A Class II Histone Deacetylase Acts on Newly Synthesized Histones in Tetrahymena

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Newly synthesized histones are acetylated prior to their deposition into nucleosomes. Following nucleosome formation and positioning, they are rapidly deacetylated, an event that coincides with further maturation of the chromatin fiber. The histone deacetylases (HDACs) used for histone deposition and de novo chromatin formation are poorly understood. In the ciliate Tetrahymena thermophila, transcription-related deacetylation in the macronucleus is physically separated from deposition-related deacetylation in the micronucleus. This feature was utilized to identify an HDAC named Thd2, a class II HDAC that acts on newly synthesized histones to remove deposition-related acetyl moieties. The THD2 transcript is alternatively spliced, and the major form contains a putative inositol polyphosphate kinase (IPK) domain similar to Ipk2, an enzyme that promotes chromatin remodeling by SWI/SNF remodeling complexes. Cells lacking Thd2, which retain deposition-related acetyl moieties on new histones, exhibit chromatin and cytological phenotypes indicative of a role for Thd2 in chromatin maturation, including the proteolytic processing of histone H3.

In eukaryotes, DNA complexes with an octamer of histone proteins (two each of histones H2A, H2B, H3, and H4) to form a repeating unit of chromatin called a nucleosome (23, 29, 50). The precise positioning of nucleosomes can regulate DNAtemplated processes, such as replication, transcription, and recombination. Nucleosome assembly and initial positioning occur during the S phase of the cell cycle and are closely coupled to passage of replication forks (28). During replication, the existing nucleosomes are randomly segregated onto the newly synthesized DNA (47), while new nucleosomes are formed de novo through the assembly of free histones in a specific order. First, two H3/H4 heterodimers are deposited onto the DNA, followed by two H2A/H2B dimers (14). Prior to their deposition, free histones H3 and H4 are acetylated on lysine (Lys) residues by cytoplasmic histone acetyltransferases (HATs) in patterns distinct from transcription acetylation patterns (2, 21, 42, 53). The deposition acetylation promotes histone assembly into nucleosomes through interactions with histone chaperones, such as chromatin assembly factor 1 (CAF-1), Hif1, and Asf1, that incorporate new H3 and H4 specifically onto newly replicated DNA (1, 14, 45, 52). The deposition-related pattern on histone H4 is highly conserved from yeasts to humans and consists of diacetylated Lys5 and Lys12, which correspond to Lys4 and Lys11 in the ciliate Lys12, which correspond to Lys4 and Lys11 in the ciliate Tetrahymena thermophila (2, 8, 46). Recently, acetylation of Lys91 in the globular domain of H4 was identified as another modification important for nucleosome assembly (60). The acetylation pattern on newly synthesized histone H3 is more variable between organisms. In Tetrahymena, Lys9 and Lys14 are acetylated, whereas in Drosophila, Lys14 and Lys23 are the preferred sites (46), and in budding yeast, most new H3 molecules are monoacetylated on Lys9, Lys14, Lys23, Lys27, or Lys56 (25, 32). In contrast to H3 and H4, nascent H2A and H2B histones do not display any acetylation patterns that are distinct from parental forms.

Once assembled into nucleosomes, new histones H3 and H4 are rapidly deacetylated (2, 21), an event important for chromatin maturation. When chromatin is replicated in vivo in the presence of sodium butyrate to inhibit deacetylation, the resulting nucleosome structure and distribution on the chromatin fiber appear normal, but the “mature” fiber is abnormally hypersensitive to DNase I (7, 44). Chromatin assembled in vitro with hyperacetylated histones is also more nuclease sensitive (24). Aside from a recent demonstration that deacetylation of Lys91 on histone H4 facilitates the formation of a salt bridge to histone H2B, little is known about the role of histone deacetylation in chromatin maturation. Further studies in this area have in part awaited identification of the histone deacetylases (HDACs) involved.

Most HDACs identified to date fall into three phylogenetic classes, depending on their homology to the yeast deacetylases Rpd3 (class I), Hda1 (class II), or NAD-dependent Sir2 (class III). Enzymes in these classes can differ in localization and tissue-specific expression (11). Generally, class I HDACs reside in the nucleus, while many class II enzymes shuttle between the nucleus and cytoplasm in response to cellular signals. Some class I yeast HDACs that regulate transcription deacetylate Lys5/Lys12, in addition to other residues on H4, and thus may also play a role in chromatin maturation (41). However, their transcription function complicates investigations into possible maturation functions, since the two processes occur simultaneously in the same organelle. Most systems share the difficulty of separating chromatin maturation-related from transcription-related deacetylation events. To circumvent this bar-
rier to identifying a maturation-related HDAC, we utilized the ciliated protozoan *T. thermophila* for its two distinct nuclei, called the macronucleus and the micronucleus. Global chromatin in the macronucleus is highly acetylated, while that in the micronucleus is entirely unacetylated. Both nuclei divide and assemble new chromatin, but transcription-related acetylation occurs only in the macronucleus during vegetative growth; the micronucleus is transcriptionally silent throughout the cell cycle (2, 51). The only acetylation observed in micronuclei is highly transient and occurs in deposition-related patterns (8). Thus, HDACs that act on micronuclear chromatin during vegetative growth must function in the removal of deposition acetylation. Compelling evidence for such a micronuclear HDAC activity was observed during a period in the sexual-conjugation pathway when the macronucleus remains in a nonreplicative state (G₀) while the micronucleus undergoes successive rounds of mitosis and DNA replication (12). Throughout this period, newly synthesized histones were deacetylated by an unknown micronuclear HDAC (2, 8). Following deacetylation, micronuclear chromatin is thought to mature through evolutionarily conserved pathways, but one unique feature is the proteolytic processing of a fraction of new histone H3 molecules into a truncated form (3, 5, 6). Production of this form is related to mitotic chromosome condensation and segregation in the micronucleus (54).

