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Proteolytic removal of core histone amino termini and dephosphorylation of histone H1 correlate with the formation of condensed chromatin and transcriptional silencing during *Tetrahymena* macronuclear development

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During the sexual cycle in *Tetrahymena*, the germ-line micronucleus gives rise to new macro- and micronuclei, whereas the former somatic macronucleus ceases transcription, becomes highly condensed, and is eventually eliminated from the cell. With polyclonal antibodies specific for acetylated forms of histone H4, immunofluorescent analyses have demonstrated that transcriptionally active macronuclei stain positively at all stages of the life cycle except during conjugation, when parental macronuclei become inactive and are eliminated from the cell. In this report using affinity-purified antibodies to either the acetylated or unacetylated amino-terminal domain of H4, immunofluorescent analyses suggest that the acetylated amino-terminal tails of H4 are proteolytically removed in "old" macronuclei during this period. This suggestion was further confirmed by biochemical analyses of purified old macronuclei that revealed several polypeptides with molecular mass 1–2 kD less than that of intact core histones. These species, which are unique to old macronuclei, are not newly synthesized and fail to stain with either acetylated or unacetylated H4 antibodies. Microsequence analysis clearly shows that these polypeptides are proteolytically processed forms of core histones whose amino-terminal "tails" (varying from 13 to 21 residues) have been removed. During the same developmental period, histone H1 is dephosphorylated rapidly and completely in old macronuclei. These results strongly suggest that the developmentally regulated proteolysis of core histones and dephosphorylation of histone H1 participate in a novel pathway leading to the formation of highly condensed chromatin and transcriptional silencing during *Tetrahymena* macronuclear development.

[*Key Words*: Proteolysis; core histone amino termini; histone acetylation; dephosphorylation; histone H1; condensed chromatin; *Tetrahymena* macronuclear development]

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Nucleosome octamers contain two molecules of each of the core histones, H2A, H2B, H3, and H4. Each of these four core histones can be divided into three domains: a hydrophilic amino-terminal tail, a hydrophobic globular domain, and a short hydrophilic carboxy-terminal tail. The central hydrophobic domain has been shown to be involved in histone–histone interactions and is required for octamer assembly in vitro (McGhee and Felsenfeld 1980; van Holde 1988). In support of these biochemical data, recent genetic studies have shown that deletions into the hydrophobic domain of histone H4 are lethal in vivo (Schuster et al. 1986; Kayne et al. 1988).

The amino-terminal domain, on the other hand, is highly positively charged and accessible to post-translational modification. In vitro studies have shown that the amino termini of core histones in chromatin are labile when treated with proteolytic enzymes (Böhm et al. 1980, 1981, 1982; Dumuis-Kervabon et al. 1986) and are not required for nucleosome core particle assembly or stability (Whitlock and Stein 1978; Ausio et al. 1989). However, several studies have suggested that the core histone tails, in combination with linker histones, are involved in stabilizing the 30 nM chromatin solenoid (Allan et al. 1982; Annunziato et al. 1988; Norton et al. 1989; Oliva et al. 1990; Hill and Thomas 1990). While deletion of the amino-terminal sequences of yeast H2A, H2B, and H4 is not lethal (Wallis et al. 1983; Schuster et

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al. 1986; Kayne et al. 1988), the amino terminus of H4 is essential for repressing the silent mating loci (Kayne et al. 1988). This suggests that the positive charge contained in the amino terminus of H4 contributes to the formation or stability of heterochromatin in yeast (for a recent review and references, see Grunstein 1990; Smith 1991.).

Histone acetylation, the acetylation of specific lysines located within the amino-terminal domains of each core histone, is an active process whose precise role(s) remains controversial. Because the acetylation of lysine residues neutralizes their positive charge, it has long been suggested that this modification modulates the interaction of amino termini with the negatively charged DNA backbone (see Allfrey 1977; Hebbes et al. 1988). Strong correlative data exist implying that histone acetylation is involved in transcription, chromatin assembly, and histone replacement (for references and a review of histone acetylation, see Matthews and Waterborg 1985; van Holde 1988). Furthermore, recent genetic experiments have suggested that the ability to modulate the positive charges on amino termini of histone H4 by acetylation is more important than the presence of positive charge at these highly conserved positions (Megee et al. 1990; Park and Szostak 1990).

Like many ciliated protozoa, vegetative cells of *Tetrahymena thermophila* contain two distinct nuclei, macro- and micronuclei. Macronuclei are transcriptionally active somatic nuclei that govern the phenotype of the cell, whereas micronuclei act as transcriptionally inactive germ-line nuclei (Gorovsky 1973). Despite these differences in structure and function, macro- and micronuclei are developmentally related. During the sexual phase of the life cycle—conjugation—micronuclei undergo meiosis, exchange, fertilization, and postzygotic divisions to eventually produce new macronuclei and micronuclei. As new macronuclei (anlagen) become transcriptionally active, the parental "old" macronucleus in each cell becomes highly condensed, ceases transcription, and undergoes a process of degeneration and elimination from the cell (Wenkert and Allis 1984; Weiske-Benner and Eckert 1987).

One of the more striking differences between macro- and micronuclear core histones is the steady-state level of postsynthetic histone acetylation. Biochemical (Vavra et al. 1982; Chicoine and Allis 1986) and immunofluorescent (Lin et al. 1989; Pfeffer et al. 1989) analyses with antibodies specific to acetylated H4 have demonstrated that histone acetylation is specific to macronuclei at all stages of the life cycle except when micronuclei undergo periods of rapid replication and chromatin assembly. As expected, transcription-related acetylation begins selectively in new macronuclei as soon as these nuclei begin to differentiate from micronuclei. Unexpectedly, however, antibody staining is suddenly lost from parental macronuclei as soon as these nuclei become condensed, cease transcription, and are eliminated from the cells (Lin et al. 1989; Pfeffer et al. 1989). The mechanism(s) responsible for this sudden turnover of acetyl groups in "old" macronuclei during this stage of the life cycle is not known.

In this report biochemical and immunocytological evidence is presented documenting a developmentally regulated proteolytic processing event that specifically removes the amino terminus of each macronuclear core histone (with the possible exception of H2A). One consequence of this proteolysis is the removal of most of the lysines that undergo reversible acetylation. Data are also presented that histone H1 is present in old macronuclei during this time. Interestingly, H1 is rapidly and completely dephosphorylated, precisely as the macronucleus begins to condense and inactivate. To our knowledge, this report describes the first example of an in vivo proteolytic event that removes the amino termini of core histones in a chromatin containing intact H1. These data point toward a novel pathway of chromatin condensation and transcriptional silencing in *Tetrahymena* macronuclei. Whether such a pathway applies to other biological situations where condensed chromatin is known to exist is not known but exists as an intriguing possibility.

