

# Histone Acetylation in Conjugating *Tetrahymena thermophila*

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**Abstract.** We have monitored histone acetylation during conjugation of the ciliated protozoan *Tetrahymena thermophila* using antibodies against the tetraacetylated form of H4 histone (Pfeffer, U., N. Ferrari, and G. Vidali. 1986. *J. Biol. Chem.* 261:2496–2498). During meiosis, the three prezygotic divisions, fertilization, and the first postzygotic division, micronuclei, do not contain highly acetylated forms of H4 histone. However, after the second postzygotic division, when anteriorly located micronuclei begin to develop into new macronuclei, they are strongly stained by the anti-tetraacetylated H4 histone antibody. In the old macronucleus, histones are actively deacetylated when

it has ceased to transcribe but before it is eliminated. Histone acetylation processes analyzed here appear to be correlated to the commitment to transcription rather than to the transcription process itself. This is in good correlation with evidence we have obtained in chick erythrocyte nuclei during reactivation upon fusion with mammalian cells (Pfeffer, U., N. Ferrari, F. Tosetti, and G. Vidali. 1988. *Exp. Cell Res.* 178:25–30). Furthermore, it becomes clear from our data that histone acetylation occurs in close correlation to the position of nuclei within the cytoplasm of *T. thermophila*. Mechanisms that control differential histone acetylation and deacetylation are discussed.

**S**INCE the original observation, histone acetylation has been correlated to transcription and considerable evidence has accumulated indicating that this reversible postsynthetic modification of lysine residues in the amino-terminal portion of core histones plays a key role in the regulation of gene expression (for a review see reference 2).

Different methods have been developed to fractionate chromatin depending on its transcriptional activity and accordingly, it has been found that fractions containing actively transcribed genes were enriched in highly acetylated histones (1, 17, 33). Recent analyses using anti-acetyl-lysine antibodies have established that a given gene is associated with highly acetylated histones when it is transcribed (23), although it cannot be ruled out that genes competent to be transcribed, but inactive at the moment of analysis, may also contain acetylated histones.

Nucleosomes undergo reversible conformational changes during transcriptional activation (10, 32), histones are absent from promoter sequences of active genes (25), nucleosomes inhibit initiation of transcription but do not inhibit elongation of initiated transcripts (27, 28) and at least H4 histone is retained on a highly transcribed gene (35). The contact points between nucleosomal DNA and H4 histone have been analyzed and a highly basic cluster in the amino-terminal portion has been shown to be involved in sharp bending of the DNA double helix (18). Interestingly, this cluster encompasses lysine at position 16 which is the first to be acetylated *in vivo* in calf thymus (15) and, moreover, this region is in close contact to a site 55–65 bp from the end of the nucleosomal DNA, which becomes available to nucleases upon histone acetylation (34).

Taken together, these results strongly suggest a correlation between histone acetylation and transitions in the nucleosomal structure which in turn promote transcription. The processes controlling histone acetylation and the involvement of this modification in the sequence of events leading to a competent chromatin structure are far from being completely understood.

In previous analyses we have shown that in transcriptionally silent chick erythrocyte nuclei, which do not contain highly acetylated forms of the core histones, histone acetylation is induced during reactivation upon fusion with HeLa cells leading to high acetylation levels at early stages of reactivation when the nuclei still have not undergone gross morphological changes and when there is still little, if any, transcription (31).

Here we present the study of histone acetylation processes during conjugation of *Tetrahymena thermophila*. As most of the ciliated protozoan *T. thermophila* contains a transcriptionally active somatic macronucleus and an inactive germlinal micronucleus both generated from a common ancestor during the sexual phase of the life cycle. During conjugation, the micronucleus undergoes meiosis and in two prezygotic divisions four micronuclei are formed, three of which are successively eliminated. The fourth nucleus divides again generating two haploid pronuclei, one of which migrates into the cytoplasm of the conjugation partner and fuses to the stationary pronucleus. The fertilized nucleus divides twice forming four diploid micronuclei: two of them migrate to the posterior part of the cell and differentiate into new micronuclei whereas the two anterior nuclei develop into new macronuclear anlagen which become actively synthesizing RNA.

Later they substitute the degenerating old macronucleus which migrates to the posterior part of the cell and ceases to transcribe (for a review of cytological events in *T. thermophila* see reference 8).

*T. thermophila* thus offers an interesting model system for the study of chromatin reorganization and nuclear differentiation. We have followed histone acetylation during conjugation in this protozoan using an antibody recently produced in our laboratory which specifically recognizes the tetraacetylated form of H4 histone. In previous studies we have shown that the antibody recognizes its antigen in nucleosomes (30) and in whole chromatin by in situ immunofluorescence (37). We have already shown that hyperacetylated forms of H4 histone can be found in macronuclei of vegetatively growing cells (37) of *T. Thermophila* and this is in agreement with electrophoretic analyses where the patterns of histones extracted from macro- and micronuclei are compared (21). This paper gives details of acetylation processes during the development of the new macro- and micronuclei in conjugating *Tetrahymena thermophila*.

