyclin A, $p33^{CDK2}$/cyclin A, $p34^{CDC2}$/cyclin B, and $p34^{CDC2}$/complexed with unknown protein(s). For simplicity these enzymes are referred to by the one letter abbreviations A, B, C, and M, respectively.

Histone H1 Purification. Histone H1 was isolated from CHO cells grown in culture as previously described (Gurley et al., 1995). Briefly, H1 was extracted from crude chromatin by extraction in 5% perchloric acid. Following extraction, histone H1 was precipitated in 20% trichloroacetic acid and lyophilized. Histone H1 was dissolved in water containing 0.2% trifluoroacetic acid, applied to a reverse phase CN HPLC column (Waters μBondapak 3.9 $\times$ 150 mm), and separated from contaminants using an acetonitrile gradient of 5 to 35%. The fractions containing H1 were pooled, lyophilized and stored at -20 °C.

Perchloric acid extracts of CHO cells contain, in addition to histone H1, other H1 variants such as H1° and HMG proteins (D'Anna et al., 1985). The CN reverse phase column removes the H1° and HMG proteins from the H1 (Gurley et al., 1990). Thus, H1° was not involved in the phosphorylation analyses in this work.

CHO H1 consists primarily of one H1 subtype, H1a (74% of total H1). However, it does contain small amounts of a minor subtype H1b (17%) and trace amounts of two other subtypes, H1c and H1d (8% combined) (Gurley et al., 1975). The CN column does not subfractionate these subtypes (Gurley et al., 1990). Previous work has shown that the phosphopeptides from the minor subtypes can be distinguished from those of the major subtype when the tryptic digests of whole H1 are subjected to HPLC analysis (Gurley et al., 1995). Thus, the phosphorylation analyses discussed in this work refer to H1a.

Phosphorylation of Histone H1. H1 was phosphorylated *in vitro* by the four kinases using the following conditions. HPLC purified, lyophilized H1 was dissolved in ddH2O at a concentration of 5 μg/μL. For AU-PAGE (acid-urea polyacrylamide gel electrophoresis) analysis, 10 μg of histone H1 was phosphorylated in a volume of 50 μL in H1 phosphorylation buffer (50 mM Tris, pH 7.4, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 75 mM NaCl, containing 0.1 mM ATP and 25 μCi -γ [^{32}P]ATP) using 5 μL of purified enzyme fraction. Reactions were stopped by precipitation in 20% TCA. Precipitated histone was resuspended in AU-PAGE loading buffer (5% acetic acid containing 8 M urea and 25% glycerol) and loaded onto a 12% 45 cm long AU-PAGE slab containing 8 M urea and run at 300 V (constant voltage) for 24 h. The gels were stained with Coomassie blue, dried, and exposed to Kodak film (XAR-5) for autoradiography.

When histone H1 was phosphorylated *in vitro* for use in tryptic digestion experiments, reactions were scaled up 10-fold more than that used in the AU-PAGE analysis. H1 (100 μg) was phosphorylated in H1 phosphorylation buffer (as above) in a volume of 500 μL containing 0.1 mM ATP and 25 μCi-γ [^{32}P]ATP and up to 50 μL of purified enzyme fraction. The reaction mixture was incubated at 32 °C for 20 min and stopped with the addition of phosphoric acid to a final concentration of 75 mM. The samples were then precipitated in 20% TCA at -20 °C for 2 h. The precipitate, containing H1, was recovered by centrifugation, washed 3 times in acidified acetone (acetone containing 0.1 M HCl), once in acetone, and dried under vacuum. The dried H1 samples were then used for tryptic digestion.

Trypsin Cleavage of Histone H1. Trypsin digestion and analyses of tryptic peptides were carried out by the method of Stone et al. (1989) as previously described (Gurley et al., 1995). Briefly, HPLC purified and lyophilized H1 samples

were dissolved in 8 M urea containing 0.4 M NH_4HCO_3. Dithiothreitol was added and the mixture heated to 50 °C to facilitate protein denaturation. After cooling, iodoacetamide was added and the mixture incubated at room temperature for 15 min. Following this step, the urea concentration is diluted with water to 2 M. This concentration keeps the carboxyamidomethylated proteins in solution but does not significantly inhibit trypsin digestion (Stone et al., 1989). The trypsinization occurs for 24 h at 37 °C and the reaction stopped by freezing or by direct injection onto a reverse-phase HPLC system.

HPLC Separation of Tryptic Peptides. The tryptic peptides were resolved using an HPLC system equipped with dual UV and radioactivity detection. The peptides were separated on a C18 Deltapak steel column using an acetonitrile gradient running from 1.6 to 30% in 120 min, followed by a 30 min 30 to 60% gradient and a 15 min 60 to 78% gradient, as described in Gurley et al. (1995). The peptides eluting from the column were detected by UV absorption at 210 nm. The eluent was mixed with scintillant (Radiomatic flowscint A) and detected using an HPLC radioactivity monitor (Berthold, LB 504). The UV and radioactivity data was quantitated, integrated, and analyzed as described in Gurley et al. (1995).

