MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4305–4311 Vol. 16, No. 8
0270-7306/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Essential and Nonessential Histone H2A Variants in *Tetrahymena thermophila*

XIUWEN LIU, BING LI, AND MARTIN A. GOROVSKY*

Department of Biology, University of Rochester, Rochester, New York 14627

Received 15 March 1996/Returned for modification 6 May 1996/Accepted 28 May 1996

Although variants have been identified for every class of histone, their functions remain unknown. We have been studying the histone H2A variant hv1 in the ciliated protozoan *Tetrahymena thermophila*. Sequence analysis indicates that hv1 belongs to the H2A.F/Z type of histone variants. On the basis of the high degree of evolutionary conservation of this class of histones, they are proposed to have one or more distinct and essential functions that cannot be performed by their major H2A counterparts. Considerable evidence supports the hypothesis that the hv1 protein in *T. thermophila* and hv1-like proteins in other eukaryotes are associated with active chromatin. In *T. thermophila*, simple mass transformation and gene replacement techniques have recently become available. In this report, we demonstrate that either the *HTA1* gene or the *HTA2* gene, encoding the major H2As, can be completely replaced by disrupted genes in the polyploid, transcriptionally active macronucleus, indicating that neither of the two genes is essential. However, only some of the *HTA3* genes encoding hv1 can be replaced by disrupted genes, indicating that the H2A.F/Z type variants have an essential function that cannot be performed by the major H2A genes. Thus, an essential gene in *T. thermophila* can be defined by the fact that it can be partially, but not completely, eliminated from the polyploid macronucleus. To our knowledge, this study represents the first use of gene disruption technology to study core histone gene function in any organism other than yeast and the first demonstration of an essential gene in *T. thermophila* using these methods. When a rescuing plasmid carrying a wild-type *HTA3* gene was introduced into the *T. thermophila* cells, the endogenous chromosomal *HTA3* could be completely replaced, defining a gene replacement strategy that can be used to analyze the function of essential genes.

Although histone proteins are highly conserved, nonallelic variants have been identified for every class of histone (35, 39), raising the possibility that different subtypes have different functions. Initial analyses on the function of histone subtypes were done in the yeast *Saccharomyces cerevisiae* (17–19), which has two copies of the genes for each of the core histones (27). Grunstein and colleagues demonstrated that yeast cells with only one of the two nonallelic subtypes for any of the core histones are still viable (reviewed in references 17 to 19). These results argue that the functions of histone variants are largely redundant in *Saccharomyces cerevisiae*, although when each of the 2A-2B gene pairs in *Saccharomyces cerevisiae* was deleted, some phenotypic differences were observed; most (but not all) of these differences could be corrected by doubling the dose of the remaining pair (26). Another line of evidence against assigning specific functions to histone variants comes from the study of sea urchin histones. Developmental switching of histone subtypes in sea urchins is a classic example that, on first analysis, suggests that different histone subtypes may play different roles during different developmental stages. However, it has been shown for one sea urchin species that the H3 and H4 genes expressed during early or late stages of development actually encode identical proteins (8). Since developmental changes in these cases are not related to the expression of distinct protein subtypes, it is possible that developmental switching of nonidentical, nonallelic subtypes simply reflects the accumulation of neutral mutations in these genes.

On the basis of these studies, it could be concluded that the presence of histone subtypes is of little functional significance. However, recent studies have suggested that at least some histone variants may have specific functions. Thus, in vertebrate cells a protein (CENP-A) that specifically localizes to centromeric regions has been shown to be nucleosomal and to be an unusual H3 variant (30), while mutations in a gene (CSE4) encoding a similar protein in *Saccharomyces cerevisiae* cause chromosome nondisjunction and cell cycle arrest (29), suggesting that each of these proteins functions to set up a special chromatin structure at the centromere. Studies on histone H2A variant hv1 in *Tetrahymena thermophila*, and on related H2A.F/Z variants in other organisms, argue strongly that histone H2A variants can have distinct functions. In vegetatively growing *T. thermophila*, hv1 is found in the transcriptionally active macronucleus but not in the transcriptionally inactive micronucleus (1, 2). Recently, it has been shown that hv1 appears in the micronuclei of conjugating cells at (or just preceding) a period during which they become transcriptionally active (28). Thus, in *T. thermophila*, there is a strong developmental correlation between the presence of hv1 and transcription. An hv1-specific antiserum stained nucleoli of mammalian cells (presumably the actively transcribed ribosomal gene containing chromatin [3]), and the same antiserum also stained a subset of loci on *Drosophila melanogaster* polytene chromosomes that are active, were active, or will be active during the third larval instar or prepupal stage of development (11). These studies suggest that hv1 and proteins antigenically related to it in mammalian cells and in *D. melanogaster* are preferentially associated with transcriptionally active or potentially active (competent, poised, activated) chromatin.

Evolutionary analyses also support the hypothesis that quantitatively minor hv1-related proteins in diverse species have distinct functions. H2A.F/Z variants have been found in representatives of three of the four eukaryotic kingdoms (none have yet been described for plants): hv1 in *T. thermophila* (1, 2, 36), the *pht1* gene product in *Schizosaccharomyces pombe* (7), H2AvD in *D. melanogaster* (32–34), H2A.F/Z in sea urchins

* Corresponding author.

(12), H2A.F in chickens (9, 20), and H2A.Z in mammals (5). Comparison of the deduced protein sequences of the major H2As and the H2A.F/Z variants led to the surprising observation that these variants in different organisms are more similar to each other than each is to the major H2A in the same organism. A detailed evolutionary analysis of H2A protein sequences (31) demonstrated that the major H2As and the H2A.F/Z variants diverged early in eukaryotic evolution and, surprisingly, the H2A.F/Z proteins show less evolutionary divergence than the major H2As. These results argue strongly that there were two types of H2A genes in primitive eukaryotes before the divergence of ciliates, fungi, and animals and that the major and variant H2As have been under different selective pressures since that time. Therefore, the major H2As and H2A.F/Z variants must have distinct and important functions.

