Identification and Mutation of Phosphorylation Sites in a Linker Histone

PHOSPHORYLATION OF MACRONUCLEAR H1 IS NOT ESSENTIAL FOR VIABILITY IN TETRAHYMENA*

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Linker histone phosphorylation has been suggested to play roles in both chromosome condensation and transcriptional regulation. In the ciliated protozoan Tetrahymena, in contrast to many eukaryotes, histone H1 of macronuclei is highly phosphorylated during interphase. Macronuclei divide amitotically without overt chromosome condensation in this organism, suggesting that requirements for phosphorylation of macronuclear H1 may be limited to transcriptional regulation. Here we report the major sites of phosphorylation of macronuclear H1 in Tetrahymena thermophila. Five phosphorylation sites, present in a single cluster, were identified by sequencing 32P-labeled peptides isolated from tryptic peptide maps. Phosphothreonine was detected at two non-proline-directed sites that do not resemble known kinase consensus sequences. Phosphoserine was detected at two non-proline-directed sites that do not resemble known kinase consensus sequences. Phosphorylation at the two noncanonical sites appears to be hierarchical because it was observed only when a nearby p34cdc2 site was also phosphorylated. Cells expressing macronuclear H1 containing alanine substitutions at all five of these phosphorylation sites were viable even though macronuclear H1 phosphorylation was abolished. These data suggest that the five sites identified comprise the entire collection of sites utilized by Tetrahymena and demonstrate that phosphorylation of macronuclear H1, like the protein itself, is not essential for viability in Tetrahymena.

In eukaryotes, the association of DNA with histones in nucleosomes and the folding of nucleosomal filaments within chromatin can restrict the accessibility of DNA sequences to factors required for gene expression and DNA replication (1, 2). Crystallographic analyses have led to detailed appreciation of the arrangement of core histones within nucleosomes and the histone-histone and histone-DNA interactions responsible for the stability of nucleosomal structure (3, 4). Many aspects of nucleosome structure are expected to be common to all eukaryotes because core histones are among the most highly conserved proteins known (1, 2). However, the structure and occurrence of proteins, collectively referred to as H1 or linker histones that bind the outer surface of nucleosomes and portions of the linker DNA extending between adjacent nucleosomes, are more variable (1, 2, 5, 6).

Data from recent biochemical and molecular genetic analyses have firmly established that chromatin structure plays a fundamental role in the regulation of gene expression. Acetylation of conserved lysine residues within the amino termini of core histones, for example, is a major pathway for modulating transcriptional activity (7, 8). In contrast, the function of linker histones and their various post-translational modifications in vivo remain unclear (5, 6, 9). Previous notions that H1 acts globally to repress transcription (10, 11) and that H1 phosphorylation is involved in mitotic chromosome condensation (9) are in contrast to evidence that linker histones can affect transcription positively or negatively in gene-specific fashion in vivo (12, 13) and that linker histones are not required for mitotic chromosome condensation in vitro (14) or in vivo (15). Furthermore, recent observations suggest a role for H1 phosphorylation in transcriptional regulation (16, 17).

Tetrahymena thermophila is a model organism that offers several advantages for investigations of the function and metabolism of linker histones. Like other ciliated protozoa, vegetative Tetrahymena contain two nuclei, a transcriptionally inactive, germ-line micronucleus, which divides mitotically, and a transcriptionally active, somatic macronucleus, which divides mitotically (18). Macronuclear H1 shares the overall amino acid composition and acid solubility characteristics of eukaryotic H1, in general, but lacks the central globular domain found in H1 of metazoans (6, 19, 20). In contrast to the multiplicity of H1 genes found in most eukaryotes, the Tetrahymena haploid genome contains only one copy of the macronuclear H1 gene (20), facilitating methods of genetic transformation we have utilized previously to demonstrate that although macronuclear H1 is not required for viability, it is required for normal compaction of interphase chromatin (15) and can function as a positive or negative regulator of expression in a gene-specific fashion in vivo (12). Like H1 of multicellular eukaryotes, macronuclear H1 is a substrate for p34cdc2 in vitro, and we have presented evidence previously that suggests that this H1 is phosphorylated by a homolog of Cdc2 in vivo (21, 22). However, unlike many eukaryotes in which high levels of H1 phosphorylation occur mainly during mitosis, the majority of macronuclear H1 molecules are highly phosphorylated in unsynchronized, growing cultures of Tetrahymena (19, 21–23). Given the absence of mitotic chromosome condensation in the amitotically dividing macronucleus (18), these differences likely reflect the high percentage of the macronuclear genome that is transcribed (24). Thus, in Tetrahymena macronuclei, potential relationships between macronuclear H1 phosphorylation and chromatin transcription can be investigated apart from effects

