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Phosphorylated and Dephosphorylated Linker Histone H1 Reside in Distinct Chromatin Domains in *Tetrahymena* Macronuclei

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Phosphorylated and dephosphorylated isoforms of *Tetrahymena* macronuclear H1 were separated from each other by cation-exchange high performance liquid chromatography and used to generate a pairwise set of antisera that discriminate the phosphorylation state of this linker histone. Affinity-purified antibodies from each sera recognize appropriate H1 isoforms and stain macronuclei under appropriate physiological conditions. Immunogold localizations demonstrate that phosphorylated and dephosphorylated H1 localize nonrandomly in distinct subdomains of macronuclear chromatin. Dephosphorylated H1 is strongly enriched in the electron-dense chromatin bodies that punctuate macronuclear chromatin. In contrast, phosphorylated H1 isoforms, as well as an evolutionarily conserved H2A.F/Z-like variant (hv1) believed to function in the establishment of transcriptionally competent chromatin, are modestly enriched at the periphery of chromatin bodies and in the surrounding euchromatin. Using antibodies against TATA-binding protein, we show that transcriptionally active chromatin lies outside of the chromatin bodies in an area relatively devoid of H1. Antibodies against general core histones are more or less evenly distributed across these domains. Together, these data are consistent with a model in which phosphorylation of H1, perhaps in association with hv1, loosens the binding of H1 in chromatin leading to chromatin decondensation as part of a first-step mechanism in gene activation. In contrast, our data support the view that dephosphorylation of this linker histone facilitates or stabilizes condensed, transcriptionally silent chromatin.

INTRODUCTION

Several levels of organization are required to permit the packaging of eukaryotic genes within nuclei. Beyond the nucleosomal thin filament, a second level of folding occurs giving rise to higher-order 30-nm chromatin fibers (van Holde, 1989; Wolffe, 1992). Proteins of the linker histone (H1/H5) family are required for this higher level of folding in vitro and as such are generally believed to play a fundamental role in mediating chromatin condensation (Thoma *et al.*, 1979). Most H1s have a tripartite structure consisting of a globular domain flanked by lysine-rich amino- and carboxy-terminal tails. The central domain interacts with the nucleosome core particle while the positively charged tails interact with linker DNA and are thought to be required for proper chromatin folding (Allan *et al.*, 1980; Staynov and Crane-Robinson, 1988).

The presence of H1 or post-translational modifications affecting H1 and its interaction with DNA are likely to play a pivotal role in regulating gene expression. Studies have suggested that H1 is missing, reduced, or modified in genes undergoing active transcription. From these observations, H1 is generally thought to exert a repressive influence on transcrip-

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tion, by inducing the formation of condensed chromatin or by preventing the binding of transcription factors (for references see Juan *et al.*, 1994). However, the exact relationship of H1 to transcription remains an enigma (Garrard, 1991; Zlatanova and van Holde, 1992).

Phosphorylation of specific serine and threonine residues located in the tail domains of H1 has been observed in a number of different cell types, ranging from protozoa to mammals (Hohmann, 1983). Addition of negatively charged, phosphoryl groups should weaken the interaction of H1 with negatively charged DNA, and in vitro evidence consistent with this hypothesis has been provided (Suzuki, 1989; Churchill and Travers, 1991; Hill *et al.*, 1991; Green *et al.*, 1993). Given this, the high level of H1 phosphorylation observed during mitosis (for review see Bradbury, 1992) is surprising because phosphorylation of the H1 tails might be expected to decondense the chromatin fiber (Clark and Kimura, 1990). In some systems, H1 phosphorylation is uncoupled from mitosis and highly condensed, transcriptionally inactive chromatin is tightly correlated with dephosphorylated H1 (Roth and Allis, 1992). Recent studies also indicate that mitotic chromosome condensation can occur in extracts lacking H1 (Ohsumi *et al.*, 1993) and that other polypeptides play an important role in this process (Hirano and Mitchison, 1994; Peterson, 1994). Thus, the functional relationship, if any, of H1 phosphorylation to condensed chromatin remains unresolved.

To gain insights into the biological function of H1 phosphorylation, we continue to exploit strengths of the ciliated protozoan *Tetrahymena*. Like other ciliates, *Tetrahymena* contain two functionally distinct types of nuclei, macro- and micronuclei (for review see Gorovsky, 1980). Macronuclei are transcriptionally active, somatic nuclei that divide amitotically. Micronuclei, in contrast, are transcriptionally inactive, germline nuclei that divide mitotically. One of the more prominent differences between macro- and micronuclei concerns polypeptides associated with linker DNA. Macronuclei contain a H1-like histone with phosphorylation characteristics typical of that histone (Allis and Gorovsky, 1981; Roth *et al.*, 1988). Although mitosis is missing in macronuclei, macronuclear H1 is phosphorylated by a Cdc2-like kinase activity (Roth *et al.*, 1991) and most of the in vivo phosphorylation occurs on Cdc2 kinase consensus sites (Dadd *et al.*, 1993).

Because macronuclei are amitotic and divide without chromosomes, H1 phosphorylation in macronuclei cannot play an exclusive role in chromosome condensation. Instead, we have argued that H1 phosphorylation is positively correlated with chromatin decondensation and genome reprogramming in macronuclei (Roth *et al.*, 1988; Roth and Allis, 1992). To test this hypothesis, antibodies highly selective for phosphorylated and dephosphorylated H1 have been generated and characterized. Using these antibodies as a pairwise set in immunogold localizations, we found that condensed, transcriptionally inactive macronuclear chromatin is highly enriched in dephosphorylated H1. In contrast, phosphorylated H1 isoforms and a relatively minor H2A variant hv1 are enriched in what appears to be a transition zone between active and inactive chromatin. These results suggest that phosphorylation of macronuclear H1 plays an important role as part of a gene activation process and that dephosphorylation of this histone may facilitate or stabilize condensed chromatin domains.

