Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*

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Vegetative cells of the ciliated protozoan *Tetrahymena thermophila* contain a transcriptionally active macronucleus and a transcriptionally inactive micronucleus. Although structurally and functionally dissimilar, these nuclei are products of a single postzygotic division during conjugation, the sexual phase of the life cycle. Immunocytochemical analyses during growth, starvation, and conjugation were used to examine the nuclear deposition of hv1, a histone H2A variant that is found in macronuclei and thought to play a role in transcriptionally active chromatin. Polyclonal antisera were generated using whole hv1 protein and synthetic peptides from the amino and carboxyl domains of hv1. The transcriptionally active macronuclei stained at all stages of the life cycle. Micronuclei did not stain during growth or starvation but stained with two of the sera during early stages of conjugation, preceding the stage when micronuclei become transcriptionally active. Immunoblot analyses of fractionated macro- and micronuclei confirmed the micronuclear acquisition of hv1 early in conjugation. hv1 staining disappeared from developing micronuclei late in conjugation. Interestingly, the carboxy-peptide antiserum stained micronuclei only briefly, late in development. The detection of the previously sequestered carboxyl terminus of hv1 may be related to the elimination of hv1 during the dynamic restructuring of micronuclear chromatin that occurs as the micronucleus enters a transcriptionally incompetent state that is maintained during vegetative growth. These studies demonstrate that the transcriptional differences between macro- and micronuclei are associated with the loss of a chromatin component from developing micronuclei rather than its de novo appearance in developing macronuclei and argue that hv1 functions in establishing a transcriptionally competent state of chromatin.

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Meiosis and gamete formation, followed by fertilization, determination, and differentiation are programmed developmental events in higher eukaryotes, where individual gametes unite and the zygote proceeds through embryogenesis to produce morphologically and functionally distinct tissues. These developmental events also occur during sexual reproduction in the unicellular ciliate, *Tetrahymena thermophila*. However, as opposed to the

morphologically and functionally distinct tissues generated at the endpoint of sexual reproduction in higher eukaryotes, conjugation in *Tetrahymena* generates morphologically and functionally distinct nuclei that reside in a single cell.

Tetrahymena, like all ciliates, have two types of nuclei: a diploid micronucleus that serves as the germ line of the cell, and a polyploid somatic macronucleus that controls the phenotype of the cell and is destroyed during the sexual process of conjugation (Gorovsky 1973,

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1980). During vegetative growth, the micronucleus divides mitotically and is transcriptionally inert while the macronucleus divides amitotically and is transcriptionally active. Differences in chromatin composition between the two types of nuclei are associated with their functional dichotomy. Although both nuclei possess the four core histones (H2A, H2B, H3, and H4; for review, see Gorovsky 1986), these proteins are highly acetylated in macronuclei while histone acetylation is greatly reduced in micronuclei (Vavra et al. 1982; Chicoine et al. 1987). In addition, the macro- and micronuclei contain dramatically different linker proteins. Macronuclei have an H1-like protein that has many properties characteristic of H1 in higher organisms (Gorovsky et al. 1974). H1 is lacking in micronuclei (Gorovsky et al. 1975; Johmann and Gorovsky 1976). Instead, micronuclei contain a set of unique linker-associated polypeptides (α , β , γ , and δ) that lack typical H1-like properties (Allis et al. 1984).

Macronuclei also contain two minor histone variants, hv1 (an H2A variant) and hv2 (an H3 variant), which are absent from micronuclei of vegetative cells (Allis et al. 1980b). Antibodies specific to hv1 cross-react on Western blots with acid-soluble nuclear proteins from animal (mouse), plant (wheat), and fungal (*Saccharomyces cerevisiae*) kingdoms (Allis et al. 1986). Sequence analysis (Allis et al. 1986; White et al. 1988; Van Daal et al. 1990) indicates that the central core region of hv1 is strikingly similar to H2A.F of chicken (Harvey and Whiting 1983; Dalton et al. 1989), H2A.Z of mammals (Hatch and Bonner 1988, 1990), and H2A.F/Z of sea urchins (Ernst et al. 1987). An hv1 cDNA clone was used to clone H2AvD, an H2A variant of *Drosophila melanogaster* (Van Daal et al. 1988, 1990), which is encoded by an essential, single-copy gene (Van Daal and Elgin 1992). Comparison of the derived protein sequences of the major and variant H2As led to the surprising observations that the variants in different organisms are more similar to each other than each is to the major H2A in the same organism (White et al. 1988; Van Daal et al. 1990), and that the variant H2As actually show less evolutionary divergence than the major H2As. These results indicate that there were two types of H2A genes in primitive eukaryotes before the divergence of the major eukaryotic groups and that the major and variant H2As have been under different selective pressures since that time. Therefore, the major and variant H2As must have distinct and important functions.

