# Unphosphorylated H1 Is Enriched in a Specific Region of the Promoter when *CDC2* Is Down-Regulated during Starvation[▽]

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*Tetrahymena thermophila* **macronuclear histone H1 is phosphorylated by a cdc2 kinase, and H1 phosphorylation regulates *CDC2* expression by a positive feedback mechanism. In starved wild-type cells, decreased expression of the *CDC2* gene is correlated with a global reduction in the phosphorylation of H1 and reduced phosphorylation of H1 in the region upstream of the *CDC2* gene. To determine whether the reduced H1 phosphorylation upstream of the *CDC2* gene is merely a reflection of global dephosphorylation or is due to specific targeting of dephosphorylation of H1 to the *CDC2* promoter during starvation, the *CDC2* promoter was mapped, and the distributions of phosphorylated and unphosphorylated H1 across the *CDC2* gene were determined using chromatin immunoprecipitation. Unphosphorylated H1 is specifically enriched in a region of the *CDC2* promoter when the gene's expression is reduced during starvation but not when *CDC2* is highly active in growing cells. The region of unphosphorylated H1 coincides with a region that is essential for *CDC2* expression. These studies are the first in vivo demonstration that the phosphorylation of H1 is being regulated at a fine level and that unphosphorylated H1 can be specifically targeted to a promoter, where it likely regulates transcription in a gene-specific manner.**

Linker histones (H1s) are associated with the linker DNA between nucleosome core particles in chromatin. They are less conserved than core histones and often exhibit multiple isotypes and cell type-specific variants (11). Metazoan H1s have three domains: a more conserved central globular domain and less structured N- and C-terminal tails (29). In the protozoan *Tetrahymena thermophila*, the transcriptionally active macronucleus contains a single H1 whose linker location and solubility properties are typical of other H1s. The amino acid composition of *Tetrahymena* macronuclear H1 is similar to that of the lysine-rich C-terminal domain of metazoan H1 (25, 30, 36), which controls the binding of H1 to chromatin in vivo (32, 56) and plays a major role in stabilizing chromatin secondary structure in vitro (42). However, as in some other protists, *Tetrahymena* H1 lacks a globular domain.

In vitro studies suggested that H1 is required for formation and stabilization of higher order chromatin structure (49, 60) and acts as a general repressor of transcription (21, 38, 46, 47). However, in vivo, H1 is not essential for chromosome compaction per se but is required for complete condensation of interphase and mitotic chromosomes (43, 53), and the in vivo function of linker histones on transcription is gene specific and can be either positive or negative. In *Xenopus laevis* embryos, increased amounts of an H1 variant repressed oocyte but not somatic 5S rRNA genes or other Pol III transcripts (7, 35). Also in *Xenopus*, somatic, but not maternal, H1 selectively silences the transcription of regulatory genes required for mesodermal differentiation (55). In *Tetrahymena*, knockout of the *HHO1* gene encoding ma-

cronuclear H1 had no detectable effect on the expression of most genes tested but induced the basal expression of the repressed *NgoA* gene. Surprisingly, H1 was required for the induced transcription of the *CyP1* gene (54). Overexpression of two H1 variants in mouse cultured cells increased both basal and induced expression from the mouse mammary tumor virus (MMTV) promoter (27). In yeast, disruption of the gene encoding H1 also resulted in decreased expression of specific genes (31). In *Neurospora crassa*, elimination of H1 expression resulted in specific derepression of the pyruvate decarboxylase gene (20). In mice, deletion of genes encoding specific H1 subtypes can attenuate or enhance genes subject to position effect (1). In mouse embryonic stem cells, when half of the total H1 was depleted by knocking out three H1 genes, only about 0.56% of over 4,500 genes examined showed significant differences in expression (19). Thus, the function of linker histones in vivo is usually not essential, and effects on gene expression can be positive or negative. The apparent exception to this, embryonic lethality (but not general cell-lethality) caused by reducing H1 in mice (18), could be explained by effects on the expression of specific genes critical to specific developmental stages.

In vivo studies of the effects of H1 on transcription have generally involved elimination or overexpression of H1. As a result, the specific cellular mechanism(s) by which H1 affects transcription in vivo is not known. Recent studies indicate H1 may regulate transcription by competing for nucleosome binding with site-specific factors or with more abundant, less specific high-mobility-group chromatin proteins (10), by interacting with chromosome remodeling complexes (33, 48), by effects on DNA methylation (19, 61), or by phosphorylation (4, 6, 8, 16, 34, 37, 58).

Numerous studies have suggested that global changes in H1 phosphorylation, like posttranslational modifications of core histones, can be regulated to modify histone–chromatin inter-

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actions and are correlated with changes of transcription in vivo (reviewed in reference 63). Phosphorylation of H1 also has been implicated in chromatin decondensation that occurs at sites where DNA is being replicated (2) and in reduced binding of Hp1α and disassembly of higher order chromatin structures during interphase in mammalian cells (12, 28). We have been studying H1 and its phosphorylation in the ciliated protozoan *Tetrahymena thermophila*. In *Tetrahymena*, macronuclear H1 was previously found by Edman sequencing to be phosphorylated at three major, canonical cdc2 sites and, in a hierarchical fashion, at two minor, non-cdc2 sites whose phosphorylation depends on prior phosphorylation of the three cdc2 sites. Mutation of these five sites in combination abolished all detectable $^{32}$P signal from radiolabeled H1 (44), suggesting that they represented all of the phosphorylation sites on *Tetrahymena* H1 (44). However, recent mass spectroscopic analyses identified two additional minor, non-cdc2 phosphorylation sites whose phosphorylation requires prior phosphorylation on at least one of the three cdc2 sites. Thus, elimination of the five sites that were originally described did indeed completely eliminate phosphorylation of *Tetrahymena* H1 (22).

*Tetrahymena* H1 phosphorylation levels change dramatically in response to various physiological conditions. H1 is highly phosphorylated in growing cells (25) and is hyperphosphorylated upon heat shock (23) and during prezygotic stages of conjugation (50). H1 phosphorylation decreases in stationary phase and decreases still further during prolonged starvation (22, 50), and H1 is rapidly and almost completely dephosphorylated during late conjugation when the parental macronuclei cease transcription and become highly condensed prior to their elimination from the cell (40). These changes in H1 phosphorylation in physiological and developmental conditions that alter gene expression patterns suggest that H1 phosphorylation functions in transcription in *Tetrahymena*. Indeed, phosphorylated and dephosphorylated macronuclear H1 are enriched in transcriptionally active and inactive subdomains of macronuclear chromatin, respectively (41).

