

Characterization of Phosphorylation Sites in Histone H1 in the Amitotic Macronucleus of *Tetrahymena* during Different Physiological States

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Abstract. Histone H1 is highly phosphorylated in transcriptionally active, amitotic macronuclei of *Tetrahymena* during vegetative growth. However, the level of H1 phosphorylation changes dramatically in response to different physiological conditions. H1 is hyperphosphorylated in response to heat shock and during prezygotic stages of conjugation. Conversely, H1 is largely dephosphorylated during prolonged starvation and during elimination of parental macronuclei during conjugation. Mapping of phosphorylation sites within H1 indicates that phosphorylation occurs at multiple sites in the amino-terminal portion of the molecule, predominantly at threonine residues. Two of these sites have been identified by compositional analyses and microsequencing of tryptic peptides. Interestingly, two major sites contain the sequence Thr-Pro-Val-Lys similar to that contained in the sites recognized by growth-associated histone kinase in other

organisms. No new sites are detected during the hyperphosphorylation of H1 which occurs during heat shock or in early stages of conjugation, and no sites are preferentially dephosphorylated during starvation or later stages of conjugation. Therefore, changes in the overall level of H1 phosphorylation, as opposed to phosphorylation or dephosphorylation at particular sites, appear to be important in the regulation of chromatin structure under these physiological conditions. Further, since no cell division or DNA replication occurs under these conditions, changes in the level of H1 phosphorylation are best correlated to changes in gene expression during heat shock, starvation, and conjugation. We suggest that, at least in *Tetrahymena*, H1 hyperphosphorylation is used as a rapid and transient mechanism for the cessation of transcription under conditions of cellular stress.

HISTONE H1 plays an important role in nucleosome structure as well as in the formation and maintenance of higher order chromatin structures (Allen et al., 1980, 1986; Cole, 1984; Crane-Robinson, 1984; McGhee and Felsenfeld, 1980; Pederson et al., 1986; Simpson, 1978; Thoma and Koller, 1977; Thoma et al., 1979). Modulation of higher order chromatin structures through the association or disassociation of H1 has been proposed as a mechanism for the regulation of several cellular processes including transcription and replication (Billings et al., 1979; Blumenfeld et al., 1978; Davie and Saunders, 1981; Hohmann, 1983; Kimura et al., 1983; Komaiko and Felsenfeld, 1985; Newrock et al., 1978; Reeves, 1984; Risdale and Cole, 1987; Schlissel and Brown, 1984; Weintraub, 1984, 1985). Such modulation could directly result from structural changes in the H1 molecule itself via posttranslational modifications that change the interaction of H1 with chromatin.

H1 is highly phosphorylated in a number of different cell types, ranging from protozoa to mammals (for review see Hohmann, 1983). Both the level and specific sites of phosphorylation have been observed to change in response to development (Billings et al., 1979; Blumenfeld et al., 1978; Green and Poccia, 1985; Lennox et al., 1982; Newrock et al., 1978; Talmadge and Blumenfeld, 1987) or the progression of the cell cycle (Ajiro et al., 1981a,b, 1983; Balhorn et al., 1972; Bradbury et al., 1974a,b; Gurley et al., 1975, 1978a,b,c; Hohmann et al., 1975, 1976; Langan, 1982; Lennox and Cohen, 1983). Indeed, a hyperphosphorylation of H1 during mitosis in higher eukaryotes has led to the correlation of such phosphorylation with the condensation of mitotic chromosomes (Balhorn et al., 1972; Bradbury et al., 1974a,b; Gurley et al., 1975, 1978a,b,c; Inglis et al., 1976; Krystal and Poccia, 1981; Lake et al., 1972; Matsumoto et al., 1980; Paulson and Taylor, 1982; Tanphaichitr et al., 1976). However, H1 is phosphorylated at other times during the cell cycle (Ajiro et al., 1981a,b, 1983; Gurley et al., 1975, 1978a,b; Hohmann et al., 1975; Lennox et al., 1982) and in nuclei which do not divide mitotically (Allis and Gorovsky, 1981;

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Talmadge and Blumenfeld, 1987). Therefore, phosphorylation of H1 must serve other functions as well, possibly in the regulation of transcription or replication.

The putative transcriptional and mitotic functions of H1 phosphorylation have been physically separated in the ciliated protozoan *Tetrahymena thermophila*. Like other ciliated protozoans, *Tetrahymena* possess two nuclei (for review see Gorovsky, 1986). The macronucleus is transcriptionally active and provides all gene products necessary for the cell. The micronucleus is transcriptionally inactive during vegetative growth and functions as a germline nucleus. As well, these two nuclei differ in their mode of division. Micronuclei divide mitotically while macronuclei divide amitotically without overt condensation of chromatin. Therefore, in *Tetrahymena*, phosphorylation of proteins important in the condensation of mitotic chromosomes should be restricted to the micronucleus, and phosphorylation of proteins related to transcriptional activity should be restricted to the macronucleus.

The histone components of both macronuclei and micronuclei have been well characterized (Allis et al., 1979, 1980a,b, 1984; Allis and Gorovsky, 1981; Allis and Wiggins, 1984a,b; Bannon and Gorovsky, 1984; Chicoine et al., 1984; Johmann and Gorovsky, 1976a,b). Both nuclei contain common core histones—H2A, H2B, H3, and H4. However, only macronuclei contain an H1 histone which shares some properties (size, solubility, and amino acid composition) with H1 from higher eukaryotes (Allis and Gorovsky, 1981; Gorovsky et al., 1974; Gorovsky and Keevert, 1975; Gorovsky, 1986). The micronucleus contains a unique set of linker histones, termed alpha, beta, and gamma (Allis et al., 1979, 1984; Allis and Wiggins, 1984a,b; Chicoine et al., 1984). Macronuclear-type H1 is highly phosphorylated in growing cells (Allis and Gorovsky, 1981), and the level of H1 phosphorylation has been observed to change under a variety of physiological conditions. Upon starvation, H1 is largely dephosphorylated (Allis and Gorovsky, 1981; Glover et al., 1981). Subsequent heat shock of starved (or growing) cells results in a hyperphosphorylation of H1 (Glover et al., 1981). Recent studies have shown that hyperphosphorylation of H1 is observed to an even greater extent soon after starved cells of opposite mating type are mixed and induced to conjugate (Schulman et al., 1987). Later in the sexual cycle (7.5 h), H1 is quantitatively dephosphorylated in parental macronuclei (Schulman et al., 1987).