MATERIALS AND METHODS

Cell Culture and Labeling with [^{32}P]Orthophosphate

Genetically marked strains of *Tetrahymena thermophila*, CU 427 (Mpr/Mpr[6-mp-s]VI), and CU 428 (Chx/Chx-[cy-s]VII) were used in all experiments reported here. These were provided by P. Bruns (Cornell University, Ithaca, NY). Cells were grown axenically in 1% enriched proteose peptone to log phase (< 200,000 cells/ml). Where appropriate, cells were labeled continuously during vegetative growth with [^{32}P]orthophosphate (10–20 uCi/ml). After vegetative growth, cells were either harvested immediately for nuclear isolation or were collected for starvation, conjugation, or heat shock as described previously (Roth *et al.*, 1988). Where appropriate, protein synthesis was blocked by the addition of 10 μg/ml cycloheximide. Except during heat shock (1 h at 41°C), all cultures were maintained at 30°C.

Nucleus Isolation and Extraction of H1

Macronuclei were isolated from growing *Tetrahymena* by the methods of Gorovsky *et al.* (1975) with the following changes: spermidine was omitted from all buffers and 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate, and 10 mM iodoacetamide were added to the nucleus isolation buffer. During homogenization, formaldehyde was added directly to the nucleus isolation buffer at a final concentration of 1%. Cross-links were reversed by resuspending nuclei in 4 M guanidine-HCl, 10 mM EDTA, and 0.5 M 2-mercaptoethanol (pH 8.0) and boiling for 30 min. Upon cooling, the nuclear lysate was converted to 0.4 N H_2S0_4 and dialyzed overnight against 0.4 N H_2S0_4. Acid-soluble protein generated by centrifugation of the lysate at 15,000 × *g* for 15 min was precipitated by the addition of 5% perchloric acid. Perchloric acid–soluble protein was in turn recovered by precipitation with 20% trichloroacetic acid. All precipitates were washed once in acidified acetone, once in acetone, and dried under vacuum. In some experiments, whole cell protein or total nuclear protein was prepared by solubilizing samples directly in sodium dodecyl sulfate (SDS) sample buffer.

Separation of Phosphorylated and Dephosphorylated H1 Isoforms

Macronuclear H1 was applied to a poly CAT A-high performance liquid chromatography (HPLC) column (4.6 × 200 mm or 21.1 × 250 mm, Poly LC, Columbia, MD) pre-equilibrated in 0.30 M sodium perchlorate in 10 mM phosphoric acid, adjusted to pH 6.5. Phosphorylated and dephosphorylated isoforms of H1 were eluted from the column using a linear gradient of 0.35–0.45 M sodium perchlorate in the above buffer as described previously (Lu *et al.*, 1994). Typically, 11 main peaks of macronuclear H1 are resolved under these conditions. Preliminary experiments showed that this profile is identical with that of H1 extracted directly from intact cells by

perchloric acid and that H1 is significantly less modified when formaldehyde is omitted from the nucleus isolation buffer. Individual peaks from the poly CAT A column were desalted by dialysis or reverse phase HPLC.

Generation and Affinity-Purification of Antibodies Selective for Phosphorylated and Dephosphorylated H1

Crude antisera selective for phosphorylated and dephosphorylated H1 were generated initially using hyperphosphorylated (peak 1) and dephosphorylated (peak 11) H1 recovered from the poly CAT A column. Antibodies against phosphorylated and dephosphorylated H1 epitopes were further enriched by binding to peak 1 or peak 11 affinity columns, respectively. Proteins were coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) according to the manufacturer's directions. Antibodies were eluted from each affinity column with a 5 M sodium iodide wash and dialyzed in the presence of 10% goat serum.

Macronuclear H1 is phosphorylated in vitro by HeLa Cdc2 at sites identical to those phosphorylated in vivo (Roth *et al.*, 1991). To ensure that a similar, if not identical, region of H1 was being compared in this study, a 16-amino acid peptide (#43 Ala Ser Thr Thr Pro Val Lys Lys Asp Val Thr Pro Val Lys Ala Asp), was synthesized to a domain of macronuclear H1 known to be highly phosphorylated. Peptide affinity columns were then constructed using either the unmodified peptide or the modified peptide after in vitro phosphorylation using human Cdc2 kinase (Roth *et al.*, 1991). Antibodies eluting from the H1 affinity columns described above were further purified using appropriate (dephosphorylated or in vitro phosphorylated) H1 peptide affinity columns. Antibodies bound to the peptide affinity columns were eluted as described above.

H1 Cleavage by Cyanogen Bromide

Where appropriate, H1 was cleaved chemically with cyanogen bromide (CNBr) as described previously (Roth *et al.*, 1988). Control reactions were performed in 70% formic acid alone; no cleavage of H1 was observed under this condition.

Gel Electrophoresis and Immunoblotting

SDS polyacrylamide gels were routinely stained by Coomassie blue, photographed, and where appropriate, processed for autoradiography. The specificity of antibodies used in this report was analyzed by immunoblotting analyses. Balanced protein loads were ensured by staining parallel gels of equivalently loaded samples and by staining immunoblots directly with Ponceau Red. All blots were blocked with nonfat dry milk and incubated with preimmune sera or affinity-purified IgG. All antibody reactions were detected by alkaline phosphatase-conjugated secondary antibodies after a color reaction. In some cases only the H1 region of the gel or blot is shown; in these cases no other band or cross-reacting species was observed. Antibodies against *Tetrahymena* TATA-binding protein (or TBP) were kindly provided by M. Gorovsky and have been characterized recently (Stargell and Gorovsky, 1994).

Indirect Immunofluorescence Analyses

Cells were fixed and processed for indirect immunofluorescence as described previously (Wenkert and Allis, 1984). Primary antibodies used in this report were detected by rhodamine-conjugated secondary antibodies. Cells were finally stained with the DNA-specific dye diamidinophenolindole at 1 μg/ml in Tris-buffered saline. All intervening washes after antibody incubations were in Tris-buffered saline.

Electron Microscopy and Immunogold Labeling

Vegetative cells were fixed in PLP (McLean and Nakane, 1974) overnight at 4°C. The fixative was removed by centrifugation and pellets were washed with 0.1 M sodium phosphate buffer, dehydrated through a graded series of alcohol at room temperature and embedded in LR White (Ted Pella, Redding, CA) overnight at 4°C. After centrifugation, pellets were resuspended in fresh resin and cured in gelatin capsules for 48 h at 60°C. Thin sections were cut with a diamond knife using an LKB III ultramicrotome instrument and collected on 200 mesh gold grids.