The *H2AvD* gene was cloned by homology to hv1 and shown to be present in a single copy in the haploid *D. melanogaster* genome (34). A homozygous lethal mutation due to deletion of the second exon of the *H2AvD* gene was rescued by P-element-mediated transformation using the wild-type gene (32). This was the first demonstration that histone subtypes perform an essential function in vivo that was distinct from that of the major H2A genes. These results suggested that H2A.F/Z variants had essential functions in eukaryotes. However, since *D. melanogaster* is a multicellular organism, it is possible that the essential requirement for the *H2AvD* gene is associated with its expression at a specific time in development and not with the sequence differences between H2AvD and the major H2A protein. Consistent with this hypothesis, deletion of the *pht1* gene in *Schizosaccharomyces pombe*, which encodes an H2A.F/Z variant, is not lethal, although it does result in slow growth and chromosome missegregation (7).

To determine whether the differences in the essential nature of the H2A.F/Z variants of *D. melanogaster* and *Schizosaccharomyces pombe* reflected a difference between unicellular and multicellular eukaryotes, we have examined the H2A gene family in *T. thermophila*. There are three H2A genes in *T. thermophila* (24). *HTA1* and *HTA2* encode the major H2As, which account for about 80% of the H2A protein; *HTA3* encodes the minor H2A variant, hv1, which constitutes about 20% of H2A protein (1). The two major H2As are evolutionarily related (31) and are virtually identical in their central core regions but differ markedly in their carboxy-terminal tails (24). The low copy number of the H2A genes in *T. thermophila*, plus the availability of both major and minor H2A variants, provides a unique opportunity to study the functions of both types of conserved histone H2A proteins in a eukaryotic genome. Two prerequisites for these analyses are the ability to transform *T. thermophila* cells and the targeting of the disrupted genes to the correct chromosomal loci by homologous recombination. Recent developments in *T. thermophila* molecular genetics have proven the feasibility of such studies (13, 15, 21).

In this report, using these newly developed molecular genetic tools, we demonstrate that neither the *HTA1* gene nor the *HTA2* gene, encoding the major H2A proteins, is essential for the growth of *T. thermophila*. The *HTA3* gene encoding an H2A.F/Z type variant, however, cannot be completely replaced, indicating that it is essential for the vegetative growth of *T. thermophila.*

MATERIALS AND METHODS

Construction of plasmids for the disruption of H2A genes in *T. thermophila*. A *neo* cassette (on plasmid p4T21, originally called the *H4IneoBTU2* cassette), which contains an aminoglycoside 3′-phosphotransferase gene (*Aph*, or *neo*) under the control of a histone H4 gene (*HHF1*) promoter and a β-tubulin gene (*BTU2*) terminator, was described previously (14). For *HTA1* disruption, plasmid pXL53 containing the *T. thermophila HTA1* gene (24) was first digested with *Bgl*II and *Xho*I and then blunt-end ligated to eliminate most of the 5′ upstream regions of the *HTA1* gene to make a shorter construct that is easier to manipulate. This new clone (pXL200) contains only 153 bp of upstream sequence (from the starting ATG), 417 bp of coding sequence, and about 1.6 kb of 3′ flanking sequence. A 1.4-kb *Eco*RV-*Sma*I fragment from plasmid p4T21 containing the *neo* cassette was ligated into the *Bal*I site in the *HTA1* coding region to obtain a clone (pXL201) from which a 3.6-kb *Bgl*II-*Sac*I fragment was used to disrupt the *HTA1* gene (Fig. 1). A similar construct was made for the *HTA2* gene. The 1.4-kb *Eco*RV-*Sma*I fragment of p4T21 was inserted at the *Bal*I site within the *HTA2* coding region of plasmid pXL46 (24) to obtain pXL202, from which a 3.4-kb *Sac*I-*Kpn*I fragment was used to disrupt the *HTA2* gene. The construct for *HTA3* disruption (pXL203) was made by ligating the 1.4-kb *Eco*RV-*Bam*HI fragment from p4T21 into the *Nar*I (blunted)-*Bgl*II sites of phv1 (24). A 3.2-kb *Eco*RV-*Xho*I fragment released from pXL203 was used to disrupt the *HTA3* gene.

Transformation of *T. thermophila* by electroporation. *T. thermophila* strains of two different mating types, CU428 *Mpr*/*Mpr* (mps VII) and B2086 Mpr^+/Mpr^+ (mps II), were used for conjugant electrotransformation. Growth, starvation, and mating of *T. thermophila* cells were essentially as described elsewhere (13). Plasmid DNA was digested by appropriate restriction enzymes (see above) to release H2A gene fragments interrupted by the *neo* cassette, extracted by phenol-chloroform, ethanol precipitated, and resuspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.5, at a concentration of 0.4 μg/μl. For each transformation, 125 μl (50 μg) of DNA was used. Electroporation was done with Electro Cell Manipulator 600 (BTX, San Diego, Calif.) under the conditions described previously (13).

Selection of transformants. The *neo* gene confers paromomycin resistance in *T. thermophila*. Initial selection of transformants was done with 140 μg of paromomycin (Humatin; Parke-Davis, Div. of Warner Lambert Co., Morris Plains, N.J.) per ml. Paromomycin-resistant transformants were then transferred daily to medium containing twice-higher concentrations of drug until a concentration at which they stopped dividing was reached. After the lethal drug concentration was determined, cells were transferred from lower to higher drug concentrations by 0.25-fold increments. The level of drug resistance was considered to be the highest concentration at which cultures could be maintained in continuous growth by twofold daily dilution.