The nature and function of these physiological changes in H1 phosphorylation are not understood. However, they cannot be related to mitosis since macronuclei divide amitotically. Further, cell division is arrested in starved, heat-shocked, and conjugating cells, and macronuclei do not undergo any significant DNA replication under these conditions (Allis et al., 1988; Doerder and Debault, 1975). Therefore, it seems probable that the dephosphorylation and hyperphosphorylation of H1 observed under these conditions are related to changes in gene expression that occur during starvation, heat shock, and conjugation.

To better understand the nature and regulation of these changes in H1 phosphorylation, we have compared the sites of H1 phosphorylation under these physiological conditions. Our data indicate that H1 is phosphorylated at multiple sites within the amino-terminal 69 residues of the molecule, predominantly at threonine residues. We have identified two ma-

ior phosphorylation sites, both of which contain the sequence Thr-Pro-Val-Lys. No new phosphorylation sites were observed during heat shock or in early stages of conjugation, and no sites were preferentially dephosphorylated during starvation or later in conjugation. We conclude, therefore, that changes in the level of phosphorylation within the population of H1 molecules occur via changes in the number of phosphorylated molecules and the number of phosphates per molecule. Further, phosphorylation (and dephosphorylation) of H1 occurs randomly at all sites. Possible consequences of these changes in H1 phosphorylation to chromatin structure and gene expression are discussed, and a comparison of the sites (both in sequence and location) of H1 phosphorylation identified here are compared to those previously identified in other organisms.

Materials and Methods

Cell Culture and Labeling with [³²P]Phosphate

Tetrahymena thermophila strains CU427 (mpr/mpr-[6-mp-s]VI) and CU428 (Chx/Chx-[cy-s]VII) were grown under standard conditions as previously described (Gorovsky et al., 1975). To label cells with [³²P]phosphate, shaking cultures (approximate density of 250,000 cells/ml) were harvested by centrifugation at 1,200 g and were resuspended in 10 mM Tris/HCl, pH 7.4, containing 10 μCi/ml [³²P]phosphate (ICN K & K Laboratories, Inc., Plainview, NY). After 2.5 h gentle shaking at 30°C, cells were either returned to food for a brief (1-h) recovery period followed by nuclei preparation or were transferred to fresh Tris without label for overnight starvation. Little growth occurred during starvation so that the density of starved cells was typically 250,000–300,000 cells/ml. Cells were starved for 16–24 h at 30°C with gentle agitation, and they were then either harvested for the preparation of nuclei or transferred to fresh Tris prewarmed to 41°C for 1 h of heat shock. After 1 h at 41°C, heat-shocked cells were also harvested for nuclei preparation.

Conjugation of cells was performed as previously described (Bruns and Brussard, 1974). Cells to be mated were starved in 10 mM Tris, pH 7.4 (200,000 cells/ml), containing 10 μCi/ml [³²P]phosphate without shaking at 30°C for 12–24 h. Equal numbers of cells of opposite mating type were mixed, and mating cells were harvested for nuclei preparation at either 3 or 7.5 h after mixing. More than 90% pairing of mating cells was observed in all experiments.

Nuclei Preparation and Extraction of Histones

Macronuclei were isolated as described by Gorovsky et al. (1975) except that the isolation buffer (Medium A) did not contain spermidine but did contain 10 mM Tris/HCl, pH 7.0, 1 mM iodoacetamide, 1 mM PMSF, and 10 mM butyrate. Histones were extracted in 0.4 N H₂SO₄ and subsequently fractionated with PCA as previously described (Schulman et al., 1987).

PAGE and Electroelution of H1

PCA-soluble macronuclear proteins were separated preparatively on 22% polyacrylamide-SDS gels as previously described (Allis et al., 1979, 1980a,b). After electrophoresis, gels were briefly stained and destained, and the bands corresponding to H1 were excised and subjected to electroelution (Hunkapillar et al., 1983). Electroeluted H1 was dried under vacuum, resuspended in 0.1 ml of H₂O, and precipitated by the addition of 1.0 ml of 95% ethanol prechilled to –20°C. After several hours at –20°C, the protein precipitate was collected by centrifugation at 12,000 g and dried under vacuum. Precipitation with ethanol was typically repeated at least three times to remove residual SDS before subsequent chemical or enzymatic analyses.

Acid urea polyacrylamide electrophoresis was performed as previously described (Allis et al., 1979, 1980a,b).

Cleavage by Cyanogen Bromide

Electroeluted H1 was dissolved in 70% formic acid containing 20 mg/ml cyanogen bromide. Control reactions were performed in 70% formic acid alone. Reactions proceeded for 36–48 h in the dark at 22°C, and were then

dried under vacuum and subjected to SDS electrophoresis as described above. In some cases, cyanogen bromide cleavages were performed on H1 bands excised from SDS gels without electroelution. Gel pieces were rinsed several times in 62 mM Tris/HCl, pH 6.8, and then immersed in either formic acid or formic acid containing cyanogen bromide. Reactions were performed as above. H1 and cyanogen bromide cleavage products were released from the gel pieces during the course of the reaction. After the reaction, gel pieces were removed and discarded, and the reaction liquid was dried under vacuum. To remove residual acid, dried reaction products were typically washed once in acidified acetone and once in acetone and redried before SDS electrophoresis.

Phosphoamino Acid Analysis

[³²P]Phosphate-labeled H1 was excised and electroeluted from SDS-polyacrylamide gels as described above. Eluted protein was washed several times in ethanol at -20°C to remove residual SDS, dried under vacuum, and dissolved in 5.7 M HCl. Hydrolysis was performed in vacuum-sealed tubes under nitrogen at 110°C for 2 h. After hydrolysis, samples were dried, dissolved in TLC buffer (11:10:370 acetic acid/formic acid/H₂O) and spotted onto Avicel (Analtech Inc., Newark, DE) thin layer chromatography plates with phosphoserine and phosphothreonine standards. Chromatography was performed for 1.5 h at 800 V in the above buffer. Phosphoserine and phosphothreonine standards were visualized by staining with ninhydrin, and [³²P]phosphate-labeled amino acids were detected by autoradiography.