## Materials and Methods

*Tetrahymena thermophila* strains BII and CHX were a kind gift from Dr. Günter Cleffmann (Giessen). Cells were grown in 1% tryptone/0.1% (wt/vol) yeast extract/0.001% (wt/vol) EDTA at 29°C in flasks 10× larger than the culture volume without shaking to a titer of  $4-5 \times 10^5$  cells/ml.

Nuclei were isolated and histones were extracted after the procedure described by Allis et al. (5), except that 0.25 M HCl was used instead of H<sub>2</sub>SO<sub>4</sub> and that histones were precipitated with acetone instead of TCA. Histones were separated either on 12.5% acrylamide slab gels containing Na-dodecylsulfate (26) and electrophoretically transferred to nitrocellulose sheets (36) or on Triton/urea/acetic acid gels and blotted by diffusion as described (30). Blots were immunostained following procedures described previously (30).

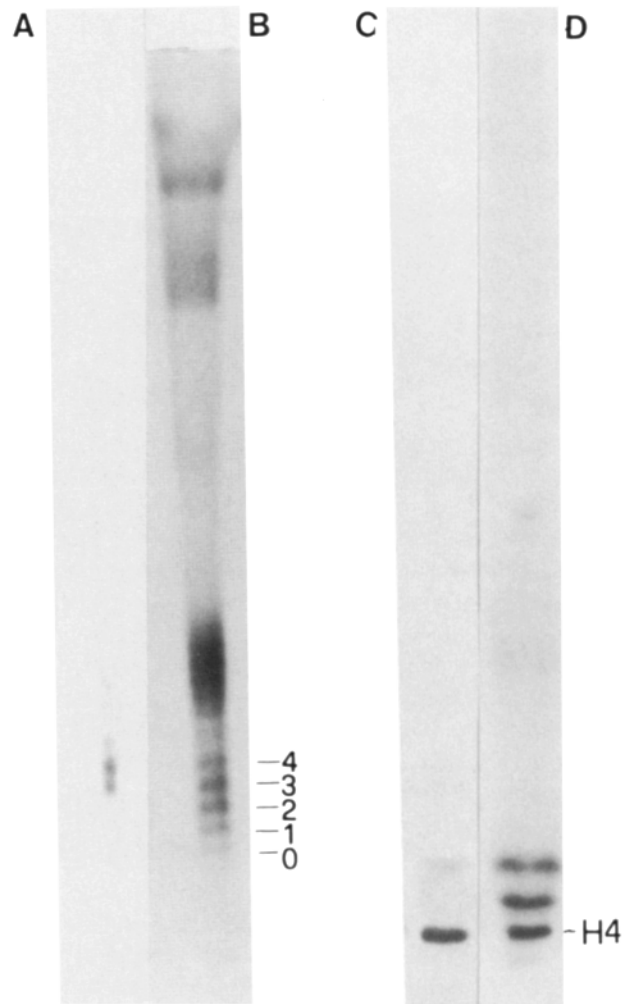
For conjugation, cells were collected by centrifugation at 300 g for 5 min, washed three times and finally resuspended in 10 mM Tris-HCl, pH 7.0, to an optical density of 0.2 at 600 nm. After 20–24 h starvation at 29°C, equal volumes of the two strains were mixed. At different times after beginning of conjugation, 0.1 ml aliquots were removed and cells were applied to microscopy slides by centrifugation in cytospin adapters (Haereus) at 100 g for 10 min. Slides were shortly dried and fixed for 5 min in methanol, 5 min in 3.7% formaldehyde in PBS, and permeabilized by treatment with 0.5% Triton X-100 in PBS for 15 min.

Normally, 60–90% of the cells conjugated and they showed good synchrony as judged from analysis of micronuclear stages. During conjugation cells were not refed.

Antibodies were raised in the rabbit and characterized as reported (30). Purified IgG fraction of the anti-(tetraacetylated) H4 antiserum was applied to the slides at 100-fold dilution (to serum concentration). Slides were incubated for 90 min at room temperature and washed three times in PBS with gentle shaking. Rhodamine-conjugated goat anti-rabbit IgG (H + L; Jackson Immuno-Research Laboratories, Inc., Avondale, PA) were diluted 1:100 in PBS containing 0.1 mg/ml nonimmune goat immunoglobulins and applied to the slides followed by 60 min incubation and washing as above.

4',6-diamidino-2-phenyl-indole (DAPI; Sigma Chemical Co., St. Louis, MO) was dissolved at 0.1 mg/ml in ethanol, 100-fold diluted in PBS and applied to the slides with 5 min incubation followed by two washes in PBS. Slides were mounted in 90% glycerol/10% PBS and examined and photographed in a Zeiss Axiophot equipped with epifluorescence illumination using filter combinations 487901 (UV-H365) for DAPI and 487915 (Green H546) for rhodamine. Kodak Ektachrome P800/1600 were exposed at 1,000 ASA, reciprocity factor 0, and developed at 1,600 ASA.