RESULTS

Methods for the determination of phosphorylation sites within CHO histone H1 *in vivo* have been established (Gurley et al., 1995). Multiple H1 kinases, however, have not been purified from CHO cells as they have from *murine* cells (Hamaguchi et al., 1992; Swank, 1995). The purified *murine* kinases provide an opportunity to compare *in vivo* and *in vitro* phosphorylation sites in histone H1. The use of the four *murine* kinases also allow an examination of possible affinity differences for specific phosphorylation sites within H1, as well as potential substrate specificity directed by the cyclin components (Peeper et al., 1993).

Kinetics of Phosphorylation for Purified CHO Histone H1. To ensure that the *in vitro* phosphorylation of H1 using the purified kinases occurred under conditions of adequate substrate, cofactors, and ATP, time course phosphorylations were performed and quantified. Purified CHO histone H1 was phosphorylated for 10, 20, 30, and 60 min. The reactions were stopped, and H1 was separated on 12% AU-PAGE gels. After Coomassie blue staining, the gels were dried and autoradiograms made. Figure 1 shows the autoradiograms of H1 phosphorylated by the four kinases.

Quantification showed that under the conditions used phosphate was incorporated in a linear manner (data not shown). Linear phosphate incorporation occurred for up to 40 min, with a plateau of incorporation evident by 60 min (data not shown). Differences in the final extent of phosphorylation was evident, as some enzymes appear to phosphorylate H1 more efficiently than others, especially at endpoints when the substrate is no longer saturating (see Figure 1; 60 min time points). Subsequent phosphorylations for mapping purposes were carried out for up 20 min, well within the linear range of phosphate incorporation for all four CDKs.

Mapping of the in Vivo Histone H1 Phosphorylation Sites. Histone H1 contains a large number of potential phosphorylation sites, up to 21 sites in rabbit [reviewed by van Holde (1989)]. However, of these, only six sites are phosphorylated *in vivo* (Hohmann et al., 1976). To determine specific *in vivo* phosphorylation sites in CHO cells, histone H1 was isolated from cultures of CHO cells grown in the presence of $^{32}P_i$. After acid extraction and HPLC purification, the histone was trypsinized. The tryptic peptides were separated by C18 reverse phase column chromatography and both the UV and radioactivity profiles were integrated (Gurley et al., 1995).

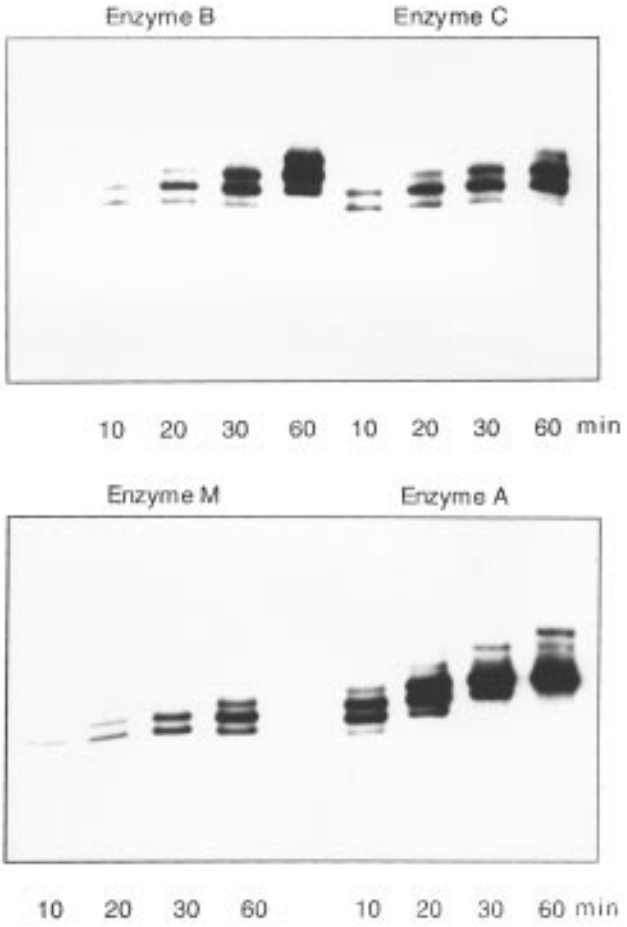

FIGURE 1: Autoradiograms showing time course phosphorylation of histone H1. Cyclin-dependent kinase preparations are referred to as Enzyme B ($p33^{CDK2}$/cyclinA); Enzyme C ($p34^{CDC2}$/cyclinB); Enzyme A ($p34^{CDC2}$/cyclin A); and Enzyme M ($p34^{CDC2}$ complexed with unidentified components). Enzyme fractions were added to purified H1 and incubated for the times indicated as described in methods. The reaction mixtures were separated by acid-urea gel electrophoreses. The gels were dried and autoradiograms made.