Results

Antibodies specific to the acetylated amino-terminal domain of histone H4 represent a powerful tool with which to probe the status of H4 acetylation in chromatin. When chromatin is refractory to biochemical purification or when cytological criteria are required to precisely define a developmental stage, indirect immunofluorescence with antibodies such as these provides an alternative means to evaluate changes in H4 acetylation. Recently, two independent studies reported the unexpected finding that staining with anti-acetylated H4 antibodies is lost from parental macronuclei during the sexual stage of the *Tetrahymena* life cycle when macronuclei condense, cease transcription, and begin to be eliminated from the cell (Lin et al. 1989; Pfeffer et al. 1989). Although neither of these studies shed any light on the mechanism(s) underlying this sudden and specific loss of acetyl groups from H4, both groups speculated that histone deacetylation was likely being induced in old macronuclei by a modulation in the enzyme activities responsible for histone acetylation and/or deacetylation.

Proteolytic processing of core histone amino termini

In this report a detailed series of immunofluorescent and biochemical analyses were carried out to study changes in histone acetylation during the development of the old macronucleus. For clarity in presentation, it is convenient to divide the development of old macronuclei into two arbitrary stages, I and II, according to their size, refractility under phase-contrast optics, and staining with the DNA-specific dye, DAPI (for a detailed ultrastructural characterization of the degradation and autolysis of parental macronuclei during the postzygotic period of conjugation, see Weiske-Benner and Eckert 1987). In stage I (which occurs ~8–9 hr after opposite mating types are mixed) parental macronuclei move into the posterior cytoplasm and begin to become pycnotic, but stain brightly with DAPI. In contrast, old macronuclei in

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stage II (after 9.0 hr) are considerably smaller and stain much more weakly with DAPI than do stage I macronuclei.

Figure 1 shows typical pairs at either of these stages examined by phase-contrast, DAPI–UV, and immunofluorescence microscopy. In both stages I (a–c) and II (d–f), developing new macronuclei (labeled an for macronuclear anlagen) stain positively with affinity-purified, anti-acetylated H4 antibodies, presumably due to the initiation of transcription-related acetylation in these nuclei (for details, see Lin et al. 1989; Pfeffer et al. 1989). In striking contrast, parental macronuclei (labeled om for old macronuclei) lose the staining with anti-acetylated H4 antibodies as they develop from stage I to stage II, suggesting a loss of acetyl groups from the amino-terminal domain of H4. However, when affinity-purified antibodies specific to the unacetylated amino-terminal domain of H4 were used in similar analyses, unexpected results were obtained. In stage I (g–i) all nuclei (including micronuclei) stain positively with these antibodies, in-

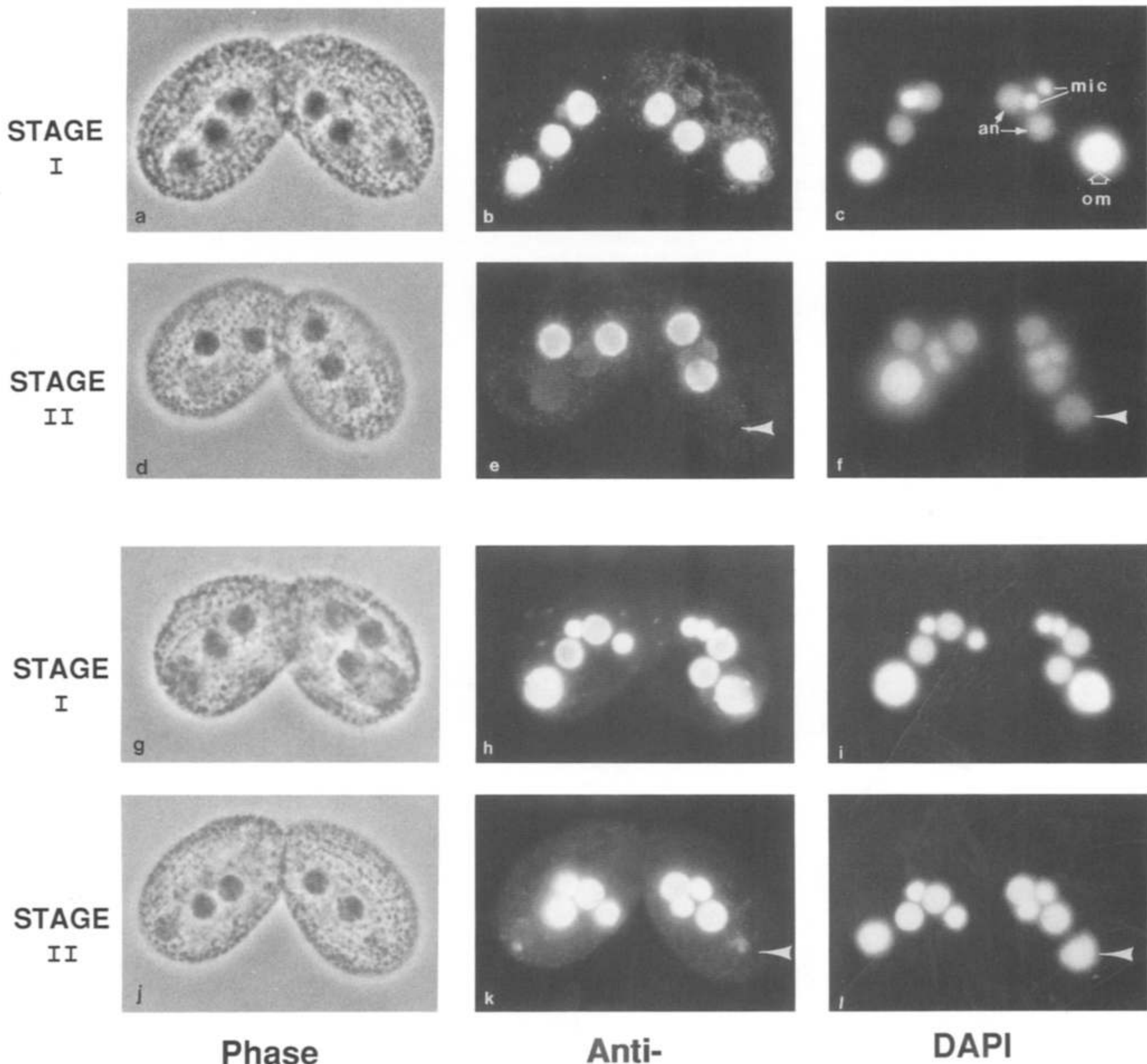

Figure 1. Immunofluorescent analyses of old macronuclei at different developmental stages with either anti-acetylated (*a–f*) or anti-unacetylated (*g–l*) H4 antibodies. Conjugating cells, at either developmental stage I [8–9 hr (*a–c, g–i*)], or II [9.5–12 hr (*d–f, j–l*)], were fixed and processed for indirect immunofluorescence as described previously (Wenkert and Allis 1984; Lin et al. 1989). The concentration of affinity-purified IgG used [anti-acetylated (*a–f*) or unacetylated (*g–l*) histone H4 antibodies] was 0.5 μg/ml in all cases. The second antibody used was rhodamine-conjugated goat anti-rabbit IgG. In addition, nuclei were stained with DAPI. Cells were examined with phase-contrast (*a,d,g,j*), or immunofluorescence (*b,e,h,k*), or DAPI-UV (*c,f,i,l*) microscopy. (an) Anlagen; (mic) micronuclei; (om) old macronuclei.