In vitro mutagenesis coupled with gene replacement studies showed that global phosphorylation of *Tetrahymena* macronuclear H1 regulates transcription of specific genes both positively and negatively in vivo and that phosphorylation of H1 mimicked the effects of H1 knockout (16, 54). More detailed mutagenic analyses indicated that phosphorylation of H1 creates a negative-charge patch (15) and causes a small increase in the rapid rate of association-dissociation of H1 on chromatin (14).

Genes whose expression is affected by H1 phosphorylation were cloned by suppression-subtractive hybridization based on their differential expression in starved A5 cells (in which the five phosphorylation sites identified by Edman sequencing were mutated to alanines, eliminating all phosphorylation) and starved E5 cells (in which these five phosphorylation sites were mutated to glutamic acid to mimic hyperphosphorylation [17]). A number of *Tetrahymena* genes were shown to be regulated by the change in H1 phosphorylation levels that occurs during starvation, including the *CDC2* gene encoding a cdc2 kinase, which was expressed more strongly in starved E5 cells than in A5 cells. In wild-type (WT) growing cells, when H1 is highly phosphorylated, the *CDC2* gene is highly expressed. During starvation of WT cells, when H1 is extensively but incompletely dephosphorylated, the *CDC2* gene is weakly expressed, but if the E5 mutant H1 is overexpressed, endogenous H1 becomes hyperphosphorylated and *CDC2* expression increases in starved cells. When H1 dephosphorylation during starvation was inhibited by okadaic acid (OA), *CDC2* expression also increased. These studies argue that there is a positive feedback between *CDC2* expression and phosphorylation of H1. Chromatin immunoprecipitation (ChIP) demonstrated that phosphorylation of H1 upstream of the *CDC2* gene was decreased in starved cells (17). These studies indicated that the phosphorylation level of H1 associated with the *CDC2* upstream region decreased in starved cells but did not determine whether this change was simply a reflection of the global dephosphorylation of H1 in the entire macronucleus, was due to specific dephosphorylation of H1 or binding of unphosphorylated H1, or reflected loss of H1 from the promoter. To distinguish among these possibilities, here we performed more extensive ChIP analyses, coupled with promoter mapping, demonstrating that unphosphorylated H1 is specifically enriched in a restricted region of the *CDC2* promoter in starved but not in growing cells. We also show that this region is essential for *CDC2* expression. These results argue that unphosphorylated H1 can be localized to a specific promoter region where it likely regulates transcription in a gene-specific manner. Coupled with studies demonstrating that Cdc45 can target Cdk2 and H1 phosphorylation to replication foci in mammalian cells (2), these studies indicate that, like posttranslational modifications of core histones, targeted regulation of the H1 phosphorylation state is a general mechanism for regulating chromatin function.

## MATERIALS AND METHODS

**Strains and culture conditions.** WT strains CU428 and B2086 were provided by P. J. Bruns (Cornell University). A5 and E5 strains were created by Y. Dou (16). Macronuclear H1 knockout strain ΔH1 was created by X. Shen (53, 54). Cells were grown in super proteose peptone (SPP) medium containing 1% proteose peptone (1× SPP) at 30°C (26) with vigorous shaking. Cell number was determined using a Coulter counter (Coulter Electronics, Inc.). For starvation, cells were washed and resuspended in 10 mM Tris (pH 7.5) at 30°C without shaking for 24 h.

**Genomic walking.** Genomic walking libraries with different restriction fragments ligated to an adaptor at both ends (13) were provided by K. Mochizuki and were used as templates for nested PCR of genomic fragments using two primers in the adaptor and two *CDC2* gene primers obtained from the cDNA sequence. The PCR products were cloned and sequenced.

**5′ RACE and 3′ RACE.** cDNA synthesized from log-phase total RNA by NotI-adaptor and oligo(dT) primers through "template switched" reverse transcription (62) by SuperScript II RNaseH⁻ RTase (Life Technologies) was provided by C. Tsao. NotI-adaptor primer and two *CDC2* primers were used for nested PCR of this cDNA. PCR products were cloned and sequenced, revealing at least four clustered transcription start sites. The most frequent start site, found in 7 of 11 clones, occurred 52 bp upstream, another two clones started 56 bp upstream, and the remaining two started at 36 and 44 bp upstream of the initiator ATG. Short 5′-untranslated regions (UTRs) and multiple start sites are typical of *Tetrahymena* genes. A similar strategy, using 3′ rapid amplification of cDNA ends (RACE), identified the 3′ UTR at a single site 226 bases downstream from the TGA.

