Antibodies Specific to Acetylated Histones Document the Existence of Deposition- and Transcription-related Histone Acetylation in *Tetrahymena*

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Abstract. In this study, we have constructed synthetic peptides which are identical to hyperacetylated amino termini of two *Tetrahymena* core histones (tetra-acetylated H4 and penta-acetylated hv1) and used them to generate polyclonal antibodies specific for acetylated forms (mono-, di-, tri-, etc.) of these histones. Neither of these antisera recognizes histone that is unacetylated. Immunoblotting analyses demonstrate that both transcription-related and deposition-related acetate groups on H4 are recognized by both antisera. In addition, the antiserum raised against penta-acetylated hv1 also recognizes acetylated forms of this variant. Immunofluorescent analyses with both antisera demonstrate that, as expected, histone acetylation is specific to macronuclei (or new macronuclei) at all stages of the life cycle except when micronuclei undergo periods of rapid replication and chromatin assembly. During this time micronuclear staining is also detected. Our results also suggest that transcription-related acetylation begins selectively in new macronuclei immediately after the second postzygotic division. Acetylated histone is not observed in new micronuclei during stages corresponding to anlagen development and, therefore, histone acetylation can be distributed asymmetrically in development. Equally striking is the rapid turnover of acetylated histone in parental macronuclei during the time of their inactivation and elimination from the cell. Taken together, these data lend strong support to the idea that modulation of histone acetylation plays an important role in gene activation and in chromatin assembly.

HISTONE acetylation, the acetylation of specific lysines located within the amino-terminal domains of non-H1 histones (H2A, H2B, H3, and H4), is an active process whose precise role(s) remains controversial. Since acetylation of lysine residues neutralizes their positive charge, it has long been suggested that this modification modulates the interaction of histone amino termini with the negatively charged DNA backbone. Indeed, strong correlative data exist implicating histone acetylation in transcription, chromatin assembly, and histone replacement (see Matthews and Waterborg, 1985, for references and a review of histone acetylation). However, genetic experiments recently carried out in yeast have shown that amino termini of individual histones are either not essential for growth or are possibly redundant (Kayne et al., 1988).

In the ciliated protozoan, *Tetrahymena*, it is clear that at least two general systems of histone acetylation exist. One system, which we refer to as transcription-related acetylation, is postsynthetic and maintains high levels of acetate on all core histones in transcriptionally active macronuclei. This system does not exist in transcriptionally inactive micronuclei (Vavra et al., 1982). A second system, which we refer to as deposition-related actylation, affects newly synthesized H3 and H4 regardless of whether they are destined for micro- or macronuclei (Allis et al., 1985). Furthermore, unique sites of acetylation have been identified which distinguish transcription from deposition-related acetylation (Chicoine et al., 1986), and it is likely that two different enzyme systems are responsible for catalyzing these distinct acetylation reactions (Chicoine et al., 1987; Richman et al., 1988).

Despite large differences in chromatin structure and function between macro- and micronuclei in vegetative cells (Gorovsky, 1973), these two nuclei are related. During the sexual phase of the life cycle, conjugation, micronuclei undergo meiosis, exchange, fertilization, and postzygotic division to eventually produce new macronuclei and micronuclei. During this stage, as new macronuclei (anlagen) become transcriptionally active, the "parental" macronucleus in each cell is degraded. Biochemical studies, using synchronous populations of developing new macronuclei, have shown that transcription-related histone acetylation is active in very early stages (4–8C) of anlagen differentiation (Chicoine and Allis, 1986). Thus, it seems likely that postsynthetic acetylation of histones plays a critical role in the sequence of events which transforms transcriptionally inactive micronuclei into transcriptionally active, new macronuclei.

In this study, we have generated polyclonal antisera specific to acetylated forms of histone H4 and a minor macronuclear histone variant, hv1, by immunizing rabbits with synthetic peptides which are identical to hyperacetylated amino-terminal domains of these histones. Immunoblotting and immunofluorescent analyses clearly document the existence of both deposition- and transcription-related histone acetylation in *Tetrahymena*. The dynamic modulation of histone acetylation that we have observed with these antibodies lends strong support to the idea that histone acetylation (and factors governing its regulation) plays a central role in modulating chromatin structure and function.

Materials and Methods

Cell Culture, Labeling, and Isolation of Nuclei

Genetically marked strains of *Tetrahymena thermophila*, CU427 (Mpr/Mpr[6-mp-s VI]) and CU428 (Chx/Chx[cys VII]) were used in all experiments reported here. These were kindly provided by P. Bruns (Cornell University, Ithaca, NY). Cells were grown axenically in 1% enriched proteose peptone as described previously (Gorovsky et al., 1975). All matings (typically 80–90% 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 increase the proportion of acetylated histone subspecies, sodium butyrate was added to cultures for 1 h at a concentration of 50 mM. In all cases, nuclei were prepared from cells using the methods of Gorovsky et al. (1975) and Allis and Dennison (1982) wherein micronuclei are purified by differential centrifugation and sedimentation at unit gravity. The purity and DNA ploidy of various nuclear preparations were determined by flow microfluorometry (Allis and Dennison, 1982). Nuclei were either used immediately or stored at −80°C for future use.

Extraction of Histones and Gel Electrophoresis

Histones were extracted from nuclei according to previously described procedures (Allis et al, 1979) taking all precautions to avoid artifactual protein losses. To obtain deacetylated histone samples, macronuclei were incubated overnight at 4°C in the EDTA buffer described by Vavra et al. (1982) before histone extraction. Acid–urea slab gel electrophoresis was performed as described by Allis et al. (1980). Gels were typically stained with Coomassie brilliant blue R, destained, and photographed.

Peptide Synthesis and Coupling to Carrier Protein

Peptides corresponding to the amino terminus of *Tetrahymena* H4 (Glover and Gorovsky, 1979) and hv1 (Allis et al., 1986) were synthesized by the solid phase procedure on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA). Where appropriate, lysine (+ charge) or acetyl-lysine (Ac) were incorporated during the synthesis to give the desired peptides shown in Fig. 1. *t*-Butyloxycarbonyl- (tBoc) N^{α}-protected amino acids were coupled to the terminal tBoc-cysteine-OCH_2-phenyl-acetoamidomethyl-polystyrene resin. All amino acids were purchased from Applied Biosystems, Inc. except for tBoc-N^{α}-acetyl-N^{ϵ} L-lysine which was from Bachem Bioscience Inc. (Philadelphia, PA). The carboxy-terminal G-G-C for the penta-acetylated hv1 peptide and the carboxy-terminal G-C for un- and tetra-acetylated H4 peptides were included to assist in coupling to the carrier protein (see below). Peptides were cleaved from the resin and deprotected using the trifluoroacetic acid-trifluoromethanesulfonic acid procedure (Bergot et al., 1986). After cleavage the peptides were analyzed by reverse phase HPLC, amino acid analysis (Pico Tag System; Waters Associates, Milford, MA) and NH_2-terminal amino acid sequence analysis (477A Protein Sequencing System; Applied Biosystems, Inc.).

Peptides were coupled to the carrier protein, keyhole limpet hemocyanin (KLH), as described by Lerner et al. (1981). For coupling to the unacetylated H4 peptide (Fig. 1), KLH was first reacted with 20 mM *N*-ethylmaleimide for 2 h at room temperature to block sulfhydryl groups in KLH. This was done to ensure that the sulfhydryl group from the terminal cysteine residue of the H4 peptide (and not the ε-amino groups on the lysines) would

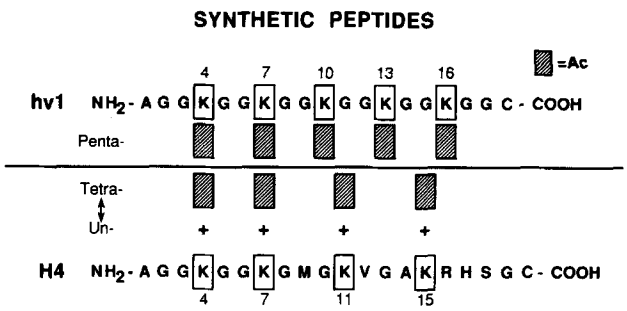


Figure 1. Synthetic peptides corresponding to the amino terminus of *Tetrahymena* H4 and hv1 were constructed using either unmodified (therefore having + charge) or acetylated (▨) lysines (see Materials and Methods for details). In each peptide the terminal glycine and cysteine residues are not in the actual protein sequence and serve a spacer and coupling function, respectively. Throughout this paper, antibodies raised to each of these peptides are collectively referred to as "penta" hv1 (raised against the hv1 peptide containing five acetylated lysines), "tetra" H4 (to the H4 peptide containing four acetylated lysines), and "unacetylated" H4 (to the H4 peptide containing no acetylated lysine) even though our immunoblotting data clearly show that more than one acetylated subspecies is recognized by each antisera.