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dicating the presence of unacetylated H4 in all nuclei. However, as parental macronuclei progress into stage II of development (j–l) staining with the unacetylated H4 antibodies is lost (cf. g–i and j–l in Fig. 1). This unexpected result suggested two possibilities. First, changes in chromatin conformation and/or permeability of the nuclear envelope between stages I and II may limit the accessibility of antibodies to the amino termini of the core histones in old macronuclei. However, antibodies raised against other macronuclear proteins such as H1 (see Fig. 4, below) stain old macronuclei in stage II, suggesting that nuclear envelope permeability is not the reason for the loss of antibody staining. Second, the amino-terminal tails of H4 and possibly other core histones are being specifically removed by a proteolytic processing mechanism during the development of old macronuclei. Proteolytic removal of amino-terminal tails would account for the loss of both anti-acetylated (Fig. 1e) and anti-unacetylated (Fig. 1k) H4 staining seen in Figure 1. Although the amino-terminal domains of histones in core particles are excised selectively by digestion with proteases in vitro (for review, see van Holde 1988), to our knowledge proteolytic removal of core histone amino termini in vivo has not been described.

The hypothesis of proteolytic processing of core histone amino termini during the development of old macronuclei suggested by the immunofluorescent data in Figure 1 prompted us to look for biochemical evidence supporting such a mechanism. Initially, we asked whether faster-migrating (hence, smaller) peptides appeared in the core histone region of an SDS–polyacrylamide gel in a manner correlating temporally with the development of old macronuclei. Figure 2A shows the core histones from macronuclei isolated from either starved nonmating cells (St) or from conjugating cells (mat) at various stages of development. These results demonstrate that prominent faster-migrating bands under H2B and H4 (arrows pointing upward) appear approximately when old macronuclei switch from stage I to stage II. Importantly, these polypeptides are not apparent in starved cells or mating cells before the stage of old macronuclear development, suggesting that their formation is being developmentally regulated and is not the consequence of artifactual proteolysis during nucleus isolation and/or histone extraction.

None of our experiments demonstrated complete conversion of any of the core histones to smaller cleaved fragments. This could result from a variety of possibilities, including the persistence of nonmated cells (usually 10–20%) in our mass matings or the inherent asynchrony of mass matings (typically 30–60% of the cells are in any one given cytological stage; Martindale et al. 1982). As well, we cannot rule out the possibility that some cleavage products comigrate with intact histones in our one-dimensional gels.

To better fractionate all of the known macronuclear

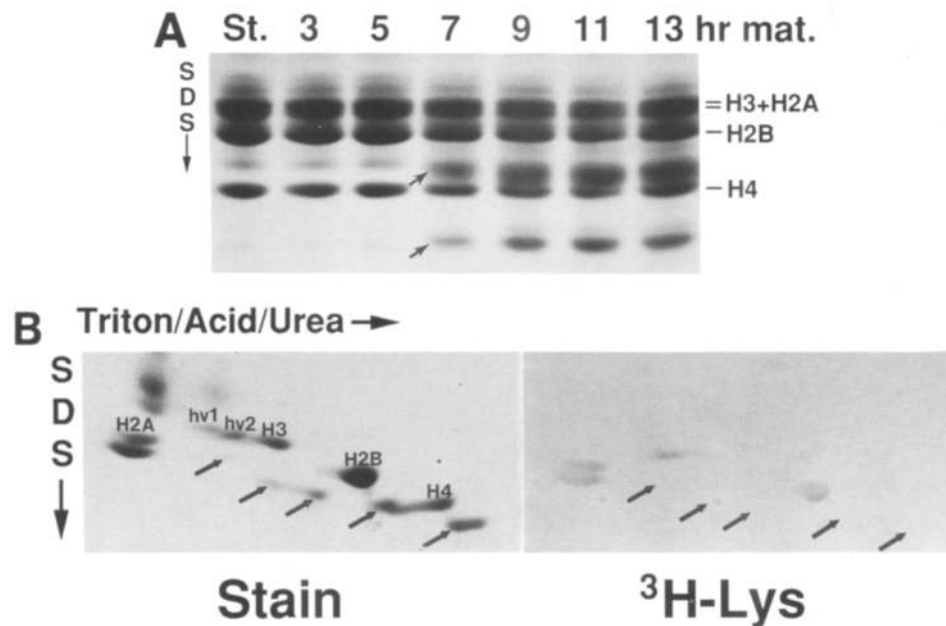

Figure 2. Developmental appearance of faster-migrating polypeptides correlates precisely with inactivation and condensation of macronuclei during late stages of conjugation. (*A*) Macronuclei or old macronuclei were purified from either starved (St) or mating (mat) cells at the times indicated by sedimentation at unit gravity. Histones were extracted and electrophoresed in a one-dimensional SDS–gel; only the core histone region of the gel is shown. Arrows indicate prominent polypeptides that appear under H4 and H2B in stages that correspond to the differentiation of old macronuclei (7–13 hr). (*B*) Ten-hour mating cells were labeled for 30 min with [^{3}H]lysine (2 μCi/ml, 50 Ci/mmole) prior to nucleus isolation and histone extraction. Acid extracts from old macronuclei (isolated by sedimentation at unit gravity and judged to be 80% pure by flow microfluorometry; Allis and Dennison 1982) were electrophoresed in a two-dimensional gel (Triton–acid–urea by SDS) and examined by staining (*left*) or fluorography (*right*). Arrows pointing upward indicate faster-migrating polypeptides under most of the core histones that are not observed in stages not containing old macronuclei. Only the core histone region of the two-dimensional gel is shown.