Isolation of total genomic DNA and Southern blot analysis. Transformants were grown at one-half the highest drug concentration they could tolerate in super Proteose Peptone (SPP) medium (1% Proteose Peptone, 0.2% glucose, 0.1% yeast extract, 0.003% Sequestrene) without shaking to about 2×10^5 cells per ml. Nontransformed control cells (strain CU428) were grown in SPP medium without drug. Total cellular DNA that included both macro- and micronuclear DNAs was isolated by a method described previously (15). Restriction digestion and Southern transfers were done according to standard protocols (4). Probes were made from gene-specific fragments by random priming (4) in the presence of [α-^{32}P]dATP. The *HTA1* gene-specific probe was made from the 1.75-kb *Hin*dIII-*Spe*I fragment from pXL52 (24) containing 20 bp of the coding region, 461 bp of the 3′ transcribed but nontranslated region, and about 1.3 kb of additional 3′ nontranscribed sequence. The *HTA2* gene-specific probe was synthesized by using the 1.65-kb *Eco*RI-*Spe*I fragment from pXL48 (24) which consists of 29 bp of the coding region, 130 bp of nontranslated sequence, and about 1.5 kb of 3′ nontranscribed sequence. The entire 2.1 kb of the phv1 insert was released by *Hin*dIII digestion and used as a probe for the *HTA3* gene.

Isolation of total RNA and Northern blotting. Total RNA was isolated by using guanidine isothiocyanate as described previously (23, 36). Ten micrograms of RNA was loaded on each lane of a 1.2% agarose-formaldehyde gel, electrophoresed and blotted, and the filter was hybridized as described elsewhere (4).

Probes were made by random-prime labeling of a PCR product of the *HTA1* coding region (24) for hybridization to both the *HTA1* message and the *HTA2* message. The *HTA3* probe was the same sequence as that used for the Southern blot described above.

Total protein preparation and Western blotting. *T. thermophila* cultures were grown as described above. About 10^6 cells were spun down and washed once in 10 mM Tris-HCl, pH 7.5. Cells were resuspended in 200 μl of 10 mM Tris-HCl, pH 7.5, and lysed by adding 200 μl of 2× sample buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% β-mercaptoethanol, and 0.01 mg of bromophenol blue per ml). Proteins from 10^5 cells (40 μl) were subjected to SDS–15% polyacrylamide gel electrophoresis (PAGE) and transferred onto an Immobilon-P membrane (Millipore, Bedford, Mass.) by using a semidry transfer unit (Gelman Biotrans, Ann Arbor, Mich.). After blocking in 1% bovine serum albumin–Tris-buffered saline, a mixture of anti-hv1 (1:20,000 dilution [28]) and anti-H2A (1:10,000 dilution [28]) was added and the blot was incubated for 1 h. A 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma, St. Louis, Mo.) was used as a secondary antibody. Blots were developed by using the ECL Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

Rescuing the *HTA3* knockout deletion. Plasmid pXL210, which carries an intact *HTA3* gene, was constructed by putting the 2.2-kb genomic clone of *HTA3*, hopefully containing all the necessary promoter sequences and polyadenylation signals, onto plasmid vector CV22 (13). This plasmid contains a mutated version of the *rpl29* gene conferring cycloheximide resistance and one copy of the rDNA

TABLE 1. Transformants and drug resistance

Transformant (type[a]) and target gene	Drug level (mg/ml)[b]
XLT1 (a), *HTA1*	3.0 (4.2)
XLT2 (a), *HTA1*	1.5 (2.1)
XLT3 (b), *HTA1*	45 (62.5)
XLT5 (a), *HTA1*	2.0 (2.8)
XLT6 (a), *HTA1*	2.0 (2.8)
XLT7 (a), *HTA2*	0.8 (1.1)
XLT9 (a), *HTA2*	0.8 (1.1)
XLT10 (a), *HTA2*	0.8 (1.1)
XLT11 (c), *HTA3*	6.0 (8.3)
XLT12 (c), *HTA3*	5.0 (6.9)
XLT13 (c), *HTA3*	5.0 (6.9)
XLT15 (c), *HTA3*	5.0 (6.9)
XLT17 (c), *HTA3*	3.0 (4.2)
XLT18 (c), *HTA3*	5.0 (6.9)

[a] a, disruption, complete replacement; b, integration, complete replacement; c, disruption, partial replacement.
[b] Highest concentration at which cells will divide at least once a day for paromomycin equivalent and paromomycin sulfate (in parentheses).

replication origin. Plasmid pXL203 (to knock out the *HTA3* gene) and pXL210 (for rescue) were then cotransformed as described above. Transformants were selected first with paromomycin at 140 μg/ml, and 44 clones were isolated. These clones were then subjected to cycloheximide selection to screen for double transformants. Two of the 44 were resistant to both drugs, and they were then further selected with increasing concentrations of paromomycin in an attempt to completely replace the endogenous *HTA3* gene. As a control, we also tried to select clones resistant only to paromomycin at the higher paromomycin concentrations. Genomic Southern blots were then performed to determine whether complete replacement of the endogenous *HTA3* locus had been achieved.

RESULTS

Transformation in *T. thermophila*. In the conjugant electrotransformation used here, the linearized DNA fragments are targeted into the transcriptionally active macronuclear genome, leaving the transcriptionally inert micronuclear genome undisturbed (13). Each gene in the macronucleus exists in ~45 copies. The initial transformants probably have only a fraction of these copies disrupted. Since macronuclei divide amitotically, distributing the multiple gene copies randomly to each daughter nucleus (a process referred to as phenotypic assortment [10, 25]), increasing drug pressure selects for cells with increasing copies of the disrupted genes in the macronuclear genome and the concomitant loss of the undisrupted genes. If a gene is nonessential, eventually this process should lead to complete replacement of intact genes by disrupted ones. For essential genes, it should be possible to obtain partial replacement of the endogenous genes. Cells growing at the highest possible drug concentration should contain the maximum number of disrupted genes and the smallest number of undisrupted genes with which the cells can still grow.