**Figure 2.** Indirect immunofluorescence of conjugating *T. thermophila*, prefertilization stages. (a and a') A single starved cell before conjugation. The micronucleus is located in an indentation of the macronucleus. (b and b') A conjugating pair in meiotic prophase after 2 h of conjugation. Crescent formation in one partner whereas in the other partner the micronucleus has just left its original position. (c and c') A pair after the second prezygotic division (5 h of conjugation). Four anteriorly located micronuclei are visible in both cells. (d and d') After the third prezygotic division (6 h of conjugation) two anterior pronuclei are visible. In the posterior part of the cells remaining

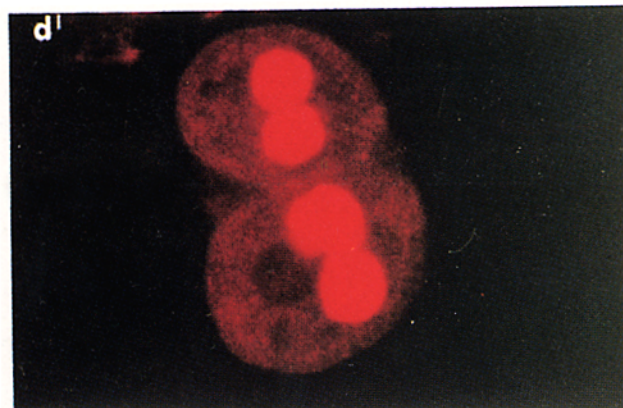
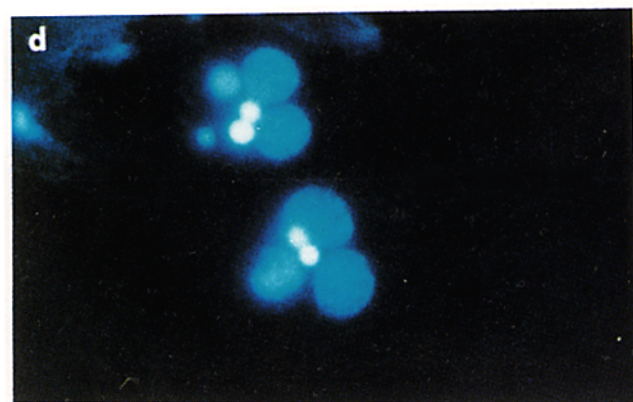
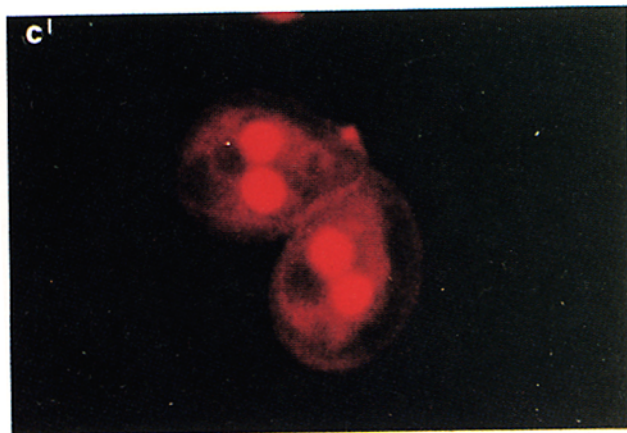
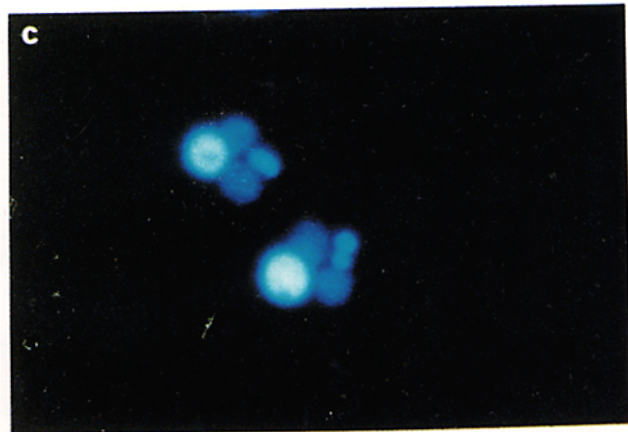
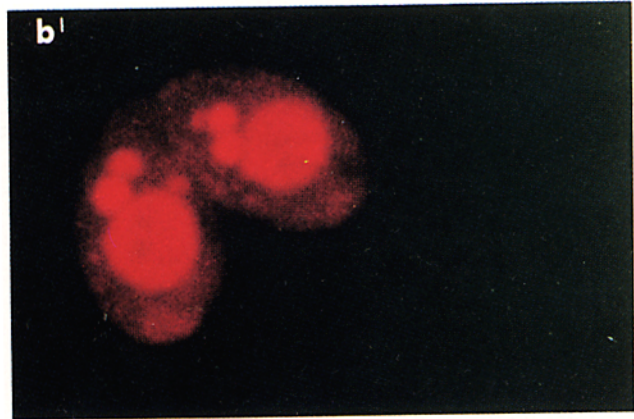
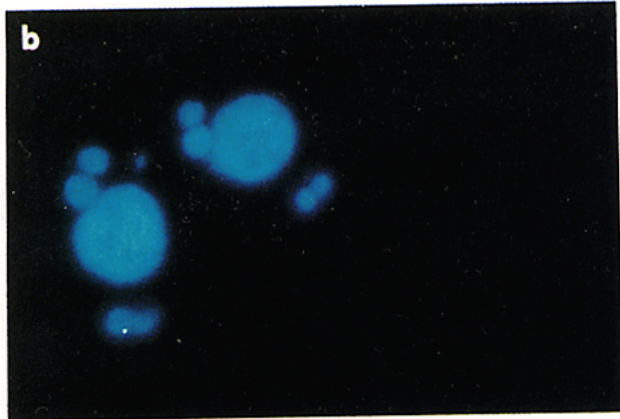
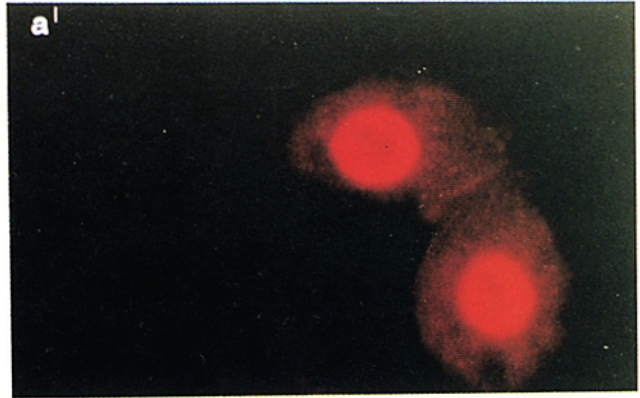
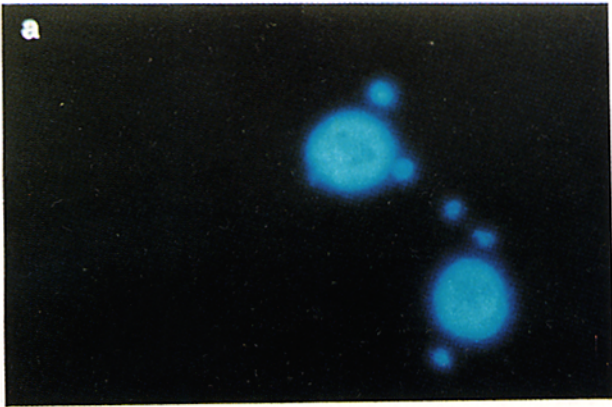