Tryptic Digestions

Electroeluted H1 was dissolved in fresh 0.1% ammonium bicarbonate and L-1-*P*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was added to a final concentration of 0.1-0.2 mg/ml. Digestions were performed at 37°C for 16-24 h. Digestion products were then dried under vacuum, dissolved in H₂O/0.1% trifluoroacetic acid, and subjected either to reverse-phase HPLC using a C8 analytical column (Aquapore octyl-RP300; Brownlee Labs, Santa Clara, CA) with a linear gradient of 0-25% acetonitrile/0.1% trifluoroacetic acid in 60 min, or to alkaline electrophoresis (West et al., 1984) followed by autoradiography. HPLC-purified phosphopeptides were subsequently subjected to microsequencing as previously described (Allis et al., 1986) except that the derivatives were identified by HPLC using a phenylthiohydantoin analyzer (model 120A; Applied Biosystems, Inc., Foster City, CA).

Results

Physiological Changes in H1 Phosphorylation

To compare known physiological changes in H1 phosphorylation (Allis and Gorovsky, 1981; Glover et al., 1981; Schulman et al., 1987), H1 molecules isolated from vegetatively growing, starved, heat-shocked, or conjugating cells were examined electrophoretically (Fig. 1). Different phosphorylated forms of H1 are easily separated by electrophoresis in acid urea or SDS-polyacrylamide gels. At least five different phosphorylated forms of H1 are resolved by acid urea PAGE (Fig. 1 A). [³²P]Phosphate-labeling experiments indicate that the fastest migrating species in this gel system carries no phosphate, and that slower migrating forms of H1 contain increasing amounts of phosphate in accordance with previously published reports (Glover et al., 1981). All phosphorylated forms of H1 are present in growing cells (Fig. 1, GR), and most molecules carry at least some phosphate as indicated by the underrepresentation of the fastest migrating, dephosphorylated form of H1 in these cells. However, after prolonged starvation (24-30 h), the less phosphorylated and dephosphorylated forms of H1 predominate, indicating that massive dephosphorylation of H1 has occurred (Fig. 1, ST). Subsequent heat shock of starved cells results in a striking shift in the mass of H1 to more highly phosphorylated forms (Fig. 1, HS). This same shift is observed to an even greater extent 3 h after starved cells of opposite mating type are

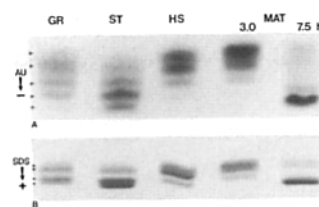


Figure 1. Physiological changes in levels of H1 phosphorylation. PCA-soluble histones extracted from growing (GR), starved (ST), heat-shocked (HS), and mating cells at 3 (MAT 3.0) or 7.5 (MAT 7.5) h were directly compared by

electrophoresis in either (A) acid urea or (B) SDS-polyacrylamide gel systems. Only the H1 regions of the stained gels are shown. Arrowheads point to different phosphorylated forms of H1 separated by these electrophoretic systems. The dephosphorylated form of H1 is the fastest migrating species in both gel systems.

mixed and allowed to conjugate (Fig. 1, MAT 3.0). Later in the sexual cycle (7.5 h after mixing), however, an almost quantitative dephosphorylation of H1 is observed (Fig. 1, MAT 7.5).

These shifts in H1 phosphorylation are also observed when H1 molecules are subjected to SDS-PAGE (Fig. 1 B). At least four electrophoretic forms of H1 are typically resolved by electrophoresis in SDS, and increased phosphorylation leads to a decreased mobility in this gel system as in the acid urea gel system (Allis and Gorovsky, 1981; Glover et al., 1981). As reported by others (Billings et al., 1979; Lennox and Cohen, 1983), this decreased mobility in SDS gels is consistent with a change in either the conformation or the distribution of local charge densities (or both) of the phosphorylated H1 molecule. To understand the nature of these changes and to possibly correlate differences in the phosphorylation of H1 molecules to different physiological conditions, the individual sites of H1 phosphorylation were examined as described below.

Cleavage of H1 with Cyanogen Bromide

The gene encoding *Tetrahymena thermophila* H1 has been cloned and sequenced (Wu et al., 1986), and the predicted amino acid sequence is shown in Fig. 2. A single methionine

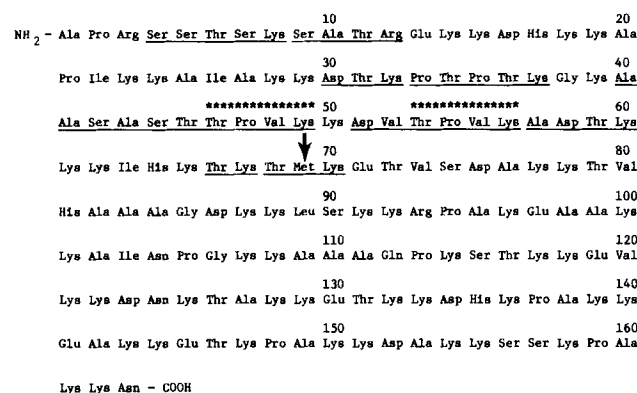
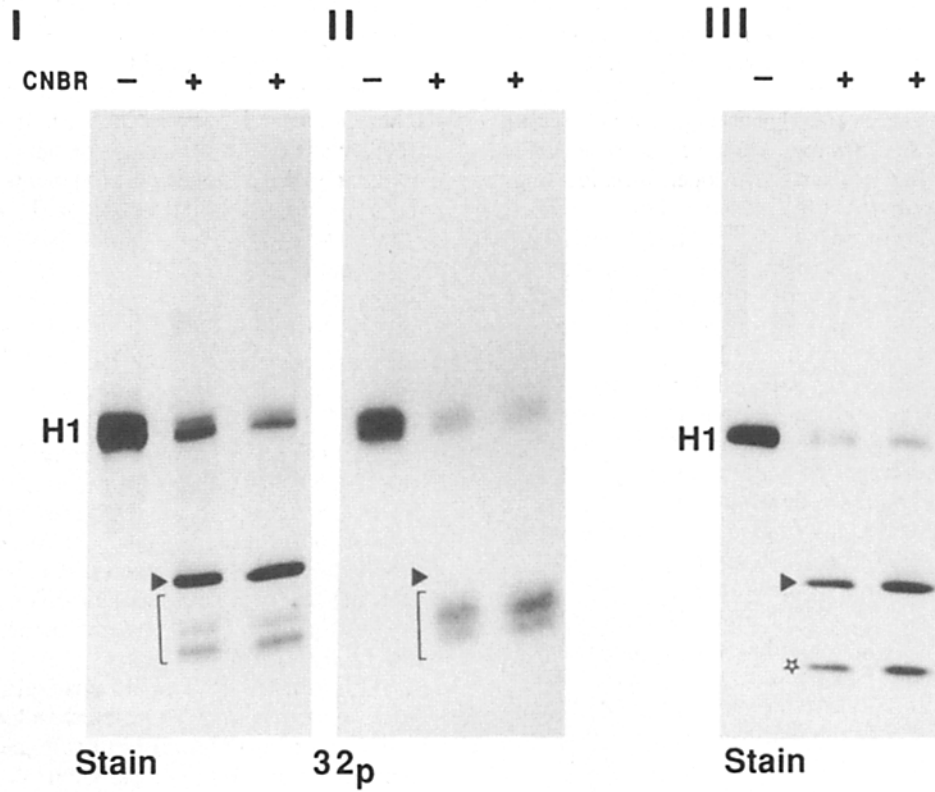


