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Deposition-related histone acetylation in micronuclei of conjugating *Tetrahymena*

(histone synthesis/chromatin assembly/histone deacetylation/ciliates)

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ABSTRACT **Macro- and micronuclei of the ciliated protozoan, *Tetrahymena thermophila*, afford a unique opportunity to study histone acetylation under conditions where acetylation associated with the regulation of transcription and acetylation associated with the deposition of histones on the DNA are separable. In this study we demonstrate that histone H3 and histone H4 synthesized in young (5 hr) conjugating *Tetrahymena* are deposited into micronuclei in acetylated forms. Most of the newly synthesized histone H3 migrates as a monoacetylated form while essentially all of the new histone H4 is deposited as a diacetylated species. Since micronuclei replicate rapidly during this stage of the life cycle, but are transcriptionally inactive, these data suggest that histone acetylation is related functionally to histone deposition and chromatin assembly. Pulse–chase experiments show that micronuclei also contain a butyrate-sensitive deacetylase activity(ies) which operates to remove the deposition-related acetate groups from newly synthesized and deposited H3 and H4. This enzymatic activity probably contributes to the steady state level of micronuclear histone acetylation that is low or nonexistent. Thus, evidence is emerging for at least two independent systems of histone acetylation in *Tetrahymena*. The first system is specific to macronuclei and may be related to gene expression. The second system is common to macro- or micronuclear histones (H3 and H4) and may be related to histone deposition during DNA replication.**

Modification of histones by the acetylation of specific internal lysine residues is an active metabolic process whose function(s) is poorly understood. In general three distinct functions for histone acetylation have been proposed. The first involves postsynthetic acetylation/deacetylation reactions which occur within the nuclei of most cells and may play a role in the regulation of transcriptional activity (1–5). The second is the cytoplasmic acetylation of histones at the time of synthesis that may be related to their deposition (6, 7). Finally, histone acetylation may play a role in histone replacement during late stages of germ cell maturation (8).

The biology of the ciliated protozoan *Tetrahymena thermophila* lends itself well to investigations aimed at addressing the first two of these possible functions of histone acetylation under conditions where they are nonoverlapping (Fig. 1). During vegetative growth each cell contains two distinct types of nuclei, a transcriptionally active macronucleus and an inactive micronucleus. Consistent with the evidence linking high levels of postsynthetic (nuclear) histone acetylation to increased transcriptional activity is the marked difference in steady state levels of acetylation between macro- and micronuclei. High levels of postsynthetic acetylation are observed with core histones isolated from macronuclei while little if any acetate is associated with similar histones from micronuclei (11). In fact, the marked difference in steady state histone acetylation between macro- and micronuclei of vegetative cells provides one of the most striking positive correlations between histone hyperacetylation and transcriptional activity in a nonperturbed biological system.

The micronuclei of *Tetrahymena* also afford a unique opportunity to investigate the relationship between histone acetylation and histone synthesis and deposition without complications arising from histone acetylation associated with the regulation of transcription. During vegetative growth, micronuclei replicate but transcribe little if any RNA. The exploitation of micronuclei as replicating but transcriptionally inactive nuclei is further enhanced when one utilizes conjugating *Tetrahymena* (Fig. 1). During early periods of conjugation micronuclei undergo a series of meiotic and mitotic (one prezygotic and two postzygotic; refs. 9 and 12) divisions as part of the normal nuclear reorganization process, which ultimately establishes new macro- and micronuclei. During these divisions, significant amounts of new histones are synthesized and deposited into micronuclei (13). At this time parental macronuclei do not divide, require little new histone, and eventually are eliminated from the cells. If significant levels of acetate are associated with histones synthesized and deposited into micronuclear chromatin during this interval, a deposition-related function for histone acetylation would be strongly suggested.

Our results show that histone H3 and histone H4 synthesized in young mating cells are deposited into micronuclei in acetylated forms. With time this new histone is deacetylated by a butyrate-sensitive deacetylase(s) to a final essentially nonacetylated state. Since parental macronuclei acquire little newly synthesized histone H3 and histone H4 during this stage of the life cycle, the acetylation we have followed under these circumstances is specific to transcriptionally inactive, replicating micronuclei and, therefore, strongly supports the idea that histone acetylation plays a role in histone deposition and chromatin assembly. These results show at least two types of histone acetylation in *Tetrahymena*. One of these is presumably identical between macro- and micronuclei of growing, starved, or mating cells and is related to histone deposition at the time of DNA replication. The other type is macronuclear-specific and may play a role in the modulation of transcriptional activity in this nucleus.

