Characterization of the Mitotic Specific Phosphorylation Site of Histone H1

ABSENCE OF A CONSENSUS SEQUENCE FOR THE p34\textsuperscript{cdc2}/CYCLIN B KINASE*

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32P-Labeled histone H1 was isolated from synchronized Chinese hamster (line CHO) cells, subjected to trypsin digestion, and fractionated into 15 phosphopeptides by high performance liquid chromatography. These phosphopeptides were grouped into five classes having different cell cycle phosphorylation kinetics: 1) peptides reaching a maximum phosphorylation rate in S and then declining in G2 and M, 2) peptides reaching a maximum phosphorylation rate in G2 and then remaining constant or declining in M, 3) peptides with increasing phosphorylation throughout S and G2 and reaching a maximum in M, 4) one peptide that was phosphorylated only in M, and 5) peptides that had low levels of phosphorylation that remained constant throughout the cell cycle. Amino acid analysis and sequencing demonstrated that the mitotic specific H1 phosphopeptide was the 16-amino acid, N-terminal, tryptic peptide AC-SETAPAAPPAAAPP KEK of the H1-1 class. This peptide, which is phosphorylated on both the Ser and Thr, does not contain the consensus sequence (S/T)P\textsuperscript{X}P\textsuperscript{Z} (where X is any amino acid and Z is a basic amino acid). This sequence is thought to be required by the p34\textsuperscript{cdc2}/cyclin B kinase that has maximum phosphorylating activity in mitosis. These data indicate that this kinase either does not have an obligatory requirement for the consensus sequence in vivo as generally believed or that it is not the enzyme responsible for the mitotic specific H1 phosphorylation.

For over 27 years, the phosphorylation of histone H1 has been thought to play a role in controlling the cell cycle (Ord and Stocken, 1968). To examine this possibility our laboratory used synchronized CHO\textsuperscript{1} cells to determine the cell cycle kinetics of histone phosphorylation during cell proliferation (see review by Gurley et al. (1978a)). In those studies it was found that histone H2A and H4 phosphorylations were cell cycle-independent and probably not involved in cell cycle control, while histone H1 and H3 phosphorylations were cell cycle-dependent and, therefore, more likely to have a role in cell cycle control.

The phosphorylation of H3 was highly restricted in the cell cycle, occurring only during mitosis. In contrast, the phosphorylation of H1 was very complex, there being 1 phosphate/molecule in late G\textsubscript{1}, 3 phosphates/molecule in S and G\textsubscript{2}, and up to 6 phosphates/molecule in M (Hohmann et al., 1976). All of the phosphates in H1 and H3 are lost during telophase, thus resetting these histones to a phosphate-free state at the beginning of the next cell cycle (Gurley et al., 1978b).

In our laboratory, Hohmann et al. (1976) demonstrated that the “superphosphorylation” of H1 in mitosis involved specific serine and threonine sites in the H1 molecule that were not phosphorylated during interphase. These sites were located in the short N-terminal tail of the H1 molecule. Because of this mitotic specificity we proposed that the phosphorylation of these sites may be necessary for the condensation of interphase chromatin into condensed chromosome structure (Gurley et al., 1974, 1978b) or for the orderly separation of chromosomes during anaphase (Gurley et al., 1974). This specificity has also led to the proposal that H1 superphosphorylation may be the “trigger” for mitosis (Bradbury et al., 1973). If this were true, the enzymes that phosphorylate the mitotic specific H1 sites would be the controlling molecules of the cell cycle. Thus, it has become important to understand the details of this molecular mechanism since the control of cell proliferation is one of the most important unanswered questions in cell growth and biological differentiation and since the disfunction of this mechanism lies at the heart of the cancer problem, i.e. uncontrolled cell proliferation.

Work on protein kinases has revealed that the kinases p33\textsuperscript{cdc2} and p34\textsuperscript{cdc2}, when complexed with various cyclins, are H1 kinases that have different cell cycle periods of maximum phosphorylating activity (Evans et al. (1983); Pagano et al. (1992); reviewed by Murray (1992)). Among these, the p34\textsuperscript{cdc2} cyclin B kinase has been found to have maximum activity at mitosis (Pines and Hunter, 1989). Thus, one might expect this kinase to be the enzyme responsible for the phosphorylation of the mitotic specific serine and threonine sites in the N-terminal portion of H1 (Langan et al., 1989). This enzyme is thought to require the consensus sequence (S/T)P\textsuperscript{X}P\textsuperscript{Z}, where X is any amino acid and Z is a basic amino acid (reviewed by Moreno and Nurse (1990)). In order to phosphorylate the serine or threonine residues in H1 during mitosis, one would expect to find two such sequences in the N-terminal portion of H1. However, this is not strictly the case. For example, in calf thymus H1–1 there is no SP\textsuperscript{X}Z sequence in this part of the molecule, and in rabbit thymus H1–3 there is no TP\textsuperscript{X}Z sequence in this part of the molecule (Liao and Cole, 1981). This raised a question of whether our understanding of the mechanism of action of p34\textsuperscript{cdc2}/cyclin B and its mitotic specific role is complete or correct.