Figure 2. Amino acid sequence and phosphorylation sites in *Tetrahymena thermophila* H1. The amino acid sequence of H1 as determined by Wu et al. (1986) is depicted in three-letter amino acid code. An arrow points to the single methionine residue and amino-terminal serine- and threonine-containing tryptic peptides are underlined. The Thr-Pro-Val-Lys motif present in the two peptides identified by microsequencing is indicated by asterisks.

A



B

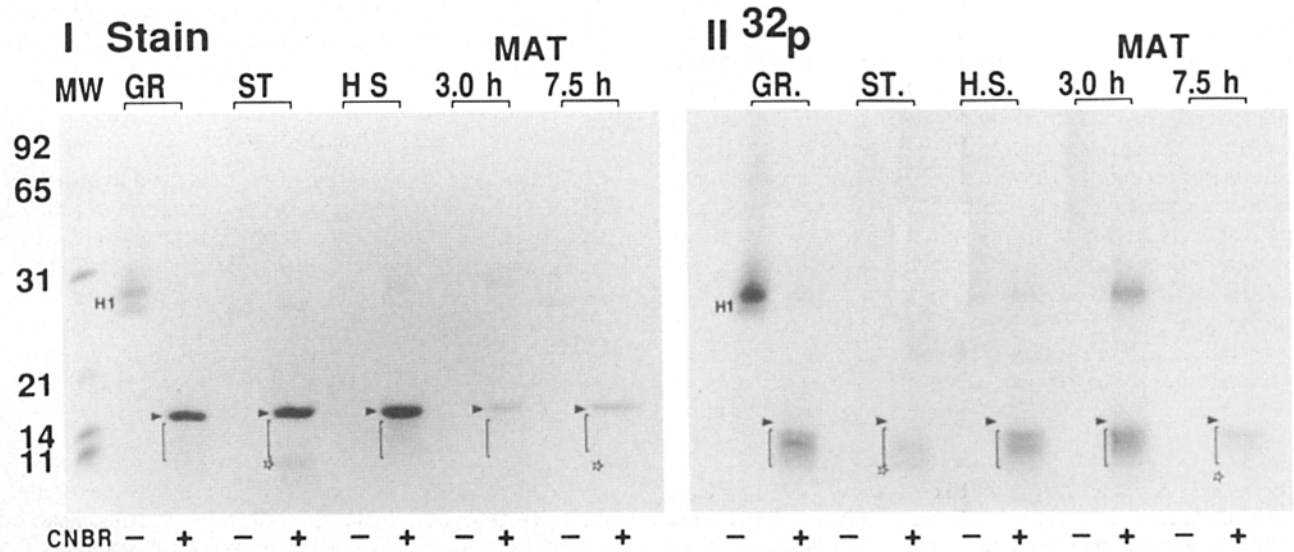


Figure 3. (A) Cyanogen bromide cleavage of H1. ^{32}P -labeled H1 isolated from growing cells was cleaved with cyanogen bromide in solution as described in Materials and Methods. Intact H1 (-) and cleavage products (+) were separated by SDS electrophoresis and visualized by staining the gel with Coomassie Blue (I). The two +’s above each panel refer to low (1 mg/ml) and high (20 mg/ml) concentrations of cyanogen bromide (CNBr). Phosphorylated fragments were identified by autoradiography of the stained gel (II). Fragments generated by CNBr cleavage of phosphatase-treated H1 are displayed in III. Phosphatase treatment efficiently removes all ^{32}P -label from H1 under the conditions described by Glover et al. (1981). Therefore, no autoradiogram of the gel shown in III is presented. The arrowheads indicate

residue at position 69 provides a convenient site for cyanogen bromide cleavage. Cleavage of H1 at this residue yields two fragments, corresponding to the amino-terminal (69 residues) and carboxy-terminal (94 residues) portions of the molecule.