MATERIALS AND METHODS

Cell Culture, Labeling, and Isolation of Nuclei. Genetically marked strains of *Tetrahymena thermophila*, Cu399 (*Chx/Chx[cys-s]VI*) and Cu401 (*Mpr/Mpr[6-mp-s]VII*) or Cu427 (*Mpr/Mpr[6-mp-s]VI*) and Cu428 (*Chx/Chx[cy-s]VII*) were used in all experiments reported here. These were kindly provided by P. Bruns (Cornell University). Cells were grown

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Expected patterns of histone acetylation in *Tetrahymena*

	Vegetative cells		Conjugating cells	
Nucleus	micro-	macro-	micro-	macro-
Transcription-related	−	+	−*	+
Replication/deposition-related	+	+	+	−

FIG. 1. Expected patterns of histone acetylation in *Tetrahymena*. Based upon the biology of *Tetrahymena*, a situation where "transcription"-related or "replication"-related histone acetylation would be expected in macro- or micronuclei is indicated (+) and a situation where histone acetylation would not be expected is indicated (−). The boxed area indicates where "transcription"- and "replication"-related acetylation would be expected to be nonoverlapping between macro- and micronuclei.
*While micronuclei are not transcriptionally active during conjugation, micronuclear specific uridine incorporation has been observed during a short interval of meiotic prophase as indicated (9, 10).

axenically in 1% (wt/vol) enriched proteose peptone as described (14). All matings were performed in 10 mM Tris·HCl (pH 7.4) according to Bruns and Brussard (15) as modified by Allis and Dennison (16). All cultures were maintained at 30°C. Growing or mating cells (typically 80–90% paired) were labeled at the times indicated with either [^{3}H]lysine (2 μCi/ml, 50 Ci/mmol; 1 Ci = 37 GBq; ICN, Irvine, CA) or sodium [^{3}H]acetate (5 μCi/ml, 5 Ci/mmol; ICN, Irvine, CA). In some experiments protein synthesis was blocked during the course of the acetate labeling as described (11). Pulse–chase experiments with [^{3}H]lysine labeled cells were carried out as described (17, 18). Sodium butyrate (50 mM) was added to some cultures according to Vavra *et al.* (11). In all cases, nuclei were prepared from cells using the methods of Gorovsky *et al.* (14) or Allis and Dennison (16) wherein micronuclei are purified by differential centrifugation and/or sedimentation at unit gravity. Nuclei were either used immediately or stored at −80°C for future use.

Deacetylation in Isolated Nuclei. In some experiments histones were deacetylated with a deacetylase that is endogenous to macronuclei isolated from growing cells (11). Deacetylation reactions were carried out as described by Vavra *et al.* (11) except that all reactions were carried out at 4°C for 12–18 hr.

Extraction of Histones and Gel Electrophoresis. Histones were extracted from nuclei according to procedures described (19) taking all precautions to avoid artifactual protein losses. In some cases where the amount of labeled material was limiting, nonradioactive histone (from macronuclei) was added as a carrier prior to trichloroacetic acid (TCA) precipitation to increase precipitation efficiency. Long acid/urea slab gel electrophoresis was performed as described by Allis *et al.* (20). Samples were electrophoresed for 11,000–13,000 V·hr at 4°C. Gels were typically stained with Coomassie brilliant blue R, destained, photographed, and processed for fluorography.

Phosphatase Treatment. Phosphatase reactions were carried out exactly as described by Glover *et al.* (21) except that whole acid-soluble/trichloroacetic acid (TCA)-precipitable nuclear protein was used, and the final enzyme concentration was increased 2-fold.

RESULTS

Newly Synthesized Micronuclear H3 and H4 Is Acetylated in Young Conjugants. Fig. 2 illustrates typical results when mating cells (>90% pairing) were labeled with [^{3}H]lysine for 30 min from 5.0 to 5.5 hr of conjugation, macro- and micronuclei were purified, and acid-soluble proteins (histones) were analyzed on long acid-urea gels by staining and fluorography. Despite more or less comparable amounts of protein being loaded (compare lanes 1 and 3), it is evident that newly synthesized histone H3 and histone H4 are deposited into micronuclei (lane 2) as acetylated species (see arrows) in a manner that cannot be explained by macronuclear contamination [little if any histone H3 or histone H4 is synthesized and deposited into macronuclei under these conditions (lane 4)]. After a 30-min labeling period, newly synthesized micronuclear H3 migrates primarily as a monoacetylated form (some di- and unmodified H3 is observed) while essentially all of the new H4 migrates as a diacetylated species. Our results also indicate that newly synthesized histone H2B and histone H2A are deposited (in micro- and macronuclei) as unmodified polypeptides.