Analysis of histone proteins in a variety of organisms has identified a variant for every major histone type (Wu and Bonner 1981; Wu et al. 1982; Zweidler 1984; Schümperli 1986; Wu et al. 1988; Sadler and Brunk 1992). The existence of histone variants with synthesis that is temporally or developmentally regulated suggests the possibility that nucleosomes containing different variants may perform distinct functions. Coupled with the fact that nucleosomes are general repressors of transcription (for review, see McGhee and Felsenfeld 1980; Grunstein 1990) and that mutations in H4 can affect repression and activation of specific genes (Park and Szostak 1990), one possible function for variant nucleo-

somes may be a role in the regulation of transcriptional activity of specific genes.

There is circumstantial evidence that hv1 plays a role in the establishment of a transcriptionally competent state of chromatin. Immunofluorescence studies using an hv1-specific antiserum demonstrated that determinants recognized by these antibodies are highly concentrated in small dots in mammalian nucleoli, presumably in the chromatin containing highly active ribosomal genes (Allis et al. 1982). hv1 determinants are also associated with a subset of loci in *Drosophila* polytene chromosomes that are, were, or will be active during the third larval instar or prepupal stage of development (S. Elgin, pers. comm.). Coupled with the specific localization of hv1 in transcriptionally active macronuclei of vegetative *Tetrahymena*, these studies suggest that hv1 and proteins antigenically related to it in mammalian cells and *Drosophila* are preferentially associated with transcriptionally active or potentially active (transcriptionally competent) chromatin.

It has been shown that micronuclei are transcriptionally active for a brief period of time, during meiotic prophase in early conjugation (Sugai and Hiwatashi 1974; Martindale et al. 1985). Recent observations show that TATA-binding protein (TBP), a component necessary for transcription by all three nuclear RNA polymerases (for review, see White and Jackson 1992; Rigby 1993), is acquired by micronuclei just before this period of transcriptional activity (Stargell and Gorovsky 1993). Anti-TBP antibodies stain micronuclei just before and continue to stain micronuclei after this period of transcriptional activity, whereas micronuclei in starved and growing cells do not stain. Previous immunocytochemical studies using anti-hv1 antibodies generated against gel-purified whole hv1 protein (anti-hv1-gel serum) demonstrated a macronuclear-specific localization of hv1 during growth and starvation; hv1 was also found to accumulate in developing macronuclei late during conjugation, approximately coincident with the acquisition of transcriptional activity in these nuclei (Wenkert and Allis 1984). However, hv1 staining during early stages of conjugation was not detected in these initial studies. Therefore, transcription in prophase micronuclei appeared to occur in the absence of hv1, casting doubt on the association of hv1 with transcriptionally competent chromatin.

We wished to reexamine the nuclear distribution of hv1 using recently obtained hv1 antisera of higher titer and the paraformaldehyde fixation technique used in the anti-TBP studies. Three polyclonal antisera were generated against different hv1 immunogens: HPLC-purified whole hv1 protein, an amino-proximal peptide, and a carboxy-terminal peptide. Mature macronuclei stain with all three antisera during most stages of the life cycle. Loss of mature macronuclear staining occurs coincident with the degradation and eventual elimination of the parental macronucleus during late conjugation. Thus, macronuclear staining is consistent with postulated association of hv1 with transcriptionally competent chromatin. The transcriptionally inert micronuclei

did not stain during growth or starvation; however, micronuclei did stain with anti-hv1-HPLC and anti-amino sera during conjugation. The onset of micronuclear staining precedes the brief window of transcriptional activity observed during meiotic prophase (Sugai and Hiwatashi 1974; Martindale et al. 1985), consistent with the hypothesis that hv1 acquisition is important for transcriptional potential. Biochemical purification of both macronuclei and micronuclei, followed by immunoblot analyses confirmed the developmentally regulated acquisition of hv1 during early conjugation. Surprisingly, the immunocytochemical staining of hv1 persisted even after micronuclei stopped synthesizing RNA and only disappeared from developing micronuclei late in conjugation, coincident with the onset of transcription in developing macronuclei and the degeneration of the parental macronucleus. In contrast, anti-carboxy antibodies stained micronuclei only briefly, late in development, just before loss of detection of hv1 by all three sera. We suggest that the carboxyl terminus of this histone is sequestered in micronuclei early in conjugation and that its detection in later stages may be attributable to a change in conformation or to loss of associated proteins preceding its degradation. These results argue strongly that hv1 is associated with transcriptional competence and demonstrate that micronuclear chromatin is actively restructured late in development to eliminate proteins specific to macronuclei in vegetative cells.

Results

Generation and characterization of hv1 antisera

Three polyclonal antisera were generated to distinct immunogens of the hv1 protein (Fig. 1a). HPLC-purified hv1 protein was used to generate an antiserum against the whole protein (anti-hv1-HPLC). Anti-amino serum was produced by immunization with a peptide corresponding to amino acids 17–28, whereas anti-carboxy serum was generated against a peptide corresponding to amino acids 129–145 (White et al. 1988). The specificity of each antibody was investigated by immunoblot assays against histone preparations separated by SDS-PAGE. Under the conditions described, all three immune antisera reacted solely with the hv1 band (Fig. 1b–d). When histone preparations were separated by triton/acid/urea-PAGE (Allis et al. 1979; Allis et al. 1980a), which alters the relative mobility of the histones, staining was again specific to the hv1 region (data not shown). These immunoblot results indicate all three sera are highly specific to the minor histone variant, hv1.