Products from cyanogen bromide cleavage of H1 isolated from growing cells labeled with [³²P]phosphate are displayed by SDS electrophoresis in Fig. 3 A. The intact molecule is split into a discrete band (Fig. 3, *arrowheads*) and a faster migrating, heterogeneous set of bands (Fig. 3, *brackets*). The larger discrete cyanogen bromide fragment has been previously identified as the carboxy-terminal fragment by microsequencing (Wu et al., 1986), indicating that the collection of faster migrating bands represent the amino-terminal portion of the molecule. The electrophoretic heterogeneity of this fragment is similar to that observed for the intact phosphorylated H1 molecule, suggesting that these heterogeneous bands represent different phosphorylated forms of the amino-terminal fragment. The lack of electrophoretic heterogeneity in the carboxy-terminal fragment suggests that this fragment is not phosphorylated. Indeed, autoradiography of the gel shown in Fig. 3 A reveals that the discrete carboxy-terminal fragment carries no phosphate (Fig. 3 A, II). As expected, phosphate is distributed exclusively among the amino-terminal fragment bands. That these heterogeneous bands represent direct phosphorylation of the amino-terminal fragment (rather than poly-ADP-ribosylation, for example) is further demonstrated in Fig. 3 A. Treatment of the intact H1 molecule with phosphatase results in complete reduction of the electrophoretic heterogeneity of this molecule, such that a single band corresponding to the dephosphorylated form of H1 is observed on SDS gels (compare intact H1 in Fig. 3 A, I to that in 3 A, III). Subsequent cleavage of the dephosphorylated molecule with cyanogen bromide results in two discrete fragments. The larger, carboxy-terminal fragment exhibits the same relative mobility as that generated from phosphorylated H1. However, upon phosphatase treatment the heterogeneity associated with the amino-terminal fragment of phosphorylated H1 is completely removed, resulting in a single band with a mobility slightly faster than that of the fastest migrating (phosphorylated) form seen in Figure 3 A, I or 3 A, II (Fig. 3 A, III, *star*). These data indicate that phosphate is localized exclusively to the amino-terminal half of H1 in growing cells.

To determine whether the carboxy-terminal fragment is phosphorylated during the hyperphosphorylation of H1 observed during heat shock or early in the sexual cycle (3 h), [³²P]phosphate-labeled H1 was isolated from cells grown under these physiological conditions and subjected to cyanogen bromide cleavage (Fig. 3 B). For comparison, dephosphorylated H1 (Fig. 1) was isolated from starved cells and

from cells 7.5 h after the initiation of the sexual cycle. As seen in Fig. 3 B, I, cyanogen bromide cleavage of H1 from heat-shocked and early conjugating cells yielded fragments very similar to those generated from H1 from growing cells. Heterogeneous bands corresponding to the amino-terminal fragment were observed (Fig. 3 B, *brackets*), but they exhibited a slightly slower mobility due to the increased phosphorylation of H1 under these conditions. Cleavage of H1 molecules from starved cells or from cells after 7.5 h of conjugation yielded two discrete fragments (Fig. 3 B, *stars*) consistent with dephosphorylation of H1 observed under these conditions. Under no condition was the carboxy-terminal fragment (Fig. 3 B, I, *arrowheads*) observed to be labeled (Fig. 3 B, II). Thus, even under conditions where H1 is maximally phosphorylated, all phosphate is associated exclusively with the amino-terminal fragment.

Phosphoamino Acid Analysis

The amino-terminal cyanogen bromide fragment of H1 contains 6 serine and 11 threonine residues (Fig. 2), providing 17 different possible sites of phosphorylation. No tyrosine residues are present in the entire molecule. To determine whether serine or threonine residues are used differentially as phosphate acceptors under the different physiological conditions described above, phosphoamino acid analysis was performed (Fig. 4).

[³²P]Phosphate-labeled H1 was isolated from cells under different physiological conditions and was subjected to acid hydrolysis and thin layer chromatography. The position of nonradioactive phosphoserine and phosphothreonine standards (included in each sample as mobility markers) was determined by staining the chromatograph with ninhydrin, and phosphoamino acids were detected by autoradiography. Typical results from these experiments are shown in Fig. 4. Unlike H1 from higher eukaryotes, phosphothreonine is the major phosphoamino acid derived from H1 under all of the physiological conditions examined. Phosphoserine is used to a much more limited extent, but was observed in all samples upon longer exposures of the autoradiograms.

Analysis of Tryptic Phosphopeptides

The 11 threonine and 6 serine residues in the amino-terminal half of the H1 molecule are predicted to reside in nine peptides generated by complete tryptic digestion of the H1 molecule according to the sequence presented in Fig. 2. To further localize the actual sites of H1 phosphorylation and to compare use of phosphorylation sites in H1 under different physiological conditions, tryptic mapping of H1 phosphopeptides was performed. ³²P-labeled H1 was isolated from growing,

the band corresponding to the carboxy-terminal cyanogen bromide fragment, and the brackets enclose heterogeneously migrating amino-terminal fragments. The star in III indicates the band corresponding to the dephosphorylated amino-terminal fragment. All samples were subjected to electrophoresis in the same gel. (B) Cyanogen bromide cleavage of H1 isolated under different physiological conditions. Products from H1 isolated from growing (GR), starved (ST), heat-shocked (HS), 3-h mating (MAT 3.0), and 7.5-h mating (MAT 7.5) cells are displayed by SDS electrophoresis as in A. In this experiment, however, cyanogen bromide cleavage of H1 was performed in gel slices (+) as described in Materials and Methods. The position of intact H1 is indicated. Because of limiting amounts of isolated H1, fivefold less material was used in the control (-) reactions. Cleavage of H1 was never observed in the absence of cyanogen bromide. Arrowheads and brackets indicate the position of the carboxy- and amino-terminal CNBr fragments, respectively, as in A. The stars denote the dephosphorylated amino-terminal fragment generated from cleavage of H1 isolated from starved and 7.5-h mating cells. The stained gel is shown in I, and the autoradiogram of this gel is shown in II.