The finding that newly synthesized histone H3 and histone H4 are deposited into micronuclei of young conjugants as species with reduced mobility on acid-urea gels (Fig. 2) does

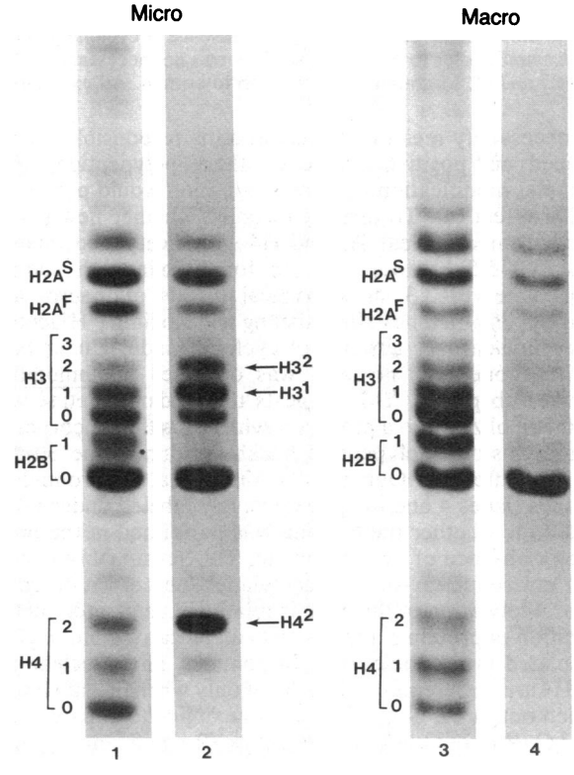

FIG. 2. Synthesis and deposition of histone in purified micro- and macronuclei isolated from 5-hr conjugating cells. Acid-soluble extracts from micro- (lanes 1 and 2) and macronuclei (lanes 3 and 4) were electrophoresed in a 30-cm long acid/urea gel and analyzed by staining (lanes 1 and 3) and fluorography (lanes 2 and 4). Mating cells (>85% pairing) were labeled for 30 min with [^{3}H]lysine from 5.0 to 5.5 hr of conjugation prior to isolation of nuclei. Numbers within brackets represent acetylation levels (e.g., 2, diacetylated subspecies) for some of the histone subspecies that are resolved in this one-dimensional gel. Only the histone region of the gel is shown. Migration was from top to bottom. All exposures were 2 days long.