Two CHO cell growth conditions were used for the *in vivo* H1 phosphorylation analysis: cells in exponential growth and cells in mitosis. Exponentially growing cells were examined for H1 phosphorylation, and the tryptic peptide UV absorption pattern and the ^{32}P-labeled phosphopeptide patterns are shown in Figure 2. The phosphopeptides found under these conditions represent the bulk of the phosphorylation sites found in G1, S, and G2 phases, because under asynchronous conditions, only ~2% of cells are found in mitosis. Asynchronous CHO cells as well as mitotic synchronized cells were grown in the presence of $^{32}P_i$ and the phosphopeptide patterns are shown in Figure 2, panels B and C. The phosphorylation pattern for mitotic blocked cells (Figure 2C) represents the largest phosphate incorporation into histone H1 and is the maximal phosphorylation state

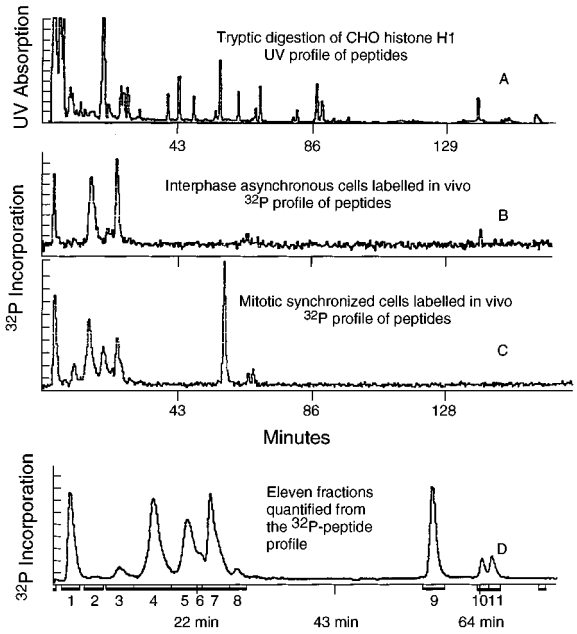

FIGURE 2: UV and 32-P incorporation profiles of peptides fractionated by HPLC after tryptic digestion. (A) UV profile of tryptic peptides from CHO histone H1. (B) 32-P incorporated tryptic peptides obtained from histone H1 isolated from asynchronous CHO cells grown in the presence of 32Pi. (C) 32-P incorporated tryptic peptides obtained from histone H1 isolated from mitotically blocked CHO cells. (D) The 11 phosphopeptides used for analysis of *in vitro* phosphorylated CHO H1 shown as an expansion of spectrum C.

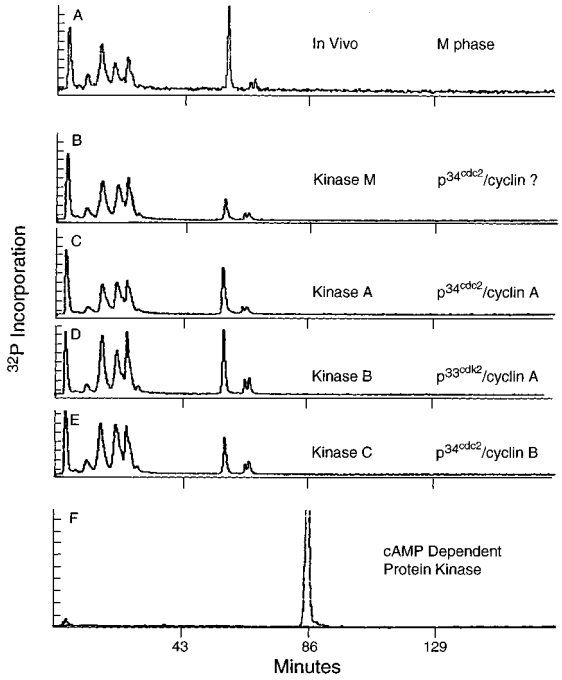

FIGURE 3: Comparison of 32-P labeled peptides obtained from *in vivo* and *in vitro* labeled CHO histone H1. (A) *In vivo* labeled peptide obtained from mitotic blocked cultures. Spectra B−E represent *in vitro* labeled peptides obtained after incubation with kinases M, A, B, C, respectively. (F) ^{32}P-labeled peptide obtained after phosphorylation with cAMP-dependent protein kinase catalytic subunit.

attainable via *in vivo* labeling (Gurley et al., 1981). The 11 phosphopeptides quantified for this study are depicted in Figure 2D.

Mapping of in vitro Phosphorylation Sites. To compare the phosphopeptide pattern found for the *in vivo* phosphorylated H1 with that of the *in vitro* phosphorylated H1, the four kinases (A, B, C, and M) were used to phosphorylate H1 as described above. All four kinases were used at amounts that resulted in the nearly the same phosphate incorporation into histone H1 and under conditions in which linear incorporation occurred. The results of the *in vitro* phosphorylation using the four kinases are shown in Figure 3, a composite of the radioactive phosphopeptide profiles of the *in vitro* and *in vivo* phosphorylated peptides (See Figure 3, panels A−E). The striking result is that all four kinases duplicate the *in vivo* mitotic phosphorylation map of histone H1, with only subtle differences in the extent of the phosphorylation as evidenced by the differential peak intensity of the phosphopeptides from the *in vitro* phosphorylations.