To examine this question we have fragmented CHO H1 with trypsin and fractionated its phosphopeptides. Using incorporation of [\textsuperscript{32}P]phosphate during various phases of the cell cycle we have identified the mitotic specific phosphopeptide and determined its sequence. This work has shown that it contains...
EXPERIMENTAL PROCEDURES

Cell Synchrony—Chinese hamster cells (line CHO) were grown exponentially in suspension culture as described previously (Gurley et al., 1980). Culture growth was monitored by measuring the culture’s cell concentration with a Coulter counter. One-liter cultures of exponentially growing cells were synchronized in various phases of the cell cycle as previously described (et al., 1978a). Briefly, briefly, the cells were first synchronized in early G1 by centrifugation for 36 h in isoleucine-deficient medium. These cells were then used for histone phosphorylation measurements in G1, or they were resynchronized near the G2/S boundary by resuspending them in complete medium containing 1 mM hydroxyurea. After 10 h in hydroxyurea, the cells were arrested near the G2/S boundary and used for histone phosphorylation measurements, or they were released from hydroxyurea blockade by resuspending in fresh medium and allowed to traverse S phase. One hour after this release the cells were used for histone phosphorylation measurements while traversing S phase. Six hours after this release the cells were used for histone phosphorylation measurements while traversing G2 phase.

To obtain cells synchronized in M, Colcemid was added to cultures 5 h after release from hydroxyurea blockade. These cells traversed S and G2 phases and were arrested and resynchronized in mitosis by metaphase arrest. Three hours after adding Colcemid, the cells entering M were used for histone measurements during mitosis.

In some experiments the cells that were released from hydroxyurea were resynchronized in G2. This was accomplished by treating the released cells with 7.5 μg of Hoechst 33342/ml of culture. This treatment permits cells to traverse S and then reversibly arrests them in late G2 (Tobe et al., 1990). After 6 h in Hoechst this culture was used for histone phosphorylation measurements in G2 phase arrest.

Cultures were also arrested in G2 by treatment with the histone kinase inhibitor staurosporine. This was accomplished by dissolving 1 mg of staurosporine lyophilized powder (Kamiya Biomedical Co.) in 2 ml of pure MeSO4 and adding 100 μl of this stock solution to 1000 ml of exponentially growing CHO cells. This 50 ng/ml treatment permits CHO cells to traverse the cell cycle and then arrests them in G2 (Crissman et al., 1991). After 10.5 h in staurosporine this culture was used for histone phosphorylation measurements in G2 phase arrest.

The fraction of cells in mitosis in the G2, and G2/S synchronized cultures was determined by staining a 1-ml aliquot of culture with acridine orange and counting the percentage of mitotic cells using a fluorescence microscope (Gurley et al., 1973).

Labeling Cells with 32P—To measure histone phosphorylation, the 950-ml synchronized cultures were treated with H3P-O4 at a concentration of 20 μCi/ml of culture. After the labeling period, the cells were removed from their growth medium by centrifugation and used for histone preparation.

Histone Preparation—Histones were prepared from the 950-ml 32P-labeled cultures containing 300,000 cells/ml as described previously (Gurley et al., 1983). Approximately 2.85 × 107 cells were centrifuged from the culture and washed once with cold saline-GM to remove the isotope-containing medium. The cell pellet was homogenized in 0.14 M NaCl containing 0.05 M sodium bisulfite, which prevents histone dephosphorylation. Crude chromatin was then centrifuged from this homogenate and histone H1 was extracted from the chromatin with 5% perchloric acid. The H1 was precipitated from the extract by adding trichloroacetic acid to a concentration of 20% (Gurley et al., 1975). The H1 was recovered by centrifugation and dissolved in 200 μl of aqueous 0.2% trifluoroacetic acid for purification by HPLC.

Following the extraction of H1, the core histones (H2A, H2B, H3, and H4) were extracted from the chromatin using 0.4 M H2SO4. These histones were recovered by acetone precipitation and dissolved in 200 μl of aqueous 0.2% trifluoroacetic acid for fractionation and purification by HPLC.