be used in the coupling reaction to KLH. This step was omitted for coupling to "tetra" H4 and "penta" hv1 peptides. KLH (20 mg in 250 μl of PBS, pH 6.5) was sonicated into a suspension to which 10 μl of *M*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (150 mg/ml in dimethyl formamide) was added. After a 30-min reaction at room temperature (with occasional vortexing), free *M*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and/or *N*-ethyl-maleimide was removed from the KLH with a Sephadex G-25 column. Protein fractions (corresponding to 5–6 mg KLH) were pooled, adjusted to pH 7.5, and reacted with 3–5 mg of the appropriate peptide (dissolved in <200 μl of PBS, pH 7.5). The coupling reaction was allowed to proceed at room temperature for several hours. Progress of the reaction was judged by the formation of a precipitate. The coupling efficiency of each reaction was determined by performing a sulfhydryl assay on aliquots of the starting peptide, KLH after reaction with *M*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, and the final product of the complete reaction of KLH with peptide as described by Ellman (1959). Typically, 30–60 molecules of peptide were coupled to 10^5 D of KLH.

Immunization

Synthetic peptides coupled to KLH (~1.2 mg/injection) were dissolved in water, emulsified by sonication with an equal volume of complete Freund's adjuvant and injected subcutaneously into rabbits. Approximately 2 wk later, this injection was repeated except that incomplete Freund's adjuvant was used. The bleeding schedule was that used previously to obtain antibodies against *Tetrahymena* tubulin (Van De Water et al., 1982). Sera were stored in aliquots at −80°C. In all experiments, preimmune serum for controls was obtained from the same animal before immunization.

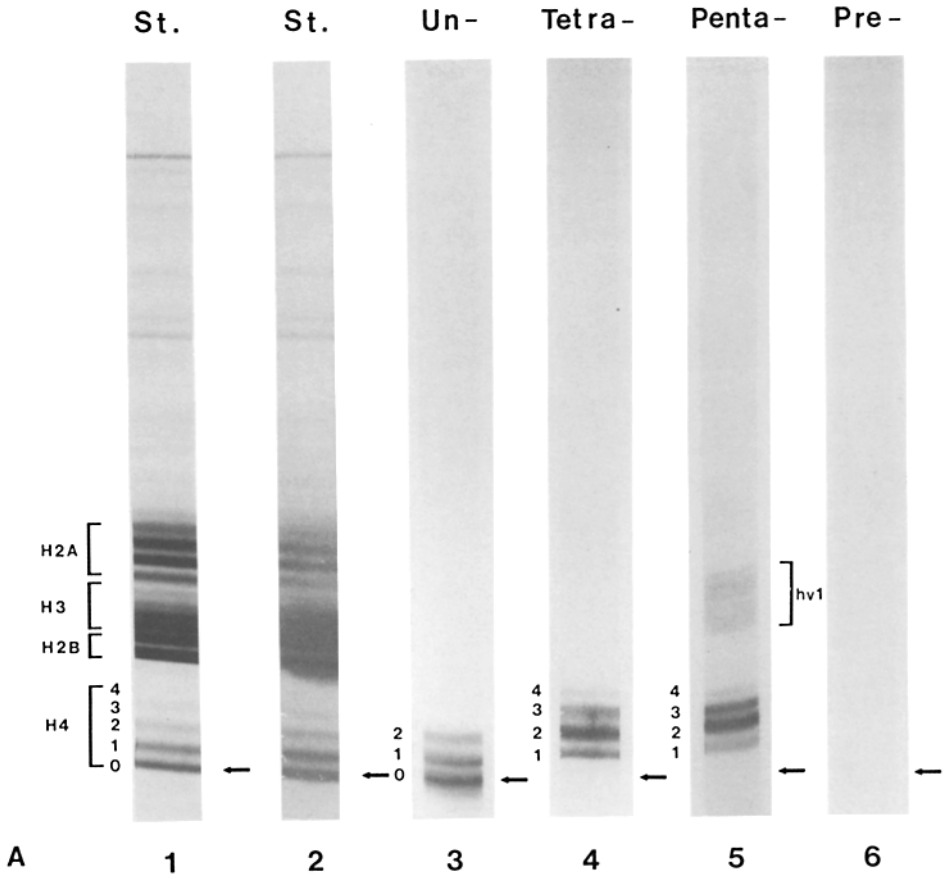
Immunoblotting

The specificities of preimmune and immune sera were assayed by immunoblotting analyses. Protein samples fractionated on acid–urea gels were transferred electrophoretically to nitrocellulose using the acetic acid transfer conditions described by Towbin et al. (1979). Routinely, one strip from each blot was stained with amido black to evaluate the efficiency of protein transfer. As well, blots were briefly stained with Ponceau red, quickly destained in water, and photographed before blocking to provide a photographic record of each blot. To ensure proper alignment of antibody reactions with the protein profile, the position of each acetylated subspecies of H4 was marked on each blot after staining with Ponceau red. Antibody reactions were detected using ^{125}I-protein A (10,000 cpm/ml) followed by autoradiography.

Immunofluorescence Microscopy

All cells (growing, starved, or mating) were fixed for immunomicroscopy as previously described (Wenkert and Allis, 1984). Cells were then in-

1. *Abbreviation used in this paper*: KLH, keyhole limpet hemocyanin.