Transformants display a broad profile of drug resistance. Initial selection yielded five clones for *HTA1* disruption, three clones for *HTA2* disruption, and six clones for *HTA3* disruption. They were then transferred to media containing increasingly higher concentrations of drug. The highest concentration of paromomycin tolerated by these transformants is defined as the maximum drug concentration at which cells can divide at least once each day, as described elsewhere (21). Table 1 summarizes these results. The expression of the *neo* gene seems to be highly position dependent. With one exception (*HTA1*-integrative transformant XLT3; see below) at the *HTA1* locus, the expressed *neo* gene product can inactivate paromomycin up to 1.5 to 3.0 mg/ml, while the *HTA2* transformants can tolerate the drug only to 0.8 mg/ml. The *HTA3* transformants give the highest resistance, up to 3.0 to 6.0 mg/ml.

All transformants obtained involved homologous recombination. Genomic Southern blots indicated that four of the five *HTA1* clones were gene replacement transformants and the other one was an integrative transformant at the *HTA1* locus. All of the *HTA2* and *HTA3* clones were the results of gene replacement. Figure 1 shows the Southern blot of two *HTA1* gene replacement transformants (XLT1 and XLT2) and the one integrative transformant (XLT3), together with two of the *HTA2* clones (XLT7 and XLT9) and two of the *HTA3* clones (XLT11 and XLT12). DNA from untransformed cells

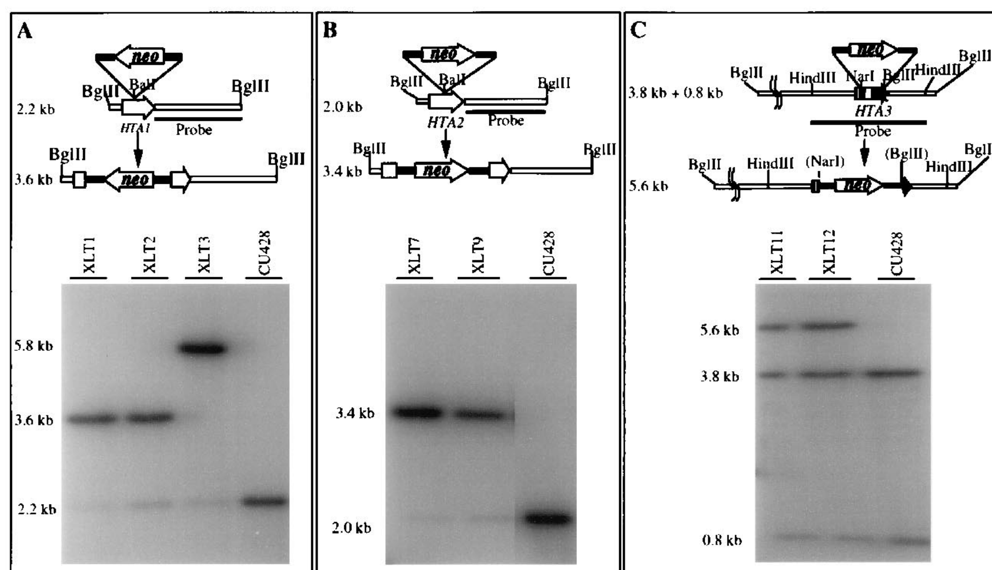

FIG. 1. Genomic Southern blots of *T. thermophila* transformants. Gene-specific probes were used for the *HTA1* (A), the *HTA2* (B), and the HTA3 (C) transformants. CU428 is one of the parental strains. The sizes of DNA fragments hybridized are indicated. Restriction maps of the original and disrupted genomic DNAs are shown at the top.

(CU428) was also run on the gel to show the sizes of the original gene fragments.

When digested with the restriction enzyme *Bgl*II and hybridized with the gene-specific probe derived from the 3′ region of *HTA1*, the endogenous *HTA1* gene produced a single 2.2-kb fragment (Fig. 1A, CU428). In the replacement transformants XLT1 and XLT2, insertion of the 1.4-kb *neo* cassette caused the band to shift to the expected size of 3.6 kb. In the integrative transformant a 5.8-kb fragment was observed instead (XLT3). When probed with a gene-specific fragment, the original *HTA2* gene gave a single 2.0-kb band (CU428), while the band shifted to 3.4 kb in the disruptive transformants XLT7 and XLT9 (Fig. 1B). When DNA from wild-type cells was digested with *Bgl*II and probed with the *HTA3* gene-specific probe, two fragments of 3.8 and 0.8 kb were observed (CU428). Insertion of the 1.4-kb *neo* cassette should eliminate the 435-bp *Nar*I-*Bgl*II fragment containing most of the *HTA3* coding region and the second intron and at the same time should destroy the internal *Bgl*II recognition sequence at the site of insertion. Therefore, the disrupted *HTA3* gene should give a 5.6-kb fragment on the Southern blot as can be seen in Fig. 1C (XLT11 and XLT12).

All these transformants can be explained by homologous recombination with the electroporated DNA fragments targeted to the correct chromosomal loci. Even for the one integrative transformant, the transforming DNA fragment must have integrated by homology at the 5′ or 3′ flanking region of the endogenous gene, as evidenced by the disappearance of the endogenous 2.2-kb *Bgl*II fragment and the appearance of a new band of the expected size on the Southern blot. The exact site of integration cannot be determined from this Southern blot and has not been mapped.

These studies demonstrate that the orientation of the *neo* cassette relative to the gene into which it inserts is unimportant because both colinear (as in the *HTA2* and *HTA3* constructs) and inverse (as in the *HTA1* construct) orientations have been shown to be functional (Fig. 1). Therefore, the 1.4-kb *neo* cassette can be used as an orientation-independent selectable marker in *T. thermophila* transformation experiments, similar to those (e.g., *URA3*, *HIS3*, *TRP1*, and *LEU2*) used in the yeast *Saccharomyces cerevisiae.*

These studies also show that genes can be correctly targeted to the right chromosomal loci, even if one end of the transforming DNA consists of a short homologous sequence. In this report, the 5′ homologous sequence for the *HTA1* construct is only 382 bp and the 5′ homologous sequence for the *HTA2* construct is 408 bp. The 3′ homologous sequence for the two is 1.8 and 1.6 kb, respectively. We have not yet investigated whether short sequences on both ends will suffice.