HPLC of Histones—Details of the fractionation and purification of histones by HPLC in our laboratory have recently been reviewed by Gurley et al. (1990). In these experiments, the H1 dissolved in 200 μl of aqueous 0.2% trifluoroacetic acid was divided into two parts, one 50-μl sample to be used for measuring the 32P incorporated into whole H1 and one 50-μl sample to be used for measuring 32P incorporated into the phosphopeptides of H1. The 50-μl sample was injected into a Waters μBondapak CN column (10-μm irregular particle size, 125-A pore size, 3.9-mm inner diameter × 15-cm length) attached to a Waters model 6000A HPLC solvent delivery system. H1 was eluted from the column with a linear gradient of acetonitrile in water containing 0.2% trifluoroacetic acid running from 5 to 35% acetonitrile in 3 h at a flow rate of 1.0 ml/min.

The H1 eluting from the column was monitored by UV absorption using a variable-wavelength detector set at 215 nm. The radioactivity of the 32P incorporated into the H1 was measured by flow liquid scintillation counting using a Berthold LB504 HPLC radioactivity monitor and Flo-Scint A liquid scintillation mixture (Packard). The UV absorption and radioactivity data were collected in a Berthold LB510 Chromatography Data System computer, which calculated the specific activity of each H1 peak. The radioactivity counts in each peak and the area of the UV peak measured in volts.

The remaining 150-μl H1 sample was then used to prepare purified H1 for tryptic peptide analysis. This was accomplished by subjecting the 150-μl H1 sample to the same HPLC procedure as the 50-μl sample except that the effluent from the column was not mixed with liquid scintillation fluid for radioactivity counting. Instead, the UV absorption of the column effluent was monitored, and the H1 peak was collected in a fraction collector. The purified H1, contained in about 10 ml of column effluent, was frozen and lyophilized to a dry powder in preparation for tryptic digestion and phosphopeptide analysis.

The core histones were fractionated by HPLC before H2B, H2A, H4, histone, and mhpH3 (where mhp refer to the less hydrophobic and more hydrophobic variants of H3, respectively). This was accomplished by injecting the 200-μl sample of core histones into the same μBondapak CN column and eluting the histones with a linear gradient of acetonitrile in water containing 0.2% trifluoroacetic acid running from 0 to 20% acetonitrile in 10 min, followed by a linear gradient running from 20 to 50% acetonitrile in 180 min at a flow rate of 1.0 ml/min. The histones eluting from the column were monitored by UV absorption, and radioactivity was measured as described above.

Trypsin Digestion of H1—The purified, dry H1 powder prepared above was subjected to trypsin digestion by a variation of the method described by Stone et al. (1989). The H1 was dissolved in 50 μl of 0.06 M HC1O4, 50 mM NH4HCO3 plus 5 mM L-cysteine. The sample was incubated at 50 °C for 15 min. After cooling to room temperature, 5 μl of 100 mM iodoacetamide was added, and the solution was incubated at room temperature for 15 min. Then 140 μl of water was added to dilute the urea, and trypsin dissolved in 5 μl of 1 M HCl was added in a ratio of 1:25 (w/w) trypsin:H1. The trypsin (Worthington), which was prepared from bovine pancreas, was L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated, chymotrypsin-fraction 94% protein and contained 240 units/mg of protein. This digestion solution was incubated at 37 °C for 24 h. The digestion was stopped by freezing.

HPLC of Tryptic Peptides of H1—The peptides of H1 generated by digestion with trypsin were fractionated by a variation of the method described by Stone et al. (1989). The 205-μl frozen sample (above) was thawed and injected (without further treatment) into a Waters Delta Pak C18 column (5-μm spherical particle size, 300-A pore size, 3.9-mm inner diameter × 15-cm length) attached to the HPLC described above. The solvents used for HPLC were 0.06% trifluoroacetic acid, H2O (Buffer A) and 0.052% trifluoroacetic acid, 80% acetonitrile (Buffer B). The tryptic peptides were eluted with linear gradients of 2.0–37.5% B from 0 to 120 min. 37.5–75.0% B from 120 to 150 min, and 75.0–98.0% B from 150–165 min at a flow rate of 0.5 ml/min. At the end of the run the column was purged with 0.05% trifluoroacetic acid in 100% acetonitrile from 165–180 min and reequilibrated with Buffer A. The eluted peptides were detected by UV absorption at 210 nm using the flow spectrophotometer, and the radioactivity of the 32P incorporated into the H1 was measured by flow liquid scintillation counting described above.