Competition experiments using hv1 synthetic peptides in immunoblot assays were performed to determine whether the three sera recognize distinct antigens of the hv1 protein. Detection of hv1 with anti-hv1-HPLC serum is not competed with amino-proximal peptide. Carboxy-terminal peptide depletes the staining significantly but does not remove it (Fig. 1b). Competition with a peptide corresponding to the variant box (amino acids 48–59) also diminishes the staining slightly. A

Figure 1. Characterization of hv1 antisera and competition with peptides in immunostaining. (a) Diagram of hv1 protein. Relative amino acid positions of the synthetic peptides are depicted by shaded boxes. (b–d) *Tetrahymena* histones were separated on a SDS–15% polyacrylamide gel and electroblotted to nitrocellulose, which was cut into strips. In some cases, as listed below, immune sera were preincubated with a 100-fold molar excess of peptide. Serum reaction mixes applied to the strips were preimmune (P); immune (I); immune plus amino peptide (A); immune plus variant box peptide (V); immune plus carboxy peptide (C); immune plus all three peptides (T). Antisera used: anti-hv1-HPLC (b); anti-amino (c); anti-carboxy (d). The positions of the histones are indicated on the Ponceau S-stained strip (M). All three immune sera (I) stain only hv1; preimmune sera (P) do not stain any histone bands.

mixture of all three peptides does not totally abolish anti-hv1-HPLC reactivity with hv1 protein, suggesting that although some of the antibodies in this polyclonal serum recognize the carboxyl terminus and variant box of hv1, others must detect different epitopes. In contrast, anti-amino and anti-carboxy antibodies can be competed completely by their respective peptides, with no effect by the alternate peptide (Fig. 1c,d).

Immunocytochemical staining of growing and starved cells with anti-hv1-HPLC is macronuclear specific

The subcellular localization of hv1 was determined by fluorescence microscopy of growing or starved cells after fixation, incubation with anti-hv1-HPLC antibodies, and detection with fluorescein-labeled secondary antibodies. Staining with anti-hv1-HPLC was found to be localized solely to macronuclei in both growing and starved cells (Fig. 2).

Anti-hv1-HPLC serum also stains micronuclei in early conjugation

During conjugation, the sexual stage of the life cycle, micronuclei undergo an extended period of meiosis, followed by nuclear exchange, fertilization, postzygotic divisions, and differentiation to eventually produce new macro- and micronuclei. At the end of this process, the parental macronucleus in each cell is degraded. As ex-

Figure 2. Macronuclear-specific localization of hv1 in growing and starved *Tetrahymena*. Immunofluorescent images of a growing cell stained with DAPI (a) and with anti-hv1-HPLC (b). A starved cell stained with DAPI (c) and anti-hv1-HPLC (d). The arrow indicates the micronucleus. Bar, 10 μ m.

pected, anti-hv1-HPLC stains the parental macronucleus throughout development until these nuclei cease transcriptional activity and begin to degenerate (Figs. 3 and 4). Surprisingly, this serum also stains micronuclei during both meiotic and gametic stages (Fig. 3). This staining appears in early meiosis (~ 1.5 hr after mixing cells of opposite mating types) and persists through crescent stage and gametogenesis. The onset of micronuclear staining with anti-hv1-HPLC precedes the onset of transcriptional activity in these nuclei, which is observable after the micronuclei begin to elongate (the stage shown in Fig. 3c; Sugai and Hiwatashi 1974; Martindale et al. 1985). Detectable levels of transcription cease by the time the nuclei are fully elongated and crescent shaped (the stage shown in Fig. 3e). However, hv1 staining persists long after micronuclear transcription ceases. Micronuclear staining is detectable in the products of the second postzygotic division, even those nuclei positioned in the posterior of the cell, which are destined to develop into mature micronuclei (Fig. 4b). Staining with anti-hv1-HPLC is lost from micronuclei and becomes specific to developing macronuclei coincident with the onset of transcription from the new macronuclei and the degradation of the parental macronucleus (Fig. 4f). The loss of hv1 staining in developing micronuclei occurs after they have assumed a position in the center of the cell, in close proximity to the still positive macronuclei.

Pre-immune serum does not stain any subcellular structures in *Tetrahymena* cells (data not shown). Furthermore, staining of all nuclei (parental macronuclei, developing macronuclei, and micronuclei) with an antibody to the major H2A is easily detectable throughout conjugation, even at late stages, indicating that micronuclear chromatin is accessible to anti-histone antibodies (data not shown).