Thin sections were blocked in sterile 1–3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min at room temperature and then incubated with primary antibody diluted in PBS overnight at 4°C. After three washes of 5 min each, sections were incubated with biotinylated secondary antibody for 1–2 h at room temperature, washed three times in PBS, and then stained with streptavidin linked to 10 nm colloidal gold particles (Sigma Chemical, St. Louis, MO) diluted in PBS. The sections were then washed twice in PBS, twice in distilled water, and stained with 2% uranyl acetate followed by lead citrate. Thin sections were observed with a Zeiss 10C transmission electron microscope (Thornwood, NY). Controls included using preimmune sera or omitting the primary antibody altogether.

Quantitative Analyses

For each antibody, quantitative analyses on the relative distribution of gold particles (number of particles/unit area) were performed from photographic enlargements of electron micrographs taken in parallel from at least two separate experiments. Gold particles were counted over equivalent areas of chromatin bodies or surrounding euchromatin. Particles associated with chromatin bodies were then subdivided into two classes: outside edge (touching or within one gold particle diameter from the outside edge of a chromatin body; see arrows in Figure 5B, inset) or inside (see white arrowheads in Figure 4B, inset). Similar analyses were performed with preimmune control sera; these values were subtracted as background from corresponding data collected with immune antibodies. Data are plotted in Figure 6 as the mean fraction (%) of gold particles scored associated with either chromatin bodies (inside and outside edge) or surrounding euchromatin. In each analysis, approximately 100–300 gold particles were scored depending upon the overall grain density.

RESULTS

Generation and Characterization of Antibodies

Even though macronuclei are amitotic, macronuclear H1 is extensively phosphorylated during vegetative growth. Much of this growth-associated phosphorylation is manifested as four or five closely spaced bands on SDS or acid-urea gels with more phosphorylated species migrating more slowly in both gel systems. Excellent resolution of this heterogeneity has recently been demonstrated when H1 is subjected to cation-exchange HPLC using a poly-aspartic acid resin (poly CAT A). From log-phase cells, roughly eleven peaks of varying heights are typically resolved (see Lu *et al.*, 1994 for details).

Using this separation, we sought to develop a set of nonoverlapping immunological reagents to probe chromatin containing H1 in different phosphorylation states. Shown in Figure 1 are SDS gel immunoblots with individual H1 isoforms probed with affinity-pu-

rified, anti-phosphorylated (Figure 1B) and anti-dephosphorylated H1 (Figure 1C) antibodies. Despite more or less even protein loads (Figure 1A), anti-phosphorylated H1 antibodies react strongly with the first five species to elute from the column. H1 recovered from later eluting peaks is weakly stained and no reaction is observed with dephosphorylated H1 (peak 11). Thus, these antibodies preferentially recognize highly phosphorylated isoforms of macronuclear, a finding similar to what is observed with human H1 (Lu *et al.*, 1994). In contrast, anti-dephosphorylated H1 antibodies react strongly with dephosphorylated and weakly phosphorylated H1 isoforms (Figure 2C). More highly phosphorylated species are not reactive with these antibodies (for clarity these antibodies will be referred to as anti-dephosphorylated H1 antibodies). Thus, this pairwise set of antibodies has nearly nonoverlapping specificity.

To further document the specificity of these antibodies, total cellular and nuclear protein from cells labeled in vivo with [^{32}P]phosphate was resolved on a one-dimensional SDS gel and analyzed by immunoblotting with the anti-phosphorylated and dephosphorylated H1 antibodies. In agreement with previous data (Lu *et al.*, 1994), strong immunoreactivity is observed with appropriate isoforms of H1. Macronuclear H1 also contains a single central methionine (residue 69) and previous experiments using CNBr digests of ^{32}P-labeled H1 have shown that all of the in vivo phosphorylation resides on its amino-terminal half (Roth *et al.*, 1988). As predicted, anti-phosphorylated H1 antibodies react only with the amino-terminal half of CNBr-cleaved H1 when it is phosphorylated (Lu *et al.*, 1994). Opposite results are obtained with the anti-dephosphorylated H1 antibodies where only the dephosphorylated form of the amino-terminal half is recognized. Neither antibody is reactive against the carboxy-terminal "half" of the molecule. Collectively, these data suggest that the antibodies employed in this study are highly selective for H1 and can discriminate phosphorylation in the appropriate domain of macronuclear H1.

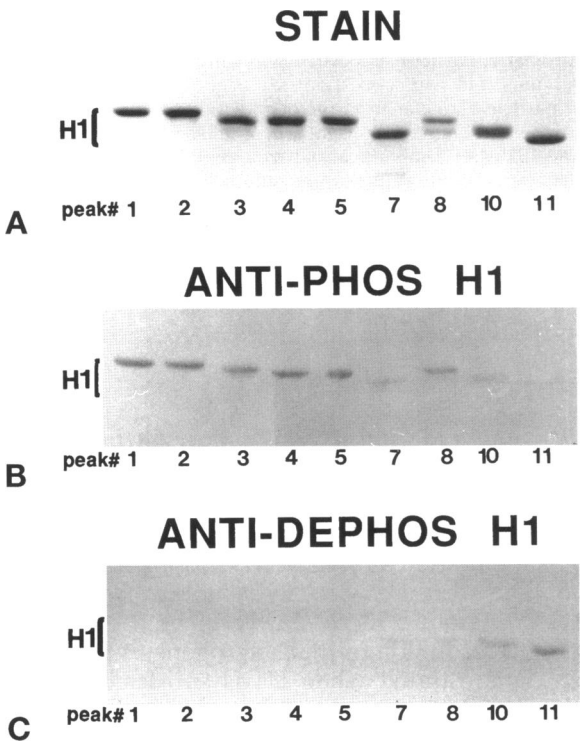

Figure 1. Antibodies generated against phosphorylated and dephosphorylated H1 recognize a distinct set of H1 isoforms. H1 recovered from each peak of the poly CAT A column was electrophoresed in an SDS gel (A), transferred to nitrocellulose, and incubated with affinity-purified anti-phosphorylated (B) or dephosphorylated (C) H1 antibodies. See Lu *et al.* (1994) for details on the fractionation of macronuclear H1 by poly CAT A. Peaks 6 and 9, which are relatively minor in vegetative cells, were not analyzed.