Measurement of Phosphorylation of Phosphopeptides—With the exception of the mitotic specific phosphopeptide, the phosphopeptides were eluted in the first 30 min of the chromatogram. As a result, their UV absorption could not be clearly resolved from the reagent peaks and hence the H1 core peptides. Therefore, to quantify the incorporated 32P in these phosphopeptides, their radioactivity was measured, and a relative specific activity for each was obtained by dividing their radioactivity by the UV absorption of the largest peptide (P1), which was completely resolved eluting at 58 min. P1 had the greatest UV absorption of all the peptides and thus provided the most accurate internal measurement of peptide load on the column. Thus, the relative specific activity of each phosphopeptide (pp1, pp2, pp3, etc.) is reported as radioactive counts in that individual phosphopeptide/total volts (area) of the largest peptide (P1), i.e. counts of ppIV of P1, counts of pp2V of P1, etc.
Amino acid analysis was performed on the P1 peptide by hydrolyzing the sample to amino acids, derivatizing the amino acids using phenyl isothiocyanate, and separating the phenyl isothiocyanate derivatives by the Pico-Tag Method of Cohen et al. (1984). The details of this procedure using a Waters “applicationspecified” reversed-phase PICO-TAG C18 column have been described by Cohen et al. (1989), and the specific instrumentation and application in our laboratory has been described previously (Gurley et al., 1991). This system has been demonstrated to give excellent linear response with very high reproducibility and a 1-pmol detection limit (Biddlemeyer et al., 1984; Cohen et al., 1984).

Sequence Analysis of the Mitotic Specific Phosphopeptide—Histone H1 was prepared by perchloric acid extraction from 4.5 × 10^9 CHO cells growing exponentially in 13 liters of suspension culture. The H1 was purified by HPLC on a large Waters Bondapak CN column (10-μm irregular particle size, 125-Å pore size, 7.8-mm inner diameter × 30-cm length) as described above. The H1 was collected in a fraction collector and lyophilized to dryness. Approximately 2,750 μg of purified H1 was subjected to tryptic digestion, and the resulting peptides were fractionated by HPLC on a large Waters Delta Pak C18 column (5-μm spherical particle size, 300-Å pore size, 7.8-mm inner diameter × 30-cm length) as described above. The mitotic specific phosphopeptide (P1) was collected in a fraction collector, frozen, and lyophilized to dryness, yielding approximately 100 μg of purified peptide.

The P1 peptide was submitted to the Protein Structure Laboratory at the University of California, Davis, for amino acid sequence analysis. This analysis was performed by automated Edman degradation on an ABI model 477A protein sequencer. The peptide was blocked on its N terminus and could not be sequenced as submitted. Therefore, it was digested for 2 days with V8 protease, which cleaves peptides after glutamic acid residues (Drapeau, 1977). This produced a 14-amino acid peptide, which was sequenced, and a dipeptide, which was blocked on its N terminus and could not be sequenced. The dipeptide amino acid composition was determined to be Ser and Glu by amino acid analysis, and the sequence was deduced to be SE because V8 protease cleaves on the carboxyl side of Glu residues (Drapeau, 1977).

RESULTS

Histone Phosphorylation in the Cell Cycle—The fractionation of H1-class histones into H1 and H1o by HPLC is illustrated in Fig. 1A. The fractionation of nucleosomal core histones into H2B, H2A, H4, and two variants of H3 is shown in Fig. 1J. The 2-h incorporation of [32P]phosphate into H1 during various phases of the cell cycle is shown in Fig. 1B–I, and that into the core histones is shown in Fig. 1, K–R.

As we have previously shown (reviewed in Gurley et al. (1978a)), the large amount of H2A phosphorylation and the small amount of H4 phosphorylation in the nucleosomal core does not change much throughout the cell cycle (Fig. 1, K–R), but H3 is highly phosphorylated in mitosis (Fig. 1P), and this phosphorylation is absent in all other phases of the cell cycle. (Fig. 1, L, M, N, Q, and R). The H3 phosphorylation observed in the G2–traversing culture is due to 12.4% mitotic cells that contaminate this culture. When synchronized cultures are arrested in G2 with Hoechst 33342, there are only 1.6% mitotic cells contaminating the culture, and there is no phosphorylation of H3 (Fig. 1Q), confirming that this phosphorylation is restricted to M. This absence of H3 phosphorylation was also observed when exponential cells were blocked in G2 with staurosporine (Fig. 1R).

The absence of H1 phosphorylation in early G1 is illustrated in Fig. 1C. However, beginning in mid-G1, we have previously shown that H1 phosphorylation is initiated (Gurley et al., 1975), so that cultures arrested at the beginning of S have one site being phosphorylated (Fig. 1D). When cells traverse through S and G2, their H1 phosphorylation rate increases (Fig. 1, E and F) and involves up to three phosphorylation sites (Hohmann et al., 1976). In M it reaches its maximum phosphorylation rate (Fig. 1G) and involves up to six phosphorylation sites (Hohmann et al., 1976). While arrest of cells in M did not inhibit the phosphorylation of H1 (Fig. 1G), the arrest of cells in G2 significantly inhibited interphase H1 phosphorylation (Fig. 1, H and I).

