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Constitutive Expression, Not a Particular Primary Sequence, Is the Important Feature of the H3 Replacement Variant hv2 in *Tetrahymena thermophila*

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Although quantitatively minor replication-independent (replacement) histone variants have been found in a wide variety of organisms, their functions remain unknown. Like the H3.3 replacement variants of vertebrates, hv2, an H3 variant in the ciliated protozoan *Tetrahymena thermophila*, is synthesized and deposited in nuclei of nongrowing cells. Although hv2 is clearly an H3.3-like replacement variant by its expression, sequence analysis indicates that it evolved independently of the H3.3 variants of multicellular eukaryotes. This suggested that it is the constitutive synthesis, not the particular protein sequence, of these variants that is important in the function of H3 replacement variants. Here, we demonstrate that the gene (*HHT3*) encoding hv2 or either gene (*HHT1* or *HHT2*) encoding the abundant major H3 can be completely knocked out in *Tetrahymena*. Surprisingly, when cells lacking hv2 are starved, a major histone H3 mRNA transcribed by the *HHT2* gene, which is synthesized little, if at all, in wild-type nongrowing cells, is easily detectable. Both *HHT2* and *HHT3* knockout strains show no obvious defect during vegetative growth. In addition, a mutant with the double knockout of *HHT1* and *HHT3* is viable while the *HHT2 HHT3* double-knockout mutant is not. These results argue strongly that cells require a constitutively expressed H3 gene but that the particular sequence being expressed is not critical.

Eukaryotic DNA is associated with a roughly equal mass of histones, in the form of nucleosomes. The core of the nucleosome is composed of about 146 bp of DNA wrapped in 1.75 turns around a histone octamer containing two molecules each of the four core histones: H2A, H2B, H3, and H4 (30). In most organisms, a fifth histone, called the linker histone or H1, causes an additional 20 bp of DNA to be associated with the core and also binds to the variable length of linker DNA between cores (36). Although the basic structure of the nucleosome core particle has been highly conserved throughout evolution, considerable nucleosome heterogeneity exists, which could provide a means for regulating the structural and functional state of chromatin. This heterogeneity is generated by secondary modifications such as acetylation and phosphorylation, by association with nonhistone proteins, and by nonallelic histone variants or subtypes differing in primary sequence (21, 40). Quantitatively minor, nonallelic histone variants have now been identified in a wide variety of organisms and for every histone (20, 35). However, their roles in modulation of chromatin structure or function are not clear. Some variants display distinct patterns of expression in development or during the cell cycle. In sea urchins, different forms of core histone genes are transcribed at precise periods in development (29). Similarly, at different stages in the development of mammals (26, 44) and birds (9), alterations in the relative abundance of nonallelic histone variants can occur in specific cell types.

In addition to developmental variants, at least two other classes of histone variants have been found based on their relation to cell growth: replication variants and replacement variants (44). Replacement variants are distinguished from replication-dependent histones because they are expressed constitutively, even in the absence of DNA replication. Although they are expressed at a low level, replacement variants accumulate in the nuclei of non-S-phase cells slowly, replacing their replication counterparts in nucleosomes (10, 37, 41, 44).

The best-studied replacement variants are H3.3 variants, which have been found in plants, mammals, birds, *Drosophila*, and *Tetrahymena* (reviewed in reference 34). In vertebrates, H3.3 differs from the major replication-dependent H3s by four amino acids, and the corresponding genes contain introns which are lacking in the major histone genes and produce polyadenylated mRNAs. However, the function of H3.3 variants is unknown.

Tetrahymena, like most ciliates, has two types of nuclei: a diploid germ line micronucleus and a polyploid somatic macronucleus (16, 17). During vegetative growth, the mitotically dividing micronucleus is transcriptionally inert while the amitotically dividing macronucleus is transcriptionally active (16). Thus, although the two nuclei have similar genetic complements (4, 5, 17, 42), only genes in the macronucleus are expressed in vegetative cells. Despite their differences in vegetative cells, macro- and micronuclei develop from identical mitotic division products derived from the zygotic nucleus during conjugation. Macronuclear differentiation during conjugation involves changes in DNA sequence organization, transcriptional activity, and chromatin structure. Macro- and micronuclei display striking differences in histone composition (reviewed in reference 18). Macro- and micronuclei contain different linker histones. In addition, macronuclei contain two core histone variants called hv1 and hv2, which are present at about 15 to 20% of the amount of a major core histone and are absent from micronuclei of vegetative cells (1). hv1 is an H2A variant which is closely related to the conserved H2A.F/Z variants found in multicellular eukaryotes, and it appears to be preferentially associated with transcriptionally active genes (39). hv2 is an H3 replacement variant; like the H3.3 variants of vertebrates, it is synthesized and deposited in the macronuclei of nongrowing as well as of growing cells (3).