Southern blot analysis suggests that the *HTA1* or *HTA2* gene can be completely eliminated. In all the *HTA1* and *HTA2* disruptive transformants (Fig. 1A, XLT1 and XLT2; Fig. 1B, XLT7 and XLT9), the endogenous *HTA1* or *HTA2* fragments have mostly disappeared. The faint bands of the undisrupted fragment in each of these transformants probably are due to the presence of small amounts of micronuclear DNA in the total DNA. Thus, while genomic Southern blots argue that most, if not all, of the macronuclear copies of the *HTA1* and *HTA2* genes can be knocked out, the presence of micronuclear DNA precludes the conclusion that complete macronuclear knockouts have been obtained. Other methods are required to demonstrate disruption of all copies of macronuclear genes (see below).

Southern blot analysis indicates that *HTA3* can be only partly eliminated: the disrupted *HTA3* genes can only partially replace the endogenous genes (Fig. 1C, XLT11 and XLT12).

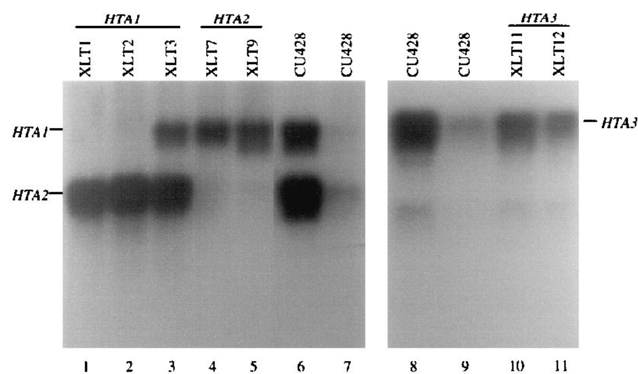

FIG. 2. Northern blot analyses of *HTA* messages in *T. thermophila* transformants containing gene disruptions. Total RNAs from the *HTA1* (lanes 1 to 3) and *HTA2* (lanes 4 and 5) transformants were probed with an *HTA1* coding sequence that recognizes mRNAs from both genes. RNAs from the *HTA3* transformants (lanes 10 and 11) were hybridized with an *HTA3* gene-specific probe. Lanes 7 and 9 contain 0.2 g of total RNA (1/45 of the amount in lanes 6 and 8). The nature of the faint, faster-migrating band in the lanes probed with the *HTA3* probe is unknown.

Judging from the intensity of the 5.6-kb band relative to the sum of the 3.8- and 0.8-kb fragments, about 50% of the *HTA3* genes are disrupted in the two clones analyzed. Thus, at the highest drug concentrations at which the *HTA3* transformants can grow, large amounts of the undisrupted gene are still present, indicating that *HTA3* is an essential gene. These studies on the *HTA3* knockouts also reveal an interesting feature of gene dosage regulation in the *T. thermophila* macronucleus. When the *HTA3* gene was disrupted, endogenous wild-type genes could be only partially replaced by genes interrupted by the *neo* cassette. This indicates that there is a mechanism limiting the number of normal *HTA3* chromosomes that a macronucleus can contain. Given the similarity in intensities of the genomic Southern blots of wild-type cells and *HTA3* transformants (Fig. 1C), that limit is probably close to the normal level of 45 copies per (G_1) macronucleus (10).

Expression of the *HTA1* or the *HTA2* gene but not the *HTA3* gene is abolished in the gene replacement transformants. In vegetative cells, genes in macronuclei are transcribed while those in micronuclei are not (reviewed in references 6 and 16). Thus, if a complete knockout of macronuclear genes has been obtained, no mRNA should be produced from the disrupted genes. Northern blots were hybridized with a probe containing the *HTA1* coding region that recognizes both *HTA1* and *HTA2* transcripts (Fig. 2). As expected, both *HTA1* (~950-nucleotide) and *HTA2* (~600-nucleotide) messages hybridized in the parental strain CU428. The *HTA1* messages are not detectable in the *HTA1* gene replacement transformants XLT1 and XLT2, and *HTA2* messages are not detectable in *HTA2*-disrupted clones XLT7 and XLT9. Under these conditions, 1/45 of the CU428 RNA is still detectable for *HTA1* and *HTA2* messages (lane 7), suggesting that *HTA1* and *HTA2* messages, if present, must be transcribed from less than one copy of the corresponding gene in the macronucleus. These results argue strongly that the macronuclear *HTA1* or *HTA2* genes have been completely knocked out in these strains. *HTA1* messages are still present in strain XLT3, which is an integrative transformant in which the endogenous sequence is still intact. As expected, the *HTA3* messages are also detectable in all *HTA3* disruption clones in which the intact gene has only been partially replaced.

Western blotting demonstrates the absence of H2A1 or H2A2 protein in the complete knockouts. When total *T. thermophila* proteins were separated by SDS-PAGE and probed

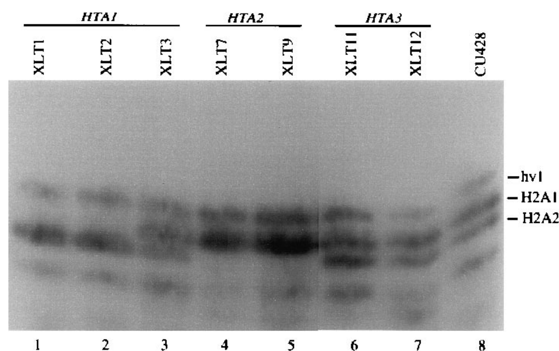

FIG. 3. Western blot analyses of histone 2A proteins in *T. thermophila* knockout strains. *T. thermophila* cell lysates from two of the *HTA1*-disruptive transformants (lanes 1 and 2), the one integrative clone (lane 3), two of the *HTA2* transformants (lanes 4 and 5), two of the *HTA3* transformants (lanes 6 and 7), and the parental strain CU428 (lane 8) were run in SDS-PAGE. The blot was probed with a combination of anti-H2A and anti-hv1 antisera to detect all the three types of H2A proteins. H2A1 and H2A2 proteins are not detectable in the *HTA1*- and *HTA2*-disruptive transformants, respectively. The unlabeled, fastest-migrating band comigrates with histone H4, whose amino-terminal sequence is similar to that of hv1 (2).