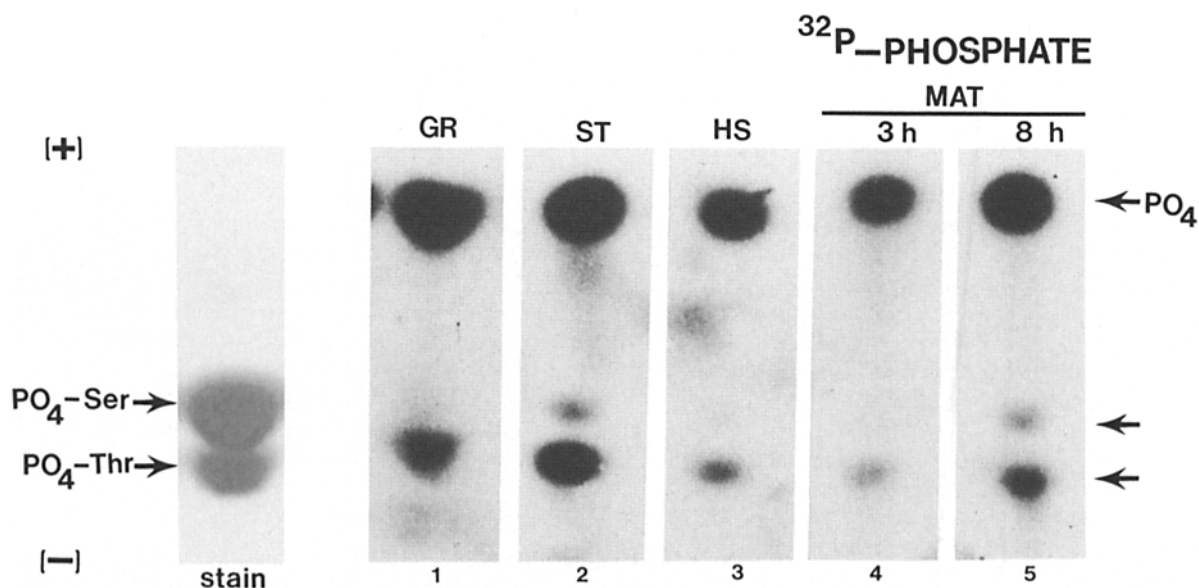


Figure 4. Phosphoamino acid analysis of H1 isolated from different physiological states. ^{32}P -labeled H1 molecules isolated from cells grown under the indicated physiological conditions were hydrolyzed, and phosphoamino acids were separated by thin layer chromatography with phosphothreonine ($\text{PO}_4\text{-Thr}$) and phosphoserine ($\text{PO}_4\text{-Ser}$) standards. Positions of standards were visualized by staining with ninhydrin (e.g., see the first lane labeled *stain*). ^{32}P -labeled amino acids and free phosphate (PO_4) released by hydrolysis were detected by autoradiography of the chromatograms (lanes 1-5). All samples analyzed were labeled in the same experiment, and all except the sample from growing cells were analyzed on the same chromatogram. Chromatograms were aligned by the migration of phosphothreonine and phosphoserine standards. More material (~ 10 -fold) derived from starved cells was loaded onto the chromatogram resulting in a stronger autoradiographic signal (lane 2). The autoradiographic signal in one sample (lane 4) is unusually weak due to loss of H1 during electroelution from the gel.

starved, heat-shocked, or conjugating cells and was digested exhaustively with trypsin. Tryptic phosphopeptides were subsequently separated (by differences in size and charge) by alkaline electrophoresis (West et al., 1984) and visualized by autoradiography. Typical results from such experiments are shown in Fig. 5.

11 phosphorylated bands were resolved from tryptic digests of H1 from growing cells (Fig. 5, GR). At least some of the observed bands represent different phosphorylated forms of the same peptide. For example, microsequencing of the two slowest migrating bands (Fig. 5, bands *a* and *b*) revealed that both bands represent a peptide with the sequence Ala-Ala-Ser-Ala-Ser-Thr-Thr-Pro-Val-Lys (Fig. 2, residues 40-49). The position of the phosphate within this peptide is unknown, but the two phosphorylated bands in Fig. 5 must represent different phosphorylated forms (either in the number or position of phosphorylated residues) of this sequence. Only one other peptide was successfully purified and subjected to microsequencing (Fig. 5, band *c*). This peptide was identified as residues 51-56 with the sequence Asp-Val-Thr-Pro-Val-Lys. Phosphorylation must occur at the single threonine residue within this peptide. Both phosphotryptic fragments identified contain the sequence Thr-Pro-Val-Lys, similar to sites phosphorylated by H1 kinases from higher eukaryotes (Masaracchia et al., 1979; Ajiro et al., 1981b; Cicirelli et al., 1988).

The dramatic shifts in H1 phosphorylation observed during starvation, heat shock, and conjugation (Fig. 1) could result from differential use of individual phosphorylation sites under these physiological conditions, and some changes in the intensities of individual bands were repeatedly observed.

For example, one of the most highly labeled bands generated from H1 isolated from growing cells (Fig. 5, band *c*) is greatly diminished in the digests of the hyperphosphorylated H1 molecules. More striking, however, is the fact that the same set of 11 phosphorylated bands was consistently generated from tryptic digests of H1 isolated from growing, starved, heat-shocked, or conjugating cells. The overall similarity in the patterns of the tryptic maps of H1 isolated under these conditions indicates that the same set of peptides is phosphorylated under these various conditions of cell growth. That is, no new sites were observed after hyperphosphorylation of H1 during heat shock or early in conjugation, and no sites were preferentially dephosphorylated during starvation or later in conjugation. The absence of new sites of phosphorylation during heat shock and early conjugation, and the apparent random use of phosphorylation sites indicate that overall changes in the level of phosphorylation within the population of H1 molecules are more important to changes in chromatin structure under different physiological conditions than is phosphorylation or dephosphorylation at any particular site. The almost quantitative conversion of the H1 population to hyperphosphorylated or dephosphorylated forms in response to different physiological conditions (Fig. 1) suggests that H1 phosphorylation is related to large-scale changes in higher order chromatin structure under these conditions.