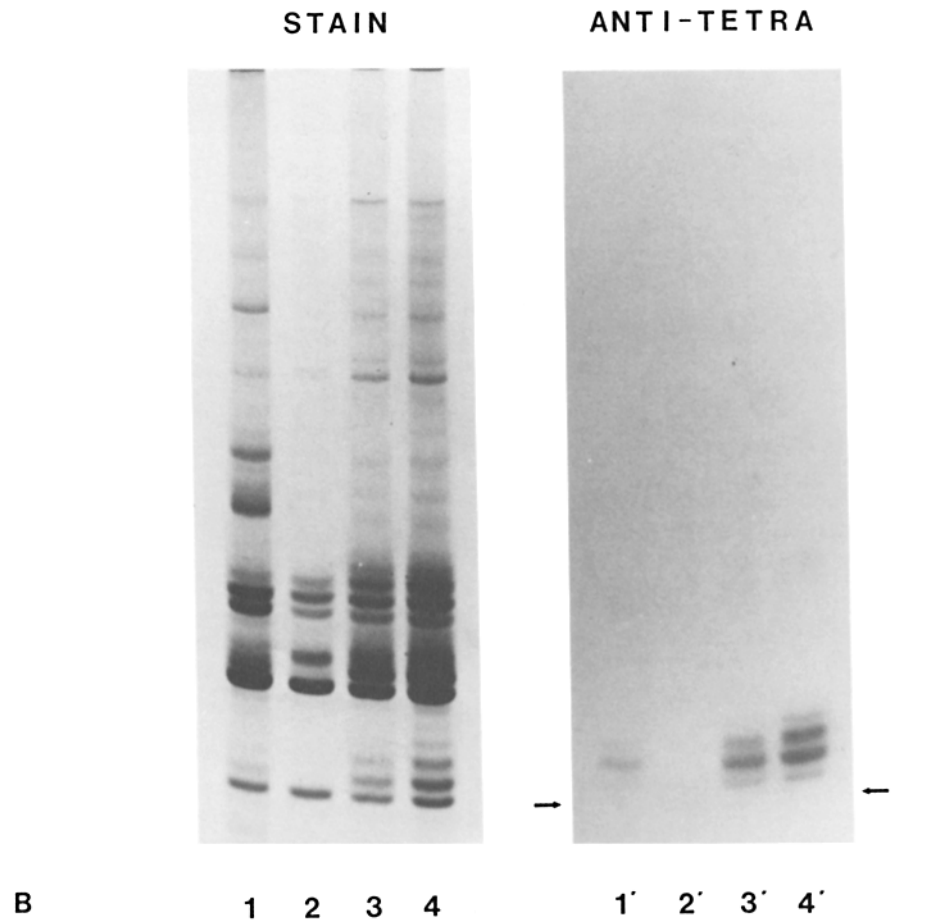


Figure 2. Specificity of antisera. (*A*) Acid-soluble protein from macronuclei was electrophoresed preparatively across an acid–urea gel. A strip from this gel was stained directly with Coomassie blue (lane *1*) and the remainder was transferred electrophoretically to nitrocellulose. One strip from this blot was stained with Amido black to evaluate efficiency of transfer (lane *2*). Remaining strips from the blot were blocked and reacted with antisera raised against the unacetylated H4 peptide (1:75 dilution, lane *3*), tetra-acetylated H4 peptide (1:100 dilution, lane *4*), penta-acetylated hv1 peptide (1:100, lane *5*), and one representative preimmune antiserum (1:100 dilution of rabbit immunized with the tetra-acetylated H4 peptide, lane *6*). All of these antibody reactions were detected with ^{125}I–protein A. hv1 (see bracket next to lane *5*) comigrates with H3 on acid–urea gels (Allis et al., 1980). hv1 staining with "penta" hv1 antibodies has been confirmed by two-dimensional (triton-acid-urea by SDS) gel immunoblots (data not shown). Arrows next to lanes *1–6* denote the position of unacetylated H4. (*B*) Acid-soluble protein from micronuclei (lanes *1* and *1'*), deacetylated macronuclei (lanes *2* and *2'*), macronuclei from normal vegetative cells (lanes *3* and *3'*), and macronuclei isolated from cells grown in the presence of sodium butyrate (lanes *4* and *4'*) were electrophoresed in an acid–urea gel and either stained (lanes *1–4*) or transferred to nitrocellulose and reacted with the "tetra" H4 antibodies (lanes *1'–4'*). Detection of antibodies was with ^{125}I–protein A.

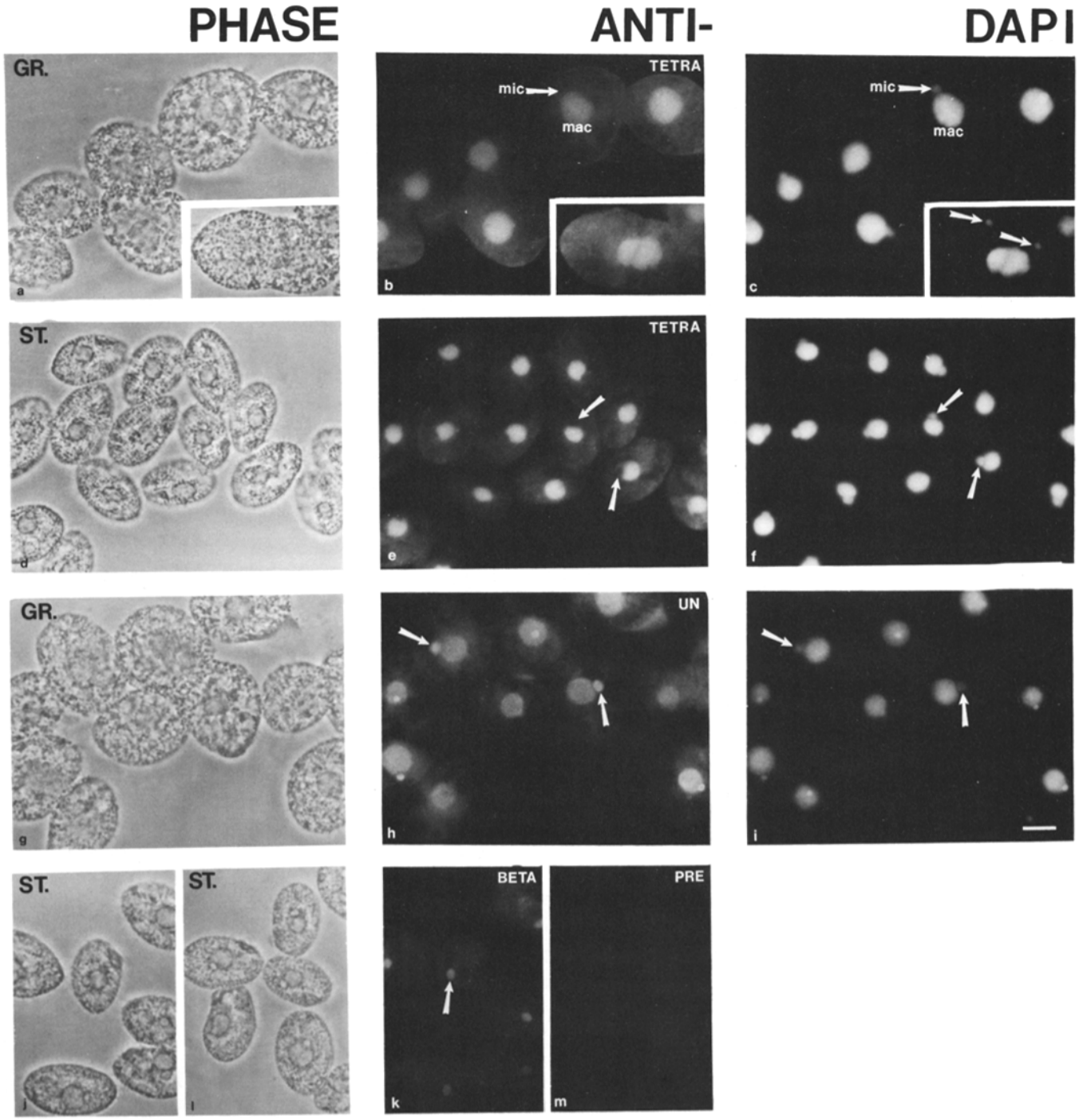


Figure 3. Selective staining of macronuclei in growing and starved cells reacted with tetra-acetylated H4 antiserum. Growing (*a–c* and *g–i*) and starved (*d–f* and *j–m*) cells were fixed and processed for immunofluorescence as previously described (Wenkert and Allis, 1986). First antibody incubations were as follows: "tetra" H4, 1:50 dilution (*b* and *e*); preimmune serum from the rabbit immunized with the "tetra" H4 peptide, 1:50 dilution (*m*); unacetylated H4, 1:50 dilution (*h*); and beta, 1:100 dilution (*k*) (beta is a micronuclear-specific H1-like polypeptide; see Allis et al., 1979 for details). Second antibody incubations used were either rhodamine- (*b*, *e*, and *h*) or fluorescein- (*k* and *m*) conjugated goat anti-rabbit sera (diluted 1:20). All dilutions and intervening washes were with PBS. In cases where rhodamine-conjugated second antibodies were used, cells were also stained with DAPI. Cells were examined using phase-contrast (*a*, *d*, *g*, *j*, and *l*) or fluorescence (*b*, *e*, *h*, *k*, and *m*) microscopy. Where appropriate cells were also examined with DAPI (*c*, *f*, and *i*) using an appropriate uv-filter cassette. Shown as an inset in *a–c* is a dividing cell which is known to contain micronuclei undergoing S phase DNA replication (see Wu et al., 1988). Arrows point to the position of some micronuclei which can be readily identified by DAPI staining. Bar, 10 μm.

cubated with immune or preimmune serum (diluted in PBS as indicated in the figure legends), followed by incubation with fluorescein- or rhodamine-conjugated goat anti-rabbit sera (Miles Scientific Div., Naperville, IL). In cases where rhodamine-conjugated goat anti-rabbit second antibodies were used, cells were subsequently stained with the DNA-specific dye, DAPI. In that way, antibody reactions as well as the distribution of total nuclei could be studied in the same cell using appropriate uv-filter cassettes. In one experiment (shown in Fig. 7), cells stained with first and fluorescein-conjugated second antibodies were stained additionally with ethidium bromide as described by Wenkert and Allis (1984). This enabled antibody-positive nuclei to be stained green and antibody-negative nuclei to be stained red. Intermediate shades were detected as well. All intervening washes and dilutions were in PBS.

Results and Discussion

Antibody Characterization

To evaluate the specificity of antibodies raised against hyperacetylated H4 and hv1 peptides (see Fig. 1), macronuclear acid-soluble protein was fractionated on long acid–urea gels, electrophoretically transferred to nitrocellulose, and probed with preimmune (all preimmune reactions were identical; one example is shown in Fig. 2 *A*, lane *6*) or various immune sera (Fig. 2 *A*, lanes *3–5*). Under these electrophoretic conditions, unacetylated and slower migrating acetylated subspecies of each histone are well-separated (see the numbers within the brackets beside each histone in lane *1*), leading to characteristic "ladders" of acetylated species. Corresponding immunoblots clearly demonstrate that "tetra" H4 immune serum contains antibodies which specifically recognize all of the acetylated forms of H4 (Fig. 2 *A*, lane *4*). Mono-, di-, and tri-acetylated H4 are easily recognized by these antibodies. While tetra-acetylated H4 is only faintly stained, this is presumably because only trace amounts of tetra-acetylated H4 exist in macronuclei isolated from vegetatively growing cells (see Fig. 2 *A*, lane *1*, and Fig. 2 *B*). Importantly, these antibodies fail to react with unacetylated H4 (see leftward-pointing arrow next to all lanes) or with any other acid-soluble macronuclear protein. To ensure proper alignment of our immunoblots with the corresponding staining pattern, another strip from the same blot was reacted with serum generated against unacetylated H4 peptide (see Fig. 1). Unlike the "tetra" H4 antibodies, this antiserum reacts well with the unacetylated form of H4 (H4°) as well as the mono- and di-acetylated forms of H4 (Fig. 2 *A*, lane *3*). Tri- and tetra-acetylated H4 are not recognized by this antiserum. The simplest interpretation of this result is that the "unacetylated" H4 antibodies recognize features of the H4 polypeptide backbone which are not accessible to the antibodies when it is tri- or tetra-acetylated or that the polypeptide undergoes a conformational change when it is hyperacetylated which prevents antibody recognition.