**Gene replacement.** To analyze the function of the promoter in its endogenous location, upstream deletions of the *CDC2* 5′-flanking region were used to drive *GFP* expression. The Cd$^{2+}$-inducible *neo3* drug selection cassette (52) was inserted downstream of the 3′ UTR to enable selection of cells containing the transformed genes. Transformation of *Tetrahymena* macronuclei occurs by homologous integration. The macronucleus is polycopy (~45 ploid) and divides amitotically: chromosomes assort randomly without segregation of sister chromatids. As a result, initial replacement of 1 of the 45 copies of the *CDC2* gene by the transforming *GFP* can be selected at increasing concentrations of paramomycin sulfate (pm; Sigma) to partially replace the endogenous *CDC2* gene while cells remain viable, even if the gene has an essential phenotype.
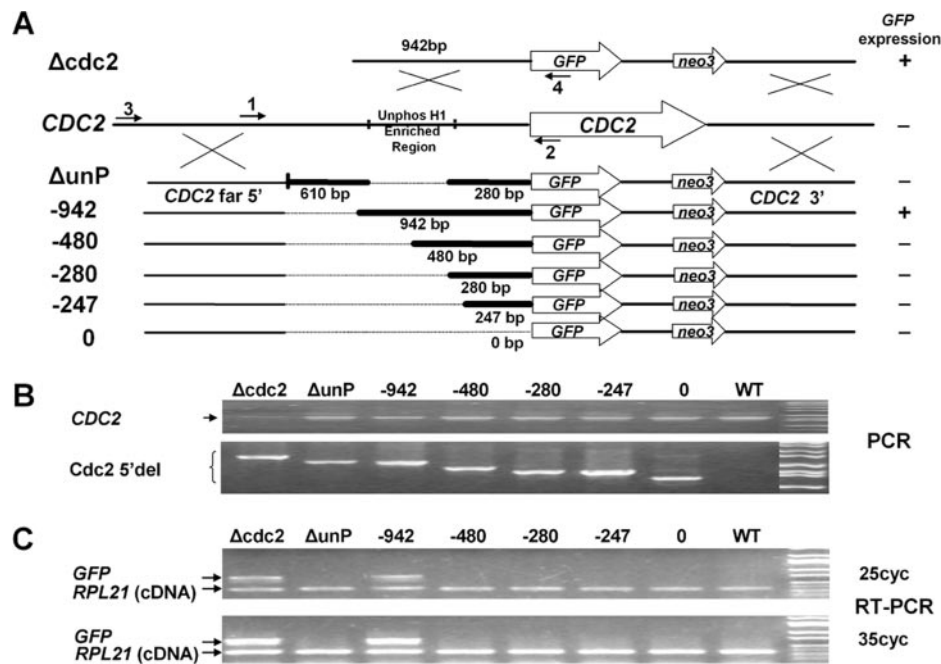
FIG. 1. Mapping the *CDC2* promoter at the *CDC2* locus using *GFP* fusions. A. Serial deletion constructs were made using *GFP* as a reporter and a *neo3* selectable marker inserted downstream of the 3′ UTR. Homologous recombination occurs in the 5′ region flanking the deletion (*CDC2* far 5′) and the distal 3′-flanking region of the *CDC2* gene to insert the reporter constructs into the endogenous *CDC2* gene during somatic transformations. The *CDC2* knockout construct, Δcdc2, lacking any 5′ deletion is shown at the top of the diagram. Below the endogenous *CDC2* locus are the deletion constructs, with the heavy lines indicating the bases immediately 5′ of the initial ATG that are retained in the constructs. The numbered arrows indicate the primers used in panels B and C. The *GFP* expression status determined by RT-PCR shown in panel C is listed to the right of the deletion constructs. B. Genomic PCR using primers 1 and 2 showed the presence of the endogenous *CDC2* gene (*CDC2*), and genomic PCR using primers 3 and 4 demonstrated that the various deletion constructs (Cdc2 5′del) localized to the endogenous *CDC2* locus in the transformed cells. Shown are the ethidium bromide (EB)-stained PCR products separated on agarose gels. C. *GFP* expression was analyzed by RT-PCR using total RNA extracted from the above-mentioned transformants in log phase. Shown are the EB-stained RT-PCR products from 25 or 35 cycles of PCRs separated on agarose gels. The two primers for the ribosomal protein *RPL21* gene span an intron, so true RT-PCR products are distinguishable from contaminating genomic DNA. Unphos H1, unphosphorylated H1.

To create the deletions, the *Tetrahymena* macronuclear genome sequence (http://www.tigr.org/tdb/e2k1/ttg/index.shtml) was used to construct primers to obtain the 5′ upstream regions of the deletions (*CDC2* far 5′) and the distal 3′ downstream region, enabling homologous recombination with the endogenous *CDC2* gene during somatic transformations (Fig. 1). *CDC2* knockout and promoter-mapping constructs were digested with KpnI and SacI and transformed into 15- to 17-h conjugating CU428 and B2086 cells using the Biolistic PDS-1000/He particle delivery system (Bio-Rad) as described previously (9). Transformants were selected initially at 70 μg/ml pm and 1 μg/ml Cd$^{2+}$ and were serially transferred every 2 to 3 days to fresh media containing 0.5 μg/ml Cd$^{2+}$ with increasing concentrations of pm.

**RT-PCR.** Total RNA (3 μg) isolated with Trizol (Life Technologies), oligo-(dT) primer, and Stratascript RTase (Stratagene) were used for reverse transcription (RT) according to the manufacturer's instructions. The cDNA was amplified by gene-specific primers GFP-N and GFP-C, together with L21 FW and L21 RV. The L21 primers span an intron, and the PCR product is 850 bp from genomic DNA and 360 bp from cDNA.

**H1 extraction and gel electrophoresis.** H1 was extracted from whole cells using 5% perchloric acid (PCA [25]). PCA-soluble proteins were analyzed on both sodium dodecyl sulfate (SDS)–12% polyacrylamide and 15% acid-urea polyacrylamide gels (23). To dephosphorylate H1, 25 μg was treated with λ-protein phosphatase (10 U/μl for 5 h at 30°C; New England Biolabs, Inc.) and precipitated with 20% trichloroacetic acid.

**Purification of anti-unphosphorylated H1 antibody.** Anti-unphosphorylated H1 was affinity purified by a modification of the method of Olmsted (45). About 40 μg of highly phosphorylated H1 extracted from heat-shocked (40°C for 10 min) CU428 cells was separated by SDS–12% polyacrylamide gel electrophoresis (PAGE). The gel was electroblotted to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) with transfer buffer (48 mM Tris, 39 mM glycine, 18% methanol, 0.1% SDS) at 15 V for 1.5 h. The portion of the membrane containing phosphorylated H1 was cut into small pieces, blocked with 5% nonfat dry milk in TBST (100 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.5) for 1 h, and washed with Buffer 1 as described previously (45). The blot fragments were then mixed with 10 μl unphosphorylated H1 antiserum (originally called dephosphorylated H1 antiserum in reference 41) and 490 μl 1% bovine serum albumin in TBST and incubated at 4°C overnight with gentle shaking, and the supernatant was saved. Adsorption was repeated two more times by treating the blot fragments with 0.2 M glycine–HCl (pH 2.8) to remove the antibodies and using them to retreat the supernatant. The purified anti-unphosphorylated H1 antibody was assayed by Western blotting and used for ChIP.

**Chromatin immunoprecipitation.** ChIP was performed as described previously (17). Primer pairs were designed to amplify overlapping fragments with similar GC contents of different sizes along the *CDC2* gene 5′-flanking and coding regions to enable multiplex PCR.