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related to the hyperphosphorylation of linker histones at mitosis that occurs in other eukaryotes. In pursuit of this goal, we have mapped the major sites in macronuclear H1 that are phosphorylated in vivo in *T. thermophila* to enable a molecular genetic approach employing mutagenesis of sites to investigate the function of macronuclear H1 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Strains Employed—** *T. thermophila* strains CU427 (Mpr/Mpr [6 mp-s VII]) and CU428 (Chx/Chx-cy-S VII) were grown in 1% enriched proteose peptone (SPP) as described previously (25). The creation of strain Δ1–5 will be described in detail elsewhere. Briefly, site-directed mutagenesis was employed to convert the five phosphorylation sites to alanines in a construct containing the *HHO1* gene coding region as well as 5' and 3'-flanking sequences, with a single copy of the neomycin resistance cassette (26) containing the drug resistance marker neo inserted within the *HHO1* gene 3'-flanking sequence. Complete replacement of the wild type *HHO1* gene in polyplid macronuclei was obtained by homologous recombination following biolistic transformation and selection of transformants with paromomycin.

**Metabolic Labeling—** Cultures were labeled continuously during growth overnight (14–16 h) in SPP containing 5–25 μCi/ml [32P]orthophosphate (ICN Catalog No. 64041) and harvested at a density of 2.5–3.0 × 10^7 cells/ml.

**Preparation of Macronuclear H1—** Incubation of *Tetrahymena* at 4°C rapidly induces dephosphorylation of macronuclear H1. Accordingly, except where noted, cells were collected by centrifugation at room temperature (1000 × g for 5 min) prior to disruption and extraction at 4°C as described below. Macronuclei were isolated as described previously (27) using 0.1 M p-chloromercuriphenylsulfonic acid in the homogenization and wash buffers to inhibit phosphatases (28). Macronuclei were extracted with 0.2 M HSO_4, and crude macronuclear H1 was prepared by selective precipitation of other proteins with 5.4% (w/v) per centic acid (PCA). Crude macronuclear H1 was recovered from the PCA-soluble fraction by precipitation with trichloroacetic acid (29% w/v final) per centic acid (PCA). Crude macronuclear H1 was prepared by selective precipitation of other proteins with 5.4% (w/v) per centic acid (PCA). 2 C. A. Mizzen and C. D. Allis, unpublished observations.

**Phosphorylation Site Mapping—** [32P]-labelled macronuclear H1 (1–10 μg) was electrophoresed in polyacrylamide gels containing acetic acid and urea or SDS. Only the portions of the stained gels containing macronuclear H1 and corresponding autoradiograms are shown. Arrowheads denote the position of phosphoprotein macronuclear H1 in each gel system. *B*, [32P]-labeled macronuclear H1 was digested extensively with trypsin, and the resulting peptides were resolved on polyacrylamide gels at alkaline pH. Nine peptide species (labeled 1–9 on the left) were consistently observed (lanes marked digests). Aliquots of the eight peptide fractions recovered from bands excised from preparative gels were resolved in the same gel system to assess their purity prior to microsequencing (lanes 1–7 and 9). The recovery of peptide 8 was insufficient for subsequent analysis. Only the autoradiogram is shown.

**RESULTS AND DISCUSSION**

Macronuclear H1 prepared from *Tetrahymena* labeled with [32P]orthophosphate during vegetative growth was resolved into five distinct bands upon electrophoresis in polyacrylamide gels containing acetic acid and urea (AU-PAGE) and in gels containing SDS (SDS-PAGE) (Fig. 1A). Essentially all the electrophoretic heterogeneity associated with macronuclear H1 in this species appears to be due to phosphorylation because it is abolished by incubating extracts with alkaline phosphatase prior to electrophoresis (19, 21, 23, 32). Careful alignment of gels and corresponding autoradiograms established that the

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1. Y. Dou, C. A. Mizzen, C. D. Allis, and M. A. Gorovsky, manuscript in preparation.
2. C. A. Mizzen and C. D. Allis, unpublished observations.
3. The abbreviations used are: PCA, per centic acid; AU-PAGE, acetic acid-urea-polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high performance liquid chromatography.
fastest migrating species in each gel system represented non-
phosphorylated macronuclear H1 (arrowheads, Fig. 1A). The observation that the AU-PAGE mobility of H1 proteins is
versely related to the degree of phosphorylation (33) suggests that the most slowly migrating form in AU-PAGE represented tetraphosphorylated macronuclear H1.

Although 6 serine + threonine residues occur within the first 11 residues of this H1 (Fig. 3), phosphorylation was not detected between residues 1 and 12 when macronuclear H1 prepared from cultures grown overnight in the presence of \([^{32}P]\)orthophosphate was sequenced (data not shown). Due to the absence of potential sites between residues 12 and 30, we inferred that no phosphorylation occurs within the first 30 residues of this protein. We have shown previously that all phosphorylation in this species occurs in the amino-terminal fragment liberated upon CNBr cleavage of the methionine residue at position 692 (21, 23) (Fig. 3). Together, these data suggest that all sites of phosphorylation can be localized to the segment extending from Thr-31 to Thr-68. Eleven potential sites of phosphorylation, comprising 2 serine and 9 threonine residues, together with numerous lysine residues are found within this 37-residue segment.