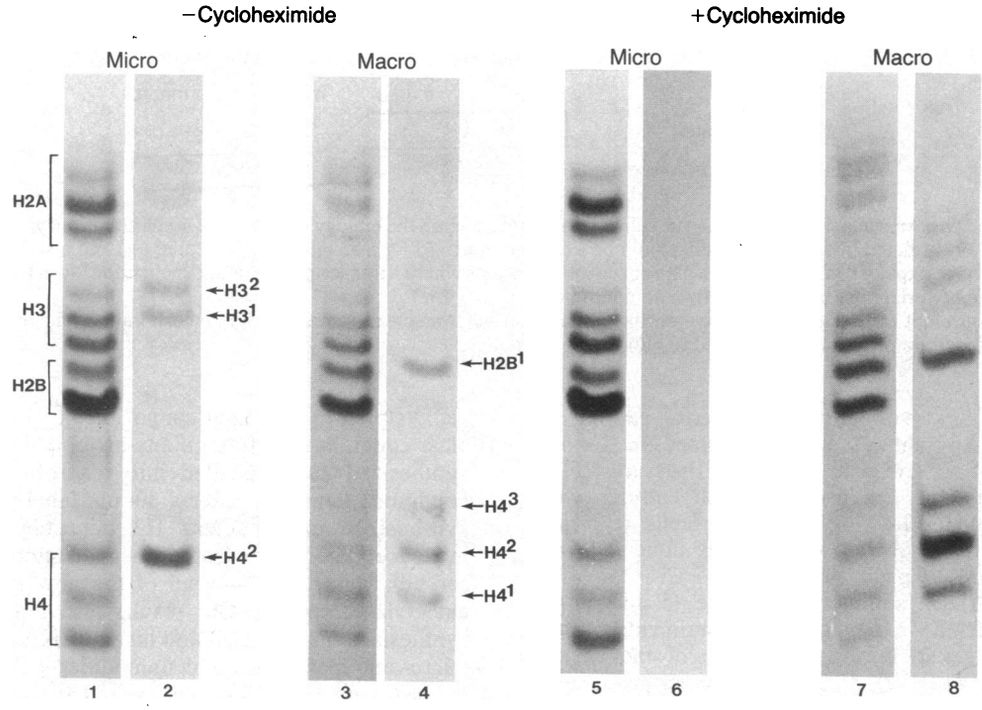

FIG. 3. Acetate incorporation into micro- and macronuclear histone isolated from 5-hr conjugating cells. Acid extracts from micro- (lanes 1, 2, 5, and 6) and macronuclei (lanes 3, 4, 7, and 8) were electrophoresed in a long acid/urea gel and analyzed by staining (lanes 1, 3, 5, and 7) and fluorography (lanes 2, 4, 6, and 8). Mating cells (>85% paired) were labeled for 30 min with sodium [^{3}H]acetate from 5.0 to 5.5 hr of conjugation in the presence (lanes 5–8) or absence (lanes 1–4) of cycloheximide (10 μg/ml) prior to nucleus isolation. Due to a low recovery of micronuclei from cells labeled in the absence of cycloheximide (lanes 1 and 2), nonradioactive macronuclear histone was added as a carrier to the sample prior to precipitation. This was not necessary with the micronuclear sample from cells labeled in the presence of cycloheximide (lanes 5 and 6). Migration was from top to bottom. All exposures were 14 days long.

not necessarily mean that acetylation is responsible for the reduced net positive charge on these polypeptides. For example, modification by phosphorylation would produce a similar effect (6). To directly examine whether newly synthesized micronuclear H3 and H4 were acetylated, mating cells (5.0–5.5 hr) were labeled for 30 min with sodium [^{3}H]acetate. Fig. 3 shows typical results of macro- and micronuclear histones after labeling with sodium [^{3}H]acetate in the presence or absence of cycloheximide. Under both labeling conditions no label was observed in unmodified histone subspecies. This suggests that sodium acetate was not metabolized into a precursor which was then incorporated into histone polypeptide backbone during the 30-min labeling interval. Our results show that macronuclear histones (lanes 4 and 8) are extensively labeled under these conditions whether the labeling was performed in the presence or absence of cycloheximide. This presumably results from macronuclear-specific acetylation/deacetylation reactions. Acetylation of this type is relatively unaffected by the inhibition of protein synthesis and can be carried out *in vitro* in isolated macronuclei (11). In contrast, micronuclear H3 and H4 are preferentially acetylated only when the labeling is carried out in the absence of cycloheximide (compare lanes 2 and 6). Without cycloheximide (lane 2) significant amounts of acetate were observed in H3 (mono- and diacetylated) and H4 (diacetylated, see arrows) subspecies which are not likely to have resulted from macronuclear contamination because little if any monoacetylated H2B is observed. Essentially no acetate was associated with micronuclear histone when cells were labeled in the presence of cycloheximide (lane 6), therefore, this acetylation reaction requires ongoing protein (histone) synthesis.

Further evidence that acetylation is the primary (if not exclusive) modification of newly synthesized H3 and H4 (Fig. 2) is that the mobility of H3 and H4 pulse-labeled with [^{3}H]lysine was unaffected by extensive phosphatase treatment, but was partially converted to an unmodified form when micronuclei were incubated in "deacetylation buffer," which has been shown to activate or enhance a deacetylase activity in isolated macronuclei (11) (data not shown). Direct evidence for a micronuclear deacetylase activity(ies) operating *in vivo* will be presented in the next section.

These data show that acetylation of newly synthesized micronuclear H3 and H4 occurs in young conjugating cells. Since micronuclei are not transcriptionally active, these data strongly support the idea that acetylation of the arginine-rich histones plays a significant role in the deposition of histone and chromatin assembly. As far as we are aware, this is the first study demonstrating significant levels of acetate associated with micronuclear histones in *Tetrahymena*.