Here, we report the identification of a class II HDAC called Thd2 (*Tetrahymena* histone deacetylase 2) that localizes to the micronucleus, where it removes deposition-related acetylation from histones H3 and H4. Cells lacking Thd2 display micronuclear chromatin phenotypes, including reduced H3 proteolysis. *THD2* is an alternatively spliced gene, the first reported for a coding sequence in *Tetrahymena*. Of the two resulting splice variants, the predominant form encodes an HDAC enzyme containing a putative inositol polyphosphate kinase (IPK) domain that in other systems has been implicated in the regulation of chromatin remodeling. From this study, possible roles for HDACs in the maturation of new chromatin are revealed.

**MATERIALS AND METHODS**

**Bioinformatics.** Eighteen HDACs (Thds) were identified as containing putative HDAC domains from The Institute for Genomic Research-annotated genes in the *Tetrahymena* macronuclear genome database (http://www.ciliate.org). Their respective gene sequence identification numbers are listed in Table 1. Since experimental confirmation of the coding sequences was lacking, only the HDAC domains were used for phylogenetic analysis. The sequences of the 18 *Tetrahymena* HDAC domains were aligned with the *S. cerevisiae* histone deacetylase 2 (*HST2*) that localizes to the nuclear and mitotic chromosomes and the *H. sapiens* HDAC 6 (HDAC6). The sequences were then used for phylogenetic analysis. The robustness of the tree topology was determined using MEGA4, and the robustness of the tree topology was assessed using 1000 bootstrap replicates.

**Strains and cell culture conditions.** *T. thermophila* strains B2086 (II), CU426 (chi+/chi- [I-sy +]), and SB1869 (mp+/mp- [I-sy]) were used as the wild-type strains (*Tetrahymena* Stock Center, Cornell University). Unless otherwise indicated, cells were grown at 30°C with shaking in 1% (wt/vol) enriched proteose peptone (17) or 2% proteose peptone, 0.2% yeast extract, and otherwise indicated, cells were grown at 30°C with shaking in either 1% (wt/vol) medium with 1X penicillin, streptomycin, and fungizone (Gibco-BRL) to a density of 1 × 10⁵ to 3 × 10⁵ cells/ml for all experiments. The cells were starved in 10 mM Tris·HCl (pH 7.4) for 14 to 20 h at a density of 3 × 10⁵ cells/ml at 30°C with no shaking.

**RT-PCR.** Genomic DNA was isolated as described previously (61). Total RNA was isolated from vegetatively dividing, starved, and conjugating cells using the RNeasy Total RNA kit (Qiagen, Valencia, CA). The cDNA was made as previously described (31) using 2 μg of total RNA for each reaction. PCR was performed on dilutions of the cDNA to determine the linear range of conditions for the sample set (data not shown). Experiments were performed using conditions that were within the linear amplification range to obtain semiquantitative results. PCR and reverse transcription (RT)-PCR were performed using 2X GoTaq master mixture (Promega, Madison, WI) following the manufacturer’s directions and using the following primer sets: THD2a (+) (5′-GTTATTITTTGTATATGCTGC-3′) and THD2b (+) (5′-TTAGACTCATTAAATAGATAC-3′); THD2 (−) (5′-CTAAATGTGATCTTTAATCT-3′) and HHP1 (−) (5′-TAGCAATGAACAACCTTTAACAATC-3′); THD1 (−) (5′-TGGTTAGGGGATACCTT-3′) and ACT1 (−) (5′-TGTAGGAAAGAGAGTTCATC-3′); ACT1 (−) (5′-GGAAGAGGAGGAGGAGTACTC-3′) and THD2 (−) (5′-AACTTTTTTATGACACAGAGGAATGGAAGAGCAGGAGGAGAGTACTC-3′). ACT1 (−) (5′-GGAAGAGGAGGAGGAGTACTC-3′) and THD2 (−) (5′-AACTTTTTTATGACACAGAGGAATGGAAGAGCAGGAGGAGAGTACTC-3′).

**THD2** gene and splice variant analysis. The 5′ terminus of the *THD2* mRNA was deduced by RT-PCR with the THD2 (−) primer listed above and the following primers upstream of the predicted translation start site: THD2c (−) (5′-CCAAACAGAACAACATTGGCAG-3′), THD2-132 (−) (5′-ATTCTGGATGTAAGATGAAAGATGTTAC-3′), THD2-219 (−) (5′-CTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT
Plasmid construction. The NEO construct used to replace THD2 was made following a previously described method (56), except that the THD2 3′ and 5′ flanking regions were amplified by PCR using the following primers: 5′ FlankBamHI (+) (5′-GAGGGATCCAAAGTGTGATGTGTTTGAAG-3′), 5′ FlankXmaI (+) (5′-GTCATCGATAAGAAACAACTCCTGTCTGTC-3′), and 5′ FlankXhoI (+) (5′-AGCTCATCAGATAACAACTCTTCTGTGC-3′). The PCR products were digested with appropriate restriction enzymes and ligated into pT2-1 containing the Neo' drug resistance cassette to make pTHD2-NEO (56). For transformation, pTHD2-NEO was digested to produce a putative HDAC domains of the 18 Thd protein sequences.