**Figure 1.** Western blot analysis of *Tetrahymena thermophila* histones. Coomassie brilliant blue stain of TAU gel (lane B) and SDS gel (lane D) and immunostain of the corresponding blots using anti-tetraacetylated H4 histone antibodies (lanes A and C). The antibody stains the band corresponding to H4 histone on the blot derived from the SDS gel and bands corresponding to the tetra- and triacetylated forms of H4 on the TAU blot, indicating that the antigenic determinant is conserved in *T. thermophila*.

## Results

### Immunoreactivity of *T. thermophila* H4 Histone to Antibodies against the Tetraacetylated Form of Mammalian H4 Histone

For the experiments to be described we have used antibodies against the tetraacetylated form of H4 histone elicited in the rabbit employing the chemically acetylated peptide 1–37 isolated from calf thymus H4 histone (30). The sequence of this histone shows two amino acid substitutions in the amino-terminal portion and an insertion of an arginine residue at position three as compared to the corresponding histone of *T. thermophila*. This region encompasses four lysine resi-



micronuclei are eliminated. (a-d) DAPI stain revealing the nuclear stage. (a-d') Indirect immunofluorescence using anti-tetracetylated H4 histone antibodies and rhodamine-conjugated second antibody. Note that no reaction occurs in micronuclei during the prefertilization stages indicating that tetraacetylation of H4 histone is not correlated to deposition of histones during replication or to the chromatin reorganizations occurring during meiosis and prezygotic divisions.



dues which are the target for postsynthetic acetylation and this pattern is conserved between *T. thermophila* and mammals (lysines at positions 4, 7, 11, 15 and 5, 8, 12, 16, respectively) (20, 24).

By immunoblotting of histones separated on Triton/acetic acid/urea gels we have previously shown that our antibody reacts only with the tetraacetylated and to a lesser extent with the triacetylated form of H4 histone (30). Here we have tested the reactivity of the antibody with histones extracted from isolated macronuclei of *T. thermophila*, separated by SDS and by Triton/urea/acetic acid gel electrophoresis and blotted onto nitrocellulose sheets. The binding of the antibody has been revealed by anti-rabbit immunoglobulins antibodies conjugated to horseradish peroxidase. The results are depicted in Fig. 1. The antibody stains specifically the band corresponding to H4 histone on blots from SDS-gels and bands corresponding to the tetra- and triacetylated form of this histone on blots from TAU gels. This indicates, as expected, that the antigenic determinant, that is the pattern of  $\epsilon$  acetyl-lysines in the amino-terminal portion of the molecule, is not immunogenically distinct from the corresponding pattern in mammalian H4 histone.