Antibodies generated against penta-acetylated hv1 behave quite similarly in immunoblotting analyses to the "tetra" H4 antibodies (compare lane *4* with lane *5*). Like the "tetra" H4 antiserum, all acetylated forms of H4 (but not unacetylated H4) are recognized by the "penta" hv1 antibodies. Unlike the "tetra" H4 antiserum, acetylated subspecies of hv1 are also recognized (see the bracket next to lane 5 in Fig. 2 *A*). The strong similarity between these two antisera is not surprising when one compares the amino-terminal sequences of hv1 and H4 (see Fig. 1). The first eight residues (A GGK GGK G) of each protein are identical. Furthermore, much of the remaining sequence included in the synthetic peptides is similar between the two polypeptides as are the number and spacing of acetyl-lysines. The stronger immune reaction of the "penta" hv1 antibodies with acetylated H4 subspecies (as compared to acetylated forms of hv1 itself) could be explained by the fact that hv1 is a minor histone variant in macronuclei comprising only ~10% of the H2A histone class (Allis et al., 1980). However, in some immunoblots from SDS gels approximately equal staining of hv1 and H4 has been observed (data not shown). Despite this difference, it is clear that both "tetra" H4 and "penta" hv1 antisera react with acetylated species of H4.

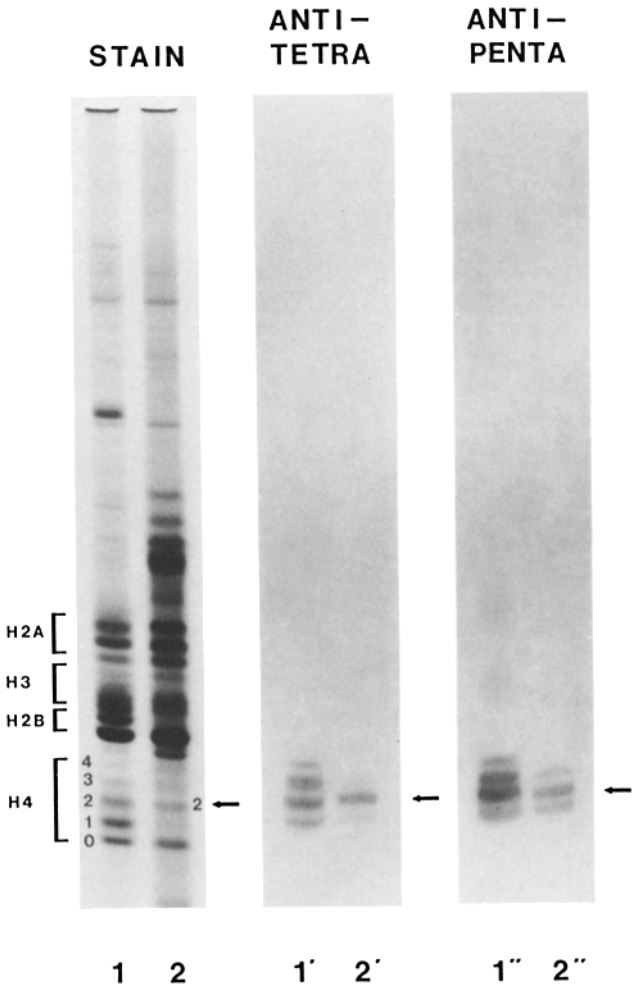


Figure 4. Reactivity of "tetra" H4 and "penta" hv1 antisera to deposition-related, diacetylated H4. Acid-soluble macro- (lanes *1*, *1′*, and *1″*) and micronuclear (lanes *2*, *2′*, and *2″*) protein prepared from 5.5-h conjugating cells (percent pairing = 85%) was electrophoresed in an acid–urea gel and either stained (lanes *1* and *2*) or transferred to nitrocellulose and reacted with "tetra" H4 (lanes *1′* and *2′*) or "penta" hv1 (lanes *1″* and *2″*) antisera as in Fig. 2. Antibody reactions were detected with ^{125}I–protein A. The arrow beside each panel points to the diacetylated form on H4 which is pronounced in micronuclei at this stage of conjugation. Earlier studies have shown that essentially all of the newly synthesized H4 entering micronuclei during this period is diacetylated (Allis et al., 1985). Since micronuclei undergo extensive DNA replication and chromatin assembly during this stage to support numerous meiotic and mitotic divisions (see text) and yet are transcriptionally inactive, this H4 diacetylation is thought to play a role in histone deposition.