Quantification of Phosphorylation Differences. In order to examine the phosphorylation differences between the four CDKs, the radioactivity profile of each phosphopeptide was integrated and expressed relative to the H1 column load measured by the UV absorption of the large N-terminal peptide eluting at 58 min (9) as described (Gurley et al., 1995). Such a comparison allows the determination of the relative levels of phosphorylation for each peptide. High levels of phosphorylation of a particular peptide would indicate a preferred phosphorylation site for that kinase. The summary of this analysis is shown in Figure 4. The results indicate that there are no major differences between the four kinases in their ability to phosphorylate histone H1. There

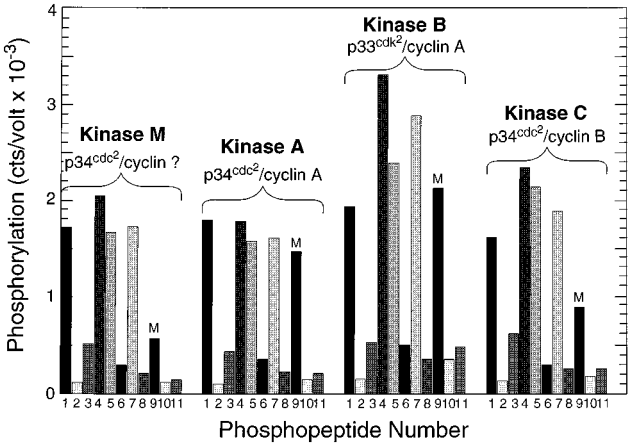

FIGURE 4: Relative phosphorylation rates of the 11 peptides analyzed from in vitro labeled histone H1 using four different cyclin dependent kinases. The phosphorylation of each peptide within a set is expressed relative to the HPLC column load as measured by the UV absorption of the large N-terminal peptide eluting at 58 min (9). This normalization permits comparison of the phosphorylation levels of each phosphopeptide within that set, and with phosphopeptides from phosphorylation by the other kinases [see Gurley et al. (1995)].

are, however, subtle differences in the phosphorylation of specific sites when the four kinases are compared.

A comparison of the major phosphorylated peptides from the *in vivo* and *in vitro* analyses is shown in Table 1. The three major interphase phosphorylation sites on peptides 1, 4, and 7 show very little variation. Expressed as a percent of total incorporation into H1, peptide 4 yields incorporation levels of 21.7, 26.2, 26.4, and 26.5% for kinases A, B, C, and M, respectively. The maximum difference for peptide 4 incorporation is less than 5%. Of these three interphase peptides, the maximal variation in incorporation is 1.2−1.5-

Table 1: Comparison of Peptide Phosphorylation Levels

	in vivo		*in vitro*			
peptide	exp	mitotic	A	B	C	M
			^{32}P Incorporation (cts/volt)			
1	10.70	34.54	1804	1944	1625	1724
4	27.93	43.35	1790	3319	2353	2051
5	3.72	25.25	1581	2397	2146	1671
7	20.29	25.68	1614	2888	1894	1731
9	0.96	38.35	1477	2135	895	570
total	63.60	167.17	12 683	8266	8912	7747
			% of Total			
1	16.8	20.7	21.8	15.3	18.2	22.3
4	43.9	25.9	21.7	26.2	26.4	26.5
5	5.8	15.1	19.1	18.9	24.1	21.6
7	31.9	15.4	19.5	22.8	21.3	22.3
9	1.5	22.8	17.9	16.8	10.0	7.4
total	100	100	100	100	100	100

fold. The mitotic-specific peptide 9 shows the largest variation in incorporation of all the peptides examined, with a 2.4-fold difference between kinases A and M. This, however, is small compared to the 15-fold difference in phosphate incorporation found for this peptide *in vivo* when comparing exponential and mitotic incorporation (Table 1).

cAMP-Dependent Protein Kinase Phosphorylates Only One Site. The observation that all four CDKs phosphorylated the same peptides (Figure 3, panels B−E) raised concerns that the preparation of H1 may have affected the structure and therefore the specificity of its phosphorylation. To ensure this had not occurred, phosphorylation was done using cAMP-dependent protein kinase, known to phosphorylate only one site. Cloned, overexpressed catalytic subunit was used to phosphorylate 100 μg of histone H1. The H1 was treated identically as the samples phosphorylated by the CDKs. The results of the tryptic digestion and HPLC analysis are shown in Figure 3, panel F. This chromatogram shows that only one peptide is phosphorylated in histone H1 when treated with cAMP-dependent protein kinase. This phosphorylated peptide eluted at 86 min and was not observed in growing cultures of CHO cells (see Figure 2, panels B and C). cAMP-dependent phosphorylation sites have been described in histone H1 [reviewed by van Holde (1989)]. One of the cAMP-dependent phosphorylation sites is Ser 37, which was found to be phosphorylated only in regenerating liver. The *in vivo* mapping results indicate that this phosphorylation is lacking in both rapidly growing interphase and mitotic blocked CHO cells. The lack of any overlap of cAMP phosphorylation sites with CDK sites indicate that the H1 site specificity was maintained.

DISCUSSION

The enzyme thought to be responsible for the high levels of histone H1 phosphorylation at mitosis was first described by Lake and Salzman (1972). Later, this enzyme, called growth-associated H1 kinase, was found in many types of dividing mammalian cells (Schlepper & Kippers, 1975; Langan, 1978) and in *physarum* (Chambers et al., 1983; Chambers & Langan, 1991). This enzyme is cAMP independent, chromatin bound, and specific for H1 (Chambers & Langan, 1991), and the phosphorylation sites *in vitro* match the *in vivo* sites (Langan, 1978; Langan et al., 1980). Purification of growth-associated H1 kinase (Chambers & Langan, 1991) revealed that it shared homology with the CDK first identified by genetic methods in yeast, p34^{CDC2} kinase. The p34^{CDC2} kinase also phosphorylated H1 at the same sites as the growth associated kinase (Langan et al., 1989). As a result, H1 has often been used as a substrate when examining p34^{CDC2}/cyclin complexes and other closely related CDKs.