**Isolation of macronuclei and micrococcal nuclease digestion.** Macronuclei were isolated as described previously (26), washed three times with RSB buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride), and resuspended with 600 μl RSB buffer with 0.1 mM CaCl₂ at $5 \times 10^7$ macronuclei/ml. Micrococcal nuclease (MNase; Worthington) was added to 50 U/ml, and the nuclei were digested for 0, 1, 2, 3, 4, and 5 min at 37°C. Digestion was stopped by addition of 3.5 volumes of stop buffer (1% SDS, 0.5 M EDTA, 18 mM Tris, pH 9.5; prewarmed to 65°C), followed by incubation at 65°C for at least 20 min. Proteinase K was then added to 1 mg/ml, and incubation continued at 55°C for 4 h. An equal volume of water was added to the digestion, and the DNA was extracted with phenol-chloroform, precipitated with ethanol in the presence of 0.3 M sodium acetate, and resuspended in double-distilled water. As a control, an equal amount of genomic DNA was resuspended in RSB and treated with 5 U/ml MNase for 0, 0.5, 1, and 2 min at 37°C, and DNA was reisolated as described above for macronuclei. The purified MNase-digested DNA was then digested with TaqI, and digests were separated on a 1.2% agarose

gel, blotted, and hybridized (3). Probe DNA was PCR products amplified from *Tetrahymena* genomic DNA by primer sets CDC2 840 up and CDC2 1105 down, labeled with [$\alpha$-$^{32}$P]dATP by random priming.

**Primers used in this study.** The *CDC2* primers used for nested PCR and sequencing in genomic walking were the following: CDK1, 5′-AGATTTCTCC GATTGCGGTAGAGGG-3′; CDK5, 5′-CGCTCAAGCTTGCTATTTTTCAT AACC-3′; CDK10, 5′-TGAATAAGCTCGAAGGTAGC-3′. The adaptor primers were the following: L1, 5′-GTTCATCTTTACAAGCTAGCG-3′; L2, 5′-TC CTGAACAATGCTGTGGAC-3′. The NotI-adaptor primer used in 5′ RACE was 5′-GAGGCGGCCGCAATGAACACTGCGTTTGCGGG-3′. The *CDC2* primers used in 5′ RACE were CDK1 and CDK5 (see above). Gene-specific primers used in RT-PCR were the following: GFP-N, 5′-ATGAGTAAAGGA GAAGAACTTTTC-3′; GFP-C, 5′-TTATTTGTATAGTTCATCCATGCC-3′; L21 FW, 5′-AAGTTGGTTATCAACTGTTGCGTT-3′; L21 RV, 5′-CCCAGA AAGTTCCTGCTGCAT-3′. Primers used to amplify the different fragments in ChIP were the following: 181-bp fragment, CDK 65 down (5′-ATAAATACTT ATCAAAAAGCAAATG-3′) and CDK 245 up (5′-TACCTCCTAATTTTAGT TTATCTAC-3′); 310-bp fragment, CDK 243 down (5′-GTAAATGAAGAAC ATTAAGAATGC-3′) and CDK 552 up (5′-ATATACTTGGATAACAAAAA GAGG-3′); 222-bp fragment, CDK 524 down (5′-TATAGCCTCTTTTTGTTAT CCAAG-3′) and CDK 745 up (5′-TAAAAATTTCCTATCATTCTAGTAC-3′); 199-bp fragment, CDK 727 E down (5′-AGTCCGGAATTCGAATGATAG GAAATT TTTACTAAGC-3′) and CDK 913 up (5′-TTGCTTAAAGGTACTTT AACCTTC-3′); 242-bp fragment, CDK 901 down (5′-TACCTTTAAGCAATTA AGAAAGTTG-3′) and CDK 1142 up (5′-AAATATATTTGATTAAAAACCA ACC-3′); 260-bp fragment, CDK 1130 down (5′-ATCAAATATATTTATTAG TAACCAC-3′) and CDK 8 (5′-TTGATAATCAATAAAGAGAGACAC-3′); 308-bp fragment, CDK 1389 down (5′-AATAGTTCAAGAAGTTCTATTAGT C-3′) and CDK 5 (5′-CGCTCAAGCTTGCTATTTTTCATAACC-3′); 343-bp fragment, CDC 1646 coding (5′-AATAAATAATCTGACAGTAAAAATGG-3′) and CDK 1 (5′-AGATTTCTCCGATTGCGGTAGAGGG-3′); 472-bp fragment, CDC 1989 coding (5′-TCTCTCTTAAAGGAGTTATAACATC-3′) and CDC 2225 coding (5′-TTGAGGCTTCAAATCTCTATGAGG-3′); 539-bp fragment, CDK 1130 down (5′-ATCAAATATATTTATTAGTAACCAC-3′) and CDK 5′f EcoRI (5′-AGTCCGGAATTCTTTTACTGTCAGATTATTTAT TTGC 3′); *GTU1* 280-bp fragment, GTU C13AL (5′-CTTGGCCGACTTAAA GTGTAATGATAT CTCTAGGC-3′) and GTU 521 (5′-AATGTGAGGAGTG AGTGAG-3′). Primers used to amplify the DNA fragment used for the probe template in MNase mapping were CDC2 840 down (5′-CGATCAGAAAGCA AATTAAAAATATA-3′) and CDC2 1105 up (5′-TGAAACACTCAAAAAAG CCTCTC-3′).

**Nucleotide sequence accession number.** The GenBank accession number for *CDC2* is AY706655.



FIG. 2. Specificity and purification of anti-H1 antibodies. A. Macronuclei were isolated as described previously (26) from heat-shocked (40°C for 1 h) cells. A total of $3 \times 10^6$ macronuclei/lane were subjected to SDS–12% PAGE, transferred to PVDF membranes, and probed with antisera that were produced against phosphorylated H1 (phos-H1), unphosphorylated H1 (unphos-H1), or H2A. Blots were stripped and reprobed with anti-H2B, -H3, or -H4 antisera. B, C, and D. Histone H1 was extracted with 5% PCA from WT cells in log phase (log) or cells heat shocked at 40°C for 10 min (hs). Aliquots of H1 were treated with $\lambda$-protein phosphatase (+P). H1 samples were subjected to SDS-PAGE, transferred to a PVDF membrane, and then probed with antisera produced against phosphorylated or unphosphorylated H1 peptides. The blot was stripped and reprobed for each successive analysis. Phosphorylated H1 and unphosphorylated H1 isoforms are labeled. B. Phosphatase treatment abolishes the signal recognized by anti-phosphorylated H1 antiserum. C. Unpurified anti-unphosphorylated H1 antiserum recognizes both phosphorylated and unphosphorylated H1s. D. Anti-unphosphorylated H1 antibodies purified by immunoadsorption demonstrate high specificity for unphosphorylated H1.