A slight cross-reaction is observed with the band corresponding to H2a and H3 histones which are not separated on this SDS gel and with a band probably corresponding to H1 histone on blots from TAU gels. The different cross-reaction on blots from different gel systems indicates that only the binding to acetylated forms of H4 histones is specific.

### Immunofluorescence Analysis of Conjugating *T. thermophila*

The antibody has been used in immunofluorescence analyses of vegetatively growing, starved, and conjugating *Tetrahymena thermophila*. The results are shown in Figs. 2 and 3. For conjugation, cells with different mating types have been mixed after initialization under starving conditions. At different times, samples have been removed and prepared for indirect immunofluorescence staining, using the anti-tetraacetylated H4 histone antibody and a rhodamine-conjugated second antibody (*red stain*, sections on right in Figs. 2 and 3). The nuclear stage of the cells has been revealed by counterstaining the same cells with DAPI, a DNA specific fluorescence dye (14) (*blue stain*, sections on left in Figs. 2 and 3).

In starved nonconjugating cells, the micronucleus is located in an indentation of the macronucleus and shows no staining with the antibody whereas the macronucleus strongly reacts (Fig. 2, *a* and *a'*), indicating that only the macro-

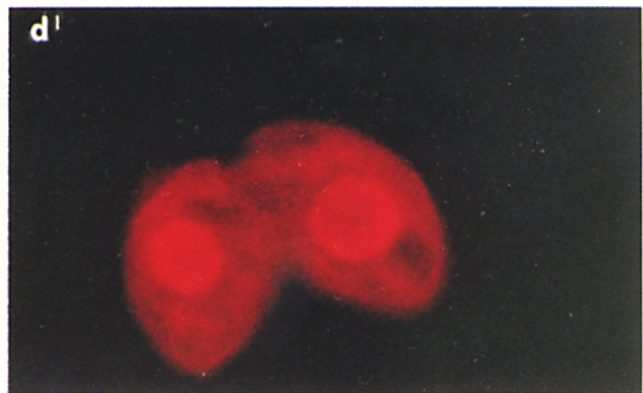
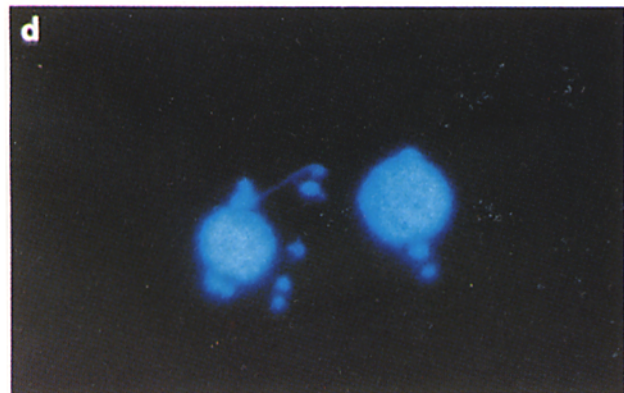
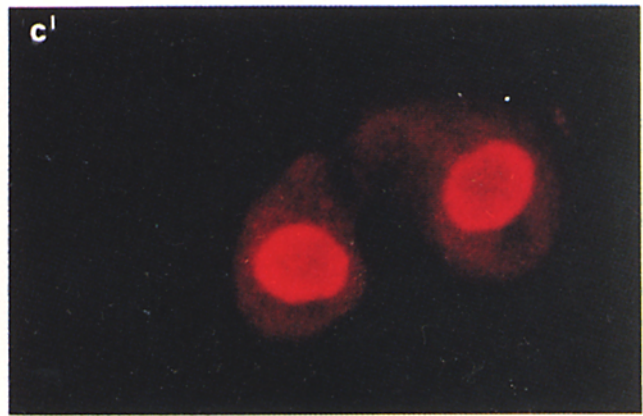
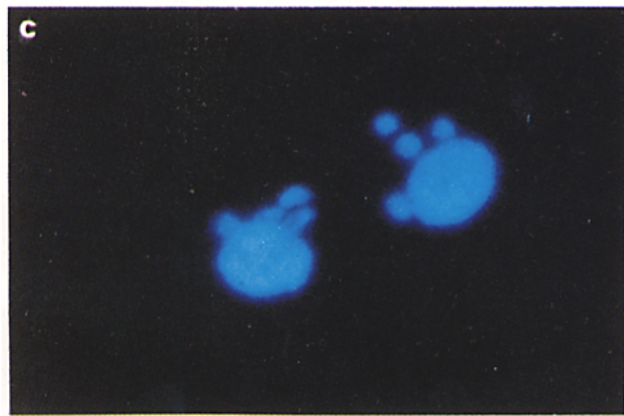
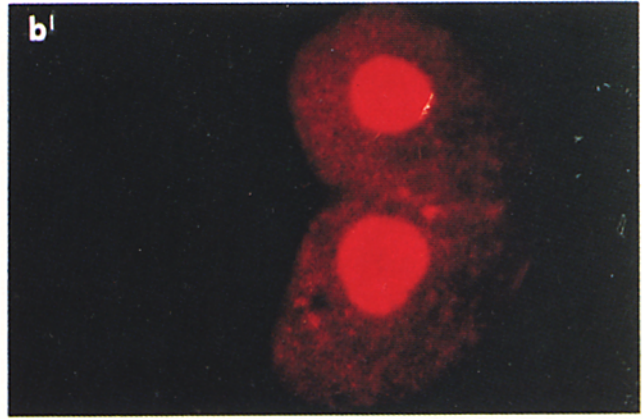
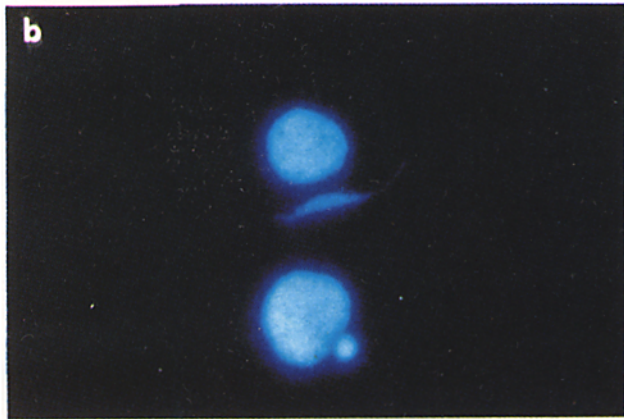
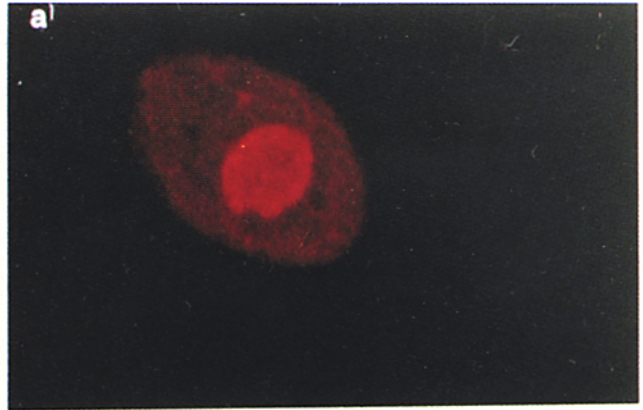
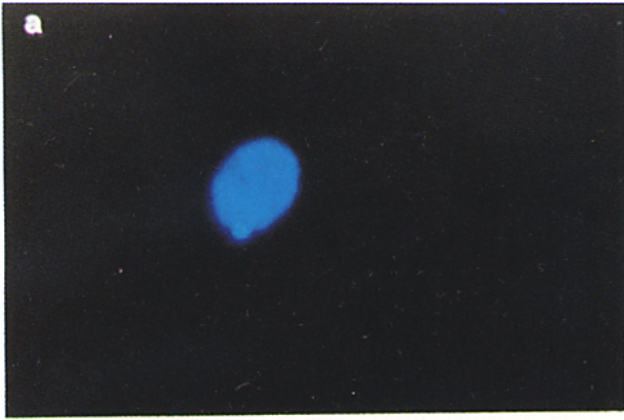
nucleus contains highly acetylated forms of H4 histone. The same is observed in vegetatively growing cells (data not shown).