To construct the tree with yeast HDAC domains that are less similar to Thd proteins than those of humans.

FIG. 1. Phylogenetic tree of the putative ThiDs (unweighted-pair group method using average linkages). Shown is an alignment of the putative HDAC domains of the 18 Thd protein sequences. S. cerevisiae Rpd3 (class I), Hda1 (class II), Sir2, Hst2, and Hst4 (class III) were used as references to sort the putative ThiD proteins into their respective classes (boldface). The positions of human homologs are shown in light-gray capital letters. (Note: HDAC6 and SIRT5 appear twice due to construction of the tree using the following primers: α-histone H4 (15,000; a gift from C. D. Allis), α-histone H3 (15,000; a gift from C. D. Allis), α-H4K16ac (1:2,000; Upstate Biotechnology catalog no. 06-762), and α-histone H3S10ph (1:5,000; a gift from C. D. Allis).

Lack of THD2 expression in strain JS01 was confirmed by RT-PCR using the THD2a (+) and THD2 (−) primers listed above.

Immunoblot analysis. Nuclei from B2086 (wild-type) and JS01 (thd2a) were isolated as previously described (17). The nuclei (1 × 10⁸) were lysed by incubation in 30 μl of sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Micronuclei (5 × 10⁴) and macronuclei (1.6 × 10⁶) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 15% (for histone H4) or 22% (for histone H3) polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the following antibodies: α-histone H4 (1:5,000; a gift from C. D. Allis), α-histone H3 (1:5,000; a gift from C. D. Allis), α-H4K16ac (1:2,000; Upstate Biotechnology catalog no. 06-762), and α-histone H3S10ph (1:5,000; a gift from C. D. Allis).

Indirect immunofluorescence and DAPI staining of cells. B2086 (wild-type) and JS01 (thd2a) cells in logarithmic growth (1 × 10⁵ to 3 × 10⁵ cells/ml) were fixed in paraformaldehyde and processed for immunofluorescence as previously described (9). For detection of acetylated histones, cells were incubated with α-acetylated histone H4 antisera (1:200; Upstate Biotechnology catalog no. 06-598), α-acetylated histone H3 antisera (1:200; a gift from C. D. Allis), or α-histone H3K9ac antisera (1:200; Upstate Biotechnology catalog no. 06-942), which also detects H3K14ac in Tetrahymena. For detection of only micronuclei, cells were incubated with anti-micronuclear linker histone 1 (α-H1K [micro-nuclear-specific linker histone]) antisera (1:200; a gift from C. D. Allis). Primary antibodies were detected with rhodamine-conjugated goat anti-rabbit immunoglobulin G (1:100; Jackson Immunoresearch catalog no. 111-025-003). The cells were counterstained with 0.1 μg/ml 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Sigma Chemicals) in 0.1% bovine serum albumin–phosphate-buffered saline for 10 min according to the common protocol (37).

Nucleotide sequence accession numbers. The cDNA sequences for the splice variants were submitted to GenBank under accession numbers EU254713 (Thd2a) and EU254714 (Thd2b).

RESULTS

Thd2 localizes to the micronucleus. Thds were first identified by searching the Tetrahymena Genome Database to find predicted open reading frames that contained putative HDAC domains. Eighteen putative HDAC proteins were identified (Table 1) and classified by their similarity to the yeast HDACs Rpd3 (class I), Hda1 (class II), and Sir2 (class III) (Fig. 1). The HDAC domains fell into all three classes—3 in class I (Thd1, Thd3, and Thd6), 2 in class II (Thd2 and Thd4), and 11 in class III (Thd8 through Thd18)—and 2 were HDAC-like (Thd5 and Thd7). Most of the putative Tetrahymena class III HDACs
(Thd8 through Thd18) were more closely related to human sirtuin homologs than to the yeast sirtuins Sir2, Hst2, and Hst4 (Fig. 1).

An HDAC that resides in the micronucleus would likely remove deposition-related acetyl marks from newly synthesized histones, since only deposition acetylation occurs on histones in this nucleus. To identify a micronuclear HDAC, several of the putative HDAC genes (Fig. 1) were cloned in frame with GFP coding sequence at their 5' ends and expressed from the metallothionein promoter. The resulting amino-terminal GFP fusion proteins were induced, and their localization in live cells was assessed by fluorescence microscopy. Of four tested, only GFP-Thd2 localized to the micronucleus in addition to the macronucleus (Fig. 2); the other putative HDACs tested localized exclusively to the macronucleus or to the cytoplasm (data not shown) (57).