## RESULTS

**Mapping the promoter of the *CDC2* gene using *GFP* fusions.** Prior to analyzing H1 phosphorylation at the *CDC2* gene, we characterized the *CDC2* mRNA and mapped the promoter at its endogenous locus using *GFP* fusions. A total of 1.6 kb of *CDC2* 5′-flanking sequence was cloned by genomic walking, and the transcription start sites and 3′ UTR were determined by 5′ and 3′ RACE. A series of upstream deletions of the *CDC2* 5′ region attached to the *GFP* coding region were introduced into WT cells (Fig. 1A). Since we showed previously that *CDC2* expression is linked to H1 phosphorylation (17), we reasoned that some endogenous cdc2p would be necessary for H1-regulated expression of *GFP* in the *CDC2* locus, so the promoter mapping was done in transformants whose endogenous *CDC2* gene was only partially replaced (Fig. 1B, upper panel). The introduced genes containing the 5′ deletions and the *GFP* reporter were easily detected by PCR of genomic DNA in these transformants (Fig. 1B, lower panel), and RT-PCR was done to detect *GFP* mRNA (Fig. 1C). Note that we have restricted our analyses to log-phase cells where the *CDC2* gene is normally highly expressed and have used a sensitive RT-PCR assay to determine whether any mRNA was detectable rather than measuring the precise amount of *GFP* mRNA.
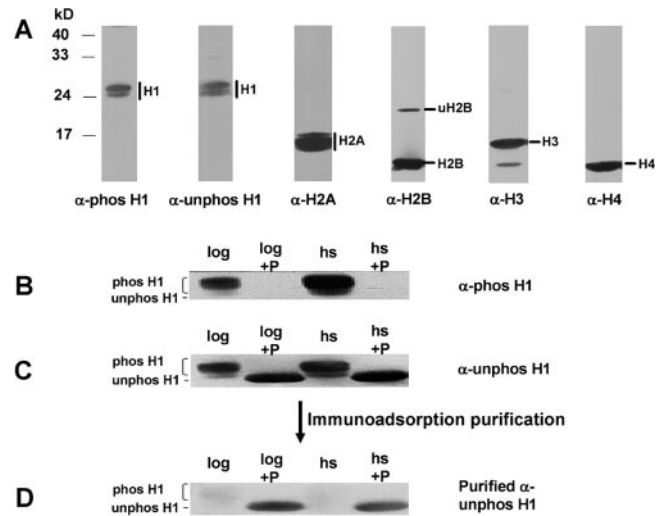
Note also that mRNA from ribosomal gene *RPL21* serves as a positive control for each PCR. A strain with an intact promoter in which most copies of the *CDC2* coding regions have been replaced with *GFP* ($\Delta$cdc2) is the positive control for the *GFP* message. A region required to drive *GFP* expression in log-phase cells was mapped to between $-480$ and $-942$ bp upstream from the initial ATG (about 430 to 890 bp upstream of transcription start sites). A region ($-280$ to $-942$) that was found to be enriched in unphosphorylated H1 in starved cells (see below) was also shown to be required for *GFP* expression (Fig. 1).

**Specificity of the anti-phosphorylated and anti-unphosphorylated H1 antibodies.** We showed previously that a 539-bp region upstream of the *CDC2* coding sequence was enriched when immunoprecipitated by anti-phosphorylated H1 antibody in log-phase cells when *CDC2* is highly expressed but not in starved cells when *CDC2* is weakly expressed (17). There are three possible explanations for this finding: (i) H1 associated with this region could simply mirror the behavior of global H1, which is highly phosphorylated in growing cells and largely dephosphorylated in starved cells (25, 50); (ii) unphosphorylated H1 is specifically localized in this region or becomes inaccessible to the antibody (possibly due to interference from other proteins) in starved cells; and (iii) H1 is present in this
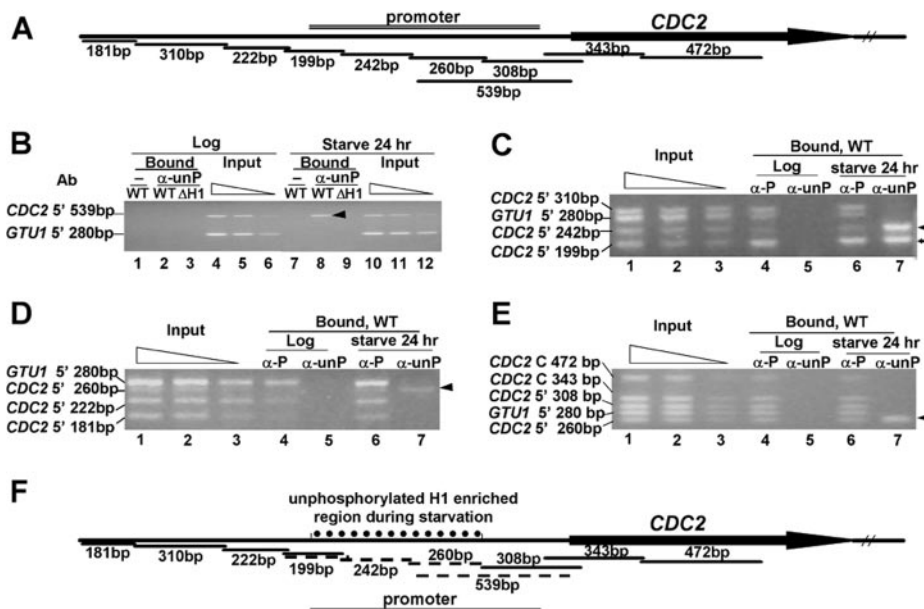
FIG. 3. Unphosphorylated H1 is specifically localized to a restricted region of the *CDC2* promoter during starvation when *CDC2* is weakly expressed. A. Diagram of the *CDC2* PCR fragments analyzed in the ChIP assay. Primer sets that amplify adjacent fragments differing in length by 19 to 130 bp were designed across the 5′ and coding regions of the *CDC2* gene. The 539-bp fragment overlaps two fragments (260 bp and 308 bp) that were also analyzed. Different fragments were amplified in multiplex PCR of input or immunoprecipitated (Bound) DNA together with a 280-bp control fragment immediately upstream of the *GTU1* coding region. *GTU1* gene expression is not affected by H1 phosphorylation. B. Chromatin in the *CDC2* promoter is enriched in unphosphorylated H1 in starved cells. ChIP was done on log-phase or 24-h-starved WT CU428 cells or H1 knockout cells (ΔH1) using purified anti-unphosphorylated H1 antibody (α-unP) or no antibody (−). Input DNA and immunoprecipitated DNA were purified and used in multiplex PCR to amplify a 539-bp fragment immediately upstream of the *CDC2* coding region and the *GTU1* 280-bp fragment. The input sample was diluted in a 2.5-fold series to serve as a quantitation control. Shown are the PCR products from the bound or input DNA analyzed on a 3% agarose gel and stained with ethidium bromide. The arrowhead shows the *CDC2* 539-bp fragment that was enriched by immunoprecipitation with purified anti-unphosphorylated H1 antibody only in starved-cell chromatin. No PCR fragments were detectable in the absence of antibody or in chromatin from cells lacking H1. C, D, and E. Mapping the region enriched in unphosphorylated H1 in starved cells. ChIP was done on log-phase or starved cells, and the immunoprecipitated DNA was analyzed as described above. The input results are for the starved cells, where the critical observations have been obtained. The inputs from the growing cells are indistinguishable from those of starved cells (data not shown). Arrowheads indicate the fragments that were enriched by purified anti-unphosphorylated H1 antibody in starved cells. The arrow (←) in panel C points to a fragment that was enriched by immunoprecipitation with both anti-phosphorylated H1 (α-P) and purified anti-unphosphorylated H1 (α-unP) antibodies. F. Summary of the ChIP results. Fragments reproducibly enriched by immunoprecipitation with anti-unphosphorylated H1 antibody in 24-h-starved cells are indicated by dashed lines. Fragments that were not enriched were labeled by solid lines. The left boundary of the region containing unphosphorylated H1 resides within the 199-bp fragment, since it was enriched by both anti-phosphorylated and anti-unphosphorylated H1 antibodies. The 539-bp fragment contains the right-hand boundary, indicated by the fact that it can be split into one (260-bp) fragment that is enriched in unphosphorylated H1 and another (308-bp) fragment that is not.