In this paper, four different highly purified CDKs were examined: the interphase-specific p33^{CDK2}/cyclin A (kinase B), the late interphase p34^{CDC2}/cyclin A (kinase A), the mitotic-specific p34^{CDC2}/cyclin B (kinase C), and the p34^{CDC2}/unknown cyclin (kinase M). These CDKs provide examples of the various cell-cycle-dependent classes of growth-associated H1 kinase that would be expected to facilitate the cell-cycle-specific phosphorylation of histone H1: early interphase single-site phosphorylation, late interphase three-site phosphorylation, and mitotic-specific six-site phosphorylation (Gurley et al., 1978a,b, 1981, 1995).

All Four CDKs Are Authentic Growth-Associated H1 Kinases. H1 contains 21 serines and threonines that are potential phosphorylation sites for kinases (van Holde, 1989). However, in CHO H1, only the phosphorylations of six of these sites are growth-associated *in vivo* (Hohmann et al., 1975, 1976). Comparison of the H1 phosphopeptides produced *in vivo* from mitotic CHO cells with those produced *in vitro* by the four different CDKs indicate that only the growth-associated sites are phosphorylated by these kinases and none of the other 15 potential sites are phosphorylated. Control experiments showed that the cAMP-dependent protein kinase was specific for only one site and that site was different from the CDK sites. This result indicates that the H1 substrate used in these experiments had maintained its site specificity in isolation. It also shows that the cAMP-dependent protein kinase does not phosphorylate H1 in exponential growing interphase or mitotic cells (see Figure 2, panels B and C, and Figure 3F). These experiments demonstrate that the four CDKs are authentic growth-associated H1 kinases responsible for H1 phosphorylation during the cell cycle.

All Four CDKs Phosphorylate All Cell-Cycle Specific Sites in Histone H1. Because of the cell cycle fluctuations in the activities of the various CDKs, it was expected that the different kinases would demonstrate specificity in phosphorylating the various cell-cycle specific sites on H1. For example, kinase B (p33^{CDK2}/cyclin A) was expected to phosphorylate only one, two, or three interphase-specific sites and expected to not phosphorylate the mitotic-specific sites in the N-terminal portion of H1. Similarly, it was expected that kinase C (p34^{CDC2}/cyclin B) would phosphorylate only the mitotic-specific sites in the N-terminal portion and not the interphase sites in the C-terminal tail. Surprisingly, all four of these cell-cycle-specific kinases phosphorylated all the cell-cycle-specific sites. Not only was the complete spectrum of phosphorylation sites duplicated qualitatively by all the kinases, but so was the relative quantitative labeling of the specific phosphopeptides. This data clearly demonstrate that these CDKs are capable of phosphorylating any available growth-associated H1 site. Indeed, it shows that all four CDKs are capable of producing the maximum phosphorylation of H1 achieved at mitosis.

CDKs Phosphorylate Cell-Cycle-Specific Sites with Only Minor Affinity Differences. The initial characterization of these CDKs used a synthetic peptide substrate containing only one phosphoacceptor site. This peptide, S1 (Hamaguchi

et al., 1992), has the sequence AAKAKKTPKKAKK and is based on the accepted consensus amino acid motif (S/T)-PXK. Using this S1 peptide, large quantitative differences were found between the four purified CDKs. For example, the K_m differences between kinases B and M was nearly 20-fold (Swank 1995). Therefore, it was anticipated that large differences would also be found for the phosphorylation of H1 by the CDKs. Such differences would help in understanding the *in vivo* cell-cycle dependent phosphorylation sites within H1. Surprisingly, quantification of the phosphorylated H1 peptides revealed that only small differences were measured in the ability of each kinase to phosphorylate specific H1 sites (Figure 4; Table 1). This contrasts with results from telomere binding protein (TBP) phosphorylation using these same kinases (Hicke et al., 1995) in which TBP was phosphorylated by kinases A, B, and C, but not kinase M. Together with the synthetic peptide studies, this indicates that some substrates are specific for the CDKs used here, whereas H1 is nearly equally phosphorylated by all four CDKs studied. Therefore, it appears that the structure of H1 in chromatin at different stages of the cell cycle is involved in determining the selection of H1 sites for phosphorylation.