Nuclear fractionation and immunoblotting demonstrates the acquisition of hv1 by micronuclei during early conjugation

The unexpected finding that micronuclei stain with anti-hv1-HPLC during early conjugation prompted the biochemical purification of these nuclei to confirm the sudden developmentally regulated acquisition of hv1. Nuclei were harvested from cells in log phase growth, starvation, or 2.5 hr after the initiation of conjugation, formaldehyde fixed, and subjected to sedimentation at

Figure 3. During conjugation both macronuclei and micronuclei are stained with anti-hv1-HPLC serum. Mating cells were visualized with DAPI stain (a,c,e,g) and with anti-hv1-HPLC serum (b,d,f,h). Mating pairs from early conjugation: The micronucleus has just moved away from the macronucleus, at ~ 1.5 hr after mixing (a,b); meiotic prophase, stage II, at 2.5 hr (c,d); crescent stage of meiotic prophase, at 3.5 hr (e,f); third postzygotic division, ~ 5 hr after mixing (g,h). Times given are approximate and are the earliest point after mixing cells when the stages can generally be observed (for a complete description of the conjugation process in *Tetrahymena*, see Martindale et al. 1982). Bar, 10 μ m.

Figure 4. Later stages of conjugation show loss of anti-hv1-HPLC staining from micronuclei. Immunofluorescent images of mating pairs were stained with DAPI (a,c,e) or anti-hv1-HPLC (b,d,f). (a,b) Early macronuclear development showing developing macronuclei in the anterior end of the cells and micronuclei in the posterior end, ~7 hr after mixing; (c,d) macronuclear development II, micronuclei and developing macronuclei have migrated to the middle of the cell while the parental macronucleus has moved to the posterior, at ~8 hr; (e,f) late macronuclear development II showing anti-hv1-HPLC staining is lost from micronuclei and diminishes in the parental macronucleus, which is beginning to degrade, at ~8.5 hr. Bar, 10 μ m.

unit gravity, which separates the nuclei primarily on the basis of size (Wang and Allis 1993). The samples were adjusted to produce approximately equivalent histone loads and analyzed by SDS-PAGE followed by immunoblotting with anti-hv1-HPLC. As expected, macronuclei from all three stages contained significant amounts of hv1 (Fig. 5a). Micronuclei from growing or starved cells contained trace amounts of hv1, probably attributable to small amounts of contaminating macronuclei. However, micronuclei isolated from conjugating cells clearly possessed a significant amount of hv1. The amount of hv1 in micronuclei from early conjugants cannot be explained by extensive macronuclear contamination, as staining of the same blot with antibodies against the macronuclear-specific linker histone H1 (Chicoine et al. 1985) showed little or no staining of any of the micronuclear preparations (Fig. 5b). Final staining of this blot with anti-H4 serum demonstrated nearly equivalent loads for all samples (data not shown).

Estimates of the abundance of hv1 in conjugating micronuclei were made by comparing the Ponceau S stained with the immunostained blot. We estimate that micronuclei from early stage conjugants contain one-fourth to one-third the amount of hv1 as growing cell macronuclei. Because ~15% of the total H2A in growing cell macronuclei is hv1 (Allis et al. 1980b), ~5% of the H2A in micronuclei of early conjugants is hv1. Depending on whether nucleosomes contain two molecules of hv1 or one molecule of hv1 and one of either of the two major H2As, 5–10% of the micronuclear nucleosomes contain hv1 at this stage. Although these estimates are approximate, it seems clear that hv1 is associated with more than a few micronuclear DNA sequences but not with the majority of the micronuclear genome.

These results confirm the developmentally regulated acquisition of hv1, initially observed via immunocytochemical techniques. They also demonstrate that not all macronuclear proteins accumulate in the micronuclei at this stage of development because H1 does not.

Staining of conjugating cells with anti-peptide antisera

Staining of conjugating pairs with anti-amino serum parallels the staining observed with the anti-hv1-HPLC serum. This serum stains parental macronuclei throughout development (Fig. 6). In addition, this serum stains meiotic (Fig. 6b) and gametic micronuclei (data not shown), as well as postzygotic nuclei (Fig. 6d,f). Staining is restricted to macronuclei late during development (Fig. 6h).

Although anti-carboxy serum stains the parental ma-

Figure 5. Biochemical purification of fixed nuclei and immunoblot analyses demonstrates the sudden acquisition of hv1 in micronuclei during early conjugation. Nuclei were isolated from growing, starved, and conjugating (2.5 hr postmixing) cultures and fractionated. (a) Macronuclear (mac) and micronuclear (mic) samples were separated on a SDS-12% polyacrylamide gel and electroblotted to nitrocellulose. hv1 was detected after incubation with anti-hv1-HPLC serum. (b) Subsequently, incubation with anti-H1 serum was used to assay for macronuclear contamination. Multiple bands represent previously described phosphorylated forms of H1 (Roth et al. 1988).