Upon conjugation, the micronucleus undergoes meiosis and Fig. 2 *b* shows early meiotic prophase with crescent formation in one partner whereas the other one shows a slightly earlier stage where the micronucleus has just left its pouch. Fig. 2 *c* shows the situation after the second prezygotic division and Fig. 2 *d* shows the result of the third prezygotic division where pronuclei are seen in the anterior part of the cells and micronuclei which will be eliminated are located in the posterior part of the cells. During all these nuclear events which include DNA synthesis in meiotic prophase and between nuclear divisions (7), the anti-tetraacetylated H4 histone antibody does not stain micronuclei whereas macronuclei are labeled (Fig. 2, *a'-d'*), indicating that the antibody used does not reveal histone deposition related acetylation processes which occur during DNA-synthesis. Deposition-related histone acetylation leads to the diacetylated form of H4 histone showing a different acetylation site usage (13). No diacetylated forms are recognized by the antibody used here (Fig. 1).

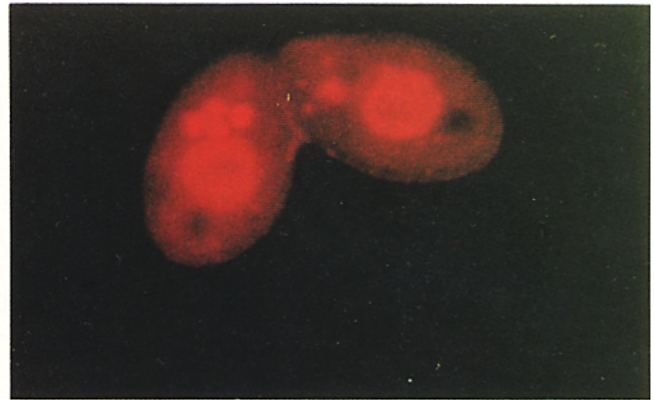
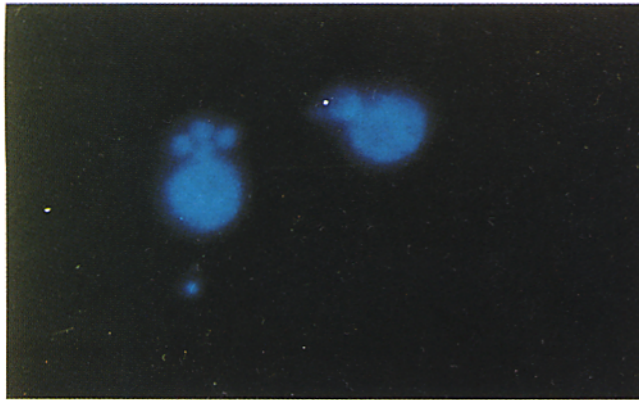
This situation persists up to the first postzygotic division (Fig. 3, *a* and *a'*) where none of the two micronuclei in each partner is stained by the antibody. After the second postzygotic division two micronuclei migrate to the posterior part whereas the other two micronuclei of each cell remain in the anterior part and begin to swell. Over these young macronuclear anlagen a strong label is now observed whereas the posteriorly located micronuclei show no staining with the antibody (Fig. 3, *b* and *b'*) indicating that at this stage differential acetylation occurs in different nuclei depending on their position within the cytoplasm. The importance of the position is emphasized by the fact that in the few cases where we observed an apparent migration failure of micronuclei, H4 histone is acetylated in all four micronuclei (Fig. 4), although the cause of this event remains unknown and its frequency does not allow statistical evaluation.

In different analyses it has been shown that at this cytological stage during conjugation, transcriptional activity is still carried out by the centrally located macronucleus whereas the young macronuclear anlagen are still silent (38, 39). As the onset of transcription may depend on refeeding (9), it must be pointed out that in the experiments reported here conjugating pairs have not been refed. Moreover, histone acetylation appears to peak rapidly as it is difficult to see intermediate states. As judged from the position and size of the labeled macronuclear anlagen this process clearly precedes

**Figure 3.** Indirect immunofluorescence of conjugating *T. thermophila*, postfertilization stages. (*a* and *a'*) Conjugating pair after the first postzygotic division (6.5 h of conjugation). Two micronuclei have formed from the fertilized micronucleus. (*b* and *b'*) Second postzygotic division (7 h of conjugation). Two anteriorly located micronuclei (also referred to as young macronuclear anlagen) begin to swell and will later on develop into new macronuclei whereas two products of the second postzygotic division have migrated to the posterior part of the cell and will become new micronuclei. The old macronucleus is still centrally located. (*c* and *c'*) Macronuclear anlagen development (8 h of conjugation). The old macronucleus has migrated to the posterior part of the cells but is only slightly pyknotic. Macronuclear anlagen have enlarged and are now centrally located whereas micronuclei have not undergone morphological changes. (*d* and *d'*) Macronuclear anlagen development after 9 h of conjugation. The old macronucleus is in an advanced stage of elimination whereas new macronuclei continue to enlarge. (*a-d*) DAPI stain revealing the nuclear stage. (*a'-d'*) Indirect immunofluorescence using anti-tetraacetylated H4 histone antibodies and rhodamine conjugated second antibody. Note that up to the first postzygotic division of the fertilized nucleus no acetylation takes place in the micronuclei. After the second division, when also morphological differentiation begins, anteriorly located young macronuclear anlagen are heavily stained by the antibody indicating the onset of histone acetylation which rapidly reaches maximum levels. Acetylation of H4 histone persists during macronuclear anlagen development, whereas no reaction occurs in the old macronucleus in its final position in the posterior part of the cell. New micronuclei are never stained by the antibody.







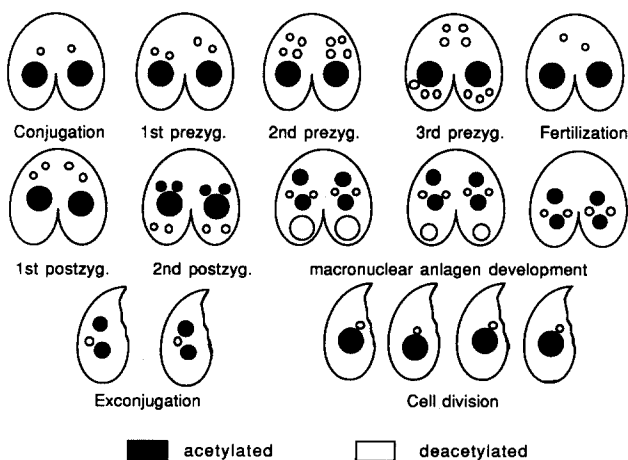
**Figure 4.** Migration failure of micronuclei after the second postzygotic division. Cells are stained with DAPI (*left*) and with anti-tetraacetylated H4 antibodies (*right*). When micronuclei fail to migrate to the posterior part of the cells, histones of all four products of the second postzygotic division become acetylated.

the deposition of histone variant 1 in the developing macronuclei which has been found to correlate well with the onset of transcription (39).