CDKs Do Not Require the Consensus Amino Acid Motif to Phosphorylate H1. It has previously been thought that the consensus sequence (S/T)PXK was required in a substrate, particularly H1, in order for the serine or threonine to be phosphorylated by a CDK. However, sequence analysis identified that one growth-associated phosphopeptide in H1 did not have such a consensus sequence. This peptide was identified as the N-terminal tryptic peptide of CHO H1: Ac-SETAPAAPAAAPPAEK (Gurley et al., 1995). Two phosphoacceptor residues were identified within this N-terminal fragment, Ser 1 and Thr 3. We have demonstrated that both of these amino acids are phosphorylated only during mitosis (Hohmann et al., 1975, 1976). Until recently it was assumed that the mitotic-specific serine phosphorylation site was Ser 18, a residue contained within the N-terminal H1 fragment obtained by NBS cleavage and also present in a consensus sequence. However, Gurley et al. (1995) have clearly shown that the mitotic-specific phosphorylation sites are Ser 1 and Thr 3, neither of which are contained in a CDK consensus sequence. This report unequivocally demonstrates that all four CDKs, both the interphase and mitotic-specific kinases, phosphorylate this mitotic-specific site. Two conclusions must be drawn from these observations: none of these CDKs exclusively require the consensus sequence for phosphorylation and any of these CDKs can phosphorylate the mitotic-specific H1 phosphorylation sites.

The Ser 1 and Thr 3 phosphorylation sites are the last phosphorylations to occur on H1 during the cell cycle (Gurley et al., 1978a, 1995), and its potential functional relevance has been the subject of much consideration (Bradbury et al., 1973, 1974a,b; Hohmann et al., 1976; Gurley et al., 1978a,b, 1981). As the last cell cycle phosphorylation event to occur on H1, this modification may play a role in triggering mitosis, or in modulating other proteins or factors involved in chromatin condensation. If phosphorylation at these sites is responsible for structural changes that occur upon, or prior to, chromatin condensation, then phosphorylation at these two sites at times other than mitosis may be responsible for the mitotic catastrophe observed under conditions leading to hyperphosphorylated H1 during interphase (Th'ng et al., 1994). The results presented here indicate that this is possible since interphase-specific CDKs, like kinase B ($p33^{CDK2}$/cyclin A), can phosphorylate the normally mitotic-specific sites if it is presented with an accessible N-terminal portion of H1.

CDKs Phosphorylate H1 Sites Randomly. The work of Gurley et al. (1978a,b, 1981) demonstrated that increasing numbers of H1 sites are phosphorylated as a cell traverses its cell cycle, suggesting sequential phosphorylations of the growth-associated sites. However, the above results indicate that all growth-associated sites are phosphorylated by all CDKs, indicating that random phosphorylation is occurring *in vitro*. This latter conclusion is supported by time-course phosphorylation experiments using less enzyme, which also resulted in full mitotic-level phosphorylation and corresponded to only one or two radiolabeled bands when analyzed by AU gel analysis (data not shown). A CDK requirement for sequential phosphorylation would yield altered phosphate occupancy at specific sites when examining time or enzyme dependent phosphorylation. Instead, such analysis resulted in increased occupancy of all sites, thus, indicating random phosphorylation of the sites. These results suggest that the sequential phosphorylation in CHO cells *in vivo* is only observed when H1 is bound to nucleosomes as part of chromatin.

Implications for Control of H1 Cell-Cycle-Dependent Phosphorylation. The ability of all four CDKs to duplicate the full growth-related phosphorylation pattern of H1 *in vitro* raises many questions about the *in vivo* control and function of the H1 phosphorylation process in the cell cycle. A CHO cell in G1 contains only one phosphate on the C-tail of H1, and as the cell progresses into S phase, two additional phosphates are added, both on the C-tail of H1 (Hohmann et al., 1975, 1976; Gurley et al., 1978b, 1981). During S phase, $p33^{CDK2}$/cyclinA (kinase B in this work) is active (Murray & Hunt, 1993), while other CDKs such as $p34^{CDC2}$/cyclin B (kinase C) are still inactive. We have shown that the kinase B complex will phosphorylate not three, but all six growth-related sites within H1. As CHO cells move into G2 phase, the $p34^{CDC2}$/cyclin A (kinase A) becomes active. At this stage, the full *in vivo* phosphorylation state of H1 is still not complete and the mitotic-specific phosphorylation sites remain to be phosphorylated (Gurley et al., 1978b, 1981). Again, we have shown that the kinase A complex, which should be active at this time, will phosphorylate not three, but all six growth-related sites. The CDK normally active in mitosis, $p34^{CDC2}$/cyclin B (kinase C), matches the *in vivo* mitotic observations. Kinase C is active only in mitosis when all molecules of H1 are phosphorylated on all six growth-related sites (Gurley et al., 1978a).

Why are the cyclin-dependent kinases, even though they are capable of fully phosphorylating all growth-related sites in free H1, restricted to only a subset of sites during interphase? The most likely explanation is that H1, as a substrate, is not available for full phosphorylation. Perhaps H1, when bound to chromatin, is locked into a structure that allows only limited accessibility to such kinases. This view is supported by the work of Jerzmanowski and Cole (1992) who demonstrated that H1 must be free (unbound to chromatin) to be phosphorylated. Such a limited release of H1 from chromatin *in vivo* has been demonstrated by Gurley and Hardin (1970), who observed turnover of H1 in

exponentially growing CHO cells, but no turnover of the core histones (Gurley & Hardin, 1969).