To prepare phosphopeptides for microsequencing, \(^{32}\)P-labeled macronuclear H1 was digested extensively with trypsin, and the resulting peptides were resolved on polyacrylamide gels at alkaline pH (31). Following alignment of the autoradiograms and corresponding gels, bands of interest were excised from the dried gels and the phosphopeptides eluted by extracting excised gel pieces with water. Phosphopeptides were recovered from these eluates in salt-free form using RP-HPLC. This procedure enabled the recovery of eight phosphopeptides, purified to apparent homogeneity, representing all the major species and all but one of the minor species detected in the original digest (Fig. 1B). Single peaks of radioactivity were detected for all eight peptides when RP-HPLC eluates were monitored by liquid scintillation counting, suggesting that each band in the original gels was homogeneous (data not shown).

Each of these eight phosphopeptides was analyzed by microsequencing. Plots of the \(^{32}\)P cpm released (y axis) against the major amino acid released in each sequencing cycle (x axis) unequivocally identified 2 serine and 3 threonine residues as sites of phosphorylation (Fig. 2). Remarkably, the eight phosphopeptides represented only three peptide “families” in which either the same sequence was phosphorylated to different extents (e.g. phosphopeptides 1 and 7, phosphopeptides 3 and 6) or the same phosphorylation site was contained within both a limit trypptic peptide and a slightly longer peptide resulting from incomplete digestion (e.g. phosphopeptides 2 and 4, phosphopeptides 5 and 9, phosphopeptides 6 and 7). Three phosphorylation sites, Ser-42, Ser-44, and Thr-46, were identified during the sequencing of phosphopeptides 1, 3, 6, and 7. Three phosphorylation sites extending from Thr-31 to Thr-68. Eleven potential sites of phosphorylation, comprising 2 serine and 9 threonine residues, together with numerous lysine residues are found within this 37-residue segment.

Based on microsequencing of \(^{32}\)P-labeled tryptic peptides, phosphorylation at five sites accounts for all of the major species resolved on the phosphopeptide maps, suggesting that these five sites comprise the entire collection of sites utilized in vivo. To test this hypothesis, we analyzed macronuclear H1 phosphorylation in a Tetrahymena strain, \(\Delta1-5\), expressing macronuclear H1 in which alanine was substituted for Ser/Thr at the identified phosphorylation sites (Fig. 4A). A detailed description of the creation, molecular characterization, and phenotypic analysis of this strain will be given elsewhere.1

Initial analyses of macronuclear H1 from growing \(\Delta1-5\) cells on SDS gels revealed normal amounts of macronuclear H1 completely lacking the electrophoretic heterogeneity associated with macronuclear H1 from growing wild type cells, suggesting that phosphorylation of \(\Delta1-5\) macronuclear H1 did not occur in vivo or occurred at a limited number of sites in a manner that was not sufficient to retard migration of the protein in SDS gels. To determine whether \(\Delta1-5\) macronuclear H1 was phosphorylated to any extent in vivo, we labeled \(\Delta1-5\) and wild type cells in parallel with \([^{32}P]\)orthophosphate during growth overnight, prepared H1 from whole cell extracts, and analyzed it by autoradiography of SDS gels. Even though phosphorylation was readily detected in macronuclear H1 from wild type cells,
phosphorylation was not detected when an equivalent amount of macronuclear H1 from Δ1–5 cells was analyzed in parallel, even after lengthy autoradiographic exposures (Fig. 4B). Because macronuclear H1 from the wild type cells (but not that from Δ1–5 cells) was prepared using a method that enriched less phosphorylated forms, we conclude that macronuclear H1 is not detectably phosphorylated in growing Δ1–5 cells under the conditions employed here.

Taken together, these data suggest that the five sites identified by microsequencing represent the complete set of macronuclear H1 phosphorylation sites utilized by *T. thermophila*. Alternatively, a hierarchy of sites in macronuclear H1 may exist such that the absence of phosphorylation (or possibly the alanine substitution itself) at one or more of the sites mutated in Δ1–5 macronuclear H1 precludes phosphorylation at unidentified secondary sites. It should be noted that our analyses of phosphopeptides 1, 3, 6, and 7 indicate that, in wild type cells, phosphorylation occurs hierarchically, first at Thr-46, then at Ser-42, and last at Ser-44, *in vivo* (Fig. 2). Whether this hierarchy extends to other sites is not known at present. Regardless of whether we have identified all of the sites of phosphorylation, it is clear that eliminating the 5 sites identified here results in cells completely lacking detectable phosphorylation of macronuclear H1. Our findings that macronuclear H1 phosphorylation is not essential and that Δ1–5 strain cells are viable without marked alterations in growth, suggest either that macronuclear H1 phosphorylation does not have major, global effects on transcription or that mechanisms exist that are capable of compensating for the absence of macronuclear H1 phosphorylation in Δ1–5 cells. Further analyses of the Δ1–5 strain, in comparison with wild type *Tetrahymena* and to strains constructed to mimic constitutive phosphorylation or dephosphorylation of all or some of the five sites identified here, should define the function of macronuclear H1 phosphorylation *in vivo*.

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