**THD2 is alternatively spliced.** The THD2 coding sequence was determined by cloning and sequencing its cDNA (as described in Materials and Methods). Amplifying the cDNA with gene-specific primers revealed two cDNAs of different lengths, indicating that the THD2 gene was alternatively spliced. The most abundant splice variant, called THD2a, contained only E1, E2, and E4 (Fig. 3A), while the minor form, called THD2b, contained E1 through E4 (Fig. 3A). Sequence analysis showed that the inclusion of E3 in THD2b caused a frameshift that produced a premature stop codon within E4 and loss of a putative IPK domain (Fig. 3B). The relative transcription level of THD2a was 15- to 20-fold higher than that of THD2b during vegetative growth.
vegetative growth when normalized to the amplification of genomic DNA for each PCR (Fig. 3C and data not shown). When cells were starved and no longer progressing through the cell cycle, both \( \text{THD2a} \) and \( \text{THD2b} \) transcription levels decreased two- to threefold, suggesting that both enzyme forms may have more significant functions during periods of active growth and cell division.

The HDAC domain of Thd2 was aligned with the yeast HDACs Hda1 (class II) and Rpd3 (class I) and found to have greater similarity to Hda1 (37% identical and 56% similar amino acid residues) (Fig. 4A). Thd2 had a high degree of similarity to Hda1 in regions of the HDAC domain that are conserved in most class II enzymes (Fig. 4A) (26, 63). To further classify the putative IPK domain in Thd2, the sequence was compared to two well-characterized yeast IPKs, Ipk1 and Ipk2, the primary enzymes in the pathway for conversion of inositol triphosphate (IP\(_3\)) to inositol hexaphosphate (IP\(_6\)) (Fig. 4B). The putative IPK domain of Thd2a was more closely related to Ipk2 (27% identical and 36% similar amino acid residues) than to Ipk1 (10% identical and 22% similar amino acid residues). Notably, the cofactor binding regions and inositol polyphosphate binding region for Ipk2 were similar in Thd2a. Thd2a also contains a conserved aspartate residue that is essential for Ipk2 kinase activity (Fig. 4B) (20).

Expression of \( \text{THD2} \) coincides with DNA replication. To begin characterizing \( \text{THD2} \) function, we first examined its transcription over a normal cell cycle during vegetative growth, since cells produced two- to threefold more \( \text{THD2} \) mRNA when growing than in the noncycling, starved state. In \( \text{Tetrahymena} \) cells, the micronucleus progresses through the cell cycle without resting in G\(_1\) phase. Following mitotic division, a cell proceeds directly into S phase and then rests in an extended G\(_2\) phase while the macronucleus undergoes amitotic division followed by DNA synthesis (22, 58). Logarithmically growing cells were synchronized by isolating new daughter cells through centrifugal elutriation and then allowing them to proceed through the cell cycle. The synchrony of cells in the culture was monitored at frequent intervals throughout several cell cycles by counting the cells at different stages (Fig. 5A).

RT-PCR analysis of \( \text{THD2} \) expression at regular intervals throughout two cell cycles (from 20 min to 280 min after elutriation) revealed that \( \text{THD2} \) was expressed in a cyclical pattern (Fig. 5B). Maximum expression of \( \text{THD2} \) occurred at 80 and 200 min, which were the times when the greatest numbers of cells were in micronuclear mitosis. The expression of \( \text{ACT1} \), which does not change throughout the cell cycle, was monitored as a control (34, 62). Since micronuclear DNA synthesis occurs immediately following anaphase, the observed peak of \( \text{THD2} \) expression coincident with anaphase was consistent with the possibility that Thd2a might deacetylate newly deposited histones.

To further examine whether \( \text{THD2} \) expression coincides with DNA replication and division, transcript levels were monitored throughout the sexual-conjugation pathway. Early in this process, the transcriptionally silent micronucleus undergoes three successive rounds of DNA replication and two mitoses while the transcriptionally active macronucleus remains in G\(_1\) (Fig. 6A). Exploiting this feature, we tested whether \( \text{THD2} \) expression coincided with DNA synthesis and histone deposition in the micronucleus at the time (5 to 7 h) when a micronuclear HDAC activity that removes deposition acetylation was previously detected (2). Cells of two different mating
types were starved, mixed together, and allowed to conjugate over a 24-hour period. To monitor population synchrony, the percentage of cells in each stage was determined at regular intervals throughout the conjugation time course. By 2 hours after they were mixed, 66% of the cells were paired (Fig. 6A). By 3 hours after they were mixed, 66% were in meiotic prophase and 85% were paired. Throughout the entire conjugation time course, 60 to 70% of the cells were tightly synchronized and an additional 20 to 25% of the culture was within 60 min of this primary synchronized population. RT-PCR on cDNA made from conjugating cells harvested at 1-hour intervals revealed that THD2a was most highly expressed between 5 and 7 h after the mixing (Fig. 6B). At the peak of expression (6 h postmixing), 70% of the cells were in one of the mitotic divisions (30% in prezygotic mitosis and 40% in postzygotic mitosis) (Fig. 6A). This result demonstrated a strong correlation between THD2a expression and periods of DNA replication, evidence that THD2a encodes an HDAC that acts during this time.