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core histones and histone variants, a two-dimensional (Triton–acid–urea by SDS) gel analysis was performed on acid-soluble protein extracted from an enriched population of old macronuclei purified from 10-hr mating cells (Fig. 2B). In addition to each of the known macronuclear histones, faster-migrating polypeptides were observed under most of the core histones (Fig. 2B; arrows pointing upward). The developmental appearance (Fig. 2A), abundance, and mobility of these polypeptides relative to core histones (and variants) on the two-dimensional gel suggest strongly that each might be either histone degradation products from pre-existing macronuclear histones or newly synthesized polypeptides of unknown identity that are being developmentally targeted to old macronuclei.

To distinguish these two possibilities, mating cells were labeled for 30 min with [^{3}H]lysine during stage II of old macronuclear development, and the acid-soluble proteins displayed in the stained two-dimensional gel shown in Figure 2B were also examined by fluorography ([^{3}H]lysine). The data presented in Figure 2B demonstrate clearly that each of the polypeptides migrating under the core histones (arrows pointing upward) is not being synthesized during this time interval. Thus, it seems more likely that these new polypeptides are being generated from pre-existing protein, presumably core histones. In addition, two-dimensional immunoblots with either anti-acetylated or anti-unacetylated H4 antibodies failed to react with any of these new polypeptides (data not shown). Taken together with our immunofluorescence results, these biochemical data strongly suggest (but do not prove; see below) that amino termini are being removed by proteolytic processing during the transition between stages I and II of old macronuclear development.

To determine unequivocally whether amino-terminal proteolysis is involved in the removal of core histone amino termini during the process of macronuclear condensation and inactivation, each of the faster-migrating sub-bands generated during stage II (see Fig. 3A) was excised from several two-dimensional gels, pooled separately, and transferred to Immobilon P membranes for direct microsequencing. Figure 3B shows the amino-terminal amino acid sequence of each of these polypeptides aligned with the published sequence of each corresponding *Tetrahymena* core histone or variant. In each case, the faster-migrating sub-band under each intact histone was determined to be an amino-terminal cleavage product of the adjacent, slower-migrating histone. Whereas the number of amino acids removed from each core histone is somewhat variable (varying from 13 to 21 residues), most of the lysines known to undergo postsynthetic acetylation in *Tetrahymena* (see Gorovsky 1986; Allis et al. 1986) are removed by these proteolytic cleavages.

Dephosphorylation of linker histone H1

During the formation of old macronuclei, two changes of biological significance occur—the hypercondensation of

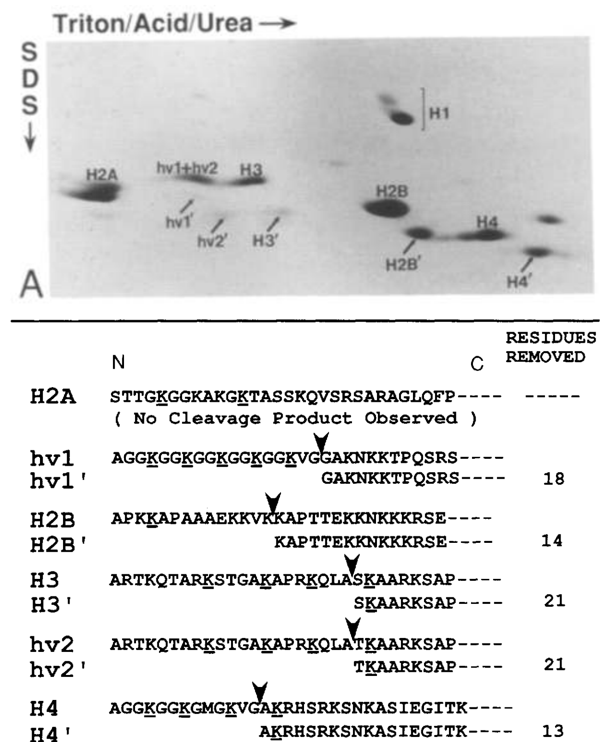

Figure 3. Proteolytic cleavage of histone amino termini in old macronuclei. (*A*) Acid extracts from purified old macronuclei of 11-hr mating cells were electrophoresed in several two-dimensional gels (Triton–acid–urea by SDS) and stained with Coomassie blue R. Each of the polypeptides (arrows pointing upward) with molecular masses 1–2 kD less than that of intact core histones was pooled separately from multiple two-dimensional gels, blotted to Imobilon P, and microsequenced directly as described in Materials and methods. (*B*) Amino-terminal sequences of intact core histones (for references, see Gorovsky 1986) and those derived from each of the faster-migrating polypeptides were aligned and are represented in single-letter amino acid code. The lysines (K) known to be acetylated in vivo (Gorovsky 1986) are underlined. Arrowheads indicate the apparent in vivo cleavage site in each core histone. (*Right*) Number of residues removed upon cleavage.

chromatin and the sudden inactivation of previous transcription—and both of these phenomena have been correlated with the presence of linker histone H1 in a wide variety of biological systems (for references, see van Holde 1988). However, H1 is known to be particularly sensitive to proteolysis in chromatin, even more so than the core histones. In light of the amino-terminal proteolytic processing of the core histones during the differentiation of old macronuclei (Figs. 1–3), it was of interest to examine the integrity of H1 in this chromatin. Figure 4 shows indirect immunofluorescent analyses with affinity-purified antibodies raised to macronuclear H1. Unlike the staining with either anti-acetylated or unacetylated H4 antibodies (Fig. 1), old macronuclei were stained positively with the H1 antibodies as long as they could be detected with DAPI staining. These data not only suggest that at least some epitopes in old macronu-

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clei are accessible to antibodies but, more importantly, that H1 is maintained in this chromatin until very late stages of macronuclear autolysis and degradation.