We have cloned all three genes encoding histone H3 proteins of *Tetrahymena* (34). Two of these genes (*HHT1* and

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HHT2) encode the same major H3 protein, a 135-amino-acid protein that is 87% identical to the major animal H3. The third gene (*HHT3*) encodes hv2, a quantitatively minor H3 which resembles the animal H3.3 in that its expression is uncoupled from the cell division cycle. Also, regulation of the synthesis of both animal H3.3 (31) and hv2 (3) is at the level of mRNA abundance. Despite these similarities, the amino acid sequence of hv2 is distinct from those of other H3 replacement variants. A rigorous evolutionary analysis (34) indicates that the H3.3 genes of animals, plants, and *Tetrahymena* evolved independently despite the similarities in their regulation. Therefore, the 16-amino-acid replacements that distinguish hv2 from the major *Tetrahymena* H3 are different from those that distinguish animal H3.3 variants from the major animal H3 histones and plant H3.3 variants from major plant H3 variants. Evolutionary comparisons suggest, therefore, that replacement H3.3 variants arose independently (at least) three times in evolution and that their common feature is not their amino acid sequence but their constitutive synthesis.

We have recently developed methods for DNA-mediated mass transformation and gene replacement in *Tetrahymena* (6, 12–14), which make it possible to study the function of *Tetrahymena* histones in vivo. To determine whether *Tetrahymena* requires an H3 variant with certain structural features or simply any H3 synthesized constitutively, we knocked out all of the expressed macronuclear copies of the *HHT3* gene encoding the H3.3-like variant hv2. As a control, we also knocked out the *HHT2* gene encoding the major H3. Both knockout strains are viable and show no obvious growth defect, demonstrating that neither *HHT3* nor *HHT2* is essential for survival. Interestingly, in cells lacking hv2, the *HHT2* gene is expressed instead of the *HHT3* gene in the absence of growth. These *HHT3* knockout cells survive prolonged starvation as well as control cells do. In addition, a mutant with a double knockout of *HHT1* and *HHT3* is viable, while a mutant with a double knockout of *HHT2* and *HHT3* is not. These results argue strongly that *Tetrahymena* cells require a constitutively expressed H3 gene but that the particular H3 sequence being expressed is not critical.

MATERIALS AND METHODS

Cells and culture conditions. *Tetrahymena thermophila* CU428 Mpr/Mpr (6-methylpurine sensitive, mating type VII) and B2086 Mpr^+/Mpr^+ (6-methylpurine sensitive, mating type II) were kindly provided by P. J. Bruns (Cornell University). The cells were grown routinely in SPP medium (19) containing 1% proteose peptone (SPP). To analyze growth rates, the cells were inoculated in 50 ml of SPP medium at a density of 10^4 cells/ml and grown at 18, 30, and 37°C with vigorous shaking. Cells were counted in a Coulter counter (Coulter Electronics, Inc.) at different time intervals. Growth data were plotted with Cricket Graph III (Computer Associates). The doubling time was calculated from the linear part of the growth curve. For starvation, the cells were resuspended at a density of 2×10^5 to 3×10^5 cells/ml in 10 mM Tris (pH 7.4) at 30°C without shaking. Short-term starvation was carried out for 18 to 22 h. To study long-term survival, the cells were counted for 22 days. To measure the survival rate during starvation, 100 single cells were isolated at different times of starvation and transferred to drops of growth medium (SPP). After 2 to 3 days, drops containing numerous cells were counted as positive for survival. To check the regrowth of transformants after starvation, the cells were starved and then transferred to 50 ml of SPP medium at a starting density of 10^4 cells/ml and counted at frequent intervals.

Plasmid constructions. pGemini/H3.3HE-5 is a Gemini plasmid (Promega) into which an *Eco*RI-*Hin*dIII macronuclear genomic fragment containing the *HHT3* gene was inserted. p4T2-1 is a pBluescript KS (+) (Stratagene) derivative containing an H4-I/Neo/BTU2 cassette, which confers paromomycin resistance on *Tetrahymena* (13). The *HHT3* knockout construct was obtained by inserting an *Eco*RV-*Sac*I restriction fragment from p4T2-1 containing the drug marker cassette into the *Hin*cII-*Sac*I sites of pGemini/H3.3HE-5, replacing almost the entire coding sequence of the *HHT3* gene. The final construct was digested with *Eco*RI and *Hin*dIII to release a 2.5-kb insert used in transformation. To disrupt the *HHT2* gene, p2C3A, a pBluescript KS (+) derivative containing a 2.1-kb *Bgl*II-*Cla*I genomic fragment of the *HHT2* gene, was digested with *Bst*BI and *Nsi*I, blunted with T4 DNA polymerase (GIBCO BRL), and ligated to an *Eco*RV-*Sma*I restriction fragment from p4T2-1 containing the *Neo* gene cassette. The resulting construct, in which the *Neo* gene was transcribed in the same direction as the *HHT2* gene, was selected, and a 3.2-kb *Spe*I-*Cla*I fragment was released for transformation. This insert contained the *HHT2*-flanking sequence and about three-quarters of the coding sequence interrupted by the drug resistance cassette at nucleotide 309 after the ATG start codon. To knock out the *HHT1* gene, a *Hin*dIII-*Sma*I fragment containing the *Neo* gene cassette was inserted into p23B4A2, a pBluescript KS (+) derivative, to replace the *Hin*dIII-*Bss*HII fragment. This insertion will delete the *HHT1* coding sequence after the *Hin*dIII site. The final *HHT1* knockout construct was released by digestion with *Spe*I and *Cla*I.