Similar analysis revealed that Thd2b expression remained low during conjugation compared to that of Thd2a. This minor variant also appeared to be more ubiquitously expressed throughout conjugation, with only a slight peak in expression at 6 h (Fig. 6B). The band corresponding to genomic DNA contamination was amplified to a greater extent at some conjugation points when THD2a levels were relatively low. Curiously, when reactions lacking RT were performed on these mRNA samples and they were used as templates for PCR, no amplification of Thd2 cDNA was observed (Fig. 3C and data not shown). This result raises the possibility that there may be yet another variant, one that retains the intron between E3 and E4. Such a variant would produce an HDAC similar to Thd2b that lacks the putative IPK domain but that has an earlier stop codon than Thd2b.

**THd2 deacetylates micronuclear histones.** To test whether Thd2 deacetylates micronuclear chromatin, a mutant cell line lacking THD2 was first engineered. A genetic construct for the somatic replacement of THD2 with a paromomycin resistance gene (NEO) was transformed into wild-type cells (Fig. 7A). Complete replacement of the THD2 allele with the NEO construct was confirmed by PCR (Fig. 7B, THD2 and NEO), as was the correct integration of the construct into the THD2 locus (Fig. 7B, THD2-NEO). The resulting cell line lacking all macronuclear copies of THD2 was called thd2Δ. RT-PCR was conducted on the thd2Δ cells to confirm the absence of THD2 expression (Fig. 7C). Obtaining a complete knockout strain indicated that THD2 was a nonessential gene in vegetatively growing cells.

The micronucleus is transcriptionally inactive and lacks acetylated chromatin in growing cells. Acetylated histone H4 in
micronuclei was previously detected only when cells were treated with general HDAC inhibitors, such as sodium butyrate and trichostatin A (2, 8, 13). To test the acetylation state of micronuclear chromatin in \textit{thd2}/H9004 cells, immunofluorescence was performed using antiserum specific for only acetylated species of histones H3 and H4 (\textit{H9251}-H3ac and \textit{H9251}-H4ac). Consistent with previous studies of wild-type cells (51), acetylated histones H3 and H4 were detected only within the transcriptionally active macronucleus and not in the micronucleus (Fig. 8, WT). In \textit{thd2}/H9004 cells, however, acetylated histones H3 and H4 were detected in the micronucleus, as well (Fig. 8, \textit{thd2}/H9004).

Micronuclear histones were acetylated in every cell and in each stage of the cell cycle observed, in contrast to wild-type cells, in which micronuclear acetylation was never detected (Fig. 8). This analysis demonstrated that Thd2 is an HDAC that normally deacetylates histones in the micronucleus. Whether it also acts on macronuclear histones was not determined.

It was deduced that the observed micronuclear acetylation in \textit{thd2}/H9004 cells resulted from the retention of deposition-related acetyl moieties. To help confirm this, we performed an immunofluorescence assay using an antiserum against acetylated lysine 9 and/or 14 on histone H3 (\textit{H9251}-H3K9/14ac), the acetyl marks found on newly synthesized \textit{Tetrahymena} histones (2, 25). Whereas this antiserum normally does not hybridize with micronuclear chromatin in wild-type cells, it did hybridize with chromatin in \textit{thd2Δ} micronuclei, indicating that H3Lys9 and/or -14 was acetylated in the mutant cells. Other deposition site-specific acetylation antisera (anti-acetylated Lys5 and anti-acetylated Lys12) were not included in this analysis due to their lack of specificity; \textit{Tetrahymena} histone H4 is shorter by 1

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**FIG. 7.** \textit{THD2} is a nonessential gene. (A) Diagram of the \textit{THD2} deletion construct used to replace \textit{THD2} with \textit{NEO} in the somatic macronucleus. Depicted in the diagram are the flanking regions (thin black lines), the coding sequence (thick dark gray lines), introns (white boxes), the histone H4 promoter (thick black lines), the \textit{NEO} promoter, and the \textit{BTU2} polyadenylation region (light gray line). The arrows represent the primers used to confirm correct integration of the replacement allele. (B) PCR amplification of genomic DNA from wild-type (WT) and \textit{thd2Δ} (Δ) cells confirmed that all \textit{THD2} alleles were replaced with the \textit{NEO} cassette. \textit{THD2} PCR was performed using Ta(+)* and T2-3 primers (WT allele), \textit{NEO} PCR with NF and NR primers (\textit{NEO} cassette), and \textit{THD2-NEO} PCR with F1 and NS primers (incorporation of the \textit{NEO} cassette in the \textit{THD2} locus). \textit{HHPI} PCR was performed as a positive control for the genomic DNA. (C) Total cDNA derived from WT and \textit{thd2Δ} cells was used in PCRs to test for the presence of \textit{THD2} mRNA in these cells. \textit{HHPI} was used as a control for cDNA synthesis and PCR amplification. Genomic DNA (G) was used as a template to control for genomic-DNA contamination in cDNA.