Further support for the presence of H1 in old macronuclei is provided in the two-dimensional gel shown in Figure 3A. In addition to the proteolytic products observed under each of the core histones, H1 is also present in an amount that approximates that observed in other stages of the life cycle (Allis and Wiggins 1984). However, the amount of electrophoretic heterogeneity typically observed with macronuclear H1 (see Roth et al. 1988) is not apparent, and most of the H1 migrates in a position corresponding to dephosphorylated H1 (dephosphorylated H1 migrates as a faster mobility subspecies in both acid urea and SDS–gels; for details, see Glover et al. 1981; Roth et al. 1988). Unfortunately, antibodies specific to phosphorylated or unphosphorylated *Tetrahymena* H1 do not exist; therefore, the status of H1 phosphorylation could not be analyzed by indirect immunofluorescence. Thus, we cannot rule out the possibility that artifactual dephosphorylation occurs during the time required to isolate old macronuclei from conjugating cells. However, methods exist to extract H1 directly from whole cells, exploiting the well-known solubility of H1 in dilute perchloric acid (PCA; see Schulman et al. 1987). Figure 5A shows whole-cell PCA-soluble proteins prepared from different time points of conjugating *Tetrahymena*. As reported previously, it is clear that H1 is hyperphosphorylated (slower migrating in SDS–gels; for details, see Schulman et al. 1987; Roth et al. 1988) during early stages of conjugation and that this level of H1 phosphorylation is maintained until 7–8 hr of conjugation, a period when stage I of old macronuclear differentiation is initiated. At 7–8 hr, a considerable amount of pre-existing H1 shifts to a faster-migrating species (Fig. 5A, arrow pointing upward), typical of fully dephosphorylated H1 (Glover et al. 1981; Roth et al. 1988). That this new polypeptide is not a proteolytic fragment generated from H1 is supported by the fact that its amino terminus, when sequenced from Immobilon blots, corresponds to that of intact macronuclear H1 (data not shown).

Further support for H1 dephosphorylation is provided by a metabolic labeling study with [^{32}P]phosphate. Cells of opposite mating type were labeled during growth for one generation with [^{32}P]orthophosphate, after which cells were starved and mated as usual. Shown in Figure 5B is the staining (bottom) and corresponding ^{32}P-labeling (top) profile of H1 extracted from macronuclei isolated from different time points of congugation. As development proceeds into stages corresponding to the formation of old macronuclei (7.5 and 8 hr), a significant fraction of the ^{32}P label is lost from the H1. Coincident

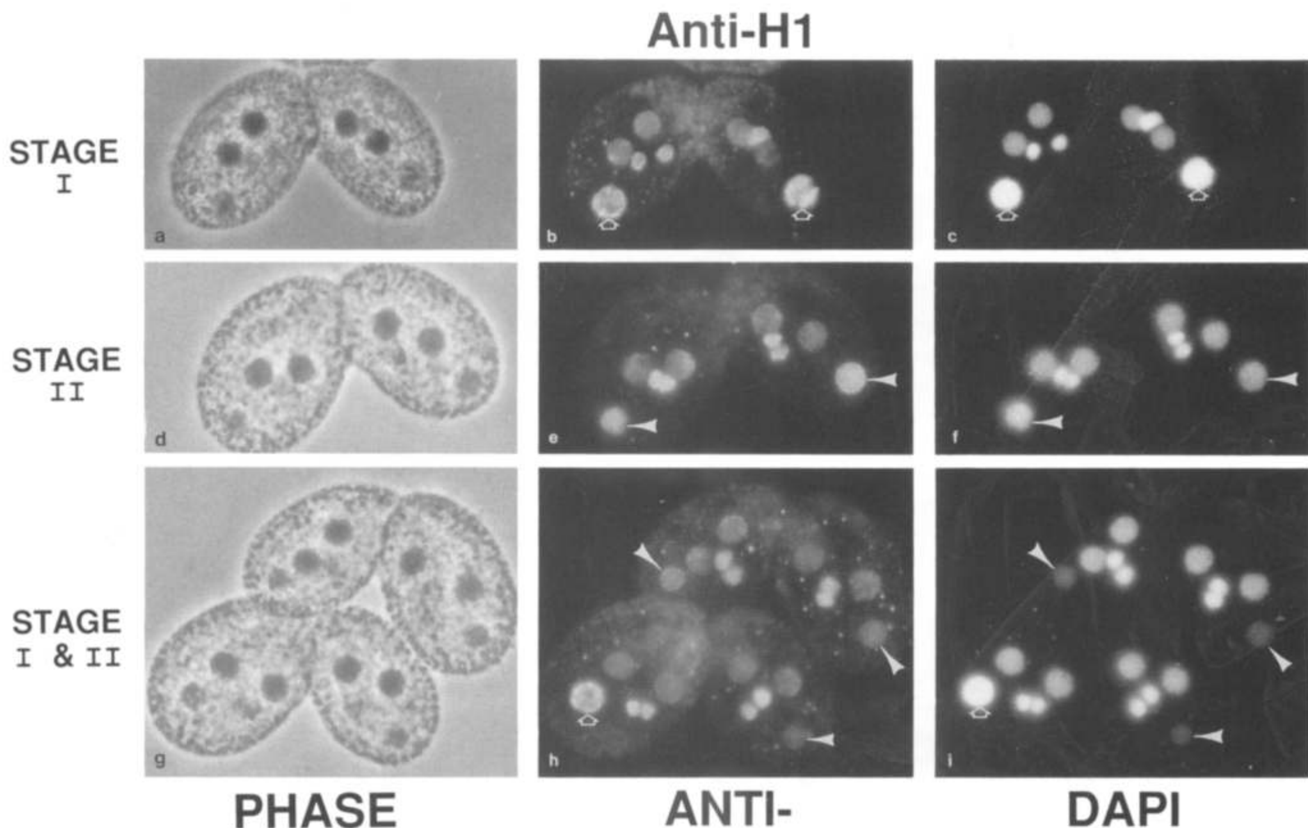

Figure 4. Retention of histone H1 in old macronuclei during stages I and II of development. Conjugating cells at either developmental stage I (*a–c*, *g–i*) or stage II (*d–f*, *g–i*) of old macronuclear development were analyzed by indirect immunofluorescence as in Fig. 1, with anti-*Tetrahymena* histone H1 antibodies.

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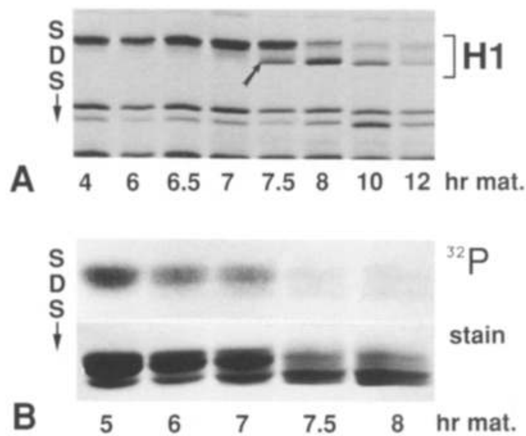

Figure 5. Dephosphorylation of histone H1 occurs precisely as macronuclei condense and inactivate during the differentiation of old macronuclei. (*A*) Total cellular PCA-soluble proteins were prepared from conjugating cells at different stages of development and analyzed on one-dimensional SDS–gels. The bracket indicates the histone H1 region. The electrophoretic heterogeneity observed results from different phosphorylated forms of histone H1; the arrow pointing upward indicates the position of dephosphorylated H1 (for details, see Glover et al. 1981; Roth et al. 1988). Identical results were obtained with purified populations of macronuclei (e.g., see Fig. 3A,B). (*B*) Growing cells were prelabeled with [^{32}P]orthophosphate before being starved and mated as usual. Shown is the staining (*bottom*) and corresponding ^{32}P-labeled autoradiograph (*top*) of H1 extracted from macronuclei isolated from different time points of conjugation. Only the H1 region of the gel is shown.

with this loss of label, a significant fraction of the stainable H1 shifts to a species with a faster SDS mobility. These data suggest that H1 is present in old macronuclear chromatin after a stage when core histone amino-terminal proteolysis has initiated. Strikingly, H1 undergoes a sudden and almost complete dephosphorylation precisely at the stage when parental macronuclei begin to condense and inactivate.