Gene replacement by conjugant electrotransformation. Conjugant electrotransformation was performed as specified by Gaertig and Gorovsky (12). *T. thermophila* CU428 and B2086 were grown in SPP medium to around 3×10^5 cells/ml, harvested, and then starved in 10 mM Tris-HCl (pH 7.4) for 12 h. At 10 h after mixing, the conjugants were washed and adjusted to 3×10^7 cells per ml in 10 mM HEPES (pH 7.4). The transforming DNA (50 μg) in 125 μl of 10 mM HEPES (pH 7.4) was mixed with 125 μl of cells and immediately subjected to electroporation with the BTX ECM 600 electroporator (BTX Inc., San Diego, Calif.). The cells were resuspended in 20 ml of SPP medium 1 min after electroporation and plated in 96-well microtiter plates. After 12 h of growth at 30°C, paromomycin sulfate (Sigma Chemical Co.) was added to a final concentration of 120 μg/ml. Transformants were apparent after 3 to 4 days of growth.

Biolistic transformation. A DNA mixture of two different knockout constructs was cotransformed into starved vegetative cells by using the Biolistic PDS-1000/He particle delivery system (Bio-Rad) as described previously (6). The transformants were selected with 200 μg of paromomycin per ml.

Southern blot analysis. Total genomic DNA was isolated as described previously (14). Genomic DNA (10 μg) was digested with *Hin*dIII and blotted onto nylon membranes as described previously (15). To probe for the *HHT3* gene, a 500-bp *Sac*I-*Hin*dIII fragment containing the 3′-flanking region of the *HHT3* gene was labeled with [α-^{32}P]dATP by random priming (2). A similarly labeled 492-bp *Spe*I-*Ssp*I fragment containing the *HHT2* 3′-flanking sequence was used as an *HHT2*-specific probe. A labeled 1.3-kb *Bss*HII-*Cla*I fragment containing the 3′-flanking region of the *HHT1* gene was used as an *HHT1*-specific probe. Hybridizations and washes were performed at 65°C.

Northern blot analysis. Total RNA was isolated with guanidine isothiocyanate as described previously (7). RNA was electrophoresed in 2.2 M formaldehyde–1% agarose gels, blotted, and hybridized as described previously (2). A 380-bp *Hin*cII-*Sac*I fragment corresponding to the coding region of the *HHT3* gene was used to detect hv2 mRNA in a Northern blot. This probe preferentially hybridizes to the *HHT3* mRNA but can recognize both *HHT1* and *HHT2* mRNAs because *HHT3* is 86.2% identical to *HHT1* (34) and 86.4% identical to *HHT2* within the coding region. In some experiments, a yeast H3 gene was used as the probe. It should recognize the three *Tetrahymena* H3 mRNAs about equally since the yeast H3 coding sequence shows a similar level of identity (72 to 76%) to the coding sequences of each gene. The yeast H3 coding sequence was PCR amplified from a plasmid provided by M. Mitchell Smith, University of Virginia (pMS191), which contains one copy of yeast H4 and H3 genes cloned into the *Hin*dIII site of pBR322. The following two primers were used in the PCR: 5′-CTATGATCTTTCACCTCTTAATC-3′ and 5′-ATGGCCAGAACA AAGCAAAC-3′. The resulting PCR product was then gel purified and labeled by random priming with [α-^{32}P]dATP (2). In some experiments, hybridization was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS

***HHT3* and *HHT2* knockout constructs transform *Tetrahymena*.** In *Tetrahymena*, there are three genes encoding histone H3. *HHT1* and *HHT2* encode the identical major H3 protein. The third gene (*HHT3*) encodes hv2, which is a quantitatively minor variant, differing at 16 residues from the major H3. Like the H3.3 genes of multicellular eukaryotes, *HHT3* is constitutively expressed (3). To knock out the *HHT3* gene, we created a construct (Fig. 1A) in which almost the entire coding sequence of the *HHT3* gene was replaced by a disruption cassette (13) containing the *Neo* coding sequence flanked by the H4-I gene promoter and the terminator of the *BTU2* gene. A 2.5-kb *Eco*RI-*Hin*dIII fragment containing the selectable marker, which confers paromomycin resistance on *Tetrahymena*, flanked by *HHT3* sequences was used to replace the macronuclear gene encoding hv2. The same strategy was used to disrupt the *HHT2* gene, which encodes the major H3 protein. A 3.2-kb linearized *HHT2* disruption construct (Fig. 1B) in which part of the *HHT2* gene coding region was replaced by the *Neo* selectable marker was prepared by digestion with *Spe*I and *Cla*I and was introduced into the developing macronuclei