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**FIG. 6.** Thd2 is expressed during conjugation coincident with micronuclear DNA synthesis and mitosis. (A) Diagram of \textit{Tetrahymena} conjugation stages. Two different mating types were mixed to initiate conjugation. Samples were taken every hour and stained with DAPI, and the percentage of cells in each stage of conjugation was determined by fluorescence microscopy, as indicated above the diagram. The black bars indicate periods of DNA synthesis and mitosis (the short bar is the prezygotic mitosis just prior to the pronuclear exchange; the long bar is the postzygotic mitoses I and II following zygotic fusion). (B) Total RNA was harvested from vegetatively growing (V) cells, starved (S) cells, and cells during conjugation (0 through 14 and 24 h after mixing) and used as a template in RT-PCRs with primers specific for \textit{THD2a} or \textit{THD2b} variants. Primers for \textit{CYP1} and \textit{HHPI}, two genes showing consistent expression throughout conjugation, were used as controls in this analysis. Genomic DNA (G) was used to control for genomic-DNA contamination in the RNA samples. (Note: \textit{THD2b} RT-PCR contained two bands; the faster-migrating band corresponds to the spliced form of \textit{Thd2b}.)

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amino acid (it is missing Arg3) at the N terminus. To help rule out possible aberrant transcription-related acetylation in the micronuclei of \textit{thd2Δ} cells, an immunoblot analysis was performed on proteins from purified micronuclei or macronuclei with antiserum detecting acetylated Lys16 (α-H4K16ac), a known transcription-specific mark found exclusively in the macronuclei of wild-type cells (8, 25). This antiserum was shown to have correct specificity for a \textit{Tetrahymena} H4 peptide acetylated at this position (E. Wiley, unpublished data). As shown in Fig. 8D, neither wild-type nor mutant (\textit{thd2Δ}) micronuclear histone H4 molecules were acetylated on Lys16. Consistent with previous studies, Lys16 acetylation was detected only in macronuclei. Due to high background, results from immunofluorescence experiments with this antiserum are not shown. Combined, these results suggest that Thd2 is normally required for maintenance of the deacetylated state of micronuclear histones H3 and H4 through removal of deposition-related acetyl modifications.

**Chromatin defects in \textit{thd2Δ} cells.** Immunofluorescence studies revealed a number of elongated micronuclei in \textit{thd2Δ} cells. Although this is the normal appearance of micronuclei in anaphase, many of these elongated micronuclei in mutant cells were closely associated with the macronucleus, an aberrant position for an anaphase nucleus (Fig. 8C, bottom right). In some cases it was difficult to define the micronuclei that stretched across portions of the macronucleus (the phenotype is depicted in the last panel). To improve the visualization of micronuclei, immunofluorescence was performed with antiserum specific for Mlh1, which is present only in the micronucleus (Fig. 9A). As expected, Mlh1 localized specifically to the micronucleus for both wild-type and \textit{thd2Δ} cells. Micronuclei were scored for elongated shape when in close proximity to the macronucleus (in the “pocket”) for both wild-type and \textit{thd2Δ} cells (Fig. 9A). The percentage of cells with abnormal micronuclei in the “pocket” for both wild-type and \textit{thd2Δ} cells (Fig. 9A). The percentage of cells with abnormal micronuclei in the pocket was approximately 10-fold higher in \textit{thd2Δ} cells than that in wild-type cells (50.5% ± 3.3% and 4.7% ± 3.2%, respectively). Micronuclei were more variable in size, as well. Although cell division appeared to progress normally, the doubling time for \textit{thd2Δ} cells was three times that of wild-type cells (9 h and 3 h,
yeast deacetylases, Rpd3, Hda1, and Sir2 (11). All three classes of HDACs (classes I, II, and III, respectively) are represented in *Tetrahymena* (Fig. 1). Interestingly, their deacetylase domains are more similar to those in human enzymes than to those in budding yeast. *Tetrahymena* Thd1, Thd3, and Thd6 enzymes are class I HDACs due to their similarity to yeast Rpd3, and their HDAC domains are most similar to human HDAC1. Thd2 and Thd4 are class II homologs with similarity to yeast Hda1 but that have deacetylase domains most similar to human HDAC6. There are two HDAC-like proteins that did not group well with any of the yeast HDACs. One of these, Thd5, is highly similar to HDAC11, a class IV HDAC thought to be a hybrid of class I and II enzymes present in primates, rodents, *Drosophila*, and plants (16, 18). *Tetrahymena* also contains 11 sirtuins (class III HDACs), more than have been reported for any other organism to date. The putative *Tetrahymena* sirtuins are similar to yeast Sir2 but are more closely related to protein sequences for the human SIRT2 (Thd16), SIRT3 (Thd13, Thd14, and Thd15), SIRT5 (Thd10, Thd17, and Thd18), and SIRT6 (Thd8, Thd9, Thd11, and Thd12) proteins. *Tetrahymena* will likely prove a useful model system for further studies addressing distinct roles for different HDACs, especially the sirtuins, within a single cell.

**DISCUSSION**

**HDACs in Tetrahymena.** Completion of the *Tetrahymena* genome-sequencing project facilitated the identification and classification of putative HDACs according to the three classical respectively) (data not shown). Together, these aberrant morphologies and weak DAPI staining are consistent with chromatin condensation abnormalities in *thd2Δ* micronuclei.