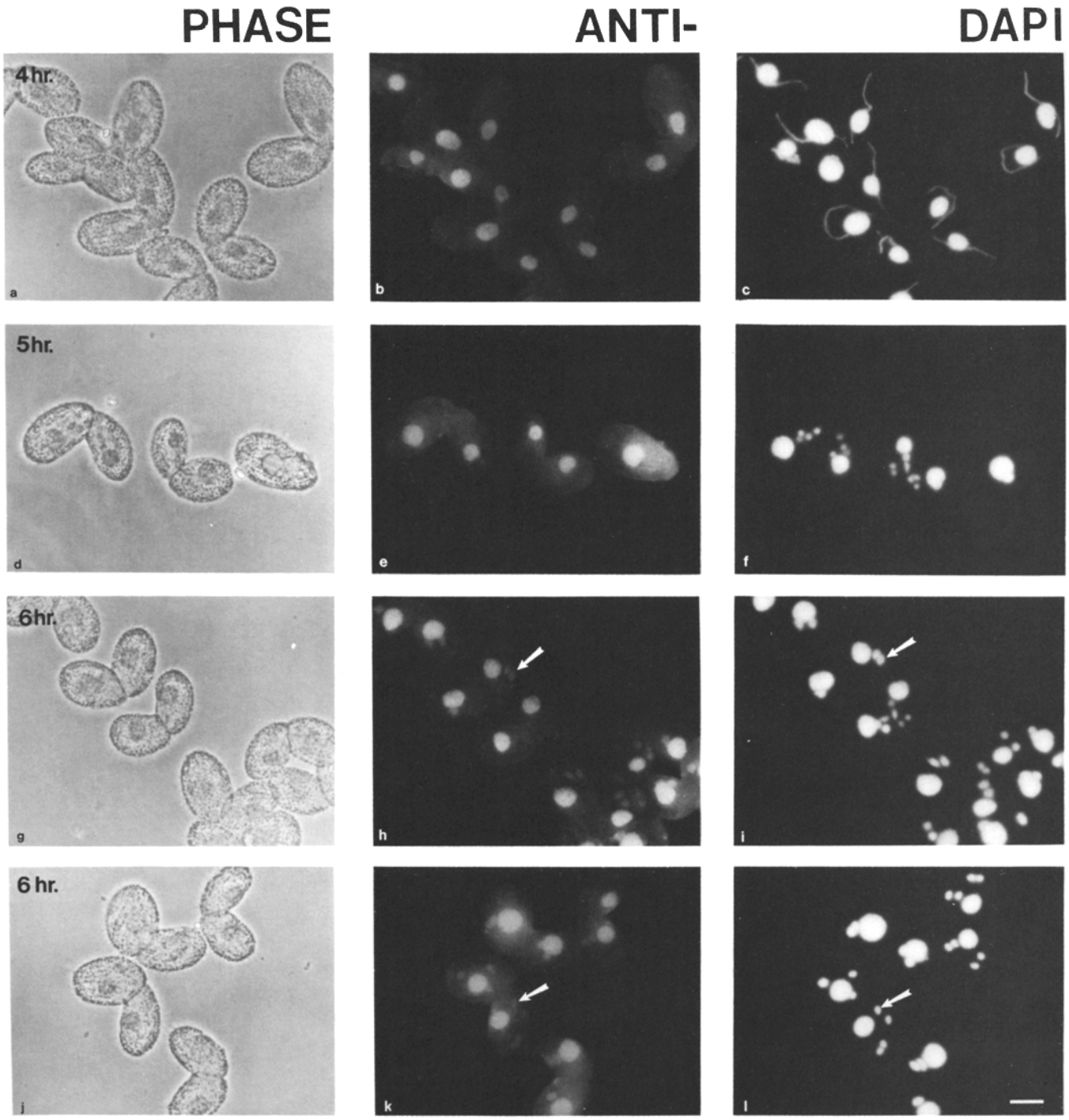


Figure 5. Immunofluorescent analyses of young conjugating cells, reacted with "tetra" H4 antibodies: evidence for deposition-related H4 acetylation. Conjugating cells in either meiotic prophase (full crescent stage: 4 h, *a–c*), at the completion of meiosis (four gametic nuclei per cell: 5 h, *d–f*), or in the process of the first or second postzygotic division (two or four micronuclei per cell: 6 h, *g–l*) were analyzed by indirect immunofluorescence using the "tetra" H4 antibodies as in Fig. 3. Arrows point to examples of positively stained micronuclei (see *h* and *k*) after the first (*h*) and second (*k*) postzygotic divisions. Bar, 10 μm.

To further demonstrate the specificity of the "tetra" H4 antiserum for acetylated forms of H4, histone samples spanning a wide range of acetylation were also analyzed in immunoblotting experiments (Fig. 2 *B*). Two sources of unacetylated histone were examined, micronuclei (lane *1*) and deacetylated macronuclear histone (lane *2*). In contrast, macronuclear histone from vegetative cells (lane *3*) and from cell grown in the presence of sodium butyrate (lane *4*) were used as two sources of highly acetylated histones. As expected for an antiserum which recognizes only acetylated forms of H4, corresponding immunoblots show only weak H4 staining in micronuclei (lane *1'*) (an amount consistent with low levels of macronuclear contamination). Macronuclear histones are completely deacetylated when nuclei are incubated overnight in EDTA-containing buffers (Vavra et al., 1982) and, as expected, no antibody staining is ob-

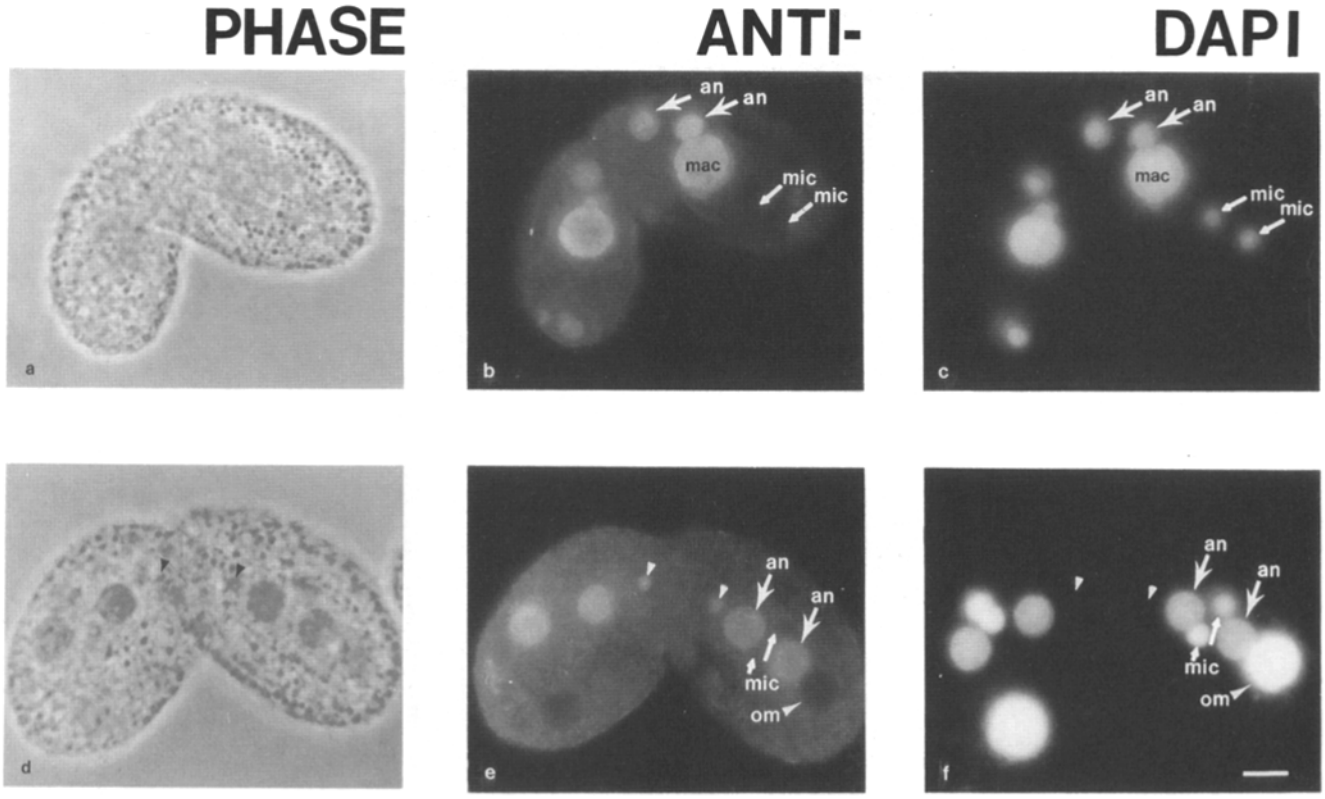


Figure 6. Selective reactivity of tetra-acetylated H4 antibodies to new macronuclei after the second postzygotic division. Examples of conjugating cells in anlagen development I (*a–c*) and anlagen development II (*d–f*; see Martindale et al., 1982, for a thorough description of these stages) are shown after reaction with "tetra" H4 antiserum. Cells were examined as in Fig. 3. Labeled are new macronuclear anlagen (*an*), micronuclei (*mic*), macronuclei (*mac*), and old macronuclei (*om*). Note not only the selective antibody staining of new macronuclei soon after the second postzygotic division, but also the rapid loss of staining in parental macronuclei (*om*). The identity of the small phase dark bodies which do not contain DNA and yet are stained with the "tetra" H4 antibodies (see small arrowheads in *d–f*) is not known. They have not been detected in preimmune serum or with the "penta" hv1 serum (see Fig. 7). Bar, 5 μm.

served in that sample (lane *2'*). This result strengthens our previous conclusion that the "tetra" H4 antibodies do not recognize the unacetylated form of H4. In contrast, strong H4 staining is seen in macronuclei from growing (lane *3'*) and "butyrate-treated" cells (lane *4'*). In the case of "butyrate-treated" macronuclei, tetra-acetylated H4 is evident in the staining profile (lane *4*), and it is easily detected by this antiserum (lane *4'*). Thus, it is clear that our "tetra" H4 antibodies specifically recognize all of the acetylated forms of histone H4 (mono-, di-, tri-, and tetra-).

Immunofluorescent Analyses

Growing and Starved Cells. Earlier biochemical studies (Gorovsky et al., 1978; Vavra et al., 1982) have clearly shown that high levels of histone acetylation are maintained in macronuclei isolated from growing *Tetrahymena*. Except under conditions where histone deposition into micronuclei is particularly active (see Allis et al., 1985 and below), little, if any, acetylated histone is observed in micronuclei. Given the acetylation-specific nature of the immune sera raised against tetra-acetylated H4 and penta-acetylated hv1 peptides (Fig. 2, *A* and *B*), we predicted that these antibodies would stain macronuclei, but not micronuclei in indirect immunofluorescent analyses. Shown in Fig. 3 are growing and starved *Tetrahymena* examined by phase-contrast (left-hand column), DAPI-uv (right-hand column), and immunofluorescent (middle column) microscopy. In most cells, micronuclei (in some cells identified with arrows) and macronuclei are easily observed with the DNA-specific stain, DAPI. However, it is clear that only macronuclei are detected in both growing (Fig. 3, *a–c*) and starved (Fig. 3, *d–f*) cells when examined by rhodamine indirect immunofluorescence using the immune serum raised against the tetra-acetylated H4 peptide. Macronuclei are not stained by preimmune control serum (Fig. 3, *l* and *m*). Furthermore, the inability of micronuclei to be stained with these antibodies is not a trivial consequence of some technical problem which selectively prevents antibody staining of micronuclei (for example, membrane impermeability). Using these fixation procedures, micronuclei are easily stained by antibodies raised to the unacetylated H4 peptide (Fig. 3, *g–i*) or to micronuclear-specific linker polypeptides (Fig. 3, *j* and *k*). Since the "tetra" H4 antibodies react well with all acetylated forms of H4 (mono-, di-, tri-, and tetra-) in immunoblotting analyses (Fig. 2, *A* and *B*), our immunofluorescent data strongly suggest that H4 is unacetylated in micronuclei during these physiological conditions. This conclusion is in excellent agreement with earlier biochemical studies (Vavra et al., 1982).