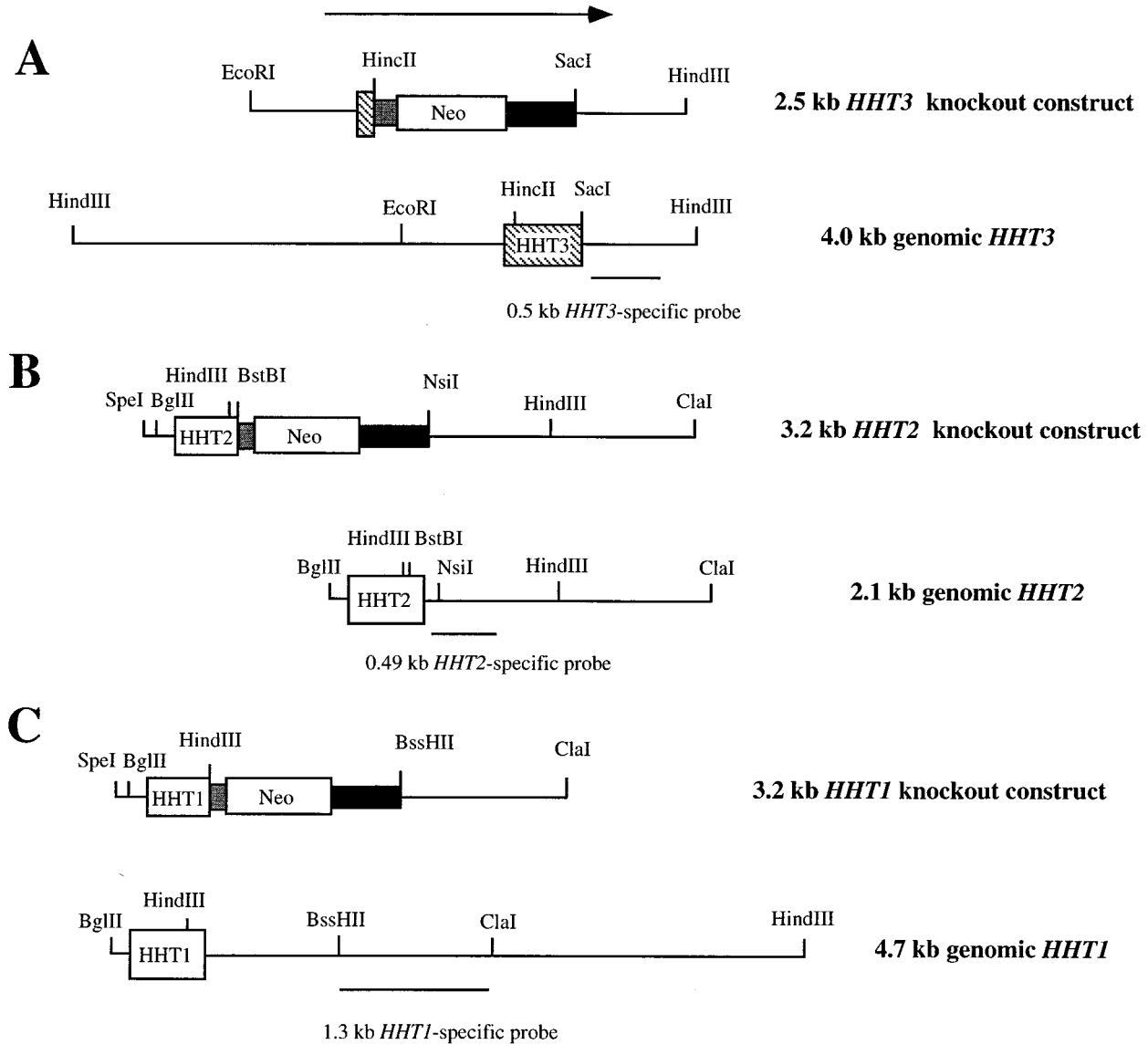

FIG. 1. Maps of knockout constructs and macronuclear genomic fragments of *HHT3*, *HHT2*, and *HHT1*. (A) Knockout construct and genomic map of the *HHT3* gene. A 2.5-kb *HHT3* knockout construct is shown. A 1.4-kb *Neo* drug resistance marker with *Eco*RV and *Sac*I ends was inserted between the *Hin*cII and *Sac*I sites of the *HHT3* gene, replacing almost the entire coding sequence. The stippled box is the H4-I promoter, followed by the *Neo* gene and *BTU2* terminator (solid box). The macronuclear genomic *HHT3* gene is shown as a hatched box in a 4.0-kb *Hin*dIII fragment. The position of the *HHT3*-specific probe is marked at the bottom. (B) Maps of the knockout construct and the genomic *HHT2* gene. A 3.2-kb *HHT2* disruption construct is shown. The *Neo* marker with *Eco*RV and *Sma*I blunt ends was inserted between the *Bst*BI and *Nsi*I sites of the *HHT2* gene, replacing about one-fourth of the coding sequence. The macronuclear genomic *HHT2* gene is shown as a 2.1-kb fragment containing the *HHT2* coding region (open box). The *HHT2*-specific probe is a 0.49-kb fragment from the 3′-flanking sequence of *HHT2* (shown at the bottom). (C) Maps of the knockout construct and the genomic *HHT1* gene. A 3.2-kb *HHT1* knockout construct is shown. The *Neo* marker was inserted between the *Hin*dIII and *Bss*HII sites. The macronuclear genomic *HHT1* gene is shown as a 4.7-kb fragment containing the *HHT1* coding region. A 1.3-kb *HHT1*-specific probe is shown at the bottom. All genes are transcribed in the direction indicated by the arrow.

to replace the endogenous *HHT2* gene. Knockout constructs were transformed into *Tetrahymena* by conjugant electrotransformation (12). By selecting cells for paromomycin resistance, two *HHT3* and three *HHT2* transformants were obtained.