Discussion

The changes in H1 phosphorylation which occur in the ami-

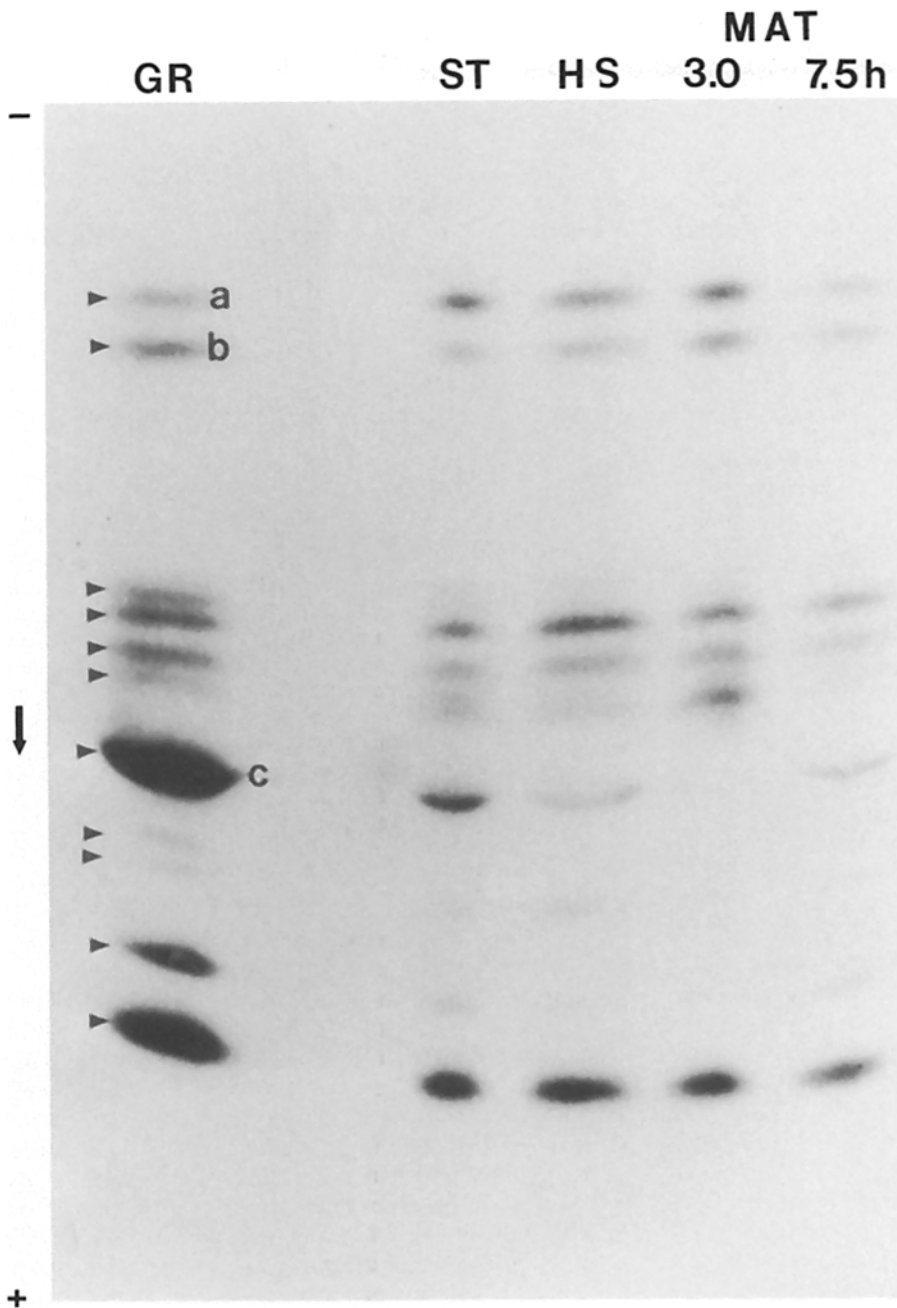


Figure 5. Electrophoretic analysis of tryptic phosphopeptides. Tryptic phosphopeptides generated by cleavage of H1 molecules isolated from cells in various physiological states were separated by electrophoresis in 40% alkaline acrylamide gels as described by West et al. (1984) and visualized by autoradiography. 11 labeled bands were resolved, as indicated by arrowheads. Those peptides identified by microsequencing are labeled *a*, *b*, and *c*. The identity and relatedness of the other bands observed have not been determined because of our inability to separate them chromatographically.

totic macronucleus of *Tetrahymena* under the physiological conditions described in this paper (in which DNA replication and cell division do not occur) are best correlated to changes in gene expression. Hyperphosphorylation of H1 in *Tetrahymena* is induced during heat shock and early in conjugation. Both are conditions in which the cell is likely to be decreasing the expression of many growth-related genes and increasing the expression of specialized gene sets (Schulman et al., 1987). The hyperphosphorylation of H1, then, could be related to either the increased expression of these few genes or the decreased expression of other genes. Given the extent of the hyperphosphorylation that occurs, we tend to favor the latter hypothesis. In this regard, it is worth noting that mitotic hyperphosphorylation of H1 in other organisms might be related to the shut off of gene expression which oc-

curs during mitosis rather than the condensation of chromosomes (Paulson and Taylor, 1982). This idea is supported by studies of early sea urchin embryos in which the hyperphosphorylation of H1 during mitosis was uncoupled from chromosome condensation (Krystal and Poccia, 1981). Mitotic phosphorylation of other chromatin-bound proteins, such as HMG 14, may also be involved in the cessation of transcription during mitosis (Krystal and Poccia, 1981).

Given that reduced gene expression occurs during starvation in *Tetrahymena* (Calzone et al., 1983; Martindale and Bruns, 1983; Martindale et al., 1985, 1987), one might expect hyperphosphorylation of H1 to occur under these conditions as well if phosphorylation is related to an overall decrease in gene expression and if such decreases are regulated transcriptionally. However, H1 is largely dephosphorylated

in these cells (Fig. 1). Unlike heat-shocked or early conjugating cells, however, gene expression has been reduced in starved cells for prolonged periods (~24 h in our experiments). Perhaps hyperphosphorylation of H1 is used primarily by the cell as a mechanism for the immediate cessation of transcription under conditions of cellular stress but either cannot be maintained for prolonged periods or is not needed once the cell has adapted to stress. In support of this hypothesis, we have observed that H1 is no longer hyperphosphorylated in cells subjected to heat shock for 24 h (data not shown). Starvation itself is a stress upon the cell, and H1 might be predicted to be hyperphosphorylated soon after cells are subjected to starvation conditions. Indeed, H1 is hyperphosphorylated from 1 to 2.5 h after the initiation of starvation and is progressively dephosphorylated after this time (data not shown). The timing of this H1 hyperphosphorylation closely coincides with the rapid decrease in gene expression that occurs within the first 2 h of starvation (Martindale et al., 1985), further indicating that hyperphosphorylation of *Tetrahymena* H1 may be related to the rapid and transient cessation of general cellular gene expression in response to stress. The subsequent dephosphorylation of H1 after prolonged starvation or heat shock, then, may be related to the adaptation of the cell to stressful conditions and the resumption of some normal (nonstress-related) cellular activities. Synthesis of non-heat-shock proteins, for example, has been observed to resume after prolonged heat shock (Hallberg et al., 1984, 1985; Kraus et al., 1987).