Figure 6. Micronuclei are stained by anti-amino antiserum but not by anti-carboxy antiserum in earlier stages conjugation; but at later stages of conjugation both antisera stain micronuclei. Immunofluorescent images of mating pairs stained with DAPI (*a,c,e,g,i,k,m,o*), anti-amino serum (*b,d,f,h*), or anti-carboxy serum (*j,l,n,p*). Micronuclei at meiotic prophase just beginning crescent formation show staining of micronuclei by the anti-amino antiserum (*b*) but not by anti-carboxy antiserum (*j*). At macronuclear development I the anti-amino serum stains both the macronuclei and the micronuclei, which are positioned in the posterior of the cell (*d*), but the anti-carboxy serum only stains the anterior developing macronuclei (*l*). At macronuclear development II anti-amino (*f*) and anti-carboxy (*n*) sera stain both micronuclei and developing macronuclei. At later macronuclear development II, when the parental macronuclei begin to degrade and lose immunostaining, neither antiserum stains micronuclei significantly (*h,p*). Bar, 10 μ m.

cronucleus throughout development, it does not detect hv1 in micronuclei during the early or middle stages of conjugation (Fig. 6j,l), suggesting that this epitope is not accessible for interaction with the antibodies. However, anti-carboxy serum does stain micronuclei briefly (Fig. 6n), late in development, just before the loss of hv1 detection by this (Fig. 6p) and the other two antisera.

Discussion

Eukaryotic DNA is packaged into nucleosomes via interaction with the four core histones, H2A, H2B, H3, and H4 (for review, see Isenberg 1979). Microheterogeneity of nucleosomes, either by secondary modifications of the histones or the incorporation of histone variants and/or nonhistone proteins, could provide a means for regulating the structural and functional state of the chromatin. Although the existence of histone variants is well established (Harvey and Whiting 1983; Grunstein et al. 1984; Zweidler 1984; Hatch and Bonner 1990; Waterborg 1991), the actual roles that these proteins play in the modulation of chromatin are not known. In an effort to gain insights into the functional significance of a histone variant, we have used immunocytochemistry to examine the nuclear distribution of hv1, an H2A variant, in

Tetrahymena. Because this variant is conserved throughout evolution, it is likely to play a fundamental role in chromatin structure and/or function.

We used synthetic peptides to the amino and carboxyl termini, as well as HPLC-purified whole protein, to generate three polyclonal antisera to hv1. Histones are comprised of three domains: an extended hydrophilic amino-terminal tail, a globular, hydrophobic core, and a hydrophilic carboxy-terminal tail (for review, see McGhee and Felsenfeld 1980). The core domain is involved largely in histone-histone interactions necessary for nucleosome stability, whereas the amino terminus is likely to interact with DNA (Hill and Thomas 1990) or with extranucleosomal proteins (Johnson et al. 1990) and serves as a site for acetylation (Bonner and Stedman 1979). The carboxy-terminal tail of histone H2A can be cross-linked to histone H1 (Bonner and Stedman 1979), and the digestion of this domain leads to decondensation of nucleosomal fibers (Hacques et al. 1990). Thus, this domain may be important for higher order chromatin structure because the presence of H1 in vitro can transform 10-nm nucleosomal fibers into 30-nm filaments (Van Holde 1989).

Immunoblotting analyses were performed to determine the specificity of the three antisera. All three were

specific for hvl under the conditions employed. Competition experiments using synthetic peptides established that anti-amino and anti-carboxy peptide antisera are specific for their immunogens. Anti-hvl-HPLC detection of hvl was only partially competed with carboxy-terminal and variant box peptides and not detectably with amino-terminal peptide. This indicates that anti-hvl-HPLC serum recognizes several regions of hvl.

Immunofluorescence analyses demonstrated that the transcriptionally active macronuclei stained at all stages of the life cycle with all anti-hvl antisera. The transcriptionally inert micronuclei did not stain during growth or starvation but stained with anti-hvl-HPLC and anti-amino sera during early stages of conjugation. The onset of micronuclear staining was ~1–1.5 hr after mixing cells, when the micronucleus migrates from its pocket-like recess in the macronuclear surface, where it usually resides in growing and starved cells. Staining by anti-acetylated histone antibodies demonstrated that at least some of the hvl present at this stage as well as at later stages is acetylated (data not shown). Acetylation of histone tails has been associated with transcription (Perry and Annunziato 1989; Ruiz and Wahl 1990) and has the potential to destabilize nucleosomes and may facilitate access of regulatory molecules (Oliva et al. 1990; Lee et al. 1993). The micronuclei at this stage swell slightly and the punctate, highly condensed chromatin observed in nonconjugating cells is transformed into a dense homogeneous core surrounded by a "halo" of less condensed chromatin (Wolfe et al. 1976), consistent with a change in chromatin structure attributable to the acquisition of hvl. The micronuclei then begin an extended period of elongation, termed the crescent stage, where partially condensed chromatin is organized into long, thin strands. The micronucleus is transcriptionally active during early crescent stage (the stage shown in Fig. 3c); this activity ceases by late crescent (Fig. 3e; Sugai and Hiwatashi 1974; Martindale et al. 1985). The detection of hvl precedes the period of micronuclear transcriptional activity and outlasts it as well. This suggests that hvl is required for the formation of active chromatin, rather than appearing secondarily as a consequence of transcriptional activity.