With this in mind, the results suggest that, *in vivo*, the binding of only the long C-terminal domain of H1 in chromatin is weakened during interphase, whereas the binding of both the C-terminal domain and the shorter N-terminal tail of H1 are weakened (and therefore accessible) during mitosis. The progressive increase in the number of sites phosphorylated as cells traverse the cell cycle (Gurley et al., 1978b, 1981) may be understood from a simple DNA binding perspective since hypophosphorylated H1 should bind DNA more tightly than hyperphosphorylated H1 (Green et al., 1993). In G1 phase, either the process of chromosome decondensation following metaphase allows access of a G1 phase CDK to the C-terminal domain of H1 or some cell-cycle-dependent factor may free the H1 C-terminal region sufficiently to permit phosphorylation of only one site. Thus, two possible scenarios can explain the cell cycle progression of phosphorylation of multiple sites on histone H1. In the first scenario, following cell division and chromosome decondensation, the first site on H1 is available for phosphorylation by G1 CDKs at the start of G1 phase. Through chromosome rearrangements initiated by the first site, subsequent sites become available at the start of S, S/G2, G2/M, and M phase. Alternatively, in the second scenario, some cell-cycle-dependent factor in G1 phase may free the H1 C-terminal region sufficiently to permit the phosphorylation of only one site. Similarly, this phosphorylation could initiate chromosomal changes and/or weaken H1 binding to DNA sufficiently to promote further dissociation so that other sites are phosphorylated in subsequent phases of the cell cycle. However, in this scenario the accessibility of additional sites could be controlled by both the phosphorylation itself and additional cell-cycle-dependent factors or events. It is possible that other histone modifications, such as acetylation of core histones, may be involved in altering the accessibility of histone H1 for modification by kinases. Post translational modifications of other nonhistone chromosomal proteins may also play a role and therefore remain a formal possibility.

The four CDKs isolated for this work are enzymes that phosphorylate only the growth-related phosphorylation sites of H1 and thus are part of the class of enzymes that are responsible for cell-cycle-related H1 phosphorylation observed *in vivo*. All four CDKs phosphorylate all of the interphase phosphorylation sites on H1 as well as the mitotic-specific phosphorylation sites. These mitotic-specific phosphorylation sites do not contain the CDK consensus, and thus, this consensus sequence does not define all potential CDK phosphorylation sites. There is no site specificity among the growth-related phosphorylation sites by any of the four CDKs indicating that cell-cycle-dependent variations in H1 phosphorylation sites observed *in vivo* are not solely due to CDK levels and types. It is therefore likely that additional factors control the accessibility of H1 when bound to chromatin. Whether this accessibility is controlled entirely or in part by the series of phosphorylations on H1 itself remains an important question. The phosphorylation of histone H1 and the molecular mechanisms enabling chromosome condensation cannot be clearly understood until H1 phosphorylation events as well as other histone modifications can be examined in a clearly defined chromosomal context.

ACKNOWLEDGMENT

We thank Ryan Mitchell (Dr. Donal Walsh's laboratory, University of California—Davis) for cloned overexpressed catalytic cAMP-dependent protein kinase.

REFERENCES

Ajiro, K., Borun, T. W., & Cohen, L. H. (1975) *Fed. Proc. 34*, 581.

Ajiro, K., Borun, T. W., & Cohen, L. H. (1981) *Biochemistry 20*, 1445−1454.

Balhorn, R., Chalkley, R., & Granner, D. (1972) *Biochemistry 11*, 1094−1098.

Balhorn, R., Jackson, V., Granner, D., & Chalkley, R. (1975) *Biochemistry 14*, 2504−2511.

Bradbury, E. M., Inglis, R. J., Matthews, H. R., & Sarner, N. (1973) *Eur. J. Biochem. 33*, 131−139.

Bradbury, E. M., Inglis, R. J., & Matthews, H. R. (1974a) *Nature 241*, 257−261.

Bradbury, E. M., Inglis, R. J., Matthews, H. R., & Langan, T. A. (1974b) *Nature 249*, 553.

Chambers, T. C., Langan, T. A., Matthews, H. R., & Bradbury, E. M. (1983) *Biochemistry 22*, 30−37.

Chambers, T. C., & Langan, T. A. (1991) *Methods Enzymol. 200*, 325−331.

D'Anna, J. A., Thayer, M. M., Tobey, R. A., & Gurley, L. R. (1985) *Biochemistry 24*, 2005−2010.

Green, G. R., Lee, H. J., & Poccia, D. L. (1993) *J. Biol. Chem. 268*, 11247−11255.

Gurley, L. R., & Hardin, J. M. (1969) *Arch. Biochem. Biophys. 130*, 1−6.

Gurley, L. R., & Hardin, J. M. (1970) *Arch. Biochem. Biophys. 136*, 392−401.

Gurley, L. R., & Walters, R. A. (1971) *Biochemistry 10*, 1588−1593.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1973) *Biochem. Biophys. Res. Commun. 50*, 744−750.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1974) *J. Cell Biol. 60*, 356−364.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1975) *J. Biol. Chem. 250*, 3936−3944.

Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., & Tobey, R. A. (1978a) *Eur. J. Biochem. 84*, 1−15.

Gurley, L. R., Tobey, R. A., Walters, R. A., Hildebrand, C. E., Hohmann, P. G., D'Anna, J. A., Barham, S. S., & Deaven, L. L. (1978b) in *Cell Cycle Regulation* (Jeter, J. R., Jr., Cameron, I. L., Padilla, G. M., & Zimmerman, A. M., Eds.) pp 37−60, Academic Press, New York.