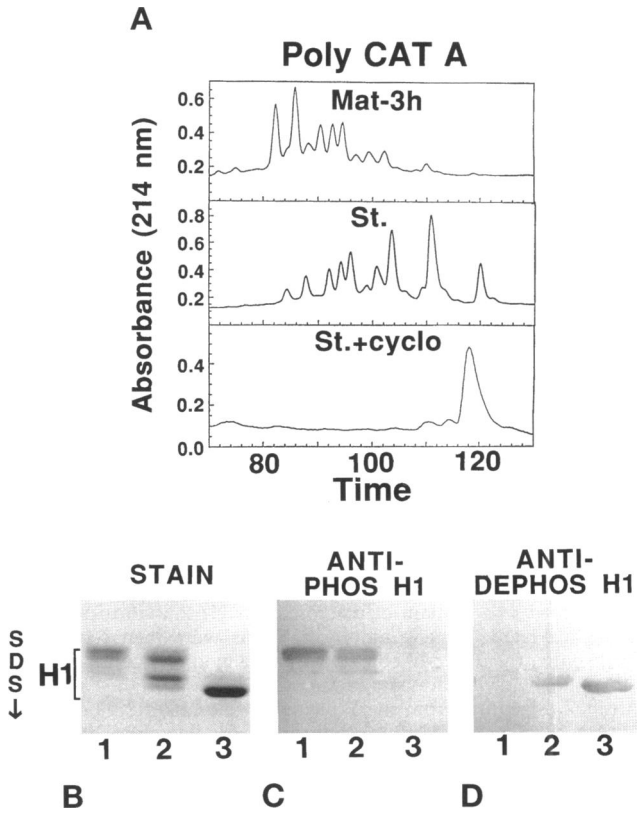

Figure 2. Differences in H1 phosphorylation under different physiological conditions. Macronuclear H1 was purified from 3-h mating cells (Mat-3h, lanes 1) or starved cells incubated in the absence (St., lanes 2) or presence of cycloheximide (St. + cyclo, lanes 3) before being subjected to chromatography using poly CAT A (A) or electrophoresis in a 12% SDS gel. Samples were analyzed by staining (B), or were transferred to nitrocellulose and probed with anti-phosphorylated (C) or dephosphorylated (D) H1 antibodies.

In Situ Analyses Distinguish Different Levels of H1 Phosphorylation

The steady state level of H1 phosphorylation in macronuclei differs dramatically in response to different physiological conditions. In particular, levels of H1 phosphorylation are low in starved, nondividing cells and are dramatically elevated when cells are induced to enter the sexual phase of the life cycle, conjugation. In agreement with previously published results (Roth *et al.*, 1988), dramatically different levels of phosphorylation are observed when H1 is purified from conjugating (Figure 2, 3 h Mat., lanes 1), starved (Figure 2, St., lanes 2), or cycloheximide-treated (Figure 2, St. + cyclo, lanes 3) cells when assayed by cation-exchange chromatography (Figure 2A) or SDS gel electrophoresis (Figure 2B).

We reasoned that our antibodies should distinguish differences in H1 phosphorylation brought about by these physiological conditions. Immunoblotting (Figure 2B) and immunofluorescence (Figure 3) analyses were performed using the above cells to test the specificity of our antibodies. As predicted, H1 is stained strongly with anti-phosphorylated H1 antibodies in 3-h mating cells when H1 is hyperphosphorylated and is only weakly stained in cells treated with cycloheximide when H1 is largely dephosphorylated. Opposite results are obtained when anti-dephosphorylated H1 antibodies are employed. With both antibodies, intermediate staining is observed with starved cells, a result anticipated from the broad distribution of H1 isoforms in these cells.

Close inspection of the macronuclei shown in Figure 3 or the insets provided in Figure 4 suggest that the subnuclear distribution of antibody staining is not uniform or equivalent with both antibodies. Numerous small punctate foci are evident in macronuclei stained with the anti-dephosphorylated H1 antibodies