Young Conjugating Cells. During the sexual stage of the life cycle, conjugation, micronuclei enter an extended period corresponding to meiotic prophase (which typically lasts up to ~4 h in our experiments). After its completion, micronuclei rapidly complete several meiotic and mitotic divisions

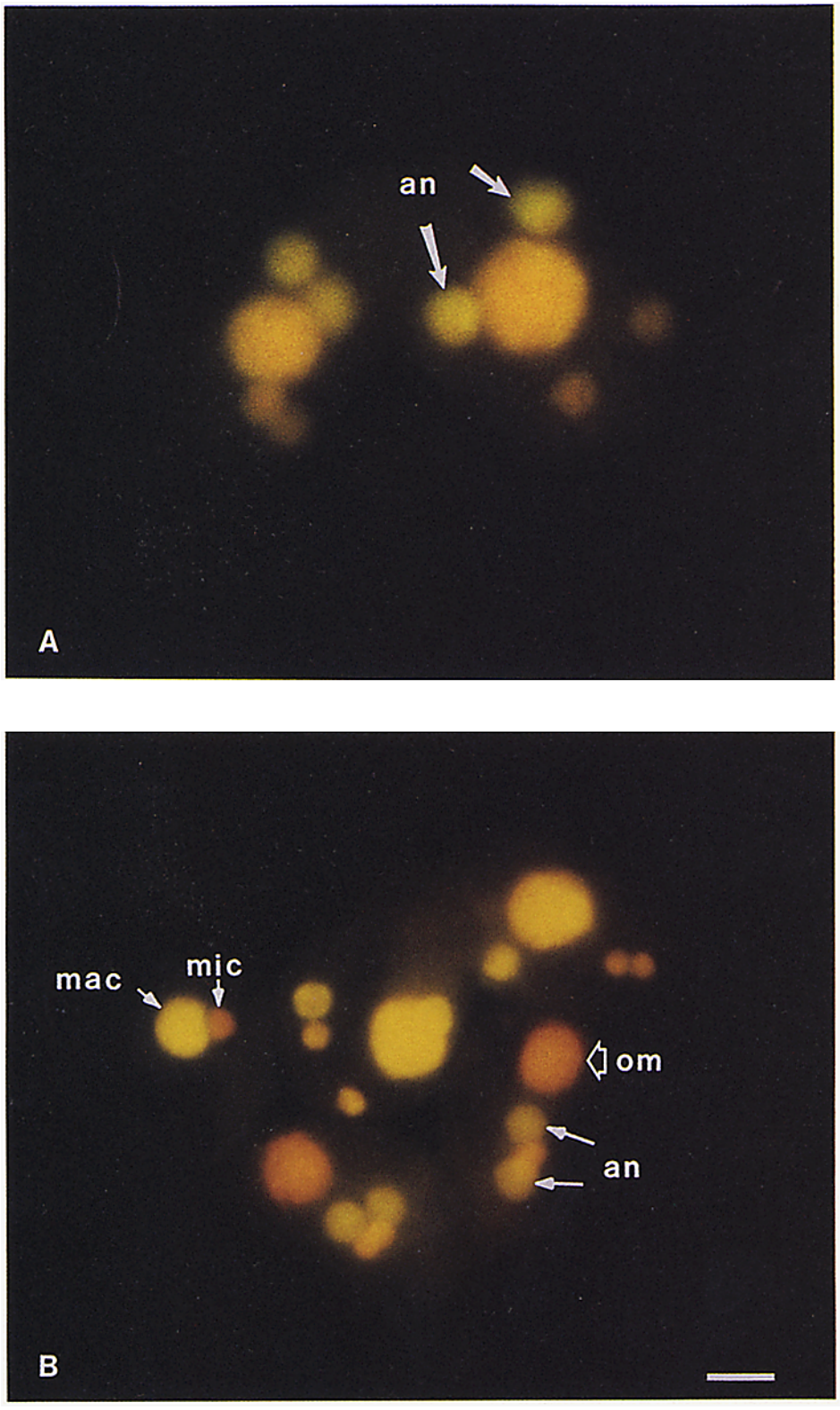


Figure 7. Deposition- and transcription-related acetylation in *Tetrahymena*: key developmental transitions. Mating cells were reacted with "penta" hv1 antibodies followed by fluorescein-conjugated second antibodies (yellow-green color). Cells were stained finally with ethidium bromide which imparts a red color to nuclei which do not react with the first antibodies (see Materials and Methods, Wenkert and Allis, 1984, for details). Note the positive (yellow-green) staining of macronuclei or new macronuclei (*an*) in all stages except when parental macronuclei (*om*) are being degraded. This staining we feel represents transcription-related histone acetylation. Micronuclei are not stained with these antibodies and are reddish except under conditions where micronuclear DNA replication and histone synthesis are pronounced. Subtle intermediate shading suggestive of nuclei first beginning to gain or lose acetylated histones is also observed (see text for details). All of these cells were taken from a time point 7 h after opposite mating types were mixed. The cell shown with a labeled *mac* and *mic* is an unpaired cell. Bar, 6 μm.

(these occur during pre- and postzygotic development; see Martindale et al., 1982 for details) to eventually produce new micro- and macronuclei. Many of these micronuclear divisions are accompanied by DNA synthesis (Doerder and DeBault, 1975; Allis et al., 1987) and, therefore, it is not surprising that most of the new histone and chromatin assembly which occurs during this interval is specific to micronuclei (Allis et al., 1985). Recently, we observed that essentially all of the newly synthesized H4 entering micronuclei during this stage is diacetylated (Allis et al., 1985). Diacetylation of newly synthesized H4 appears to be highly conserved among eukaryotes and is often referred to as deposition-re-

lated acetylation (see Richman et al., 1988 for references). Given the existence of this second system of acetylation in *Tetrahymena*, it was of interest to determine whether any of our acetylation-specific antisera ("tetra" H4 and "penta" hv1) would recognize "deposition-related" diacetylated H4.