Phosphorylation of H1 Phosphopeptides in the Cell Cycle—To measure the phosphorylation of the various H1 phosphorylation sites, the H1 peaks shown in Fig. 1 (B–I) were recovered and subjected to tryptic digestion. The resulting peptide mixture was then subjected to fractionation by HPLC as shown in Fig. 2A. The three large peaks eluting in the first 10 min and the large peak at 20 min in the UV absorption profile in Fig. 2A are tryptic digestion reagents.

The three phosphopeptides of interphase cells all eluted in the first 30 min (Fig. 2B), indicating that they were small and highly charged. None of these peptides were phosphorylated in every G1 (Fig. 2C). However, all three interphase phosphopeptides incorporated 32P in cells arrested at the G1/S boundary (Fig. 2D). Since we have previously shown by long acid–urea gel electrophoresis that the H1 of these cells contains only 1 phosphate/molecule at G1/S (Hohmann et al., 1976), these data indicate that any one of these sites is phosphorylated in late G1.
The data in Fig. 2, E and F, indicate that the same three sites that are phosphorylated singularly in late G1 are also phosphorylated in S and G2. We have previously shown that cells traversing S and G2 contain H1 with up to 3 phosphates/molecule (Hohmann et al., 1976). Thus, we conclude that there are three different major sites of interphase H1 phosphorylation, but there is no qualitative cell cycle specificity as to which site is phosphorylated first.

In addition to the three major phosphorylation sites there are some minor sites whose phosphorylation began in S phase and increased in G2. These minor phosphopeptides eluted between the three major phosphopeptides at 11 and 21 min and at 65 and 67 min (Fig. 2, E and F). The minor phosphopeptide eluting at 58 min in G2-traversing cells in Fig. 2F is really the major mitotic specific phosphopeptide. Its detection in the G2traversing culture is due to the 12.4% mitotic cells contaminating the synchronized culture as mentioned above. When the cells of this G2 culture were prevented from traversing into M by Hoechst 33342 blockade, the phosphorylation of the major sites was reduced, and that of the minor sites was inhibited below detection limits (Fig. 2H). The inhibition of interphase phosphorylation sites by the kinase inhibitor, staurosporine, was even greater (Fig. 2I). Since staurosporine blocks CHO cell cycle progression in G2 (Crissman et al., 1991), these data demonstrate a strong correlation between interphase phosphorylation of H1 and the traverse of cells from G2 into M.

Quantification of H1 Phosphorylation Sites in the Cell Cycle—Fifteen areas in the 32P incorporation profile of the tryptic phosphopeptides were sufficiently above background to quantify. These areas are indicated in the mitotic culture in Fig. 3. The three major phosphopeptides in interphase cells are phosphopeptides 1, 5, and 9 (pp1, pp5, and pp9, respectively). The mitotic specific phosphorylation sites of these phosphopeptides could be identified above background. The 32P counts in each phosphopeptide (indicated by the bar under each peak or shoulder) were quantified. Tryptic peptide P1 (panel B, upper trace) is the mitotic specific phosphopeptide pp13 (panel B, lower trace) eluting at 58 min.
phosphopeptide P1 is pp13. The lesser phosphopeptides (pp2, 3, 4, 6, 7, 8, 10, 11, 12, 14, and 15) are observed in both interphase and mitotic cells. 

The phosphorylation of these 15 phosphopeptides was normalized to column load, which was quantified from the UV absorption of peptide P1 (Fig. 3B). These relative phosphorylations in various phases of the cell cycle are presented in the histograms in Fig. 4A. Examination of these histograms indicated that the major phosphorylation sites were phosphorylated to greater than twice the extent of the minor phosphorylation sites. 

The difference in H1 site phosphorylation in cells traversing G2 and in cells arrested in G2 with Hoechst 33342 or staurosporine (Stsp) is shown in Fig. 4B. It is seen that when cell cycle traverse is arrested in G2 with Hoechst the phosphorylations of pp1, pp5, and pp9 are inhibited to 31.3, 31.3, and 33.4% of G2-traversing cells, respectively. When the cells were treated with the H1 kinase inhibitor, staurosporine, the phosphorylation of these three phosphopeptides was inhibited to 12.7, 19.4, and 17.0% of G2-traversing cells, respectively, and their cell cycle traverse was arrested in G2. These data suggest that the progression of cells from G2 to M and the phosphorylation of these three H1 sites are directly coupled. 