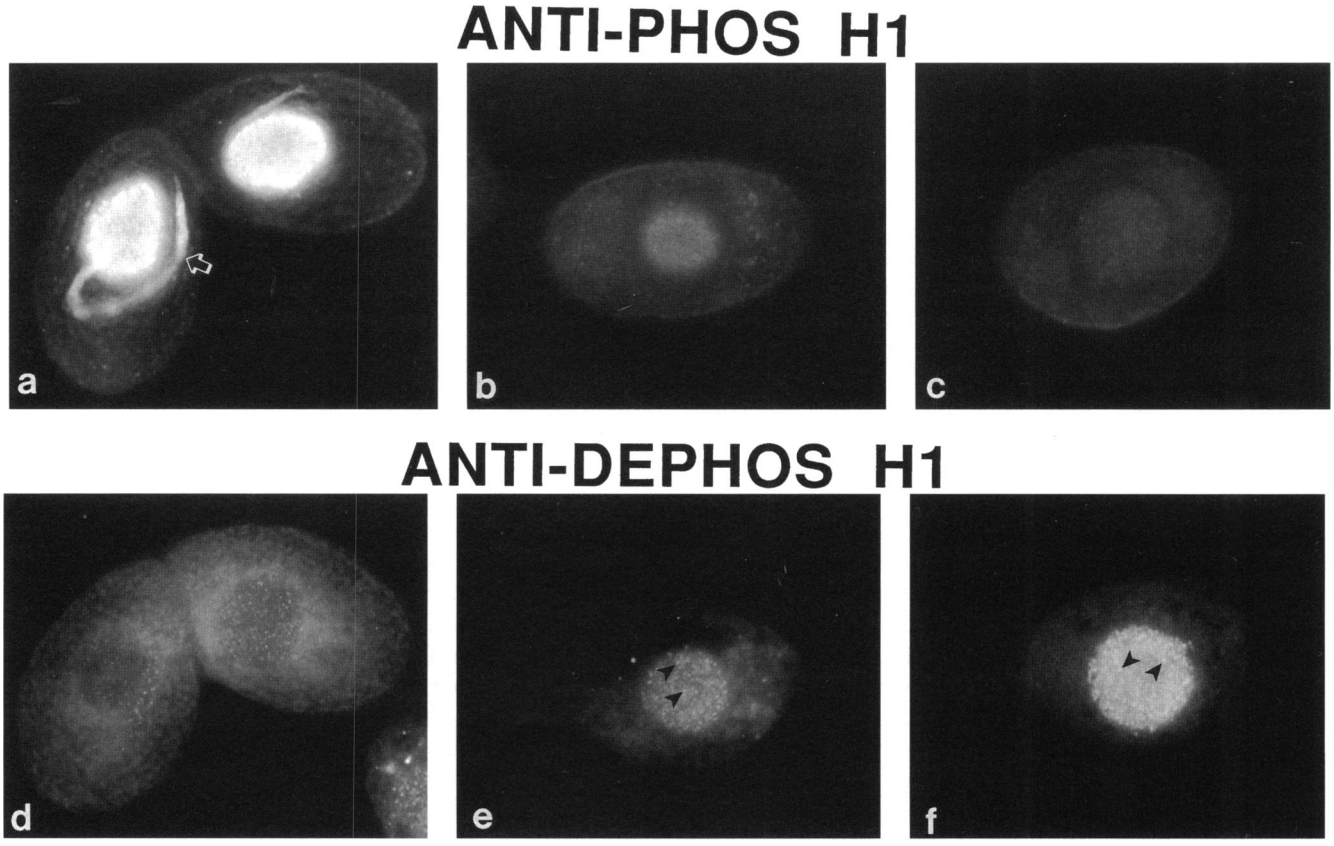

Figure 3. Anti-phosphorylated and dephosphorylated H1 antibodies discriminate differences in levels of H1 phosphorylation in situ. Aliquots of the same cells used in Figure 2 [3-h mating (a and d) or starved cells incubated in the absence (b and e) or presence of cycloheximide (c and f)] were fixed and processed for indirect immunofluorescence using anti-phosphorylated (a–c) or dephosphorylated (d–f) H1 antibodies. The open arrow in panel a identifies a crescent-stage micronucleus stained by cross-reactivity with one of the micronuclear linker histones (α), which is abundant and phosphorylated during this stage of conjugation. Arrowheads in panels e and f identify numerous small punctate dots observed at all focal planes through macronuclei stained with the anti-dephosphorylated H1 antibodies. These foci are less obvious when anti-phosphorylated H1 antibodies are used.

(see small arrowheads in Figure 3, e and f, and the inset to Figure 4B). These subnuclear dots are observed at all focal planes and are not stained with antibodies against nucleolar antigens. Thus, it is unlikely that these dots represent nucleoli (nucleoli are peripherally located in macronuclei). This staining pattern is much less evident in cells stained with the anti-phosphorylated H1 antibodies (see Figure 3, a and b and the inset to Figure 4A). These differences in immunofluorescence at the light microscopic level prompted us to explore the localization of the antibodies at an ultrastructural level.

Phosphorylated and Dephosphorylated H1 Are Localized in Distinct Regions of Macronuclear Chromatin

Macronuclear chromatin is not uniform with respect to the distribution of decondensed and condensed chromatin. In ultrastructural analyses, each macronucleus contains numerous evenly distributed patches of condensed chromatin referred to as chromatin bodies ranging from 0.1–0.3 μm in diameter (labeled CB in Figure 4; Elliot, 1973; Weiske-Benner and Eckert, 1985). In contrast, a more electron-lucent chromatin surrounds the chromatin bodies interspersed with a heterogeneous collection of interchromatin RNP particles (Swift *et al.*, 1964; Gorovsky, 1968). It is generally assumed that this hnRNP is in close association with "active" chromatin situated outside of the chromatin bodies although the location of active chromatin in macronuclei has not been rigorously established (see below). We sought to determine if phosphorylated and dephosphorylated H1 are localized differently with respect to these morphological domains.

Shown in Figure 4 are electron micrographs of macronuclei reacted with anti-phosphorylated and dephosphorylated H1 antibodies and processed for immunogold analyses. When anti-dephosphorylated H1 antibodies are utilized (Figure 4B), the vast majority of gold particles are tightly distributed with the electron-dense chromatin bodies (94% are CB-associated, see Figure 6A). Relatively few gold particles are observed in the surrounding euchromatin. A different distribution is observed with anti-phosphorylated H1 antibodies. With this antibody a smaller fraction of gold particles are chromatin body associated (73%, Figure 6B) and most often these gold particles are situated near the outside edge of the chromatin bodies (see arrowheads in Figure 4A) or reside in the chromatin immediately surrounding the chromatin bodies. No significant gold binding is observed in "minus antibody" or preimmune controls (see Figure 4C).

Transcriptionally Active Chromatin Resides Outside of Macronuclear Chromatin Bodies

The above distributions suggest a nonuniform and subtly distinct distribution of phosphorylated and dephosphorylated H1 in macronuclear chromatin, a difference that may underlie a different function for phosphorylated and dephosphorylated H1 in chromatin. To further define the subnuclear location of transcriptionally active chromatin in macronuclei, parallel immunogold analyses were performed with antibodies against the general transcription factor, TATA-binding factor or TBP (Stargell and Gorovsky, 1994), and an evolutionarily conserved H2A.F/Z-like variant hv1, thought to be enriched in transcriptionally competent chromatin (Allis *et al.*, 1980; White *et al.*, 1988). Both hv1 and TBP have recently been linked, both spatially and temporally, to the establishment of transcriptionally competent or active chromatin in *Tetrahymena* (Stargell *et al.*, 1993; Stargell and Gorovsky, 1994). Parallel reactions were also performed with anti-general H2A and H4 sera to control for general accessibility and concentration effects within distinct macronuclear subdomains.