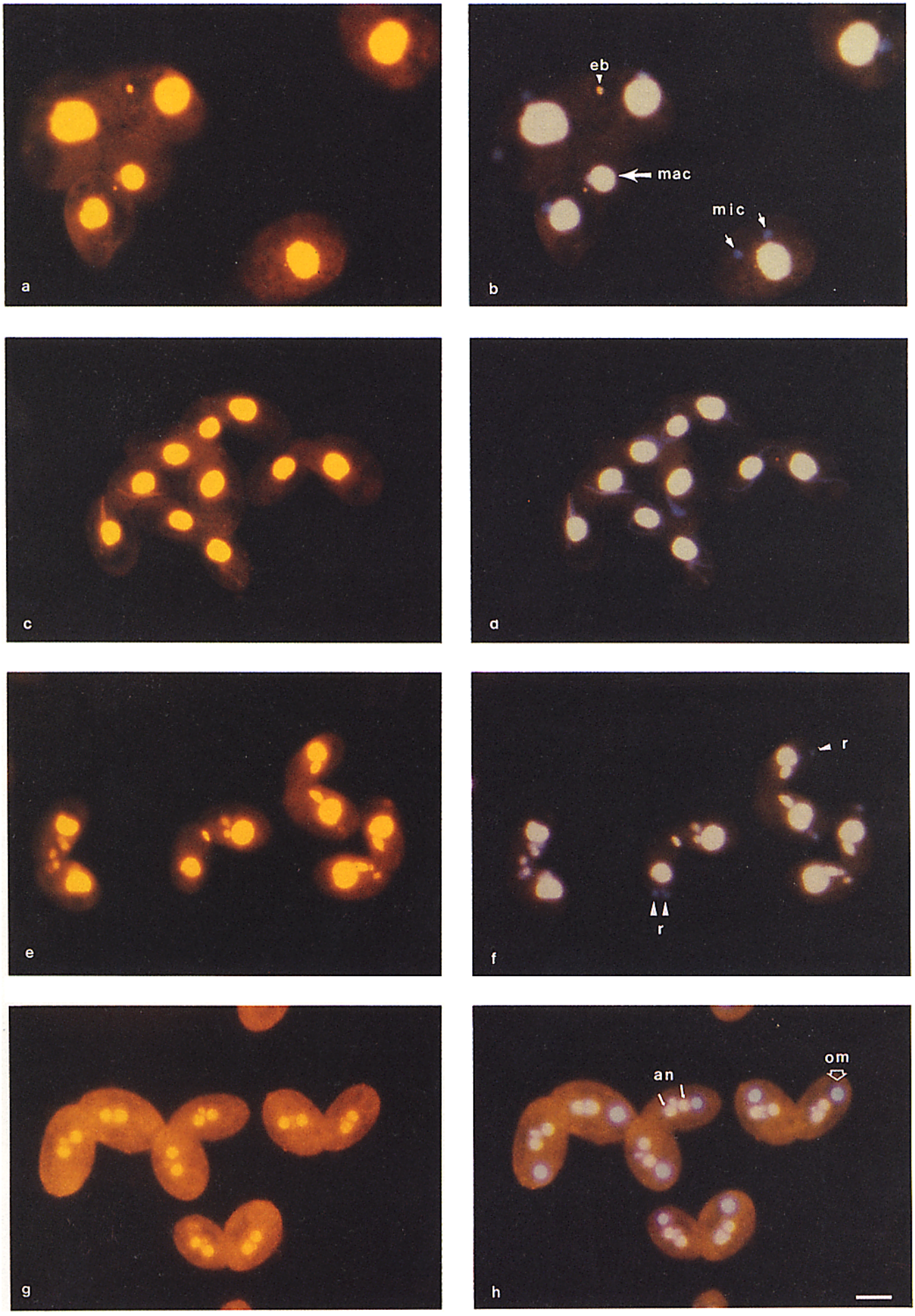
To address this question, micronuclei and macronuclei were isolated from 5- to 6-h mating cells and their histones were fractionated on acid-urea gels as shown in Fig. 4. As noted previously (Allis and Wiggins, 1984; Allis et al., 1985), unusually large amounts of diacetylated H4 are observed in the staining profile of micronuclei isolated from this stage (see leftward pointing arrow next to lane *2*), presumably resulting from deposition-related H4 acetylation. In contrast, macronuclear H4 as well as the other core histones are extensively modified by transcription-related acetylation (lane *1*). Corresponding immunoblots show clearly that the diacetylated form of H4 in micronuclei is easily detected with either "tetra" H4 or "penta" hv1 antisera. Thus it seems that both of these polyclonal antisera contain antibodies which recognize deposition- and transcription-related acetate groups on histone H4.

Given the results shown in Fig. 4, we predicted that both the "tetra" H4 and "penta" hv1 antisera would stain micronuclei in stages of the life cycle where histone deposition into micronuclei is pronounced. Fig. 5 shows typical examples of conjugating cells in meiotic prophase (*a–c*) and at the completion of the second prezygotic division (which is the second meiotic division, *d–f*). In both of these stages, only macronuclei are stained with the "tetra" H4 antibodies (similar results are obtained with the "penta" hv1 antibodies, Fig. 8, *c* and *d*). In contrast, micronuclear staining is consistently observed with the "tetra" H4 antibodies in conjugants completing the third prezygotic division, the first postzygotic division (see arrows in Fig. 5, *h* and *i*), and the second postzygotic division (see arrows in Fig. 5, *k* and *l*). Since S phase DNA replication occurs in micronuclei after each of these mitotic divisions (Doerder and DeBault, 1975; Allis et al., 1987), it seems quite reasonable to propose that the micronuclear staining seen in Fig. 5 results from deposition-related H4 acetylation. Deposition-related micronuclear staining during these periods is also observed with "penta" hv1 antibodies (see Fig. 8, *e* and *f*).

Micronuclear staining is typically low in vegetative cells (1–2%) even when cells are examined which are known to be in micronuclear S phase (see the dividing cell in the insert to Fig. 3, *a–c*). This suggests to us the possibility that deposition-related acetate groups on H4 are removed more quickly (by deacetylation) in vegetative cells than in conjugating cells. As well, it is likely that the rapid succession of multiple micronuclear DNA replications (after the third prezygotic, first postzygotic, and second postzygotic divisions) lead to an increase in the steady-state amount of deposition-related acetylated histone in micronuclei of young conjugants such that antibody staining is detected. In support of this, we point out that it is much easier to observe stainable quantities of diacetylated H4 in micronuclei from 5- to 6-h mating cells than in vegetative cells. However, in one experiment where a low density culture was used (100,000 cells/ml), 10% micronuclear staining was observed. Since micronuclear S phase occupies ~10% of the cell cycle (Wu et al., 1988), this result suggests that deposition-related histone acetylation does exist in micronuclei in vegetative cells.

Development of New Macronuclei. After the second postzygotic division, micronuclei located in the anterior cytoplasm of each pair swell and begin to differentiate into new macronuclei (often referred to as macronuclear anlagen) while posterior division products remain micronuclei (pairs in this stage have been classified as being in anlagen development I by Martindale et al., 1982). In these cells new macronuclei (labeled *an* in Fig. 6) consistently stain positively with "tetra" H4 antibodies (see the single pair shown in Fig. 6, *a–c*) while posterior micronuclei are typically not stained (or are only weakly stained). This result suggests that H4 acetylation becomes polarized very rapidly after the second postzygotic division such that only new macronuclei contain acetylated forms of H4. Since all four micronuclear division products typically stain positively with acetylation-specific antibodies immediately after the second postzygotic division (presumably because of deposition-related H4 acetylation; see the pair highlighted in Fig. 5, *k* and *l*), the selective staining of new macronuclei very early in anlagen development implies either (*a*) that deposition-related acetate groups on H4 are lost preferentially from new micronuclei after the second postzygotic division as compared to new macronuclei or (*b*) that deposition-related acetate groups on H4 are lost from both new macro- and micronuclei after this division, but that transcription-related H4 acetylation begins selectively in new macronuclei during this interval. While our immunofluorescent data do not enable us to choose which of these two possibilities (if any) is correct, we favor the latter possibility since biochemical studies have shown that very young anlagen (but not micronuclei) incorporate ^{3}H-acetate well in vivo or in vitro (Chicoine and Allis, 1986). Particularly dramatic examples of this transition are shown in color in Fig. 7. One pair is shown immediately after the second postzygotic division (Fig. 7 *A*). While all nuclei are stained somewhat yellow-green from the antibody reaction (presumably because of deposition-related acetylation), close examination shows that the posterior micronuclei are already staining somewhat red (due to staining with ethidium bromide; see figure legend for details) while the slightly swollen anterior new macronuclei (labeled *an*) are not. Thus, the distribution of acetylated histone between new macro- and micronuclei becomes asymmetric quickly after the second postzygotic division.

In older pairs (see Fig. 6, *d–f*, Fig. 7 *B*, and Fig. 8, *g* and *h*), new macronuclei are larger, become phase dark and occupy a more central position within each cell. At the same time, parental macronuclei move to the posterior of each cell, become pycnotic and are in the process of being destroyed (this stage is referred to as anlagen development II). As expected, anlagen (but not micronuclei) continue to stain with the "tetra-H4" and "penta" hv1 antibodies and continue to do so in all later stages that we have examined (we have analyzed exconjugants at 15–16 h, data not shown). Because our antibodies react with all acetylated forms of H4 (mono- through tetra-, Fig. 2), we cannot use these immunofluorescent results to evaluate the degree with which H4 is acetylated during various stages of macronuclear development. Nonetheless, earlier biochemical studies (Chicoine and Allis, 1986) have suggested all of the core histones are acetylated to an extent comparable to fully differentiated macronuclei by 15–16 h of conjugation (a time when anlagen have a ploidy of ~8C). Taken together, we conclude that H4 begins to be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acetylated quickly in new macronuclei after the second postzygotic division and continues throughout later stages of anlagen development.

One striking result to emerge from this study is the sudden loss of staining observed with parental macronuclei during stages of anlagen differentiation (see Fig. 6, *d–f* and Figs. 7 and 8). These nuclei are easily stained with antibodies to the unacetylated H4 (and antisera to other macronuclear-specific proteins; see Wenkert and Allis, 1984), which suggests that acetylated forms of H4 are quickly lost from these nuclei during their elimination from the cell. As shown in Fig. 7 *A*, a shade of red staining (indicative of a decreased ability to be stained with acetylation-specific antibodies) is first detected in parental macronuclei while they are still located centrally in each cell. As development proceeds these nuclei become very red (or dark by immunofluorescence alone, Figs. 6 and 8), indicative of a total loss of acetylated histone in old macronuclei at this time (see nuclei labeled *om* in Fig. 7 *B*). It is tempting to speculate that this loss may be partially related to the pronounced changes in chromatin morphology and loss of transcriptional activity which occurs in these nuclei during this period (Wenkert and Allis, 1986; Weiske-Benner and Eckert, 1985, 1987). As well, it would be interesting to know whether the sudden loss of acetylated H4 from "old" macronuclei during this period is brought about by an increase in histone deacetylation (as opposed to decreased acetylation) which might be sensitive to treatment with sodium butyrate.