In micronuclei, histone H3 (called H3₃) is proteolytically cleaved to form a faster-migrating form, called H3₄, which is missing 6 amino acids from the extreme amino terminus. H3₃ is the only form of histone H3 in *Tetrahymena* that is phosphorylated on Ser10, a mitotic modification necessary for chromosome condensation and segregation (4, 6, 54). In *thd2Δ* cells, the elongated micronuclear phenotype, reminiscent of either decondensed chromatin or mitotic micronuclei in anaphase, prompted us to examine H3 processing and related phosphorylation on Ser10. Immunoblot analysis revealed that while the abundance of micronuclear H3₃ was similar to that in wild-type cells, cleavage of histone H3₃ to H3₄ was greatly reduced in *thd2Δ* cells (Fig. 9B). As expected, the reduced amount of H3₄ correlated with reduced Ser10 phosphorylation, likely the result of the failed H3 processing in these cells (Fig. 9B). These results suggest that Thd2 acts upstream of the proteolytic cleavage and subsequent phosphorylation of Ser10 on histone H3₃.

**FIG. 9.** Cells lacking Thd2 exhibit chromatin phenotypes. (A) Immunofluorescence using antiserum against micronuclear linker histone H1 (α-Mlh1) was performed on wild-type (WT) and *thd2Δ* cells. DAPI staining was used to visualize both the macronucleus (M) and the micronucleus (m). A higher incidence of elongated micronuclei in close association with macronucleus was observed in the mutant cells. (B) Total proteins from purified macronuclei and micronuclei were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and hybridized with antiserum against general histone H3 (H3) or with antiserum against phosphorylated serine 10 on histone H3 (α-H3S10ph). The full-length form of histone H3 (H3₃) was detected in both macronuclei and micronuclei of all cells, but only wild-type micronuclei contained the faster-migrating proteolytically cleaved form (H3₄). Likewise, phosphorylation of Ser10, which is specific for H3₄, occurred only in wild-type micronuclei. (Note: the blot was first hybridized with α-H3S10ph and then stripped and hybridized with α-H3.)
H3 (Lys9 and Lys14 in Tetrahymena) showed that acetylation of these residues was retained in thd2Δ micronuclear chromatin (Fig. 8C). Although it is unlikely, these experiments did not rule out the possibility that micronuclear acetylation in thd2Δ cells resulted from aberrant localization of a transcription-related HAT to micronuclei. However, antiserum against the transcription-related acetyl-Lys16 on H4 (α-H4K16ac) confirmed that Lys16 remained unacetylated in thd2Δ micronuclei (Fig. 8D). Combined with the fact that only deposition acetylation has ever been observed in micronuclei, these results were taken as strong evidence that micronuclear Thd2 deacetylates newly deposited histones. Further support was provided by the finding that THD2a is maximally transcribed during periods of DNA replication (late anaphase) (Fig. 5). The clearest example of this was observed early in the sexual-conjugation cycle (hours ~5 to 7), when micronuclei undergo two rounds of DNA replication and mitotic division in rapid succession while macronuclei remain in G0. Over this period, there was a pronounced peak of THD2 transcription (Fig. 6B), the same window in which rapid deacetylation of newly synthesized histones occurs (2).

**Histone deacetylation and chromatin maturation.** Histone deacetylation is an important event in chromatin maturation (7). When chromatin is replicated and assembled in vivo in the presence of sodium butyrate to inhibit deacetylation, it retains the DNase I sensitivity typical of immature chromatin, a feature that is rapidly lost when chromatin is replicated under normal conditions (7, 44). Similarly, chromatin assembled in vitro with hyperacetylated histones displays increased sensitivity to DNase I (24). The nuclease sensitivity can be attributed in part to reduced H1-mediated internucleosomal interactions that require core histone amino termini (38, 39). Recently, it was found that deacetylation of Lys91 in the globular domain of histone H4 is important for interaction of H3-H4 tetramers with H2A-H2B dimers during nucleosome formation. Lys91 is initially acetylated prior to deposition, and Lys91 deacetylation facilitates the formation of a salt bridge between Lys91 and a glutamic acid residue on histone H2B (10, 60). Outside of these observations, little is known about the role of deacetylation in the chromatin maturation process.

Following nucleosome segregation and de novo formation on replicated DNA strands, some nucleosome remodeling is likely required to position nucleosomes for further chromatin maturation. Interestingly, the predominant splice variant of thd2Δ cells resulted from aberrant localization of a transcription-related HAT to micronuclei. However, antiserum against the transcription-related acetyl-Lys16 on H4 (α-H4K16ac) confirmed that Lys16 remained unacetylated in thd2Δ micronuclei (Fig. 8D). Combined with the fact that only deposition acetylation has ever been observed in micronuclei, these results were taken as strong evidence that micronuclear Thd2 deacetylates newly deposited histones. Further support was provided by the finding that THD2a is maximally transcribed during periods of DNA replication (late anaphase) (Fig. 5). The clearest example of this was observed early in the sexual-conjugation cycle (hours ~5 to 7), when micronuclei undergo two rounds of DNA replication and mitotic division in rapid succession while macronuclei remain in G0. Over this period, there was a pronounced peak of THD2 transcription (Fig. 6B), the same window in which rapid deacetylation of newly synthesized histones occurs (2).