region in growing cells but absent in starved cells. To distinguish among these possibilities, we wished to perform ChIP analyses with an antibody that preferentially reacted with unphosphorylated H1, in addition to the antibody against phosphorylated H1 used previously. These antibodies (kindly provided by C. David Allis) were raised against phosphorylated or unphosphorylated H1 peptides (41).

We checked the specificity of the antibodies by Western blots using both SDS-PAGE (Fig. 2) and acid-urea-PAGE (data not shown), which showed that both antisera recognized histone H1 but not the core histones (Fig. 2A). Anti-phosphorylated H1 antiserum was highly specific for phosphorylated H1 (Fig. 2B), and its reactivity was not detectable if H1 was treated with phosphatase (Fig. 2B). However, we found that this antibody could not distinguish between phosphorylated H1 isoforms differing in the number of phosphates they contained (X. Song and M. A. Gorovsky, unpublished observations). The anti-unphosphorylated H1 antiserum recognized both phosphorylated and unphosphorylated H1s

(Fig. 2C). We purified this serum (see Materials and Methods) by adsorption to phosphorylated H1 immobilized on a PVDF membrane. The antibodies remaining in the supernatant were again tested using Western blotting and now showed strong preference for unphosphorylated H1 (Fig. 2D). We used these purified anti-unphosphorylated H1 antibodies as well as the anti-phosphorylated antibodies in the subsequent ChIP assays.

**Unphosphorylated H1 is enriched in the *CDC2* promoter in starved cells.** Our previous studies had shown that the 539-bp region upstream of the *CDC2* gene was enriched when chromatin from log-phase cells was immunoprecipitated with anti-phosphorylated H1 but not when chromatin was immunoprecipitated from starved cells (17). In contrast, using anti-unphosphorylated H1 antibody (Fig. 3B, lane 8, arrowhead), this 539-bp PCR fragment within the *CDC2* promoter (Fig. 3A) was greatly enriched in DNA immunoprecipitated from 24-h-starved WT cells but was not detected in chromatin immunoprecipitated from log-phase cells (Fig. 3B, lane 2).

This result demonstrates that H1 is still associated with the *CDC2* promoter in starved cells when the gene is relatively inactive, but it is unphosphorylated. No PCR product was detected with DNA isolated from chromatin immunoprecipitated with anti-unphosphorylated H1 antibody from cells lacking H1 (Fig. 3B, lanes 3 and 9) (54) or in the absence of antibody (Fig. 3B, lanes 1 and 7), further demonstrating the specificity of the antibody. The 539-bp *CDC2* fragment and a 280-bp fragment upstream of the control *GTU1* coding region were amplified to similar extents and in proportion to the amount of the template when DNA isolated from the input chromatin prior to immunoprecipitation was used (Fig. 3B, lanes 4, 5, and 6 as well as 10, 11, and 12), validating the conditions used for the PCR.

The 280-bp *GTU1* fragment was not detectable in DNA precipitated by anti-unphosphorylated H1 from either log-phase (Fig. 3B, lane 2) or starved cells (Fig. 3B, lane 8). However, it could be immunoprecipitated from both growing and starved cells using the anti-phosphorylated H1 antibody (17). Thus, the upstream region of the *GTU1* gene probably contains little or no unphosphorylated H1 in either physiological state. In contrast, the finding that the 539-bp *CDC2* fragment is specifically immunoprecipitated with the anti-unphosphorylated H1 only in starved cells indicates that some or all of this region is more enriched in unphosphorylated H1 than the *GTU1* 5′-flanking region.

**Unphosphorylated H1 is specifically enriched in a restricted region of the *CDC2* promoter during starvation when *CDC2* is down-regulated.** The experiments described above do not allow a determination of whether the presence of unphosphorylated H1 is specific to the promoter of *CDC2* or the absence of unphosphorylated H1 is specific to the *GTU1* 5′-flanking region. To distinguish between these alternatives, we mapped the location of unphosphorylated H1 along the 5′-flanking region as well as the coding region of the *CDC2* gene using primer sets that amplify overlapping DNA fragments (Fig. 3A). In each experiment, multiplex PCR was used to amplify three to four different *CDC2* fragments together with the control *GTU1* 5′ fragment (Fig. 3C, D, and E).