The hypothesis that hvl in micronuclei is associated with transcriptional activation is supported by observations using antibodies specific to TBP. TBP is a general factor required for transcription by all three nuclear RNA polymerases (for review, see White and Jackson 1992; Rigby 1993). Anti-TBP serum stains only macronuclei during growth and starvation but stains nuclei during conjugation with a pattern similar to that observed for anti-hvl; parental macronuclei, meiotic micronuclei, gametic and postzygotic nuclei stain with anti-TBP antibody (Stargell and Gorovsky 1993). Anti-TBP staining disappears from micronuclei and is restricted to developing macronuclei late in development. Because TBP is a necessary component for transcriptional activation, it seems likely that the coincident appearance of hvl in conjugating micronuclei is associated with the transcriptional activation of these nuclei.

If hvl is solely involved in the transcription of micronuclear DNA, then why does it persist after transcription is no longer detectable? One possibility is that hvl may be required for micronuclear DNA replication, which also occurs during conjugation (Allis et al. 1987). However, because replicating micronuclei of growing cells do not stain detectably with any of the anti-hvl sera (data not shown), a function for hvl in replication seems unlikely. Another possibility is that hvl is required for the transcriptional activation of micronuclei early in conjugation and is simply not removed until late in development, after nuclear fates are determined. It may be of no consequence to the cell that this protein (and others) remains present during later stages of conjugation. However, it seems more likely that persistence of hvl in micronuclei after transcription ceases is a reflection of the fact that micronuclear chromatin remains in a transcriptionally competent state until late in conjugation. Nanney (1953) has shown that after the second postzygotic division, presumptive micronuclei, located in the posterior of the cell, can still differentiate into macronuclei if relocated anteriorly (see below).

Anti-hvl-HPLC and anti-amino sera staining disappeared from developing micronuclei late in conjugation. Interestingly, anti-carboxy antibodies stained micronuclei only briefly, just before this late stage of development. It may be that like the major H2As that have been shown to interact with H1 (Bonner and Stedman 1979), the carboxy-terminal tail of hvl in micronuclei interacts with a chromatin protein, thus obscuring this epitope from detection in situ. This hypothetical protein is not H1, as there is no detectable immunofluorescent staining of H1 in these micronuclei (data not shown). The micronuclear linker histones are likely candidates, but it is not yet known whether they are present at this stage. The detection of the previously sequestered carboxy-terminal tail of hvl during late stages of conjugation may be related to the protein's subsequent removal, as it occurs just before the loss of all hvl staining in these maturing micronuclei. An anti-hvl-gel serum described earlier (Allis et al. 1982) produced the same pattern of staining as the anti-carboxy antibodies, in which only macronuclei and some micronuclei of late-stage conjugants were stained (Wenkert and Allis 1984). This is consistent with competition experiments demonstrating that this antiserum had characteristics indistinguishable from those of the anti-carboxy serum (data not shown). The changes in accessibility revealed by these experiments suggest that the conformation of hvl in the nucleosome or its association with other proteins may be altered.

The mechanism by which hvl is eliminated from the micronucleus appears to be an active process, as micronuclei do not enlarge or divide at this time. During this transition period hvl is not the only protein to be eliminated from micronuclei. Antibodies specific to TBP (described above) and high mobility group protein B (HMG B) also cease to stain micronuclei at this transition stage (Wang and Allis 1993). Thus, the developing micronuclei appear to be undergoing a dynamic chromatin reorganization at this transition period, which may be linked to

the restriction of transcriptional and developmental potential in these nuclei, and must eventually include the acquisition of the micronuclear-specific linker histones. The developmental signals that trigger this chromatin transition are unknown. Although this restructuring occurs when the developing macronuclei and micronuclei are in close proximity in the cytoplasm, the fates of these nuclei are actually determined much earlier, after the second postzygotic division, when the products of this division are oriented such that two nuclei are located in the anterior and two in the posterior of the cell (as shown in Fig. 4a). Nanney's results (Nanney 1953) strongly support a determinative role of localized cytoplasmic regions, as nuclei relocated to the anterior region by centrifugation develop into macronuclei, whereas those relocated to the posterior region become micronuclei. At later stages developing macro- and micronuclei come together near the center of the cell, and position is no longer important. Thus, nuclear determinants must be partitioned to the anterior and/or posterior nuclei, which thereby initiate the appropriate developmental process. One of the events triggered in the micronucleus is the elimination of proteins destined to be macronucleus specific in vegetative cells. After the transition period, there must either be selective deposition of these proteins into macronuclei or selective degradation of them in micronuclei to maintain the macronuclear-specific localization observed in growing and starved cells. Although micronuclei in the later stages of conjugation are no longer pluripotent and the distinct function and compositions of macro- and micronuclei can be maintained and replicated through hundreds of vegetative divisions, this restriction of nuclear fate can be developmentally reversed. The micronuclei are responsible for the genetic continuity of the organism and, during the next cycle of conjugation, must ultimately give rise to the subsequent generation of macro- and micronuclei.