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Following nucleosome segregation and de novo formation on replicated DNA strands, some nucleosome remodeling is likely required to position nucleosomes for further chromatin maturation. Interestingly, the predominant splice variant of THD2 (THD2a) contains a putative IPK domain related to Ipk2 in yeast. This putative IPK domain is the only one of its type in the predicted Tetrahymena coding sequences (J. Smith, unpublished data). Recent studies demonstrated a functional link between the IPK signaling pathway and chromatin remodeling; Ipk2 was shown to be involved in the recruitment of chromatin-remodeling complexes for transcription (43, 48). In yeast, mutations in Ipk2, the enzyme that normally converts IP1 to IP4 and IP3, prevented specific gene induction due to failed recruitment of the chromatin-remodeling complexes SWI/SNF and INO80 (48). Thd2 contains most of the residues within the conserved cofactor binding and IP binding domains found in polyphosphate kinases (Fig. 4B). In Ipk2, a D131A mutation (underlined) within the IP kinase motif (PXXXDQXKG . . . SSLL) impaired chromatin remodeling and gene transcription in yeast (43, 48). Thd2a contains this highly conserved region and the critical aspartic residue within the IPK motif (Fig. 4B). Other than regulating chromatin remodeling, other possible nuclear roles for an Ipk2-like domain include promoting mRNA export (33, 35) and regulating nonhomologous end joining (19). Future studies will address these possibilities, especially in relation to the HDAC activity residing on the same enzyme.

Although this is the first report of an IPK motif on an HDAC enzyme, broad gene database searches revealed other Thd2 homologs with putative IPK domains nearly identical in sequence and structure across the putative polyphosphate binding and kinase motifs. Interestingly, they were found only in other protozoans (Paramaecium tetraurelia, Plasmodium falciparum, Plasmodium yoelii, Theileria annulata, and Theileria parva) (data not shown). In each case, like Thd2, the putative IPK domain was at the C terminus of the HDAC enzyme. It is possible that in other organisms, instead of residing on the same polypeptide, IPKs exist as separate polypeptides that complex with HDACs to mediate chromatin maturation.

The alternative splicing of THD2 transcripts yields a variant without the putative IPK domain, a process that could be used to regulate the IPK activity on this HDAC. Splice variant THD2a contains the putative IPK domain, whereas splice variant THD2b does not. Moreover, the two variants are differentially expressed throughout sexual conjugation. Whereas they share a common peak of transcription at 6 hours coincident with micronuclear mitosis, THD2b is expressed to almost the same degree at later times, as well (Fig. 6). In this study, GFP was cloned in frame with the THD2 genomic sequence. It is not clear which form, Thd2a or Thd2b (or both), localizes to the macronucleus; future studies will address whether these forms are differentially localized. Our experiments revealed a possible third splice variant that retains intron 3 (Fig. 3C and 6B). This form, like THD2b, would lack the putative IPK domain.

We were unable to rule out the possibility that this variant was simply an unprocessed intermediate of THD2b in which intron 3 had not yet been removed (Fig. 3A). Regardless of whether there are two or three splice variants of THD2, this is the first reported example of an alternative spliced coding sequence from Tetrahymena.

The cytological phenotypes of thd2Δ cells were suggestive of chromatin defects. Although the doubling time of thd2Δ cells was ~3 times that of wild-type cells (data not shown), nuclear division and cytokinesis appeared normal. However, 50% of elongated micronuclei (a shape typical of anaphase) in thd2Δ cells were in close proximity to or associated with the macronucleus, an aberrant position for an anaphase micronucleus. Determination of whether these micronuclei are arrested in anaphase awaits further studies. A prolonged anaphase would account for the longer population doubling time observed in mutant cells. It is noteworthy that micronuclei also elongate normally when their chromatin decondenses over a short interval early in sexual conjugation. It is thus tempting to speculate that chromatin is less condensed in growing thd2Δ cells, something that could result from defects in chromatin maturation. Although the sizes and shapes of micronuclei in mutant cells were more variable than those in wild-type cells (many were quite small), cells completely lacking a micronucleus were not observed, perhaps due to activation of a mitotic checkpoint upon loss of micronuclear DNA.
Although a more extensive analysis of cytological phenotypes in relation to chromatin maturation awaits future studies, one chromatin abnormality detected in \textit{thd2}Δ cells was defective protocytic processing of new histone H3 (H3\textsubscript{3}). It is still unclear what purpose this processing normally serves; both forms (H3\textsubscript{3} and H3\textsubscript{s}) are present in mononucleosomes (3). Specific to histone H3\textsubscript{s} is the phosphorylation of Ser10, a modification present only in micronuclei during the early stages of mitosis (54). Phosphorylation is not required for formation of the H3\textsubscript{s} species, as demonstrated with an unphosphorylated H3 mutant (H3S10A), but micronuclei in these cells displayed segregation defects (55). In contrast, protocytic processing of H3 was defective in \textit{thd2}Δ cells; H3\textsubscript{s} was greatly reduced (Fig. 9B). This result was confirmed by immunoblottting with antisera against phosphorylated Ser10 on histone H3 (α-H3S10ph). As expected, phosphorylation was greatly reduced compared to that in wild-type cells, consistent with there being less H3\textsubscript{s} in the \textit{thd2}Δ cells (Fig. 9B). These findings suggest that Thd2 is required for H3 processing and subsequent phosphorylation. As Ser10 phosphorylation is linked to chromatin condensation and progression of mitosis in many organisms, including \textit{Tetrahymena}, the reduction in phosphorylation may account for some of the observed growth and cytological phenotypes observed in the mutant cells. Future studies will explore these possible relationships in the context of roles for HDAC enzymes in chromatin maturation and function.

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