Several observations are noteworthy. First, the distribution of bound H2A or H4 antibodies is more or less equivalent across chromatin bodies and the surrounding euchromatin (Figures 5C and 6C), suggesting that antibody penetration and detection is not a significant problem in these analyses. Second, a different distribution is observed for antibodies against the minor H2A variant hv1 (Figures 5B and 6D). Here, like the distribution observed for the anti-phosphorylated H1 antibodies, a modest bias is observed for the outside edge of chromatin bodies (see arrows in the insert to Figure 5B) and the surrounding euchromatin. Third, when anti-TBP antibodies are used (Figures 5A and 6E), a significantly lower fraction of gold particles is chromatin body associated. In this case a majority of the gold ($\approx$75%) is associated with the electron-lucent euchromatin outside of the chromatin bodies. These data strongly suggest that transcriptionally active macronuclear chromatin, as defined by the presence of TBP, is enriched in the decondensed chromatin surrounding the chromatin bodies. Our analyses suggest that this domain is relatively depleted in H1, especially the dephosphorylated isoform of this protein. In contrast, transcriptionally silent macronuclear chromatin, marked by electron dense chromatin bodies, is highly enriched in dephosphorylated H1 and is markedly depleted in TBP. Although subtle, a reproducible enrichment of phosphorylated H1 and hv1 is observed at the boundary between chromatin bodies and the surrounding euchromatin (compare Figure 6, B and D).

DISCUSSION

In this report, antibodies have been generated that recognize phosphorylated and dephosphorylated isoforms of macronuclear H1 with nonoverlapping specificity. Because both antibodies were affinity purified using a short domain from the N-terminus of macronuclear H1, a domain known to be phosphorylated in vivo and in vitro by mammalian Cdc2 kinase (Roth *et al.*, 1991), it seems likely that the phosphorylation state of a similar, if not identical, H1 epitope is being directly compared in this study. This pairwise set of antibodies has been used to test the hypothesis that H1 phosphorylation acts as a first-step mechanism for inducing chromatin decondensation facilitating processes such as gene activation (Roth and Allis, 1992).

Macronuclei are well stained with anti-phosphorylated H1 antibodies during vegetative growth and in cells undergoing sudden physiological transitions where high levels of H1 phosphorylation have been reported (Roth *et al.*, 1988). Under these conditions, transcription from macronuclei is either high or novel sets of genes are being suddenly expressed (Martindale and Bruns, 1983; Stargell *et al.*, 1993). In contrast, macronuclei are strongly stained with anti-dephosphorylated antibodies in long-term starved cells, cells that are nonreplicating and support only basal transcription (Calzone *et al.*, 1983). Anti-dephosphorylated H1 antibodies, but not anti-phosphorylated H1 antibodies, also stain "old" macronuclei when parental macronuclei suddenly cease transcription and become highly condensed during the sexual stage of the life cycle (Lu and Allis, unpublished observations). During this stage, H1 is quantitatively dephosphorylated (Lin *et al.*, 1991). Collectively, these data argue that the increase in net positive charge exhibited by dephosphorylated H1 tails, like hypoacetylated core histone amino termini, may be a general feature of transcriptionally silent chromatin (see Roth and Allis, 1992).

Although subtle, our immunogold analyses suggest that phosphorylated and dephosphorylated H1 are

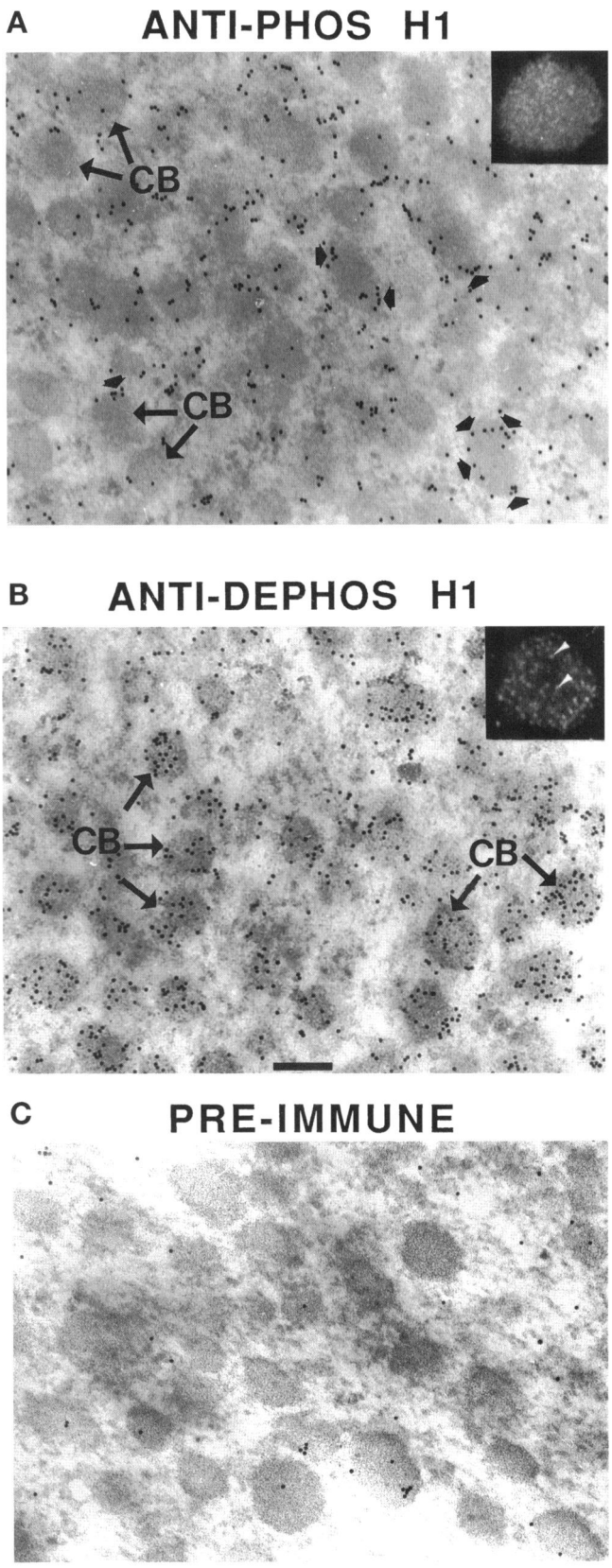

Figure 4. Phosphorylated and dephosphorylated H1 are localized in distinct structural domains of macronuclear chromatin. Thin sections of vegetative macronuclei were incubated with affinity-purified anti-phosphorylated (A) or dephosphorylated (B) H1 antibodies or pre-immune serum taken from the rabbit before immunization with hyperphosphorylated H1 (C) and processed for immunogold electron microscopy. Numerous condensed chromatin bodies (labeled CB) and surrounding euchromatin are evident. With anti-dephosphorylated antibodies, most gold particles are tightly linked to the condensed inner region of the chromatin bodies. In contrast, a more peripheral distribution is observed when anti-phosphorylated antibodies are used (see small arrowheads in A). Insets shown in panels A and B are corresponding immunofluorescent images of macronuclei stained with each antibody; small white arrowheads in panel B denote numerous submacronuclear foci that are particularly evident when macronuclei are stained with the anti-dephosphorylated H1 antibodies. Bar, 0.25 micrometers.