with a mixture of anti-H2A and anti-hv1 antisera, H2A1 or H2A2 protein, respectively, is not detectable in *HTA1* (Fig. 3, lanes 1 and 2) or *HTA2* (Fig. 3, lanes 4 and 5) gene replacement clones. Under these conditions, the parental strain CU428 (lane 8), the *HTA1*-integrative transformant (XLT3 [lane 3]), and *HTA3* partially disrupted strains (XLT11 [lane 6] and XLT12 [lane 7]) show all three types of H2A proteins (H2A1, H2A2, and hv1). Again, these studies argue that complete knockouts of the *HTA1* and *HTA2* genes, but not of the HTA3 gene, have been obtained.

Complete *HTA3* knockout can be achieved by episomal rescue. There are two possible explanations for the failure to completely knockout the *HTA3* gene. The simplest is that the hv1 protein is essential for the survival of *T. thermophila* cells. However, we cannot rule out another possibility, that introduction of the *neo* cassette itself into the *HTA3* locus is detrimental to the cells. To distinguish between those two possibilities, we sought to determine whether a plasmid carrying a wild-type *HTA3* gene would permit complete replacement of endogenous chromosomal *HTA3* genes by disrupted ones. When a rescuing plasmid carrying a wild-type *HTA3* gene was cotransformed with the knockout construct, two clones that were resistant to both paromomycin and cycloheximide were isolated. Southern blot analysis was performed after selection at the highest concentration of paromomycin at which these cells would grow. One of the two transformants showed complete replacement of the endogenous *HTA3* gene, on the basis of the disappearance of the endogenous bands and the appearance of the disrupted bands of expected size (Fig. 4). The CV22 plasmid sequences were present in high copy number but did not exist as free plasmid (data not shown). Instead, they were integrated, probably into the rDNA molecules as previously described (13), indicating that the expression of the *HTA3* gene on the rDNA molecule is sufficient to support the growth of *T. thermophila*. Interestingly, the amounts of HTA3 message and hv1 protein in this transformant were indistinguishable from the amounts in wild-type cells (data not shown). The other double-resistant clone was due to gene replacement of the chromosomal *rpl29* gene and elimination of the plasmid, so the endogenous *HTA3* gene could not be fully replaced. These results argue strongly that introduction of the *neo* cassette itself into the *HTA3* locus is not detrimental and that the *HTA3* gene is essential.

DISCUSSION

It seems likely that chromatin structure plays an important role in regulating gene expression (reviewed in reference 37). One way to modulate the structure of the nucleosome to accommodate the different roles it plays in chromatin condensation and transcription is to change its components, most notably histones. This can be achieved either by modifying the histone components or by replacement with different histone subtypes. Considerable evidence documenting the existence of histone subtypes has accumulated in recent years, and it now seems likely that, in contrast to the situation initially observed in the yeast *Saccharomyces cerevisiae*, some of these variants perform different functions (38).

The evolutionary conservation of H2A.F/Z variants argues strongly that these proteins have a unique function different from those of their major H2A counterparts (31, 33, 36). Because the yeast *Saccharomyces cerevisiae* is unusual among eukaryotes in that it does not seem to have a minor H2A variant (22), it was not possible to test this hypothesis by gene knockout experiments in that organism. Functional analysis of the H2A.F/Z variant in the fission yeast *Schizosaccharomyces pombe* by gene knockout indicated that the gene was not essential but did affect growth and chromosome segregation (7). In contrast, a deletion of the gene encoding the H2A.F/Z variant (H2AvD) in *D. melanogaster* was found to be lethal and could be rescued by transformation with a wild-type *H2AvD*

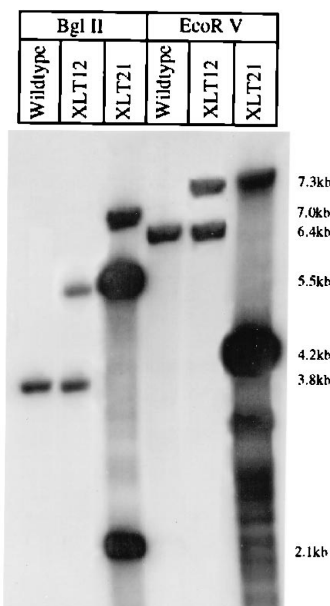

FIG. 4. Genomic Southern blot demonstrating complete elimination of the chromosomal *HTA3* gene in the presence of a plasmid containing an *HTA3* gene. Total cell DNA (10 μg) was digested with *Bgl*II or *Eco*RV and probed with a 2.2-kb *HTA3* genomic fragment. Strain XLT12 was transformed with only the *HTA3* knockout construct. Strain XLT21 was transformed simultaneously with the *HTA3* knockout construct and plasmid XL210 carrying an intact *HTA3* gene. It grows at up to 5 mg of paromomycin per ml. In the wild-type strain, 3.8-kb and 800-bp (not shown) bands for *Bgl*II digestion as well as a 6.4-kb band for *Eco*RV digestion are detected. In strains XLT12 and XLT21, these bands have been partially (XLT12) or completely (XLT21) replaced by the 5.5-kb band observed with *Bgl*II digestion and the 7.3-kb band for *Eco*RV digestion. These fragments are the sizes expected for gene replacement in the endogenous *HTA3* locus. In strain XLT21, the 7.0- and 2.1-kb fragments for *Bgl*II digestion and 4.2-kb fragment for *Eco*RV digestion indicate that the episomal *HTA3* gene has inserted somewhere else in the genome (presumably in the rDNA, but these fragments have not been mapped), where it is expressed at high enough level to enable complete replacement of the endogenous *HTA3* gene.

gene (32). The differences between *Schizosaccharomyces pombe* and *D. melanogaster* could be explained if H2A.F/Z variants were not essential for cell growth or division per se but were required at a specific developmental stage in multicellular eukaryotes.