Genomic Southern blots indicate complete disruption of the *HHT3* and *HHT2* genes. Because the *Tetrahymena* macronucleus is polyploid, only partial replacement of some of the 45 endogenous copies is obtained initially in a typical gene replacement experiment (22, 43). However, during vegetative growth, the macronucleus divides amitotically and alleles are segregated randomly at each division, a process known as phenotypic assortment (8, 27). Thus, replacement of a nonessential gene eventually can be completed by assortment (14) when the selection pressure is increased by culturing cells in the presence of increasing concentrations of paromomycin. If a gene is essential, it can only be partially replaced (25).

To determine whether transformants retained any copies of endogenous genes, cells were transferred to medium plus increasing concentrations of paromomycin for 60 to 80 generations to allow complete assortment. Both *HHT3* transformants grew in medium containing up to 2 mg of paromomycin per ml. Total genomic DNA isolated from *HHT3* transformants (Δ*HHT3*) as well as from wild-type cells was digested with *Hin*dIII and hybridized on a Southern blot (Fig. 2A) with an

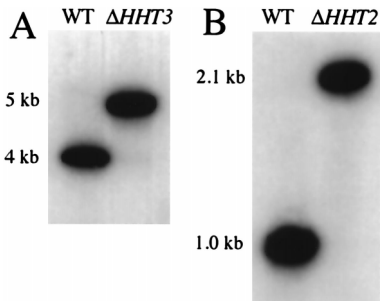

FIG. 2. Southern blot analysis of the *HHT3* and *HHT2* knockout strains indicating that complete knockout had occurred in the macronucleus. (A) Southern blot analysis of the *HHT3* knockout strain. Total genomic DNA isolated from transformants or from wild-type cells was digested with *Hin*dIII and hybridized with the *HHT3*-specific probe. In wild-type cells (WT), a 4-kb band was observed which was derived from the endogenous *HHT3* gene. In the *HHT3* knockout cells (Δ*HHT3*), only a 5-kb band was prominent, indicating that a complete replacement of the genes encoding hv2 had occurred in macronuclei. (B) Southern blot analysis of the *HHT2* knockout strain. An *HHT2*-specific probe was hybridized to *Hin*dIII-digested total genomic DNA. In wild-type cells (WT), a 1.0-kb band was detected, corresponding to the endogenous copy of the *HHT2* gene. In the *HHT2* knockout strain (Δ*HHT2*), only a 2.1-kb band was detected, indicating that the *HHT2* gene can be knocked out completely.

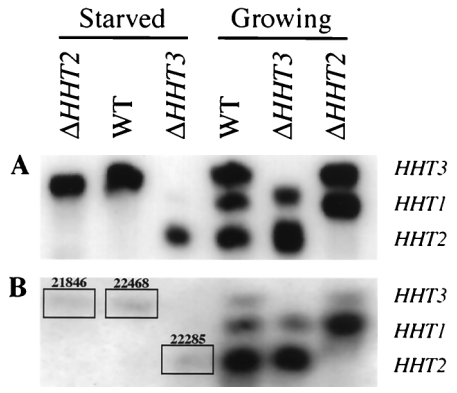

FIG. 3. Northern blot analysis of H3 mRNAs in growing and starved cells from wild-type and knockout strains. (A) A 0.4-kb probe containing the entire coding sequence of hv2 was used to analyze H3 mRNAs of growing and starved cells on a Northern blot. In wild-type cells (WT), all three H3 mRNAs were detected in growing cells while only hv2 mRNA was detected in starved cells. In *HHT2* knockouts (Δ*HHT2*), as expected, *HHT2* mRNA was undetectable in growing or in starved cells while *HHT3* mRNA was detected equally well in growing and in starved cells. In the *HHT3* knockout strain (Δ*HHT3*), *HHT3* mRNA was not detected in either growing or starved cells. However, when these cells were starved, mRNA from the *HHT2* gene, which is normally not expressed, now was easily detectable. (B) A separate experiment was done with a yeast H3 probe. Values for mRNA abundance (given above the measured box areas) were determined by PhosphorImager analysis. Little difference was observed in the H3 mRNA level in nongrowing wild-type, Δ*HHT3*, and Δ*HHT2* cells.