Gurley, L. R., D'Anna, J. A., Halleck, M. S., Barham, S. S., Walters, R. A., Jett, J. J., & Tobey, R. A. (1981) in *Protein Phosphorylation, Book B* (Rosen, O. M., & Krebs, E. G., Eds.) Vol. 8, Cold Spring Harbor Conference On Cell Proliferation, pp 1073−1093, Vol. 8, Cold Spring Harbor Laboratory, Plainview, NY.

Gurley, L. R., Spall, W. D., Valdez, J. G., Jackson, P. S., Meyne, J., Ray, F. A., Prentice, D. A, & Blumfenfeld, M. (1990) in *HPLC of Biological Macromolecules* (Gooding, K. M., & Regnier, F. E., Eds.) pp 529−570, Marcel Dekker, Inc., New York.

Gurley, L. R., Valdez, J. G., & Buchanan, J. S. (1995) *J. Biol. Chem. 290*, 27653−27660.

Hamaguchi, J. R., Tobey, R. A. Pines, J., Crissman, H. A., Hunter, T., & Bradbury, E. M. (1992) *J. Cell Biol. 117*, 1041−1053.

Hardie. D. G., Matthews, H. R., & Bradbury, E. M. (1976) *Eur. J. Biochem. 66*, 37−42.

Harris, P. (1978) in *Cell Cycle Regulation* (Jeter, J. R., Jr., Cameron, I. L., Padilla, G. M., & Zimmerman, A. M., Eds.) pp 75−130, Academic Press, New York.

Hicke, B., Rempel, R., Maller, J., Swank, R. A., Hamaguchi, J., Bradbury, E. M., Prescott, D. M., & Cech, T. R. (1995) *Nucleic Acids Res. 23*, 1887−1893.

Hohmann, P., Tobey, R. A., & Gurley, L. R. (1975) *Biochem. Biophy. Res. Commun. 63*, 126−133.

Hohmann, P., Tobey, R. A., & Gurley, L. R. (1976) *J. Biol. Chem. 252*, 3685−3692.

Hunter, T., & Pines, J. (1994) *Cell 79*, 573−582.

Inglis, R. J., Langan, T. A., Matthews, H. R., Hardie, D. G., & Bradbury, E. M. (1976) *Exp. Cell Res. 97*, 418−425.
Jerzmanowski, A., & Cole, R. D. (1992) *J. Biol. Chem. 267*, 8514−8520.
Lake, R. S. (1973a) *Nat. New Biol. 242*, 145−146.
Lake, R. S. (1973b) *J. Cell Biol. 58*, 317−331.
Lake, R. S., & Salzman, N. P. (1972) *Biochemistry 11*, 4817−4826.
Lake, R. S., Goidl, J. A., & Salzman, N. P. (1972) *Exp. Cell Res. 73*, 113−121.
Langan, T. A. (1978) *Methods Cell Biol. 19*, 143−152.
Langan, T. A. (1982) *J. Biol. Chem. 257*, 14835−14846.
Langan, T. A., Zeilig, C. E., & Leichtling, B. (1980) in *Protein Phosphorylation and Bioregulation* (Thomas, G., Podesta, E. J., & Gordon, J., Eds.) pp 70−82, S. Karger, Basel.
Langan, T. A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J., & Sclafani, R. A. (1989) *Mol. Cell Biol. 9*, 3860−3868.
Lees, E. (1995) *Curr. Opin. Cell Biol. 7*, 773−780.
Marks, D. B., Paik, W. K., & Barun, T. W. (1973) *J. Biol. Chem. 248*, 5660−5667.
Moreno, S., & Nurse, P. (1990) *Cell 61*, 549−551.
Murray, A., & Hunt, T. (1993) *The Cell Cycle*, WH Freeman and Co.
Ohsumi, K., Katagiri, C., & Kishimoto, T. (1993) *Science 262*, 2033−2035.
Ord, M. G., & Stocken, L. A. (1968) *Biochem. J. 107*, 403−410.
Peeper, D. S., Parker, L. L., Ewen, M. E., Toebes, M., Hall, F. L., Xu, M., Zantema, A., van der Eb, A., & Piwnica-Worms, H. (1993) *EMBO J. 12*, 1947−1954.
Pines, J., & Hunter, T. (1989) *Cell 58*, 833−846.
Roth, S. Y., & Allis, C. D. (1992) *Trends Biochem. Sci. 17*, 93−98.
Schlepper, J., & Knippers, R. (1975) *Eur. J. Biochem. 60*, 209−220.
Stone, K. L., LoPresti, M. B., Crawford, J. M., DeAngelis, R., & Williams, K. R. (1989) in *A Practical Guide To Protein And Peptide Purification for Microsequencing* (Matsudaria, P. T., Ed.) pp 31−47, Academic Press, New York.
Swank, R. A. (1995) Ph.D. Dissertation, University of California, Davis.
Th'ng, J. T., Guo, X. W., Swank, R. A., Crissman, H. A., & Bradbury, E. M. (1994) *J. Biol. Chem. 269*, 9568−9573.
van Holde, K. E. (1989) *Chromatin*, p 88, Springer-Verlag, New York, Inc., New York.

BI9714363