At early times of macronuclear anlagen development (Fig. 3, *b* and *b'*), the old macronucleus is still located in the center of the cell and tetraacetylated H4 histone can be detected by the antibody. Interestingly, no highly acetylated forms of H4 histone can be detected in the old macronucleus when it has reached its final position in the posterior part of the cell (Fig. 3 *c*). At this stage the old macronucleus is only slightly pyknotic and antisera against whole histone fraction and against histone variant 1, under experimental conditions essentially identical to those used by us, still stain the old macronucleus in its final position (39) where it is slowly eliminated while the new macronuclear anlagen are growing and heavily stained (Fig. 3, *c* and *c'*, and *d* and *d'*).

As in the micronuclei, acetylation of H4 histone in the old macronucleus appears to be correlated to the position within the cytoplasm. The new micronuclei, however, at later stages of conjugation migrate again to a central or even anterior position showing no induction of histone acetylation (Fig. 3, *c*, *c'*, and *d* and *d'*).

To allow a better identification of different cytological stages and to summarize the results obtained from the immunofluorescence analyses, nuclear events and acetylation processes are reported schematically in Fig. 5.



**Figure 5.** Summary of acetylation events taking place during conjugation of *Tetrahymena thermophila*.

## Discussion

In the sexual phase of the life cycle of *T. thermophila* new macronuclei and new micronuclei are generated from a common ancestor after two divisions of the fertilized nucleus. The chromatin of two formerly completely silent micronuclei is committed to transcriptional activity whereas the other two micronuclei remain inactive and develop to maintain the genetic continuity of the organism including the ability to undergo meiosis in future conjugations. These processes are accompanied by a series of events such as selective deposition of histone variants (39) and proteolytic processing of H3 and H1 like histones (3, 4, 6, 12). Furthermore, new macronuclei undergo large scale genome rearrangements accompanied by elimination of micronuclear sequences, gene amplification and formation of new telomeres (for a review see reference 4).

We show here the analysis of histone acetylation processes during conjugation up to macronuclear anlagen development under starvation conditions and the results suggest that the onset of histone acetylation is related to the commitment of chromatin to transcription and that it is the earliest event so far shown to occur during macronuclear anlagen development.

The present results appear to be in disagreement with those obtained by Chicoine and Allis (11) who have studied the level of histone acetylation in macronuclear anlagen of conjugating *T. thermophila* by electrophoretic analysis of isolated histones. These authors observed only slightly increased acetylation in young anlagen but high levels of histone acetyltransferase and histone deacetylase activities. We can explain the discrepancies with our observations as the result of different technical approaches. The preparation of nuclei, and especially of macronuclear anlagen, needs time-consuming procedures which might result in an artificial loss of acetylgroups. Our fixation protocol meets the need of immediate blocking of all metabolic processes so that the original level of acetylation is likely to be maintained.

We agree with the authors of the cited work that in young anlagen turn-over rates due to high enzyme activities may be high and we would like to stress again the model proposed by Perry and Chalkley (29) where rapid waves of acetylation and deacetylation travel through the chromatin to periodically open up the entire genome thus allowing the access of

factors that regulate transcriptional activity. We propose that this type of acetylation, corresponding to the fraction of histones acetylated with high acetate turn-over (16), is superimposed to acetylation processes with slower kinetics which may serve to maintain the structural dynamics of normally transcribed chromatin. Thus rapid acetylation processes would allow the commitment of the differentiating nucleus where, for short times, the whole genome must be available as transcription factors do not "know" in which region to bind and the completely silent chromatin does not "know" which regions are to be activated. This process, occurring at high levels in the differentiation of *T. thermophila* micronuclei and in the chick erythrocyte nuclei during reactivation, might occur at lower levels in differentiation and development of all eukaryotic cells.

Interestingly enough, deacetylation of histones in the old macronucleus appears to be a regulated event rather than a consequence of degradation of the nucleus. On the other hand, it seems unlikely that the elimination of the old nucleus is dependent on the complete deacetylation of histones. Therefore we like to suggest that deacetylation is due to the presence of specific factors that regulate enzymes involved in histone acetylation and deacetylation. These factors might be the same that induce histone acetylation in the anteriorly located developing anlagen and the cytoplasm of conjugating *T. thermophila* might contain a gradient of these factors, thus regulating differential acetylation in different regions of the cytoplasm. An alternative hypothesis is that acetylation of young macronuclear anlagen could be due to acetyltransferases which are specifically transported into the anterior nuclei. The appropriate targeting of these enzymes could be explained by the replication-expression model (22) as after the second postzygotic division DNA replication seems to occur more rapidly in macronuclear anlagen than in new micronuclei (7).

Clearly the final answer will be the identification of mechanisms which regulate histone acetylation and for studies presently in progress in this direction *Tetrahymena thermophila* appears to be the system of choice.

We wish to thank Dr. Günter Cleffman, Giessen, for providing *Tetrahymena thermophila* strains; Margherita Isola for photographic work; and Alessandra Forlani for technical assistance.

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