HHT3-specific probe derived from the 3′-flanking region of *HHT3* (Fig. 1A). In wild-type cells, a 4-kb band was observed which was derived from the endogenous *HHT3* gene. In the *HHT3* knockout transformants, a new 5-kb band was prominent, indicating that a gene replacement had occurred in the *HHT3* gene locus. A very faint band of 4 kb was also detected, which was probably due to the presence of micronuclear copies of the *HHT3* genes in the total DNA. Therefore, this result indicates that most, if not all, of the macronuclear copies of the *HHT3* gene had been knocked out. The second Δ*HHT3* clone showed the same pattern (data not shown).

Of the three *HHT2* knockout transformants, only one clone gave a band of the expected size in a PCR with one primer located in the 5′ region of *HHT2* and the other primer within the *Neo* gene sequence (data not shown). This transformant, referred to as Δ*HHT2*, was further studied by the genomic Southern blot assay. An *HHT2*-specific probe (Fig. 1B) was used to hybridize *Hin*dIII-digested total genomic DNA (Fig. 2B). In wild-type cells, a 1.0-kb band was detected, corresponding to the endogenous copy of the *HHT2* gene. In Δ*HHT2*, a 2.1-kb band was prominent. No wild-type versions of the *HHT2* gene were detected, indicating that the *HHT2* gene can be knocked out completely.

Northern blot analysis indicates up-regulation of *HHT2* mRNA in *HHT3* knockouts. In *Tetrahymena*, genes in macronuclei are transcribed while those in micronuclei are not (17). Thus, expression of endogenous genes should be abolished if a complete gene replacement has occurred in the macronucleus. To confirm the disruption of all copies of endogenous macronuclear genes in the knockout transformants, a 0.4-kb probe containing the coding sequence of hv2 was used to analyze H3 mRNAs of growing and starved cells on a Northern blot (Fig. 3A). In wild-type cells, all three H3 mRNAs were detected in growing cells, whereas the hv2 mRNA is the only one detected in starved cells (3). In the *HHT2* knockouts, as expected, no mRNA from this gene was detected in growing or in starved cells whereas mRNA transcribed by the *HHT3* gene was detected equally well in growing and in starved cells. In the *HHT3* knockout strain, the *HHT3* mRNA was not detected in either growing or starved cells, consistent with the complete gene replacement in the *HHT3* knockouts. Surprisingly, when *HHT3* knockout cells were starved, mRNA transcribed by the *HHT2* gene, which is normally not expressed, was easily detectable. A faint band the size of *HHT1* was also detected in cells lacking hv2. The up-regulation of the *HHT2* gene was further confirmed by probing with an *HHT2*-specific probe (data not shown). The probe used in this Northern blot (Fig. 3A) is the coding sequence of the *HHT3* gene, which preferentially hybridized with its own mRNA, precluding quantitative comparisons between *HHT3* mRNA in wild-type cells and the *HHT2* mRNA in *HHT3* knockout cells during starvation. To quantitate the mRNA, a yeast H3 probe, which should not preferentially hybridize to any of the three *Tetrahymena* H3 mRNAs, was used in a separate experiment and a PhosphorImager was used to quantitate H3 mRNA levels in starved cells (Fig. 3B). The amount of *HHT2* mRNA in the starved *HHT3* knockouts corresponded almost exactly to the amount of *HHT3* mRNA found in wild-type and *HHT2* knockouts. Thus, our results suggest that cells require constitutive synthesis of an H3 but that the specific protein sequence found in hv2, which differs markedly (16 residues) from the major H3, is not essential.

The *HHT1 HHT3* double-knockout mutant is viable, while the *HHT2 HHT3* double-knockout mutant is not. During starvation, the *HHT3* knockout cells showed an up-regulation of *HHT2* mRNA but little *HHT1* mRNA was detected. This suggested that the *HHT2* gene could be expressed to compensate the function of *HHT3* but that the *HHT1* gene could not. To test this, we performed double-knockout experiments by cotransforming wild-type cells with either *HHT1* and *HHT3* or *HHT2* and *HHT3* knockout constructs (Fig. 1). By selecting cells in increasing amounts of paromomycin, we obtained one Δ*HHT1*Δ*HHT3* strain which could tolerate the drug at up to 7 mg/ml, and one Δ*HHT2*Δ*HHT3* strain which could tolerate up to 10 mg/ml. These two putative double-knockout strains were analyzed on a genomic Southern blot (Fig. 4). The *Hin*dIII-digested total genomic DNA from the wild-type and Δ*HHT2*Δ*HHT3* strains was hybridized with both *HHT3* and *HHT2* specific probes (Fig. 4A). In the Δ*HHT2*Δ*HHT3* strain,