Detection of a Butyrate-Sensitive Deacetylase Activity in Micronuclei. Micronuclear histones are grossly underacetylated relative to their macronuclear counterparts (11). Furthermore, experiments with sodium butyrate, a known inhibitor of deacetylase activity, have shown that the low level of acetylation observed with *Tetrahymena* micronuclear histones is the result of low or nonexistent histone acetylation rather than deacetylation (11). Figs. 2 and 3 show, however, that new H3 and H4 are acetylated at the time of synthesis (presumably in the cytoplasm) and are deposited in that form into rapidly dividing micronuclei of young conjugating cells. If this is the case, it seems reasonable to suspect that this new histone would be deacetylated to give a final steady state level of largely unmodified histone. To look for this activity, mating cells (again at 5.0 to 5.5 hr) were pulse-labeled with [^{3}H]lysine for 30 min following which an aliquot of the cells either was killed immediately (pulse) or was chased for various lengths of time in buffer containing excess nonradioactive lysine. Fig. 4*A* shows the results obtained when micronuclear histones from these samples were fractionated

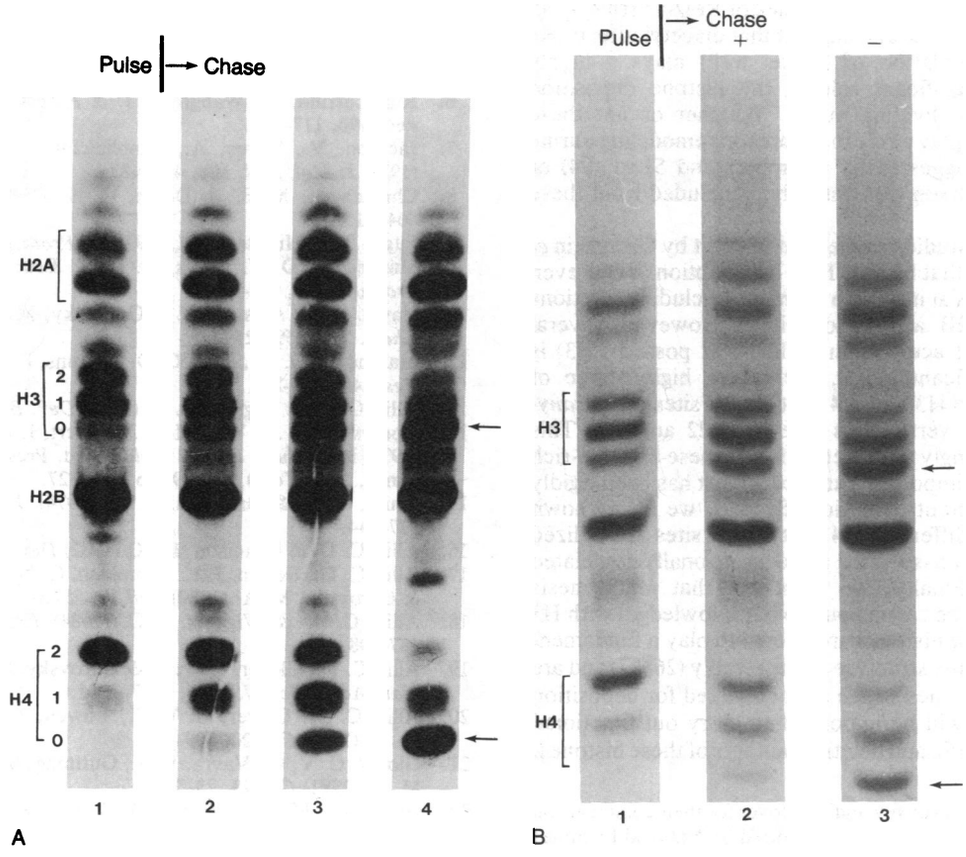

FIG. 4. Detection of a butyrate-sensitive deacetylase activity in micronuclei of 5-hr conjugating cells. (*A*) Mating cells were labeled with [^{3}H]lysine exactly as in Fig. 2. An aliquot of these pulse-labeled cells (lane 1) was removed while the remainder of the cells was chased in Tris buffer containing excess nonradioactive lysine for an additional 0.5 (lane 2), 1.0 (lane 3), or 2.0 (lane 4) hr. Micronuclei were prepared from each of these samples and their acid extracts analyzed. Since carrier protein was added to each of these samples to increase the efficiency of the trichloroacetic acid precipitation step, only the fluorographic analysis is shown. Arrows indicate the position of unmodified H3 and H4. All exposures were 5 days long. (*B*) Mating cells were pulsed and chased as in *A* above except that the chase (1.5 hr) was performed in either the presence (lane 2) or absence (lane 3) of 50 mM sodium butyrate. Migration was from top to bottom. All exposures were 2 days long.