As seen in one example of the multiplex PCR, three *CDC2* 5′-flanking fragments as well as the *GTU1* 5′-flanking fragment were similarly amplified in input DNA, and their products were proportional to the amount of template used (Fig. 3C, lanes 1, 2, and 3). Thus, relative changes in the intensities of these bands in bound DNA precipitated with anti-phosphorylated or anti-unphosphorylated H1 antibodies compared to input DNA indicate enrichment or depletion of phosphorylated or unphosphorylated H1 in all or part of the affected fragment(s). ChIP results from 24-h-starved cells show that the *CDC2* 5′ 242-bp fragment was greatly enriched by immunoprecipitation with anti-unphosphorylated H1 (Fig. 3C, lane 7, arrowhead), while it was less efficiently amplified after immunoprecipitation with anti-phosphorylated H1 (Fig. 3C, lane 6). The *CDC2* 5′ 199-bp fragment is enriched with both anti-unphosphorylated H1 (Fig. 3C, lane 7, arrow) and anti-phosphorylated H1 (Fig. 3C, lane 6) in 24-h-starved cells. Because these ChIP experiments determine whether any part of a chromatin fragment has relatively more or less affinity for the antibodies than fragments to which it is being compared, this result suggests that the 199-bp fragment contains both a region

where H1 is phosphorylated and one that is unphosphorylated. Similar ChIP experiments were done on other fragments (Fig. 3D and E).

All of the ChIP results are summarized in Fig. 3F. These studies indicate that unphosphorylated H1 is specifically enriched in a region of the *CDC2* promoter during starvation when *CDC2* expression is reduced but not in growing cells when *CDC2* is highly active. The 5′ boundary of the region preferentially associated with unphosphorylated H1 resides within the 199-bp fragment, since it was enriched by both anti-phosphorylated and anti-unphosphorylated H1 (Fig. 3C). Fragments immediately upstream of it are enriched by anti-phosphorylated H1, and those immediately downstream of it are enriched by anti-unphosphorylated H1. Enrichment of the 539-bp fragment (Fig. 3B) can be explained by the fact that it contains the right-hand boundary of the region containing unphosphorylated H1, as indicated by the observations that this fragment can be split into a 260-bp fragment that is enriched in chromatin immunoprecipitated by anti-unphosphorylated H1 (Fig. 3E) and a 308-bp overlapping fragment that is not. Thus, the region enriched in unphosphorylated H1 in starved cells is restricted to a domain of 500 to 600 bp in the *CDC2* promoter (dotted line in Fig. 3F) and does not include either the downstream coding region or regions further upstream of the *CDC2* gene. Importantly, this region is upstream of the transcription start sites that occur 36 to 52 bp upstream of the initial ATG (see Materials and Methods).

After we obtained the results localizing the region preferentially associated with unphosphorylated H1, we analyzed expression of a *GFP* fusion lacking this region (Fig. 1, ΔunP) and found it is required for *CDC2* promoter function. Thus, in starved cells, a highly specific region containing unphosphorylated H1 is localized to nontranscribed sequences upstream of the *CDC2* gene that are required for promoter activity.

**Enrichment of unphosphorylated H1 occurs without major alteration of chromatin structure.** To examine whether the chromatin structure in the *CDC2* promoter region enriched in unphosphorylated H1 differs between log-phase and starved cells, we mapped that region using MNase digestion (Fig. 4). No MNase-hypersensitive sites were detected in this region. MNase digestion of chromatin in *Tetrahymena* macronuclei has been reported to produce fuzzy bands with a spacing of ~200 bp, probably owing to the extreme AT richness of *Tetrahymena* DNA and the high level of transcription in macronuclei (24). In both growing and starved cells, a series of fuzzy bands is observed. Except for the spacing between bands 4 and 5 and bands 5 and 6, these spacings are compatible with the presence of nonrandomly positioned nucleosomes. The region between bands 1 and 2 could accommodate two tightly packed nucleosomes. No reproducible differences in the spacing of these MNase-sensitive sites were detected between growing and starved cells (Fig. 4B, region indicated by a dotted line). Thus, the differences in chromatin structure associated with the localization of unphosphorylated H1, if any, are slight.

## DISCUSSION

Links between gene expression and targeted posttranslational modifications of core histones have been extensively
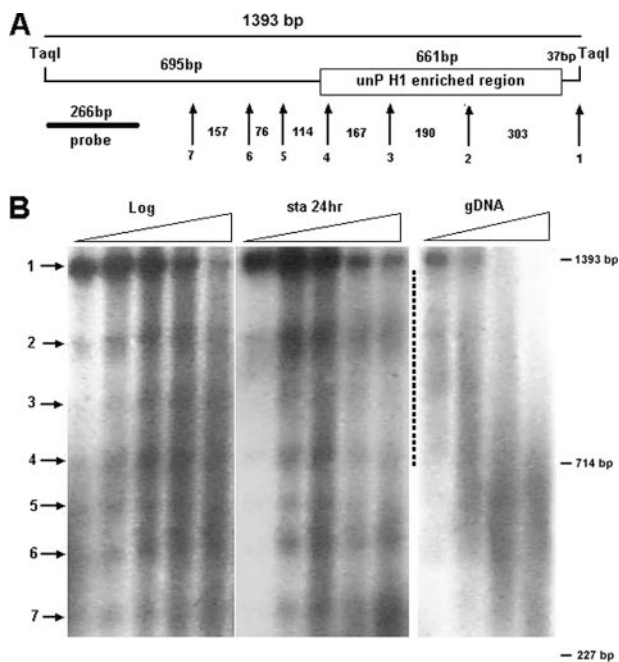
FIG. 4. Localization of unphosphorylated H1 occurs without major alteration of chromatin structure. Macronuclei from log-phase and 24-h-starved WT CU428 cells as well as genomic DNA from CU428 cells were digested with MNase, followed by TaqI digestion, and analyzed by Southern blotting. A. A diagram of the mapped region. The probe used in panel B is indicated. The numbered arrows correspond to the bands shown in panel B. The numbers in between the arrows are the spacing (in base pairs) between the bands. B. Southern blot showing DNA isolated from macronuclei from log-phase and starved cells as well as genomic DNA. All samples were analyzed on the same gel from which the last, overdigested lane of each sample set and empty lanes separating the different sets of samples were removed to facilitate visual comparisons. The dotted line indicates the position of the part of the blot corresponding to the region enriched in unphosphorylated H1.