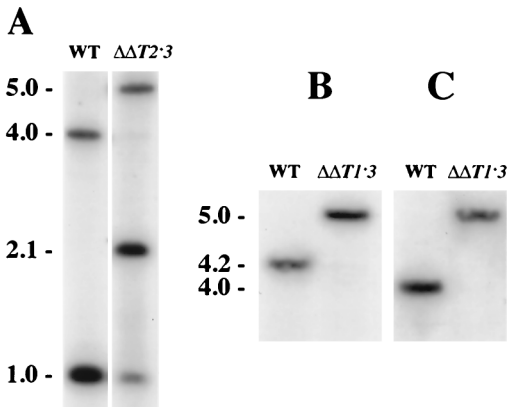

FIG. 4. Southern blot analysis of wild-type and double knockouts. (A) *Hin*dIII-digested genomic DNAs from wild-type and Δ*HHT2*Δ*HHT3* were hybridized with *HHT3* and *HHT2* specific probes. In wild-type cells (WT), a 4-kb band corresponding to the endogenous version of the *HHT3* gene was observed, while in the double knockout of *HHT2* and *HHT3* (ΔΔ*T2+3*), this band was replaced by a 5-kb band, indicating complete knockout of the *HHT3* locus. With the *HHT2* specific probe, a 1.0-kb endogenous version of the wild-type *HHT2* gene was observed in wild-type cells, while in the double knockout, a 2.1-kb band was prominent but the endogenous version of the *HHT2* gene was still easily observed, indicating that a complete double knockout of *HHT2* and *HHT3* is lethal. (B) Southern blot of wild-type and double-knockout *HHT1* and *HHT3* cells probed with an *HHT1*-specific probe. In wild-type cells (WT), a 4.2-kb wild-type version of the *HHT1* gene was observed. It was replaced by the 5-kb band in Δ*HHT1*Δ*HHT3* (ΔΔ*T1+3*), indicating complete gene knockout at this locus. (C) Southern blot of wild-type and double-knockout *HHT1* and *HHT3* cells probed with *HHT3*-specific probe. A 4-kb endogenous version of the *HHT3* gene was observed in wild-type cells (WT), which was replaced by a 5-kb band in Δ*HHT1*Δ*HHT3* (ΔΔ*T1+3*), indicating complete gene knockout at this locus. Therefore, the double knockout of *HHT1* and *HHT3* is complete for both genes.

a 5-kb band corresponding to the knockout version of the *HHT3* gene was prominent, indicating complete gene replacement at the *HHT3* locus. A faint band of 4 kb was also observed, which was probably the micronuclear copies of the *HHT3* gene, because no *HHT3* mRNA was detected in a Northern blot assay of RNA from these cells (data not shown). When the same blot was probed with the *HHT2*-specific sequence, a 2.1-kb band was prominent while a 1.0-kb band corresponding to the endogenous wild-type version of the *HHT2* gene also was easily observed, indicating that partial replacement occurred in the *HHT2* locus. Since the Δ*HHT2*Δ*HHT3* strain cannot grow in medium containing more than 10 mg of paromomycin per ml, the double knockout of *HHT2* and *HHT3* is lethal in *Tetrahymena*. Thus, when *HHT3* was knocked out, *HHT2* became essential. On the other hand, when *Hin*dIII-digested genomic DNA from the Δ*HHT1*Δ*HHT3* strain was probed with the *HHT1*-specific sequence (Fig. 4B), a prominent 5-kb band, the size expected from the knockout construct, was observed in the Δ*HHT1*Δ*HHT3* strain. No wild-type macronuclear version of *HHT1* was detected, indicating complete gene replacement at this gene locus. Again, the remaining faint band around 4.2 kb was due to the presence of micronuclear copies of the *HHT1* gene in the DNA, because no *HHT1* mRNA was detected in the Δ*HHT1*Δ*HHT3* strain (data not shown). When the same *Hin*dIII-digested genomic DNA was hybridized with the *HHT3* specific probe (Fig. 4C), a 5-kb knockout band was prominent. No wild-type version of the *HHT3* gene was observed, indicating that complete gene replacement occurred at this locus. Therefore, the double knockout of *HHT1* and *HHT3* is viable in *Tetrahymena*. These results were consistent with the observation that *HHT2* but not *HHT1* can be constitutively expressed in place of *HHT3* and argue that *Tetrahymena* needs a constitutively expressed H3 histone.

***HHT3* and *HHT2* are not essential for vegetative growth.** The growth curves of Δ*HHT3*, Δ*HHT2*, and wild-type cells are similar, although the Δ*HHT3* and Δ*HHT2* strains grow slightly slower than the wild type (Fig. 5). At 30°C, Δ*HHT3* and Δ*HHT2* have 3.1- and 3.3-h doubling times, while wild-type cells double every 2.5 h. This small difference is probably due to *Neo* gene expression, since a mutant with a knockout of the *HHF1* gene (one of two genes encoding histone H4) also grew slightly slower than wild-type cells (see Fig. 7). At 37°C (Fig. 5), little difference was found in the growth curves between the wild type and transformants. At 18°C, the growth of all three strains was also similar. Thus, neither *HHT3* nor *HHT2* is essential for vegetative growth, and their absence has only a slight effect on the growth rate.

hv2 is not essential for survival during starvation. Since hv2 is the only H3 synthesized in starved cells, we asked whether its absence affected the response of the cells to starvation. Wild-type and Δ*HHT3* cells were starved in 10 mM Tris (pH 7.4), and the cell number was measured for 22 days. To our surprise, cells lacking hv2 showed slightly increased survival during starvation compared to wild-type cells (Fig. 6A), indicating that