Conclusions

Fig. 8 summarizes the main points from our immunofluorescent analyses by showing color plates of growing (*a* and *b*) and mating *Tetrahymena* in meiosis (*c* and *d*), postzygotic division (*e* and *f*), and early stages (*g* and *h*) of anlagen development after reaction with the "penta" hv1 serum and DAPI staining (which stains all nuclei blue). Cells are shown after reaction with first and rhodamine-conjugated second antibodies (staining nuclei positive for acetylated forms of H4 and/or hv1 yellow-orange, lefthand column), or double exposures of the antibody reaction and the DAPI stain (staining acetylation-positive nuclei white and acetylation-negative nuclei blue, righthand column). Like reactions with "tetra" H4 antibodies (Figs. 3, 5, and 6), it is clear that transcriptionally active macronuclei and developing new macronuclei stain well with "penta" hv1 antibodies while transcriptionally inactive micronuclei and old macronuclei do not. This is consistent with numerous other reports which have correlated histone acetylation to transcriptional activity. Nonetheless, micronuclei are stained with these antibodies during stages when micronuclear histone deposition and chromatin assembly are pronounced (see *e* and *f*), supporting the idea that histone acetylation also plays a role in the deposition of new histone on newly replicated DNA.

This approach of raising antibodies to acetylated forms of histones has also been employed by others. Chemical acetylation of intact H4 (or a NH_2-terminal peptide of H4) has been used by other groups to obtain acetylated histone for immunization and these groups have successfully obtained antibodies which react with acetylated forms of H4 (see Pfeffer et al., 1986, 1988; Muller et al., 1987; Hebbes et al., 1988). In contrast to the antibodies that we have produced, the serum characterized by Pfeffer et al. (1986) shows high specificity for tetra-acetylated H4, while that generated by Hebbes et al. (1988) apparently recognizes acetyl-lysine on all of the core histones. Furthermore, the antibodies generated by Hebbes et al. (1988) have been used to fractionate active from inactive chromatin, strengthening the link between acetylated core histones and trancriptionally active chromatin. Thus, it seems clear that these immunological reagents represent useful new probes to investigate questions concerning both transcription- and deposition-related histone acetylation.

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References

Allis, C. D., and D. K. Dennison. 1982. Identification and purification of young macronuclear anlagen from conjugating 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. USA.* 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., L. G. Chicoine, R. Richman, and I. G. Schulman. 1985. Deposition-related histone acetylation in micronuclei of conjugating *Tetrahymena*. *Proc. Natl. Acad. Sci. USA.* 82:8048–8052.

Allis, C. D., R. Richman, M. A. Gorovsky, Y. S. Ziegler, B. Touchstone,

Figure 8. Summary of the existence of acetylated histone during the *Tetrahymena* life cycle. Growing and conjugating cells from various key stages of the life cycle were reacted with "penta" hv1 antibodies and analyzed by indirect immunofluorescence. In these analyses the same cells were examined by DAPI (which stains micro- and macronuclei blue) or by fluorescence microscopy (which stains immunopositive nuclei yellow-orange; *a*, *c*, *e*, and *g*, *lefthand column*). In double exposures of these two images (*b*, *d*, *f*, and *h*, *righthand column*), immunopositive nuclei are stained white and immunonegative nuclei are stained blue. Shown are growing cells (*a* and *b*) and mating cells in stages corresponding to meiotic prophase (*c* and *d*), postzygotic nuclear division (*e* and *f*), and macronuclear anlagen development (*g* and *h*). Note the positive staining of macronuclei or new macronuclear anlagen (*an*; *g* and *h*) in all stages of the life cycle except when parental macronuclei (*om*; *g* and *h*) are being degraded. Micronuclei are not stained (*blue, righthand column*) except under conditions where micronuclear DNA replication and histone synthesis and deposition are pronounced (*e* and *f*). The small DNA-staining body (*eb* in *b*) represents an elimination body which results from the amitotic divisions made by macronuclei in growing cells. The acetylation-negative micronuclei (*r* in *f*) probably represent "relic" micronuclei remaining from the preceding meiotic divisions (see Martindale et al., 1982). Bar, 12 μm.

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.

Allis, C. D., M. Colavito-Shepanski, and M. A. Gorovsky. 1987. Scheduled and unscheduled DNA synthesis during development in conjugating *Tetrahymena*. *Dev. Biol.* 124:469–480.

Bergot, B. J., R. L. Noble, and T. Geiser. 1986. Utility of trifluoromethane sulfonic acid as a cleavage reagent in solid-phase peptide synthesis. *Appl. Biosyst. User Bull.* 16:1–19.

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 *Tetrahymena*: evidence for control at the level of acetylation and deacetylation. *Dev. Biol.* 116:477–485.

Chicoine, L. G., I. G. Schulman, R. Richman, R. G. Cook, and C. D. Allis. 1986. Nonrandom utilization of acetylation sites in histones isolated from *Tetrahymena*: evidence for functionally distinct H4 acetylation sites. *J. Biol. Chem.* 261:1071–1076.

Chicoine, L. G., R. Richman, R. G. Cook, M. A. Gorovsky, and C. D. Allis. 1987. A single histone acetyltransferase from *Tetrahymena* macronuclei catalyzes deposition-related acetylation of free histones and transcription-related acetylation of nucleosomal histones. *J. Cell Biol.* 105:127–135.

Doerder, F. P., and L. E. DeBault. 1975. Cytofluorometric analysis of nuclear DNA during meiosis, fertilization and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. *J. Cell Sci.* 17:417–493.

Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82:70–77.

Glover, C. V. C., and M. A. Gorovsky. 1979. Amino-acid sequence of *Tetrahymena* histone H4 differs from that of higher eukaryotes. *Proc. Natl. Acad. Sci. USA.* 76:585–589.

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

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.

Hebbes, T. R., A. W. Thorne, and C. Crane-Robinson. 1988. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1395–1402.

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

Lerner, R. A., N. Green, H. Alexander, F-T. Liu, J. G. Sutcliffe, and T. M. Shinnick. 1981. Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proc. Natl. Acad. Sci. USA.* 78:3403–3407.

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 modifications of nuclear proteins and their significance. *In* The Enzymology of Posttranslational Modifications of Proteins. R. B. Freedman and H. C. Hawkins, editors. Academic Press, Inc., Orlando, FL. 125–185.

Muller, S., A. Isabey, M. Couppez, S. Plaue, G. Sommermeyer, and M. H. V. Van Regenmortel. 1987. Specificity of antibodies raised against triacetylated histone H4. *Mol. Immunol.* 24:779–789.

Pfeffer, U., N. Ferrari, and G. Vidali. 1986. Availability of hyperacetylated H4 histone in intact nucleosomes to specific antibodies. *J. Biol. Chem.* 261:2496–2498.

Pfeffer, U., N. Ferrari, F. Tosetti, and G. Vidali. 1988. Histone hyperacetylation is induced in chick erythrocyte nuclei during reactivation in heterokaryons. *Exp. Cell Res.* 178:25–30.

Richman, R., L. G. Chicoine, M. P. Collini, R. G. Cook, and C. D. Allis. 1988. Micronuclei and the cytoplasm of growing *Tetrahymena* contain a histone acetylase activity which is highly specific for free histone H4. *J. Cell Biol.* 106:1017–1026.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Van De Water, L., III, S. D. Guttman, M. A. Gorovsky, and J. B. Olmstead. 1982. Production of antiserum and radioimmunoassays for tubulin. *Methods Cell Biol.* 24:79–96.

Vavra, K. J., 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. 1985. Differentiation of nuclear structure during the sexual cycle in *Tetrahymena thermophila*. I. Development and transcriptional activity of macronuclear anlagen. *Differentiation.* 28: 225–236.

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 in developing new macronuclei of *Tetrahymena thermophila*. *J. Cell Biol.* 98:2107–2117.

Wu, M., C. D. Allis, and M. A. Gorovsky. 1988. Cell-cycle regulation as a mechanism for targeting proteins to specific DNA sequences in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. USA.* 85:2205–2209.