Both the parental macronucleus and the developing macronuclei are immune to the events that remodel chromatin in the micronuclei. Each is experiencing its own unique alterations in chromatin structure during this transition period. The parental macronucleus undergoes programmed nuclear death, a process characterized by progressive reduction in size of DNA, proteolysis of histones, and transformation of the chromatin into a highly condensed state (Davis et al. 1992). As noted previously (Orias 1986), similar events occur in nuclei of animal cells undergoing apoptosis. In contrast, the developing macronuclei continue to mature and differentiate. Macronuclear differentiation is characterized by accumulation of H1, large-scale acetylation of histones (Lin et al. 1989), chromosome fragmentation, DNA sequence elimination, rearrangement and amplification, and the onset of gene expression (for review, see Gorovsky 1980; Blackburn and Karrer 1986; Brunk 1986; Yao 1989).

The studies presented here argue that there is a tight correlation between the presence of the histone variant hv1 and transcriptionally competent chromatin. With the development of techniques for gene replacement

(Yao and Yao 1991; Kahn et al. 1993) and mass transformation of *Tetrahymena* (Gaertig and Gorovsky 1992), additional studies aimed directly at determining the *in vivo* function of this histone variant should now be possible.

Materials and methods

Cells and culture conditions

T. thermophila (strains B2086, mating type II, and CU428, mating type VII; kindly provided by P.J. Bruns, Cornell University, Ithaca, NY) were grown axenically in enriched proteose peptone at 30°C as described (Gorovsky et al. 1975). Cells were starved at a density of 2×10^5 to 3×10^5 cells/ml in 10 mM Tris-HCl at pH 7.4 for 18–22 hr, without shaking. Conjugation was induced by mixing equal numbers of cells of different mating types.

Peptide antisera preparation

Peptides corresponding to three regions of the *T. thermophila* hv1 protein were prepared by solid-phase peptide synthesis on an Applied Biosystems model 430A peptide synthesizer and subjected to amino acid analysis with a Waters Associates Pico Tag System (Fig. 1a): amino peptide, VGGAKNKKTPQSGGC; variant box peptide, GRVSAKNRVGATGGC; and carboxy peptide, CGKHSTKNRSSAKTAEPK. The underlined amino acids are not part of the protein but serve as spacers (glycines) or as a coupler (cysteine) between the peptide and the carrier protein, keyhole limpet hemocyanin (KLH, Sigma). Coupling was accomplished by a modification of described methods (Lerner et al. 1981; Doolittle 1986). Hemocyanin (20 mg) was briefly sonicated into a suspension in 0.25 ml of 50 mM phosphate, 100 mM NaCl at pH 6.5, with a Branson Sonifier model 200 and microprobe tip. Ten microliters of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce) at 150 mg/ml in dimethylformamide was added with vortexing, and the reaction allowed to proceed for 30 min at room temperature with agitation. Free MBS was removed by chromatography on a Sephadex G-25 column (Pharmacia) and the protein-containing fractions (A_{280}) were pooled and the pH adjusted to 7.5. Approximately 3–5 mg of peptide in 0.1–0.2 ml of phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate at pH 7.4) was added, and the coupling reaction was allowed to proceed for 3–4 hr. Coupling efficiency (25–50 molecules of peptide per 10^5 daltons of KLH) was determined by quantitation of unreacted sulfhydryl groups by the method of Stadtman (1957).

Immunization of New Zealand white rabbits (Dutchland) with peptide–KLH conjugants was performed by emulsifying 1 mg suspension of the peptide–KLH complex in water with an equal volume of complete Freund's adjuvant (Sigma) and injecting this subcutaneously. Secondary immunizations were performed 2 weeks later with the peptide–KLH complex in incomplete Freund's adjuvant. Blood was obtained from an ear vein weekly starting 1 week after the secondary injection. Preimmune serum was obtained before immunization. Serum was obtained by centrifugation (2200g at 4°C) of clotted blood (several hours at 4°C) and was stored at -70°C .

Histone purification

Histones were extracted from *Tetrahymena* macronuclei with 0.4 N H_2SO_4 (Allis et al. 1979) and precipitated with 0.5 N perchloric acid. The acid-insoluble pellet was dissolved in 4 M urea, 5% β -mercaptoethanol, and centrifuged for 5 min at 16,000g to

remove particulates. Two consecutive rounds of reverse-phase HPLC were performed using a C8 analytical column (Aquapore RP-300, Applied Biosystems, Santa Clara, CA). The first round employed a linear gradient of 0–60% buffer B in A over 30 min, where buffer A was 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water and buffer B was 90% acetonitrile, 0.1% TFA in water. Fractions were eluted at 1 ml/min, monitored at 214 nm, and subjected to a second round of purification using a gradient of 0–40% B in A over 15 min followed by 40–65% B over 30 min. Final histone fractions were dried under vacuum.

Histone antisera generation

hvl antibodies were obtained from rabbit immunizations using the services of the Pocono Rabbit Farm and Laboratory (Candensis, Pennsylvania). Preimmune sera from four rabbits were screened for low immunoprecipitation of chromatin (Dedon et al. 1991) and background staining on immunoblots of *Tetrahymena* macronuclear proteins. Additional preimmune serum was obtained from the rabbit with lowest background and that rabbit was subjected to the following immunization schedule: Subcutaneous injection of Freund's adjuvant was followed 1 week later by intradermal injection of 30 µg of the HPLC-purified hvl protein in Freund's complete adjuvant; this injection was repeated 2 weeks later. Bleeds were taken weekly, and an intraperitoneal boost of 3 µg of hvl protein was given 8 weeks after injections began. Sera obtained were aliquoted and stored at –70°C.