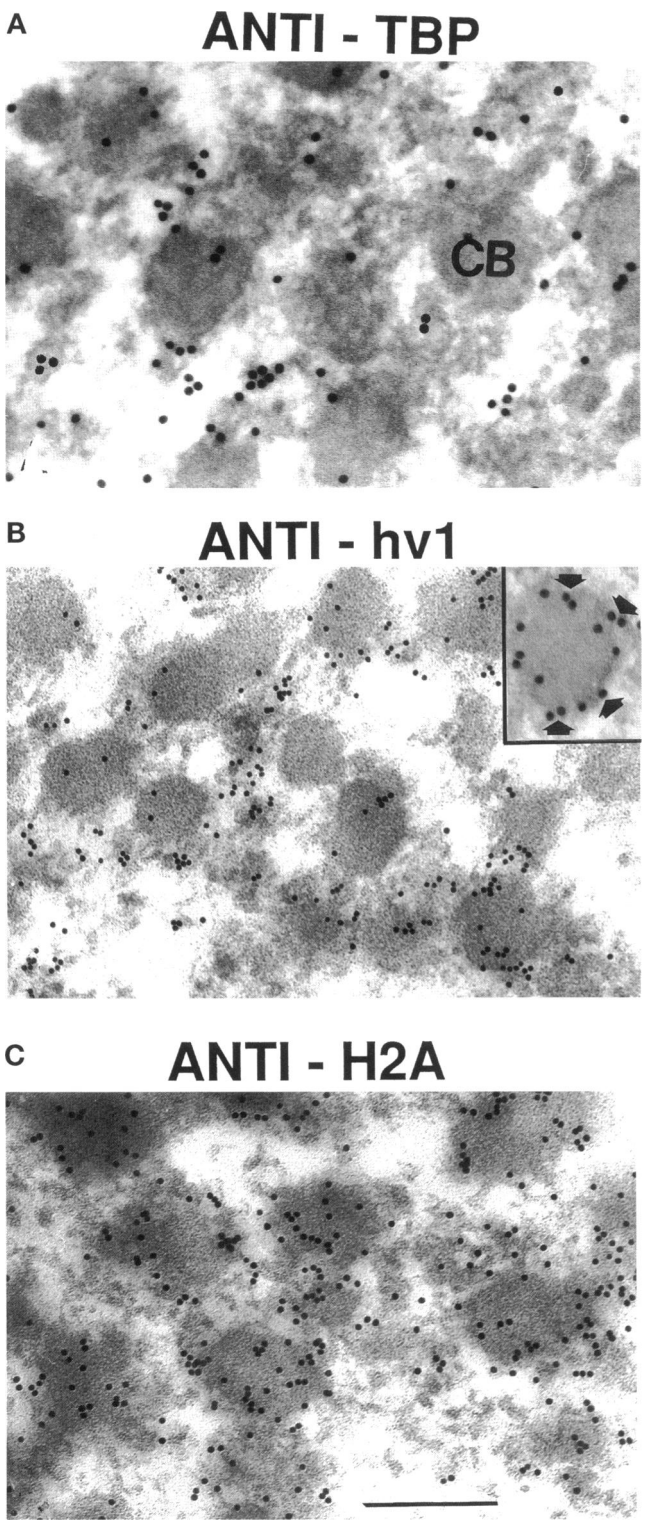

Figure 5. Transcriptionally active chromatin resides outside of the electron-dense chromatin bodies. Antibodies against TBP (A), hv1 (B), and as a control, the major H2As (C), were used in parallel immunogold analyses as in Figure 4. Numerous chromatin bodies are evident along with the surrounding euchromatin.

localized nonrandomly in distinct and potentially nonoverlapping domains of macronuclear chromatin. Our results show clearly that dephosphorylated H1 is enriched in the more condensed inner regions of macronuclear chromatin bodies and some evidence suggests that this chromatin is transcriptionally inactive. Weiske-Benner and Eckert (1985) examined the distribution of [^{3}H]uridine in macronuclei after EM autoradiography. As expected, nucleoli were highly active in RNA synthesis. Chromatin bodies, however, were largely unlabeled, a significant finding given that a majority of macronuclear DNA resides within these structures (Gorovsky, 1968). Immunogold localizations with TBP that chromatin bodies represent inactive chromatin. TBP is generally accepted to participate in basal transcription and our analyses demonstrate that this protein is strongly enriched in the decondensed euchromatin that surrounds the chromatin bodies. Our data also demonstrate that transcriptionally silent chromatin associated with chromatin bodies is facilitated or stabilized by the presence of dephosphorylated H1.

Although subtle, a reproducible fraction of the anti-phosphorylated H1 and anti-hv1 antibodies bind near the outer edge of macronuclear chromatin bodies. It is unlikely that this "outside edge" distribution results from an inability of antibodies to penetrate evenly the more condensed chromatin of chromatin bodies for several reasons. First, in all of our experiments antibodies were applied directly to thin-sectioned material (i.e., post-embedding) and therefore, epitopes situated more to the interior of the chromatin bodies should have been equally accessible for antibody binding as outer regions. Second, when anti-dephosphorylated H1 antibodies or general core histone antibodies are used in parallel assays, gold particles are distributed more or less evenly across the chromatin bodies providing a compelling argument against antibody inaccessibility. The finding that phosphorylated H1 is enriched in a "transitional zone" between inactive and active chromatin is consistent with the idea that H1 phosphorylation acts to loosen its interaction with the DNA leading to chromatin decondensation.