Examination of the data in Fig. 4A revealed that different phosphopeptides reached their maximum phosphorylation rate at different phases of the cell cycle. When the phosphorylation of each phosphopeptide was plotted versus cell cycle position it was found that the phosphopeptides could be placed in five different classes (Fig. 5). pp9 and pp15 having maximum phosphorylation in S phase (A), G2 phase (B), or M phase (C). D, pp13 was the only phosphopeptide that was phosphorylated only in M. E, five phosphopeptides had phosphorylation rates too low to accurately classify.
The Mitotic Specific Phosphorylation Site of Histone H1

**A: Amino Acid Analysis of CHO H1**

| Amino Acid | Symbol | Number/Peptide | Theoretical % | Amino Acid Analysis % |
|------------|--------|----------------|---------------|-----------------------|
| Glu        | E      | 2              | 12.50         | 12.97                 |
| Ser        | S      | 1              | 6.25          | 5.44                  |
| Thr        | T      | 1              | 6.25          | 5.90                  |
| Ala        | A      | 7              | 43.75         | 41.80                 |
| Pro        | P      | 4              | 25.00         | 29.15                 |
| Lys        | K      | 1              | 8.26          | 6.43                  |
| Total      |        | 16             | 100.00        | 98.76                 |

**B: Amino Acid Sequence**

Ac SETAAPAAPPAAPEK

Chinese Hamster CHO H1

Ac SETAAPAAPPAAPEK

Calf Thymus H1-d

Ac

Rat Liver H1-d

Ac A

Mouse H1

Ac ET TPA

Rabbit Thymus H1-3

Ac PA

Human Spleen H1-b

Ac L T PA

Human Spleen H1-c

Ac ET TPA V

Human Placenta H1-3

Ac PA

Human Placenta H1-4

**C: H1 Subfraction Classification**

Ac SETAAPAAPPAAPEK

Chinese Hamster CHO H1

Ac SETAAPAAPPAAPEK

Human Placenta H1-1

Ac L T PA

Human Placenta H1-2

Ac ET TPA V

Human Placenta H1-3

Ac PA

Human Placenta H1-4

**Fig. 6. Analysis of the N-terminal tryptic peptide (P1) of histone H1.** A, amino acid analysis of CHO P1 B, amino acid sequence of CHO P1 compared with the P1 of other H1 molecules. C, demonstration that CHO H1 P1 belongs to the H1-1 subfraction class by comparison of the amino acid sequence of CHO H1 P1 with that of the P1 peptides of human placenta H1 subtypes. Sequences of the N-terminal tryptic peptide of H1 from calf thymus (Liao and Cole, 1981), rat liver (Cole et al., 1990), human spleen (Ohe et al., 1986, 1989), mouse (Yang et al., 1987), rabbit thymus (Rall and Cole 1971; Jones et al., 1974), and human placenta (Parseghian et al., 1994) were compared with that from CHO (first line in panels B and C). In these comparisons the asterisk indicates that the amino acid in that position is the same as that in CHO H1. The dash indicates that the amino acid in that position is missing. The (Ac) indicates that the acetyl group on the N-terminal serine is assumed because the sequence was deduced from the H1's RNA sequence.

 ful, indicating that it was blocked on its N terminus. This indicated that P1 is the N-terminal peptide of H1, which is known to be blocked by an acetyl group (Phillips, 1963; Rall and Cole, 1971). To get a partial sequence on P1, the peptide was digested with V8 protease, which produced a dipeptide and a 14-amino acid fragment. The 14-amino acid fragment was sequenced and found to be TAPAAPAAPPAAPEK. The dipeptide could not be sequenced, indicating that it was blocked on its N terminus, but it was determined to contain Ser and Glu by difference from the amino acid analysis (Fig. 6A). Since the V8 protease cleaves on the carboxyl side of glutamic acid (E) residues (Drapeau, 1977), it was concluded that the dipeptide sequence must be SE with an acetyl group attached to the N terminus of the serine. The V8 protease did not cleave the 14-amino acid fragment between Glu and Lys due to steric hindrance by the lysine (K). Thus it was concluded that the sequence of P1 is Ac SETAAPAAPPAAPEK (Fig. 6B, line 1), which is consistent with the amino acid analysis in Fig. 6A.

The sequence of this N-terminal fragment of CHO H1 was compared with those of eight other mammalian H1 N-terminal fragments (Fig. 6B). Differences between CHO H1 and the others ranged from none with calf thymus H1-1 and human spleen H1-d to six with human spleen H1-a. The two phosphorylatable sites in this fragment of CHO H1 occur as a serine and a threonine at the N-terminal end of the molecule in positions 1 and 3. These two sites must be the two mitotic specific phosphorylated sites we have previously detected (Hohmann et al., 1976). The N-terminal serine has been conserved in the eight other H1 molecules shown in Fig. 6B, but the position 3 threonine has not. The threonine is present in calf thymus H1-1, rat liver H1-d, and human spleen H1-a, H1-b, H1-c, and H1-d, but not in mouse H1 and rabbit thymus H1-3.