Polyclonal rabbit anti-H2A and anti-H4 sera were generated from fractions of HPLC-purified samples at Syracuse University with the following injection schedule: An intradermal injection of 30 µg of the purified histone samples in Freund's complete adjuvant was given, and this treatment was repeated 2 weeks later using Freund's incomplete adjuvant. Bleeds were taken weekly. Serum was obtained by centrifugation (2200g at 4°C) of clotted blood (several hours at 4°C) and was stored at –70°C.

Gel electrophoresis

Tetrahymena histones were extracted and separated on SDS–triton/acid/urea–polyacrylamide gels as described previously (Allis et al. 1979, 1980a). Samples for electroblotting were run on wide preparative gels.

Immunoblotting and peptide competition

Standard immunoblotting procedures were used (Towbin et al. 1979; Harlow and Lane 1988). SDS gels were blotted to nitrocellulose using a semidry electrophoretic transfer unit (Bio-trans, Gelman, Ann Arbor, MI) with 48 mM Tris-Base, 39 mM glycine, 0.1% SDS, and 20% methanol buffer. To determine transfer efficiency, blots were stained with 0.02% Ponceau S in 0.3% trichloroacetic acid and destained in water. All incubations with sera and blocking were performed in blocking solution containing 5.0% (wt/vol) nonfat dry milk, 0.01% antifoam A (Sigma), 0.0001% merthiolate; in 1× TBS (150 mM NaCl and 50 mM Tris-HCl at pH 8.1). NaCl (300 mM) was added to the primary antibody reaction and first TBS wash to reduce nonspecific binding. A small amount of cross-reactivity to other histones was seen if the antisera were too concentrated or NaCl was omitted. Primary antibodies were used at 1:1000 dilution except for anti-hvl–HPLC, which was used at 1:10,000. Incubation was overnight at 4°C. Following reaction with peroxidase-conjugated goat anti-rabbit antiserum (Sigma, 1:2000 dilution), the blots were developed with HRP Color Development Reagent (Bio-Rad) according to the manufacturer's instructions.

Immunoblot competition was performed using 100-fold (for anti-peptide antisera) or 1000-fold (for anti-hvl–HPLC antiserum) molar excess of peptide to antibody. This ratio was calculated using an estimate of 10 mg of IgG/ml of antiserum (Harlow and Lane 1988) without determination of specific hvl IgG concentration; therefore, fold excesses given are minimum values. Peptide and serum were combined in 100 µl of blocking solution and incubated for 3 hr at 30°C. This reaction mixture was diluted in blocking solution and used for primary antibody incubation as described above.

Biochemical analyses of separated macro- and micronuclei

Purification of macro- and micronuclei by sedimentation at unit gravity, FACScan analyses, gel electrophoresis, and immunoblotting were performed as described (Wang and Allis 1993). Western blot antisera incubations were performed successively with anti-hvl–HPLC serum (diluted 1:18,000), anti-H1 serum (diluted 1:300; Chicoine et al. 1985), and anti-H4 serum (diluted 1:2000).

Cell fixation

Cells were extracted and fixed as described (Gaertig and Fleury 1992) with the following modifications. Cells were incubated for 3 min at room temperature in 0.5% Triton X-100 in PHEM buffer, washed, and then fixed in freshly prepared 1% paraformaldehyde in PBS. Fixed cells were washed in PBS and stored at 4°C for up to 1 week, and 50 µl of cell suspension was then applied to poly-L-lysine (Sigma)-coated coverslips and allowed to dry.

Indirect immunofluorescent staining and microscopy

To reduce nonspecific sticking, coverslips with dried cells were first incubated for 1 hr at 37°C in blocking solution: 10% normal goat serum (GIBCO), 3% BSA, 0.2% Tween 20 (Sigma), in PBS. Coverslips were incubated with primary antiserum overnight at 4°C. Primary antisera were diluted in blocking solution at the following concentrations: anti-hvl–HPLC (immune and preimmune), 1:500; anti-amino serum, 1:100; anti-carboxy serum, 1:100; and anti-H2A serum, 1:500. Coverslips were washed three times in PBS–0.2% Tween 20 and treated for 2 hr with secondary antibody, FITC-conjugated goat anti-rabbit antiserum (Sigma) diluted 1:160 in PBS–0.2% Tween 20. After washing once with PBS, cells were stained with the DNA-specific dye 4',6-diamidino-2-phenyl-indole dihydrochloride (DAPI, Boehringer Mannheim) at 5 ng/ml in PBS for 5 min, followed by a PBS wash. Cells were photographed using an Olympus BH-2 microscope equipped for fluorescence and filters specific for fluorescein to visualize the antisera staining or DAPI to show the position of the nuclei.

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