Discussion

Although amino-terminal domains of core histones are known to be accessible to proteolytic enzymes in vitro, this study is the first in vivo demonstration that the amino-terminal tails of core histones are proteolytically removed during the course of a "normal" program of nuclear differentiation. Although cleavage of amino-terminal tails may be a consequence of increased proteolysis from the programmed degradation of old macronuclei, H1 remains intact in these nuclei; thus, not all of the histones are undergoing random proteolysis. Core histone amino-terminal tails, along with H1, are thought to be involved in higher-order chromatin structure, and removal of these domains destabilizes chromatin fibers in vitro (Allan et al. 1982; Annunziato et al. 1988; Norton et al. 1989; Oliva et al. 1990). However, rigorous biochemical tests of these relationships are difficult because exposure of chromatin to proteases in vitro leads to the destruction of H1, which, in turn, influences the integrity of higher-order structure (Allan et al. 1982). During the formation of old macronuclei in *Tetrahymena*, intact H1 is present; thus, we speculate that H1 plays an essential role in the generation of condensed, inactive chromatin during this stage of macronuclear development.

Our in vivo data also support numerous in vitro experiments showing that amino-terminal domains of the core histones are selectively digested with proteases (van Holde 1988), presumably because these tails are both exposed and relatively unstructured in chromatin (Schroth et al. 1990). In agreement with other studies (Rill and Oosterhof 1982), no cleavage product was observed from either of the two major forms of macronuclear H2A. Interestingly, however, hv1, a relatively minor H2A variant, which is thought to associate with transcriptionally active chromatin (Allis et al. 1980; 1986), is cleaved during this stage of the life cycle (see Fig. 3). Preferential susceptibility of hv1 in chromatin could reflect a preferential distribution of hv1 in more open (unfolded) regions of chromatin. Thus, our ability to detect preferential cleavage products may reflect the distribution of histones between active and inactive regions of chromatin.

Proteolytic cleavage of the amino-terminal tails of core histones suddenly removes most of the lysine residues that undergo reversible acetylation in *Tetrahymena* (see Fig. 3B). Histone acetylation has long been believed to dampen the interaction of core histone amino termini with DNA leading to chromatin decondensation. Similarly, proteolytic removal of amino termini in old macronuclei would, like histone acetylation, abolish much of the positive charge in this domain of the core histones. Genetic experiments in yeast have shown that certain deletions of the H4 amino terminus cause specific derepression of the silent mating-type loci (Kayne et al. 1988). Therefore, it would seem that the loss of amino termini by proteolysis should cause unfolding of the chromatin and subsequent transcriptional activation rather than chromatin condensation and transcription silencing that we observe in old macronuclei. However, it is possible that amino-terminal deletions of H4 abolish an interaction with a yet unidentified repressor of the silent mating-type loci. The existence of H1-like protein(s) in yeast remains controversial (for references, see Grunstein 1990; Smith 1991). Whether mating-type repression in yeast involves a H1-like molecule is not known, but the presence of H1 in old macronuclear chromatin may explain, in part, apparent differences between the two systems.

Our data not only demonstrate that H1 is present in macronuclear chromatin as it undergoes inactivation but also that H1 is dephosphorylated during the initial stages of old macronuclear formation. Although this finding seems to contradict the popular idea that H1 phosphorylation leads to chromatin condensation during mitosis (Bradbury et al. 1974a,b), direct evidence linking H1 phosphorylation to chromatin condensation is lacking (for discussion, see Roth et al. 1988, 1991). Furthermore,

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H1 dephosphorylation has been observed in other biological systems where condensed chromatin and transcriptional silencing occur (Sung 1977; Green and Poccia 1985; Hill et al. 1990). Dephosphorylation of H1 would increase the net positive charge on the amino- and carboxy-terminal H1 tails which, in turn, may cause an increased electrostatic interaction between H1 and the negatively charged DNA backbone. Proteolytic removal of core histone amino termini during this period may promote H1–DNA interaction by exposing regions of the DNA backbone previously engaged with core histone amino termini (Hill and Thomas 1990).

If the removal of core histone amino termini plays a significant role in the formation of condensed chromatin in old macronuclei, the question remains whether this phenomenon is used in any other biological system where heterochromatin is known to exist, such as position effect variegation in *Drosophila* or X-inactivation in female mammalian cells. Inasmuch as proteolytic processing of histone amino termini is an irreversible event, it may take place only in chromatin that is being silenced irreversibly. In the case of *Tetrahymena*, macronuclei undergo complete autolysis, and degradation products from this nucleus are likely used to support the development of new macronuclei. Thus, proteolytic removal of core histone amino termini may result from a programmed degradation specific to old macronuclei in protozoa. However, it remains an intriguing possibility that proteolytic processing of histone amino termini plays a previously unrecognized role in the formation of condensed chromatin and transcriptional silencing in other biological situations. To that end, we believe that the antibodies against acetylated and unacetylated H4 amino termini used in this study represent powerful tools with which to investigate this possibility in other systems.

Materials and methods

Cell culture and isolation of nuclei

Genetically marked strains of *T. thermophila*, CU427 [Mpr/Mpr(6-mp-s VI)], and CU428 [Chx/Chx(cys VII)] were used in all experiments reported here. The strains were kindly provided by P. Brun (Cornell University, Ithaca, NY). Cells were grown axenically in 1% enriched proteose peptone, as described previously (Gorovsky et al. 1975). All matings (typically 90% pairing) were performed in 10 mM Tris (pH 7.4), according to Bruns and Brussard (1974), as modified by Allis and Dennison (1982). All cultures were maintained at 30°C. To investigate the phosphorylation profile of H1 isolated from mating cells, cells of opposite mating type were prelabeled with [^{32}P]orthophosphate (10 μCi/ml) during growth for one cell generation prior to conjugation. These cells were then starved and mated as usual. In all cases, nuclei were prepared from cells by using the methods of Gorovsky et al. (1975) and purified further by differential centrifugation and sedimentation at unit gravity according to Allis and Dennison (1982). The purity and DNA ploidy of various nuclear preparations were determined by flow microfluorometry (Allis and Dennison 1982). Nuclei were used immediately or stored at −80°C for further use.