However, in this report, using newly developed transformation and gene replacement techniques (13–15, 21), we demonstrated that, although the *HTA1* or *HTA2* gene, encoding the major H2A subtypes, can be completely knocked out, the *HTA3* gene can be only partially replaced. Thus, in *T. thermophila*, the *HTA3* gene encoding H2A variant hv1 is essential for vegetative growth. The essential nature of this class of histone variants in both *D. melanogaster* and *T. thermophila* is consistent with the evolutionary conservation of the protein sequence, which actually exceeds that of the major H2As.

Why is the H2A.F/Z variant not present in *Saccharomyces cerevisiae* and not essential in *Schizosaccharomyces pombe*? Clearly, the difference in H2A variants between these yeasts and *D. melanogaster* is not due to multicellularity, since *T. thermophila* is unicellular like the yeasts but the *HTA3* gene encoding its H2A.F/Z variant is essential as in *D. melanogaster*. We have argued elsewhere (22) that at least one function of H2A.F/Z variants in most organisms involves interaction with the linker histone H1 to create a chromatin state that is competent for transcription. Since, despite intensive study, no linker histones have been found in yeasts, we propose that the differences between them, on the one hand, and drosophila and *T. thermophila*, on the other, reflect an essential function for H2A.F/Z variants that involves interaction with linker histones.

Of the two major H2A genes *HTA1* and *HTA2*, neither is essential for the vegetative life cycle of *T. thermophila*. This may be because of the redundancy in function shared by the two proteins. It should be noted that, while these two major subtypes differ by only three conservative changes in the central core regions, the two proteins do differ considerably at their C-terminal regions. These differences could reflect neutral mutations that do not cause functional distinctions between the two. This hypothesis is supported by the fact that either of the *T. thermophila* major H2A genes can function as the sole H2A gene in yeasts and each confers a similar cold-sensitive phenotype (22). However, sequence comparisons indicate that the H2A1 or H2A2 proteins of *T. thermophila* and *T. pyriformis* are virtually identical between the two species whereas H2A1 and H2A2 within each species retain the divergent C-terminal ends (24). This suggests that the two H2A proteins may perform different functions at some stage of the life cycle other than the one (vegetative growth) examined here. Future studies of these transformants looking for more subtle phenotypic changes may help to clarify this situation. Recent studies (21a) indicate that, in a strain in which the HTA2 gene is completely knocked out, the HTA1 gene becomes essential: it can be only partially replaced. Thus, a gene encoding at least one major H2A is essential, but it is not yet known whether it is the major H2A protein sequence that is essential or whether the gene dosage has simply been reduced to levels that cannot support growth in the double-knockout mutant.

The studies described here, together with studies on the *Drosophila H2AvD* gene (32) demonstrate that histone H2A.F/Z type variants are essential in both unicellular and multicellular eukaryotes. However, we still do not know the precise function of these variants. hv1 differs from the major H2As both in primary structure and in its mode of regulation during the cell cycle. Either or both of these features could be important to its function. The finding that complete replacement of the endogenous *HTA3* genes by disrupted genes can be obtained when *HTA3* is also present on a plasmid should provide an opportunity to distinguish these possibilities. By interchanging the regulatory regions and the coding regions of hv1 and the major H2A genes and testing the ability of these hybrid genes to enable complete replacement of endogenous *HTA3* with disrupted genes, it should be possible to obtain a clear answer to the above question.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant GM-21793 from the National Institutes of Health.