Parental macronuclei are destined for destruction late in the sexual cycle, a time when striking dephosphorylation of H1 is also observed (Fig. 1, MAT 7.5). Transcription is rapidly abolished in parental macronuclei during this stage as well (Wenkert and Allis, 1984). Ultrastructural studies (Weiske-Benner and Eckert, 1987) have shown that this nucleus undergoes vast morphological changes, and biochemical analyses have detected widespread proteolytic degradation of core histones and other nuclear proteins, indicating a general destruction of chromatin structure (Allis et al., 1984; Allis and Wiggins, 1984a,b). The dephosphorylation of H1 and the lack of transcription in these nuclei, then, may be simply a consequence of these irreversible destructive events.

We have determined that phosphorylation occurs at multiple sites within the amino-terminal portion of the H1 molecule and have localized two of these sites by sequence. Recently, Hayashi et al. (1987) reported the complete H1 protein sequence in *Tetrahymena pyriformis* and identified five phosphorylation sites within this molecule. A comparison of the sequence of phosphorylated tryptic peptides from *Tetrahymena thermophila* and *T. pyriformis* is presented in Table I. Sequence differences between the two H1 molecules have led to differences in their sites of phosphorylation. Of the five phosphorylation sites identified in *T. pyriformis*, only two (residues 33–38 and 42–51) have sequences similar to corresponding (putative) sites in *T. thermophila* (residues 33–37 and 40–49). In contrast to the lack of phosphorylation in the carboxy-terminal portion of *T. thermophila* H1, *T. pyriformis* H1 is phosphorylated at two carboxy-terminal sites. Interestingly, the two phosphorylation sites that we have localized in *T. thermophila* H1 contain the sequence Thr-Pro-Val-Lys (residues 46–49 and 53–56), and a related sequence Thr-Pro-Thr-Lys is found at a third (putative) site (residues 34–37). Similar sequences are found in two of the *T. pyriformis* phosphorylation sites, Thr-Pro-Val-Lys (residues 35–38) and Thr-Pro-Ile-Lys (residues 48–51). This sequence has been identified as a growth-associated histone kinase phosphorylation site in H1 from higher eukaryotes (Ajiro et al., 1981b; Cicirelli et al., 1988; Masaracchia et al., 1979) and is similar to a consensus phosphorylation site for H1 histones, Lys-Ser/Thr-Pro-Lys (Hohmann, 1983).

Given the amount of divergence which has occurred in H1 between two strains of *Tetrahymena* (Table I), it is perhaps not surprising that *Tetrahymena* H1 has a different primary structure than H1 molecules from higher organisms. Significant sequence divergence is evident. *Tetrahymena* H1 is smaller than most other H1 molecules and appears to lack a generally conserved, central globular domain present in other H1's (Allan et al., 1986; Crane-Robinson, 1984; Hayashi et al., 1987; Wu et al., 1986). These differences make it difficult to relate the positions of phosphorylation sites within *Tetrahymena* H1's to those identified in other organisms. In higher eukaryotes, H1 phosphorylation usually occurs at specific sites near the amino terminal or the carboxy terminal of the molecule but not in the central globular do-

Table I. Comparison of Phosphorylation Sites in Histone H1 in *Tetrahymena Thermophila* and *Tetrahymena Pyriformis**

Species	Sequence†	Phosphorylation
<i>T. thermophila</i>	³³ Pro Thr Pro Thr Lys ³⁷	?§
<i>T. pyriformis</i>	³³ Ser Thr Thr Pro Val Lys ³⁸	+
<i>T. thermophila</i>	--- ³⁸ Gly Lys ³⁹	—
<i>T. pyriformis</i>	³⁹ Thr Ser Lys ⁴¹	+
<i>T. thermophila</i>	⁴⁰ Ala Ala Ser Ala Ser Thr Thr Pro Val Lys ⁴⁹	+
<i>T. pyriformis</i>	⁴² Ala Ala Pro Ala Ser Thr Thr Pro Ile Lys ⁵¹	+
<i>T. thermophila</i>	⁵¹ Asp Val Thr Pro Val Lys ⁵⁶	+
<i>T. pyriformis</i>	⁵² Asp Thr Thr Pro Val Lys ⁵⁷	—
<i>T. thermophila</i>	⁸³ Ala Ala Gly Asp Lys ⁸⁷	—
<i>T. pyriformis</i>	⁸³ Ser Ala Gly Asp Lys ⁸⁷	+
<i>T. thermophila</i>	¹¹⁵ Ser Thr Lys ¹¹⁷	—
<i>T. pyriformis</i>	¹¹⁷ Thr Ala Lys ¹¹⁹	+

* *Pyriformis* sequence and phosphorylation sites from Hayashi et al. (1987).

† Sequence location is indicated by superscripts.

§ Phosphorylation of this peptide in *T. thermophila* has not been determined.

|| Residues in the carboxy-terminal portion (70–163) of *T. thermophila* H1 are not phosphorylated (Fig. 3).

main (Hohmann, 1983). *Tetrahymena* H1 is unique in that phosphorylation occurs predominantly (and, in *T. thermophila*, exclusively) in the amino terminus of the molecule. Also, most other H1s are phosphorylated predominantly at serine residues, rather than threonine as in *T. thermophila*. The implications of these differences in H1 structure are not understood but may be related to the fact that in *Tetrahymena* H1 does not participate in mitosis-related chromatin condensation.

Although early studies of H1 phosphorylation in hamster cells indicated that particular sites within H1 were phosphorylated at specific times during the cell cycle (Gurley et al., 1975, 1978a,b,c; Hohmann et al., 1975, 1976), more recent studies in other cell types indicate that the order of phosphorylation-site use is not generally progressive (Hohmann, 1983). Instead, the proportion of phosphorylated molecules together with the number of phosphates per molecule change during the cell cycle (Ajiro et al., 1981a,b, 1983; Langan, 1982) similar to the changes we have observed in this paper under different physiological conditions. To understand how these changes in the overall level of H1 phosphorylation are regulated, it is necessary to understand the activities responsible for the phosphorylation and dephosphorylation of H1. We have recently begun to characterize these enzymes *in vitro* and to monitor changes in their synthesis or activity in response to different physiological conditions. Future studies should reveal the ways in which these enzymes are regulated and how they interact to affect changes in chromatin structure through modification of the phosphorylation state of histone H1.

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