Extraction of histone, gel electrophoresis, and amino acid sequencing

Histones were extracted from nuclei according to procedures described previously (Allis et al. 1979), taking all precautions to avoid artifactual protein losses. One-dimensional (SDS) or two-dimensional electrophoresis (Triton–acid–urea by SDS) was performed as described previously (Allis et al. 1979, 1980). PCA-soluble proteins were extracted from total cells and resolved on 12% SDS–gels as described previously (Schulman et al. 1987). Following electrophoresis, gels were stained with Coomassie brilliant blue R, destained, photographed and, where appropriate, fluorographed. Individual stained proteins were cut from two-dimensional gels, pooled separately, run through an additional 12% SDS–gel, and transferred electrophoretically to Immobilon P filters. Blots were stained with Coomassie brilliant blue R and destained before protein spots were excised and microsequenced as described previously (Allis et al. 1986).

Affinity purification of antibody

All antibodies used in this paper were purified by chromatography on protein A columns followed by affinity chromatography with specific peptide antigens. Peptides corresponding to unacetylated or acetylated amino terminus of histone H4 (Lin et al. 1989) were coupled to an agarose support via the carboxy-terminal sulfhydryl group according to the manufacturer's instructions (Sulflink Ag/Ab Immobilization kit, Pierce). *Tetrahymena* histone H1 was purified by reverse-phase (C8) high-performance liquid chromatography (HPLC) and coupled to cyanogen bromide-activated agarose beads as instructed by the manufacturer (Pharmacia LKB). The extents of purification of the different antibodies were determined by immunoblotting analyses (Lin et al. 1989).

Immunofluorescence microscopy

Mating cells of different stages were fixed for immunomicroscopy as described previously (Wenkert and Allis 1984). Cells were incubated with affinity-purified antibodies, followed by incubation with rhodamine-conjugated goat anti-rabbit antibodies (Pierce) and the DNA-specific dye, DAPI, as described previously (Lin et al. 1989).

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References

Allan, J., N. Harborne, D.C. Rau, and H. Gould. 1982. Participation of core histone "tails" in the stabilization of the chromatin solenoid. *J. Cell. Biol.* **93:** 285–297.

Allfrey, V.G. 1977. Post-synthetic modifications of histone structure: A mechanism for the control of chromosome structure by the modulation of histone-DNA interactions. In *Chromatin and chromosome structure* 2nd ed. (ed. J. Li and R. Eckhadt), pp. 167–191. Academic Press, New York.

Allis, C.D. and D.K. Dennison. 1982. Identification and purification of young macronuclear anlagen from conjugating

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

cells of *Tetrahymena thermophila*. *Dev. Biol.* **93:** 519–533.

Allis, C.D. and J.C. Wiggins. 1984. Histone rearrangements accompany nuclear differentiation and dedifferentiation in *Tetrahymena*. *Dev. Biol.* **101:** 282–294.

Allis, C.D., C.V.C. Glover, and M.A. Gorovsky. 1979. Micronuclei of *Tetrahymena* contain two types of histone H3. *Proc. Natl. Acad. Sci.* **76:** 4857–4861.

Allis, C.D., C.V.C. Glover, J.K. Bowen, and M.A. Gorovsky. 1980. Histone variants specific to the transcriptionally active, amitotically dividing macronucleus of the unicellular eucaryote, *Tetrahymena thermophila*. *Cell* **20:** 609–617.

Allis, C.D., R. Richman, M.A. Gorovsky, Y.S. Ziegler, B. Touchstone, W. A. Bradley, and R.G. Cook. 1986. hv1 is an evolutionarily conserved H2A variant that is preferentially associated with active genes. *J. Biol. Chem.* **261:** 1941–1948.

Annunziato, A.T., L.-L.Y. Frado, R.L. Seale, and C.L.F. Woodcock. 1988. Treatment with sodium butyrate inhibits the complete condensation of interphase chromatin. *Chromosoma* **96:** 132–138.

Ausio, J., F. Dong, and K.E. van Holde. 1989. Use of selectively trypsinized nucleosome core particles to analyze the role of the histone "tails" in the stabilization of the nucleosome. *J. Mol. Biol.* **206:** 451–64.

Böhm, L., C. Crane-Robinson, and P. Sautiere. 1980. Proteolytic digestion studies of chromatin core-histone structure. Identification of a limit peptide of histone H2A. *Eur. J. Biochem.* **106:** 525–530.

Böhm, L., P. Sautiere, G. Briand, and C. Crane-Robinson. 1981. Proteolytic digestion studies of chromatin core-histone structure. Identification of a limit peptide of histone H3 and H4. *Eur. J. Biochem.* **119:** 67–74.

Böhm, L., G. Briand, P. Sautiere, and C. Crane-Robinson. 1982. Proteolytic digestion studies of chromatin core-histone structure. Identification of limit peptides from histone H2B. *Eur. J. Biochem.* **123:** 299–303.

Bradbury, E.M., R.J. Inglis, and H.R. Matthews. 1974a. Control of cell division by very lysine rich histone (fl) phosphorylation. *Nature* **247:** 257–261.

Bradbury, E.M., R.J. Inglis, H.R. Matthews, and T.A. Langan. 1974b. Molecular basis of control of mitotic cell division in eukaryotes. *Nature* **249:** 553–556.

Bruns, P.J. and T.B. Brussard. 1974. Pair formation in *Tetrahymena pyriformis*, an inducible developmental system. *J. Exp. Zool.* **183:** 337–344.

Chicoine, L.G., and C.D. Allis. 1986. Regulation of histone acetylation during macronuclear differentiation in Tetrahymene. Evidence for control at the level of acetylation and deacetylation. *Dev. Biol.* **116:** 477–485.

Chicoine, L.G., D. Wenkert, R. Richman, J.C. Wiggins, and C.D. Allis. 1985. Modulation of linker histones during development in *Tetrahymena*: Selective elimination of linker histone during the differentiation of new macronuclei. *Dev. Biol.* **109:** 1–8.

Dumuis-Kervabon, A., I. Encontre, G. Etienne, J. Jauregui-Adell, J. Mery, D. Mesnier, and J. Parello. 1986. A chromatin core particle obtained by selective cleavage of histones by clostripain. *EMBO J.* **5:** 1735–1742.