REFERENCES

1. **Allis, C. D., C. V. D. Glover, J. K. Bowen, and M. A. Gorovsky.** 1980. Histone variants specific to the transcriptionally active, amitotically dividing macronucleus of the unicellular eukaryote, *Tetrahymena thermophila*. Cell **20:**609–617.
2. **Allis, C. D., R. Richman, M. A. Gorovsky, Y. S. Ziegler, B. Touchstone, W. A. Bradley, and R. G. Cook.** 1986. hv1 is an evolutionarily conserved H2A variant that is preferentially associated with active genes. J. Biol. Chem. **261:**1941–1948.
3. **Allis, C. D., Y. S. Ziegler, M. A. Gorovsky, and J. B. Olmsted.** 1982. A conserved histone variant enriched in nucleoli of mammalian cells. Cell **31:**131–136.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1988. Current protocols in molecular biology. Wiley Interscience, New York.
5. **Ball, D. L. J., C. A. Slaughter, P. Hensley, and W. T. Garrard.** 1983. Amino acid sequence of the N-terminal domain of calf thymus histone H2A.Z. FEBS Lett. **154:**166–170.
6. **Bruns, P.** 1986. Genetic organization of *Tetrahymena*, p. 27–44. *In* J. G. Gall (ed.), The molecular biology of ciliated protozoa. Academic Press, Inc., Orlando, Fla.
7. **Carr, A. M., S. M. Dorrington, J. Hindley, G. A. Phear, S. J. Aves, and P. Nurse.** 1994. Analysis of a histone H2A variant from fission yeast: evidence for a role in chromosome stability. Mol. Gen. Genet. **245:**628–635.
8. **Childs, G., C. Nocente-McGrath, T. Lieber, C. Holt, and J. A. Knowles.** 1982. Sea urchin (*Lytechinus pictus*) late-stage histone H3 and H4 genes: characterization and mapping of a clustered but nontandemly linked multigene family. Cell **31:**383–393.
9. **Dalton, S., A. J. Robins, R. P. Harvey, and J. R. Wells.** 1989. Transcription from the intron-containing chicken histone H2A.F gene is not S-phase regulated. Nucleic Acids Res. **17:**1745–1756.
10. **Doerder, F. P., J. C. Deak, and J. H. Lief.** 1992. Rate of phenotypic assortment in *Tetrahymena thermophila*. Dev. Genet. **13:**126–132.
11. **Elgin, S. C. R., V. Dietrich, E. K. Steiner, J. B. Olmsted, C. D. Allis, and M. A. Gorovsky.** Unpublished data.
12. **Ernst, S. G., H. Miller, C. A. Brenner, C. Nocente-McGrath, S. Francis, and R. McIsaac.** 1987. Characterization of a cDNA clone coding for a sea urchin histone H2A variant related to the H2A.F/Z histone protein in vertebrates. Nucleic Acids Res. **15:**4629–4644.
13. **Gaertig, J., and M. A. Gorovsky.** 1992. Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. Proc. Natl. Acad. Sci. USA **89:**9196–9200.
14. **Gaertig, J., L. Gu, B. Hai, and M. A. Gorovsky.** 1994. High frequency vector-mediated transformation and gene replacement in *Tetrahymena*. Nucleic Acids Res. **22:**5391–5398.
15. **Gaertig, J., T. H. Thatcher, L. Gu, and M. A. Gorovsky.** 1994. Electroporation-mediated replacement of a positively and negatively selectable β-tubulin gene in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA **91:**4549–4553.
16. **Gorovsky, M. A.** 1980. Genome organization and reorganization in *Tetrahymena*. Annu. Rev. Genet. **14:**203–239.
17. **Grunstein, M.** 1990. Nucleosomes: regulators of transcription. Trends Genet. **6:**395–400.
18. **Grunstein, M.** 1990. Histone function in transcription. Annu. Rev. Cell Biol. **6:**643–678.
19. **Grunstein, M., M. Rykowski, D. Kolodrubetz, J. Choe, and J. Wallis.** 1984. A genetic analysis of histone protein subtypes in yeast, p. 35–63. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), Histone genes. John Wiley & Sons, New York.
20. **Harvey, R. P., J. A. Whiting, L. S. Coles, P. A. Krieg, and J. R. Wells.** 1983. H2A.F: an extremely variant histone H2A sequence expressed in the chicken embryo. Proc. Natl. Acad. Sci. USA **80:**2819–2823.
21. **Kahn, R. W., B. H. Andersen, and C. F. Brunk.** 1993. Transformation of *Tetrahymena thermophila* by microinjection of a foreign gene. Proc. Natl. Acad. Sci. USA **90:**9295–9299.

21a. **Li, B., X. Liu, and M. A. Gorovsky.** Unpublished observations.

22. **Liu, X., J. Bowen, and M. A. Gorovsky.** 1996. Either of the major H2A genes but not an evolutionarily conserved H2A.F/Z variant of *Tetrahymena thermophila* can function as the sole H2A gene in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2878–2887.
23. **Liu, X., and M. A. Gorovsky.** 1993. Mapping the 5′ and 3′ ends of *Tetrahymena thermophila* mRNAs using RNA ligase mediated amplification of cDNA ends (RLM-RACE). Nucleic Acids Res. **21:**4954–4960.
24. **Liu, X., and M. A. Gorovsky.** Cloning and characterization of the major histone H2A genes completes the cloning and sequencing of known histone genes of *Tetrahymena thermophila*. Unpublished data.
25. **Nanney, D. L.** 1980. Experimental ciliatology. John Wiley & Sons, New York.
26. **Norris, D., and M. A. Osley.** 1987. The two gene pairs encoding H2A and H2B play different roles in the *Saccharomyces cerevisiae* life cycle. Mol. Cell. Biol. **7:**3473–3481.
27. **Smith, M. M.** 1984. The organization of the yeast histone genes, p. 3–33. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), Histone genes. John Wiley & Sons, New York.
28. **Stargell, L. A., J. Bowen, C. A. Dadd, P. C. Dedon, M. Davis, R. G. Cook, C. D. Allis, and M. A. Gorovsky.** 1993. Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*. Genes Dev. **7:**2641–2651.
29. **Stoler, S., K. C. Keith, K. E. Curnick, and M. Fitzgerald-Hayes.** 1995. A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes Dev. **9:**573–586.
30. **Sullivan, K. F., M. Hechenberger, and K. Masri.** 1994. Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. J. Cell Biol. **127:**581–592.
31. **Thatcher, T. H., and M. A. Gorovsky.** 1994. Phylogenetic analysis of the core histones H2A, H2B, H3, and H4. Nucleic Acids Res. **22:**174–179.
32. **Van Daal, A., and S. C. R. Elgin.** 1992. A histone variant, H2AvD, is essential in *Drosophila melanogaster*. Mol. Biol. Cell **3:**593–602.
33. **Van Daal, A., E. M. White, S. C. R. Elgin, and M. A. Gorovsky.** 1990. Conservation of intron position indicates separation of major and variant H2As is an early event in the evolution of eukaryotes. J. Mol. Evol. **30:**449–455.
34. **Van Daal, A., E. M. White, M. A. Gorovsky, and S. C. R. Elgin.** 1988. *Drosophila* has a single copy of the gene encoding a highly conserved histone H2A variant of the H2A.F/Z type. Nucleic Acids Res. **16:**7487–7498.
35. **Van Holde, K. E.** 1989. Chromatin. Springer-Verlag, New York.
36. **White, E. M., D. L. Shapiro, C. D. Allis, and M. A. Gorovsky.** 1988. Sequence and properties of the message encoding *Tetrahymena* hv1, a highly evolutionarily conserved histone H2A variant that is associated with active genes. Nucleic Acids Res. **16:**179–198.
37. **Wolffe, A.** 1995. Chromatin structure and function. Academic Press, London.
38. **Wolffe, A. P., and D. Pruss.** 1996. Deviant nucleosomes: the functional specialization of chromatin. Trends Genet. **12:**58–62.
39. **Wu, R. S., H. T. Panusz, C. L. Hatch, and W. M. Bonner.** 1986. Histones and their modifications. Crit. Rev. Biochem. **20:**201–263.