Our data suggest that linker histone is relatively depleted in domains that are transcriptionally active as defined by the presence of TBP. These results agree with cross-linking and immunoprecipitation experiments using general H1 antibodies where an inverse relationship between gene transcription and the presence of macronuclear H1 was reported (Dedon *et al.*, 1991). Taken together, these data are consistent with a

Figure 5 cont. Examples of peripherally located hv1 antibodies are indicated with small arrows in the inset to panel B. Bar, 0.3 micrometers.

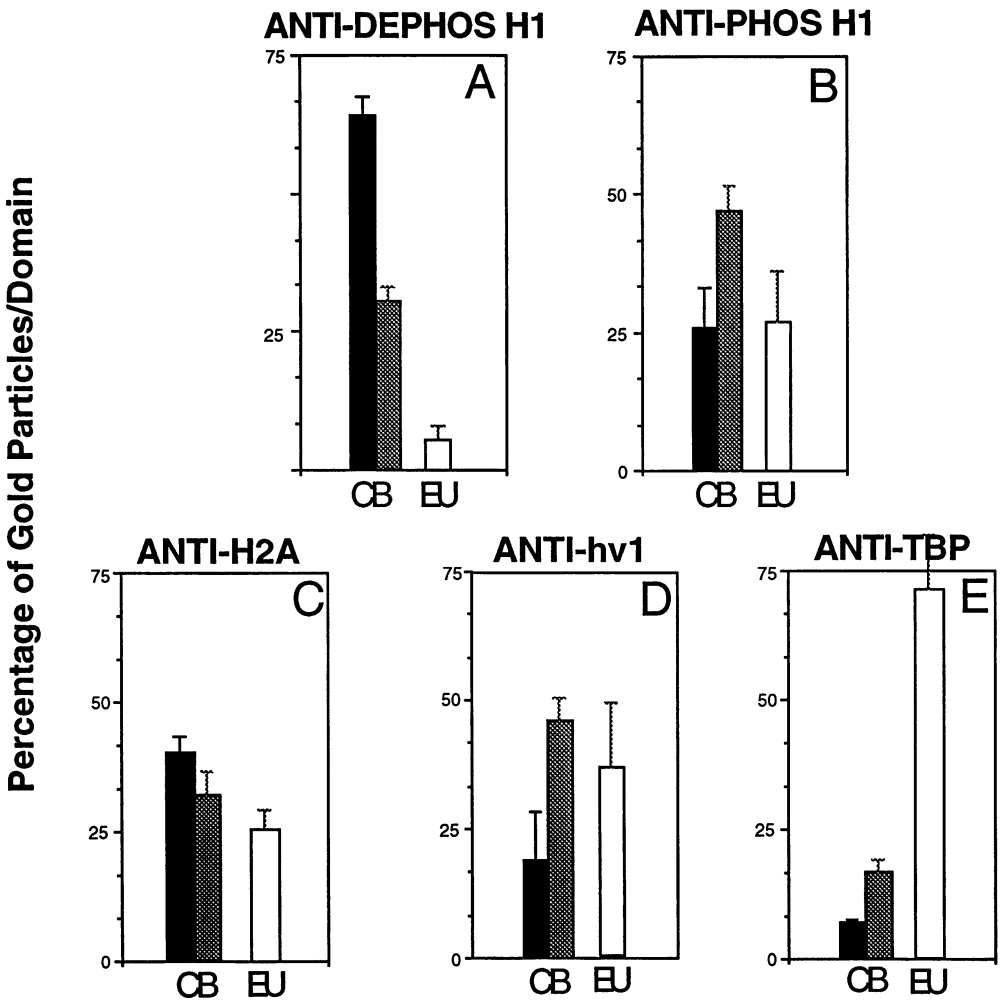

Figure 6. Quantitative analyses of gold particle distributions. For each antibody used in this study (anti-dephosphorylated H1, A; anti-phosphorylated H1, B; anti-H2A, C; anti-hv1, D; and anti-TBP, E), gold particles were counted over equivalent areas of chromatin bodies or surrounding euchromatin. Gold particles associated with chromatin bodies (CB) were then arbitrarily subdivided into two classes: outside edge or inside (refer to MATERIALS AND METHODS for details) or were scored as being euchromatin (EU)-associated. Similar analyses were performed with preimmune control sera; these values were subtracted as background from corresponding data collected with immune antibodies. Data are plotted as the mean percent of gold particles scored; error bars represent the range about the mean from at least two independent experiments. In each analysis, approximately 100–300 gold particles were scored. Solid bars, inside chromatin bodies; stippled bars, outside edge of chromatin bodies; open bars, surrounding euchromatin.

model that phosphorylation of H1 is used in a first-step mechanism to decondense the chromatin fiber after which H1 is "displaced" from its chromatin-bound position. By weakening H1:DNA interactions, phosphorylation of H1 may facilitate H1 displacement from repressed chromatin domains leading to destabilization of higher-order chromatin fibers and subsequent gene activation.

Our results also demonstrate that hv1, an evolutionarily conserved H2A.F/Z variant, is enriched at the boundary between TBP-associated euchromatin and the denser inner chromatin of chromatin bodies. These data agree with a large body of circumstantial evidence that hv1 and related H2A variants from other sources play a fundamental, yet undetermined, role in establishing a transcriptionally competent state in chromatin, a role distinct from the major H2As (see Stargell *et al.*, 1993 for references). Strong support for this idea has recently been provided by genetic experiments demonstrating that hv1, unlike H1, is an essential core histone in *Tetrahymena* (Shen *et al.*, 1995; Gorovsky, personal communication).

In higher cells, H1 is known to contact H2A in the nucleosomal core particle (Bonner and Stedman, 1979; Bouliakas *et al.*, 1980). Our quantitative analyses detect a subtle enrichment of both hv1 and phosphorylated isoforms of H1 for the outside edge of chromatin bodies. Whether a functional correlation exists between hv1 and the displacement of H1 from repressed chromatin domains, an association perhaps weakened or facilitated by the phosphorylation of H1 itself, is unclear. Given the obvious similarity between our postulated phosphorylation-induced H1 dissociation from active chromatin and the recent removal of H1 from *Tetrahymena* by gene disruption (Shen *et al.*, 1995), it will be important to determine if the loss of H1 affects the repression of specific genes. Whether the essential nature of hv1 is obviated in an H1 knockout stain also remains to be determined.

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