Glover, C.V.C., K.J. Vavra, S.D. Guttman, and M.A. Gorovsky. 1981. Heat shock and deciliation induce phosphorylation of histone H1 in *T. pyriformis*. *Cell* **23:** 73–77.

Gorovsky, M.A. 1973. Macro- and micronuclei of *Tetrahymena pyriformis*: A model system for studying the structure and function of eukaryotic nuclei. *J. Protozool.* **20:** 19–25.

——— 1986. Ciliate chromatin and histones. In *The molecular biology of ciliate protozoa* (ed. J.G. Gall), pp. 227–261. Academic Press, London.

Gorovsky, M.A., M. -C. Yao, J.B. Keevert, and G.L. Pleger. 1975. Isolation of micro- and macronuclei of *Tetrahymena pyriformis*. *Methods Cell Biol.* **9:** 311–327.

Green, G.R. and D.L. Poccia. 1985. Phosphorylation of sea urchin sperm H1 and H2B histones precedes chromatin decondensation and H1 exchange during pronuclear formation. *Dev. Biol.* **108:** 235–245.

Grunstein, M. 1990. Histone function in transcription. *Annu. Rev. Cell Biol.* **6:** 643–678.

Hebbes, T.R., A.W. Thorne, and C. Crane-Robinson. 1988. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* **71:** 1395–1402.

Hill, C.S. and J.O. Thomas. 1990. Core histone-DNA interactions in sea urchin sperm chromatin. The N-terminal tails of H2B interacts with linker DNA. *Eur. J. Biochem.* **1987:** 145–153.

Hill, C.S., L.C. Packman, and J.O. Thomas. 1990. Phosphorylation at clustered -Ser-Pro-X-Lys/Arg- motifs in sperm-specific histones H1 and H2B. *EMBO J.* **9:** 805–813.

Kayne, P.S., U.-J. Kim, M. Han, J. Mullen, F. Yoshizaki, and M. Grunstein. 1988. Extremely conserved histone H4 amino terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. *Cell* **55:** 27–39.

Lin, R., J.W. Leone, R.G. Cook, and C.D. Allis. 1989. Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in *Tetrahymena*. *J. Cell Biol.* **108:** 1577–1588.

Martindale, D.W., C.D. Allis, and P.J. Bruns. 1982. Conjugation in *Tetrahymena thermophila*: A temporal analysis of cytological stages. *Exp. Cell Res.* **140:** 227–236.

Matthews, H.R. and J.H. Waterborg. 1985. Reversible modification of nuclear proteins and their significance. In *The enzymology of post-translational modifications of proteins* (ed. R.B. Freedman and H.C. Hawkins), pp. 125–185. Academic Press, Orlando.

McGhee, J.D. and G. Felsenfeld. 1980. Nucleosome structure. *Annu. Rev. Biochem.* **49:** 1115–1156.

Megee, P.C., B.A. Morgan, B.A. Mittman, and M.M. Smith. 1990. Genetic analysis of histone H4: Essential role of lysines subject to reversible acetylation. *Science* **247:** 841–845.

Norton, V.G., B.S. Imai, P. Yau, and E.M. Bradbury. 1989. Histone acetylation reduces nucleosome core particle linking number change. *Cell* **57:** 449–457.

Oliva, R., D.P. Bazett-Jones, L. Locklear, and G.H. Dixon. 1990. Histone hyperacetylation can induce unfolding of the nucleosome core particle. *Nucleic Acids Res.* **18:** 2739–2747.

Park, E.C. and J.W. Szostak. 1990. TI point mutations in the yeast histone H4 gene prevent silencing of the silent mating type locus HML. *Mol. Cell. Biol.* **10:** 4932–4934.

Pfeffer, U., N. Ferrari, E. Tosetti, and G. Vidali. 1989. Histone acetylation in conjugating *Tetrahymena*. *J. Cell Biol.* **109:** 1007–1014.

Rill, R.L. and D.K. Oosterhof. 1982. The accessibilities of histones in nucleosome cores to an arginine-specific protease. *J. Biol. Chem.* **257:** 14875–14880.

Roth, S.Y., I.G. Schulman, R. Richman, R.G. Cook, and C.D. Allis. 1988. Characterization of phosphorylation sites in histone H1 in the amitotic macronucleus of *Tetrahymena* during different physiological states. *J. Cell Biol.* **107:** 2473–2482.

Roth, S.Y., M.P. Collini, G. Draetta, D. Beach, and C.D. Allis. 1991. A cdc^2-like kinase phosphorylate histone H1 in the amitotic macronucleus of *Tetrahymena*. *EMBO J.* **10:** 2068–2075.

Schroth, G.P., P. Yau, B.S. Imai, J.M. Gatewood, and E.M. Brad-

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

bury. 1990. A NMR study of mobility in the histone octamer. *FEBS Lett.* **268:** 117–120.

Schulman, I.G., R.G. Cook, R. Richman, and C.D. Allis. 1987. *Tetrahymena* contain two distinct and unusual high mobility group (HMG)-like proteins. *J. Cell Biol.* **104:** 1485–1493.

Schuster, T., M. Han, and M. Grunstein. 1986. Yeast histone H2A and H2B amino termini have interchangeable functions. *Cell* **45:** 445–451.

Smith, M.M. 1991. Histone structure and function. *Curr. Opin. Cell Biol.* **3:** 429–437.

Sung, M.T. 1977. Phosphorylation and dephosphorylation of Histone V (H5): Controlled condensation of avian erythrocyte chromatin. *Biochemistry* **16:** 286–290.

van Holde, K.E. 1988. *Chromatin*. Springer-Verlag, New York.

Vavra, K.K., C.D. Allis, and M.A. Gorovsky. 1982. Regulation of histone acetylation in *Tetrahymena* macro- and micronuclei. *J. Biol. Chem.* **257:** 2591–2598.

Weiske-Benner, A. and W.A. Eckert. 1987. Differentiation of nuclear structure during the sexual cycle in *Tetrahymena thermophila*. II. Degeneration and autolysis of macro- and micronuclei. *Differentiation* **34:** 1–12.

Wenkert, D. and C.D. Allis. 1984. Timing of the appearance of macronuclear-specific histone variant hv1 and gene expression of developing new macronuclei of *Tetrahymena thermophila*. *J. Cell Biol.* **98:** 2107–2117.

Whitlock, J.P. and A. Stein. 1978. Folding of DNA by histones which lack their NH_2-terminal regions. *J. Biol. Chem.* **253:** 3857–3861.

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Proteolytic removal of core histone amino termini and dephosphorylation of histone H1 correlate with the formation of condensed chromatin and transcriptional silencing during Tetrahymena macronuclear development.

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