The most important observation is that the CHO H1 mitotic specific phosphorylation site does not contain the consensus sequence (S/T)PXXZ for phosphorylation by the p34<sup>cdc2</sup>/cyclin B kinase (Fig. 6B). Thus it is concluded that either this kinase is not the enzyme responsible for mitotic specific H1 phosphorylation, or it does not have an obligatory requirement for the consensus sequence as generally believed.

Recently Parseghian et al. (1994) have proposed a classification of H1 subfractions based on the amino acid sequence of human placenta H1 subtypes. When the sequence of CHO H1 P1 was compared with that of human placenta H1 P1, the CHO N-terminal peptide was found to be identical to H1-1 of human placenta (Fig. 6C). The Ser and Thr residues are both conserved at the N-terminal end of all the human H1 subtypes in both spleen (Fig. 6B) and placenta (Fig. 6C), and like CHO there is no consensus sequence in any of these peptides.

**DISCUSSION**

Histone H1 is divided into three structural domains consisting of an apolar core, which interacts with the nucleosome and (like an elephant) has a long highly charged nonstructured polar “trunk” on the C-terminal end and a shorter nonstructured “polar “tail” on the N-terminal end (Hartman et al., 1977). Some think the trunk and tail may interact with the linker DNA as it enters and exits the nucleosome (reviewed by Moreno and Nurse (1990)).

Recent work on protein kinases has suggested that the p34<sup>cdc2</sup> kinase, which is thought to be responsible for some cell cycle-dependent H1 phosphorylations, requires a consensus sequence (S/T)PXXZ (Langan et al. 1989; Moreno and Nurse, 1990). Sequence data on rabbit H1 show that the trunk of H1 contains four such consensus sites and that the tail contains one (reviewed by van Holde (1989)). The apolar body of H1, which contains many serines and threonines, contains no such consensus sites. This suggests that cell cycle-dependent H1 phosphorylation occurs near the DNA entry and exit sites on the nucleosome and promotes structural changes there during S and G<sub>2</sub>, which leads to the ultimate condensation of chromatin into chromosomes during mitosis (Gurley et al., 1978a). To test this model we initiated a study of the cell cycle kinetics of H1 phosphorylation sites. In this work we hoped to determine if the consensus sequences are the correct in vivo phosphorylation sites and also to determine the cell cycle order in which the sites of phosphorylation occurred.

To do this we used HPLC to quantify tryptic phosphopeptides in H1 isolated from synchronized CHO cultures. Two surprising things were observed in these studies. First, three major phosphopeptides were observed at the G<sub>2</sub>/S boundary, although only 1 phosphate/molecule has been observed at this cell cycle stage using acid/urea gel electrophoresis (Hohmann et al., 1976). This indicates that, while only one phosphate is added to H1 during late G<sub>2</sub>, it is added to any one of the three interphase phosphorylation sites on the C-terminal trunk of H1. Thus, there does not appear to be an absolute order for which site is phosphorylated first, second, or third.

The other surprise was that the mitotic specific phosphopeptide eluted late during HPLC like a large peptide rather than among the smaller peptides predicted for the consensus site in the N-terminal tail of H1 (SPAK in rabbit thymus H1-3 or TPVK in calf thymus H1–1 (Liao and Cole, 1981)). Sequence analysis demonstrated that the mitotic specific phosphorylation site of CHO H1 is on the end of the N-terminal tail and...
does not contain the consensus sequence for the p34<sup>cdc2</sup> kinase. Thus, the p34<sup>cdc2</sup>/cyclin B kinase found exclusively in mitotic cells (Pines and Hunter, 1989) is not responsible for mitotic specific H1 phosphorylation, or this enzyme does not have an exclusive requirement for the consensus sequence as previously thought.

In this work, 15 peaks and shoulders of 32P radioactivity were quantified. Five major phosphorylated peaks were observed rising and falling during the cell cycle: pp1, pp5, pp7, pp9, and pp13. Five lesser phosphorylated peaks were also observed undergoing similar fluctuations (pp3, pp6, pp10, pp14, and pp15), and five very small peaks were detected just above the baseline of detection (pp2, pp4, pp8, pp11, and pp12). We have previously shown that CHO H1 consists primarily of one variant of H1 (74%), but it does contain small amounts of a second variant (17%) and trace amounts of two other variants (about 8% combined) (Gurley et al., 1975). Thus, we suspect that the five major phosphorylated peaks are from the major variant, the five minor phosphorylated peaks are from the minor variant, and the trace phosphorylated peaks are from the trace H1 variants.