documented (for recent global analyses of acetylation and methylation and for references, see references 5 and 51). There is less information concerning the relationship between H1 phosphorylation and transcription. In vitro studies have shown that H1 facilitates binding of steroid hormone receptor to MMTV promoter-containing minichromosomes, which in turn results in promoter-specific phosphorylation of H1 (37, 58), and that phosphorylated H1 inhibits the activity of ATP-dependent chromatin-remodeling enzymes (34). Lee and Archer (39) demonstrated that, in tissue culture cells, histone H1 on the MMTV promoter adjacent to the hormone response element was dephosphorylated upon prolonged hormone treatment and that specific H1 isoforms were dephosphorylated (4). However, the loss of histone H1 phosphorylation under these conditions was global, and there was no direct demonstration of the localization of differentially phosphorylated H1 to specific sites on chromatin. Interestingly, the expression of different genes responds differently to altered levels H1 phosphorylation in both mammalian cells (6) and in *Tetrahymena* (16), indicating that there is no simple relationship between H1 phosphorylation state and the transcriptional state of genes.

We showed previously that the 539-bp fragment derived

from the *CDC2* 5′ region is phosphorylated (precipitatable with anti-phosphorylated H1 antibody) in growing cells (17) and that OA prevents the starvation-induced repression of *CDC2*, as does E5, a constitutively phosphorylated H1 (17). Together with the studies described here, these observations argue that H1 in this regulatory region of *CDC2* was phosphorylated in growing cells and becomes dephosphorylated upon starvation. We also showed that there was a small but reproducible reduction in the precipitation of this fragment with anti-phosphorylated H1 antibody in starved cells. Consistent with this observation, this fragment can be divided into two regions, one of which is enriched in starved-cell chromatin immunoprecipitated with anti-unphosphorylated H1 antibody and one which is not. However, no other fragment that was enriched in unphosphorylated H1 in starved cells showed a similar reduction in phosphorylated H1. We believe that this reflects the fact that the anti-phosphorylated H1 antibody cannot differentiate different isoforms of phosphorylated H1 and thus cannot distinguish, for example, when a 5-phosphate isoform changes to a 2-phosphate isoform. As a result, the lack of a reduction in signal for the anti-phosphorylated precipitated material after starvation seen in this work does not preclude that there was reduced phosphorylation; it only means that there was not enough complete dephosphorylation to make a detectable difference with the anti-phosphorylated H1 antibody. What is being measured by the multiplex PCR approach utilized here is relative enrichment (or depletion, although that is more difficult to demonstrate). If part of a fragment has more unphosphorylated H1 than other regions and part of it has more phosphorylated H1 than other regions, it will be enriched in both precipitations, as is the case for the 199-bp fragment. We wish to emphasize that it is the positive result with the anti-unphosphorylated antibody that is critical and that is easily detected.

The detailed mechanism whereby unphosphorylated H1 replaces phosphorylated H1 in this region is not known. There is little evidence that phosphorylated or unphosphorylated H1 can bind to specific DNA sequences, although some preferential binding to four-way junctions and preferential binding based on GC content have been reported (57, 59). We have examined the *CDC2* upstream sequence that is associated with unphosphorylated H1 in starved cells for these features and found it lacks inverted repeats and binding sites for known transcription factors and that it is similar to adjacent sequences in its GC content. Another possible mechanism is that alteration of chromatin composition or structure causes unphosphorylated H1 to bind to this region more strongly than phosphorylated H1. However, as described above, the changes in chromatin structure in this region, if any, are small. In addition, fluorescence recovery after photobleaching analyses indicate that while unphosphorylated H1 exchanges more slowly than phosphorylated H1, the rate of difference is not large (14), making it unlikely that differential binding of one over the other could result in the striking localization of the unphosphorylated isoform that we have observed here. An alternative mechanism for regulating localization of unphosphorylated H1 upstream of the *CDC2* gene during starvation is the targeting of the catalytic and/or a regulatory subunit of an H1 phosphatase (14). Consistent with this, a temporally regulated, large-scale, but incomplete dephosphorylation of H1 in *Tetra-*

*hymena* cells has been shown to occur between 6 and 12 h of starvation (16), and treatment of starved cells with a phosphatase inhibitor prevents both H1 dephosphorylation and reduction of *CDC2* expression during starvation (17). Finally, we cannot rule out the possibilities that the region enriched in unphosphorylated H1 is produced by recruitment of new, unphosphorylated H1 to this region either to replace phosphorylated H1 that has been displaced by transcription initiation as has been proposed for the MMTV promoter (see Fig. 6 in reference 58) or to occupy regions that are devoid of H1 in growing cells.

An important question is whether the alteration in H1 phosphorylation upstream of the *CDC2* gene is the cause or the consequence of transcription. The unphosphorylated region is upstream of the region encoding the 5′ end of the *CDC2* mRNA and is unlikely to be transcribed. Thus, the change in H1 phosphorylation state is unlikely to be produced by transcription per se, and its position suggests it is more likely to play a role in transcription initiation than elongation. Also, replacement of WT H1 with a mutant H1 that mimics constitutive phosphorylation and treatment of starved cells with a phosphatase inhibitor both enhance *CDC2* gene expression (17). These observations argue that the targeting of unphosphorylated H1 plays an important role in regulating transcription from the *CDC2* promoter rather than vice versa. Thus, the studies described here provide evidence indicating that the phosphorylation of H1 is being regulated at a very fine level and that unphosphorylated H1 can be specifically targeted to a promoter in vivo, and they suggest it likely regulates transcription. These results, coupled with recent studies showing that Cdk2 and H1 phosphorylation are targeted to replication foci in mammals (2), where they result in chromatin decondensation and probably facilitate the progression of replication forks, argue that the targeted regulation of the phosphorylation state of H1 is a general mechanism for regulating chromatin function.

## ACKNOWLEDGMENTS

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