on a long acid/urea gel and the newly synthesized histones analyzed by fluorography. In agreement with the results presented in Fig. 2, newly synthesized micronuclear H3 and H4 was deposited initially (after a 30 min labeling interval, lane 1) as acetylated subspecies; mono- and diacetylated H3 as well as diacetylated H4 were evident. During the *in vivo* chase, however, a steady shift in the distribution to unmodified forms was observed. After a 2 hr chase (lane 4), much of the newly synthesized micronuclear H3 and H4 migrated as though it was completely deacetylated (see arrows by lane 4).

Deacetylase activity has been shown to be sensitive to sodium butyrate in several eukaryotic systems including *Tetrahymena* (11). To investigate whether the deacetylase activity(ies) described above is sensitive to butyrate, we repeated the pulse–chase experiment except that the chase was performed in the presence or absence of 50 mm sodium butyrate. These results (Fig. 4*B*) show that in the presence of sodium butyrate (lane 2) little if any deacetylation of newly synthesized micronuclear H3 or H4 occurs (compare with the pulsed, nonchased cells, lane 1). Significant deacetylation is observed under conditions where sodium butyrate is omitted from the chase media (lane 3). From these results we conclude that micronuclei (at least in young conjugating cells) contain a butyrate-sensitive deacetylase activity(ies) that operates to remove the deposition-related acetate groups from newly synthesized histone H3 and histone H4. With time this enzyme contributes to the low (or nonexistent) steady state level of micronuclear histone acetylation.

SUMMARY AND CONCLUSIONS

We have been able to study histone acetylation in *Tetrahymena* under conditions where the acetylation of histones associated with histone deposition and chromatin assembly and the acetylation of histones associated with the regulation of transcription are nonoverlapping. Evidence is presented for two general systems of histone acetylation in *Tetrahymena* (for a review, see ref. 22). The first of these is clearly specific to macronuclei and may be related to gene expression (11). It operates in the absence of protein synthesis and has been demonstrated in isolated macronuclei (11). The second system is likely to be located in the cytoplasm and appears to be related to histone synthesis and deposition. Our data show that acetate is associated with micronuclear H3 and H4 during periods of active histone synthesis and deposition (early periods of conjugation). Since micronuclei are transcriptionally inactive during this interval (9), strong evidence for deposition-related acetylation is provided. Similar results have been obtained with new histones deposited into macro- and micronuclei isolated from vegetative cells (23; Dimitsopoulos and Gorovsky, personal communication).

Our results show that newly synthesized H4 is deposited almost exclusively in a diacetylated form. This is in excellent agreement with the recent studies by Chambers and Shaw (24) in which high levels of diacetylated H4 in rapidly dividing sea urchin embryos have been reported. Our results suggest that H3 acetylation may also be used as part of the same phenomenon (at least in *Tetrahymena*). These results strong-

ly support the early pioneering studies of Ruiz-Carrillo *et al.* (6) and Jackson *et al.* (7) and suggest that diacetylation of H4 (and possibly acetylation of H3 as well) plays a highly conserved and significant role in the histone deposition process in rapidly dividing nuclei. Whether or not these modifications also play a role in chromatin remodeling during development as suggested by Chambers and Shaw (24) or Christensen and Dixon (8) cannot be concluded from these studies.

Elegant genetic studies carried out in yeast by Grunstein *et al.* (4) have shown that histone H2B can function *in vivo* even with large deletions at its amino terminus (including deletions which remove H2B acetylation sites). However, several points suggest that acetylation of H4 (and possibly H3) is functionally significant. First, there is a high degree of conservation in the H3 and H4 acetylation sites of *Tetrahymena* and several vertebrates (see refs. 22 and 25). This finding argues strongly that acetylation of these arginine-rich histones serves an important function(s) that has been rigidly maintained throughout evolution. Second, we have shown that specific (and different) H4 acetylation sites are utilized for transcriptionally associated and depositionally associated acetylation (28). Finally, we point out that mutagenesis studies have yet to be carried out (to our knowledge) with H3 and H4. Since these histones are known to play a fundamental role in nucleosome structure and assembly (26, 27) and are specifically the histones which are acetylated for deposition onto the DNA, it will be important to carry out functional tests with site-specific and deletion mutants of these histones.

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