Analysis of each phosphopeptide with respect to the cell cycle showed that four different types of cell cycle kinetics existed: peptides whose phosphorylation peaked in S, those that peaked in G2, those that rose during interphase and peaked in M, and the one peptide that was phosphorylated only in M. These preferences for different phosphorylation sites during the cell cycle probably reflect fluctuations in the various kinases and cyclins that are known to occur during the cell cycle (Evans et al., 1983; Pagano, et al. 1992; Murray, 1992). However, there did not appear to be any cell cycle exclusiveness for any phosphorylation site except the mitotic specific ones in pp13. This means that either there is not great site specificity for the various kinase/cyclin combinations, or there is significant overlap of the various kinase/cyclin types during different phases of the cell cycle. We have recently demonstrated that the former is certainly true.2

Previous analysis of CHO H1 phosphorylation using acid/urea gel electrophoresis has shown that H1 contained six phosphates during mitosis (Hohmann et al., 1976). The demonstration of both a serine and a threonine in the same N-terminal tryptic peptide of H1 explains why we see six phosphates in mitotic H1 but only detect five major phosphopeptides. Comparison of CHO H1 with other mammalian species shows that the threonine site is not conserved. Therefore, its phosphorylation at mitosis is not an exclusive necessity for chromosome condensation but may simply reinforce the negative charge function of the N-terminal serine phosphate, which is conserved in mammals.

The fact that only one mitotic specific phosphopeptide was detected suggests that all of the CHO H1 variants probably have identical N-terminal tryptic peptides. Comparison with human placenta H1 variants suggested that all of the CHO H1 variants are of the H1-1 subtype at the N-terminal end of the molecule (or the H1-d subtype from human spleen). However, since this H1 N-terminal peptide homogeneity has not been conserved among various mammalian species, it is difficult at this time to determine what the universally important sequence is for normal function in the H1 tail.

Examination of the amino acid sequence of rabbit H1 revealed that it contains five consensus sequences, one in the tail of H1, four in the trunk, and none in the nonpolar “body” (reviewed by van Holde (1989)). In our laboratory, we have been unable to detect any phosphorylation in the tail of H1 except at mitosis, and all of that phosphorylation was in the non-consensus sequence of pp13 (P1). If CHO H1 is found to have a similar consensus sequence in the H1 tail following the P1 tryptic peptide (like rabbit and calf H1 (Liao and Cole, 1981)), it will demonstrate that the consensus sequence is not a requirement for H1 phosphorylation.

Using acid/urea gel electrophoresis we have previously shown that interphase H1 can have up to 3 phosphates/molecule, and all three of these phosphates occur in the C-terminal trunk of H1 (Hohmann et al., 1976). In the present work we have fractionated three different major phosphopeptides from H1 of interphase cells, which must correspond to those three interphase phosphates (pp1, pp5, and pp9). In our earlier work we also demonstrated an additional phosphothreonine in the C-terminal trunk of CHO H1, which was only detectable in mitotic cells. The work in this report suggests that phosphothreonine probably is in pp7, which reaches a maximum phosphorylation during mitosis. Our present work indicates that the phosphorylation of this site actually begins in S phase but remains at a low level until mitosis, where its phosphorylation rate then approaches that of the other major phosphopeptides.

In conclusion, this work clearly demonstrates that the consensus sequence (S/T)P(X)Z is not necessary for the mitotic specific phosphorylation of H1. The cell cycle kinetics of phosphorylation at various H1 sites indicates that there is no absolute cell cycle-specific sites of phosphorylation during interphase. This suggests that the various cyclin-dependent kinases overlap significantly in the cell cycle and do not have different cell cycle site specificities. It appears that, during interphase, the number of phosphates per H1 molecule is more important than which sites are phosphorylated. In contrast, during mitosis there is clearly a site specificity for the N-terminal serine and threonine sites. Recent experiments in our laboratory with individual H1 cyclin/kinases have demonstrated these kinases’ lack of site specificity. Also, Jerzmanowski and Cole (1992) have demonstrated that H1 must be partially displaced from its chromatin-binding site before it can become a preferred substrate for mitotic-type phosphorylation. Thus, it appears that the displacement of the N-terminal tail of H1 from the chromatin may be sufficient for p34<sup>cdc2</sup>/cyclin B to perform mitotic specific H1 phosphorylation and that the consensus sequence is irrelevant for this particular cell cycle activity.

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Characterization of the Mitotic Specific Phosphorylation Site of Histone H1: ABSENCE OF A CONSENSUS SEQUENCE FOR THE p34cdc2/CYCLIN B KINASE

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