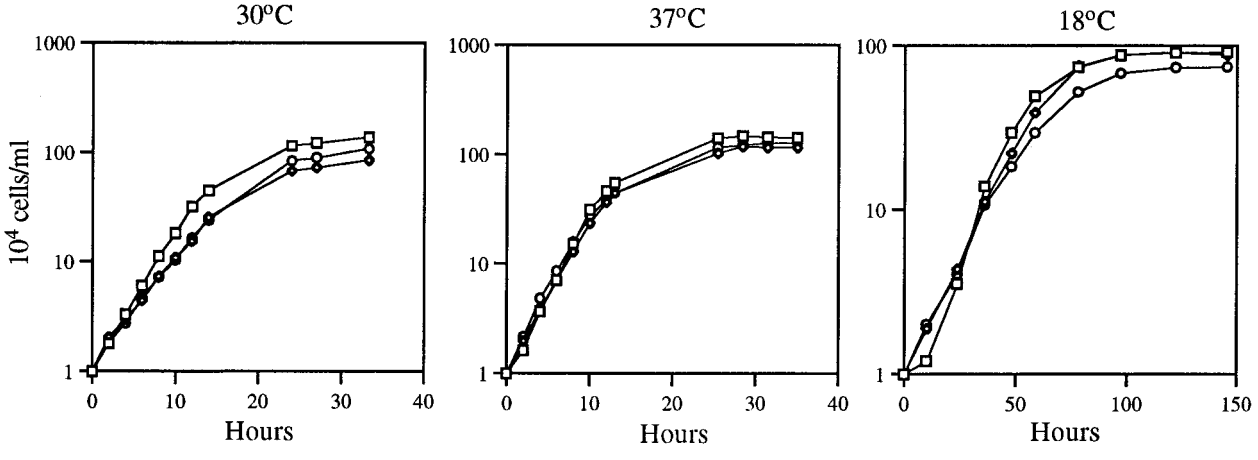

FIG. 5. Growth curves of wild-type (□), Δ*HHT3* (◇), and Δ*HHT2* (○) cells at 30, 37, and 18°C. Cells were grown in SPP medium at a starting density of 10^4 cells/ml and were counted at frequent intervals. Cell density is plotted on a log scale.

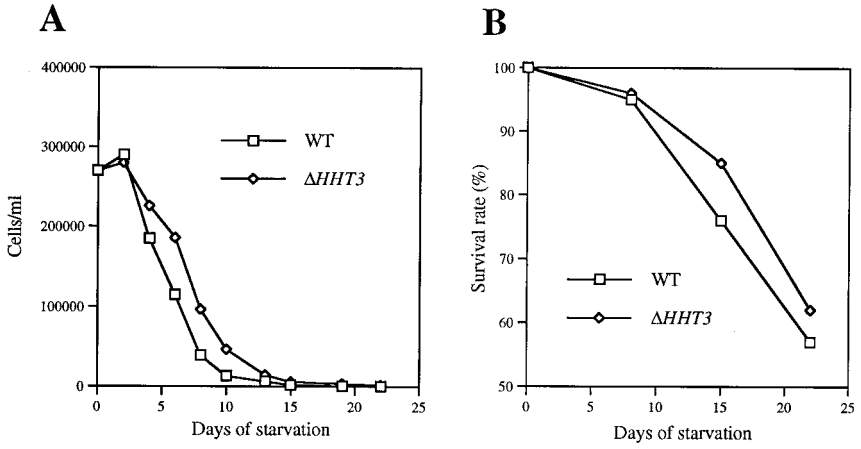

FIG. 6. Viability of wild-type (WT) and Δ*HHT3* cells after prolonged periods of starvation. (A) Changes in the number of wild-type and Δ*HHT3* cells during starvation. The cells were starved at a starting density of 2.7×10^5 cells/ml and were counted at the indicated times. (B) Survival of wild-type and Δ*HHT3* cells during prolonged periods of starvation. The cells were starved at 30°C for the indicated times, and then 100 single cells were isolated and transferred to SPP medium. After 2 to 3 days, drops containing numerous cells were counted as positive for survival.

hv2 is not essential for survival during starvation. We then tested the survival rate by measuring the fraction of cells which could resume growth after prolonged periods of starvation (Fig. 6B). We found that the survival rates of wild-type and Δ*HHT3* cells were very similar for the 22 days and, again, that Δ*HHT3* cells showed a slightly higher survival rate. In addition, we checked the rate of regrowth of transformants after 16, 40, 64, and 88 h of starvation. We found that wild-type, Δ*HHF1* (Δ*HHF1* is a strain in which the *HHF1* gene encoding H4 has been knocked out), and Δ*HHT3* cells recovered almost equally well from starvation (Fig. 7). Thus, *HHT3* knockouts show no obvious starvation-associated defect, probably because one of the two genes (*HHT2*) encoding the major H3 histone is turned on in the starved cells lacking hv2.

DISCUSSION

Replacement variants for H3 histone have been found in a wide variety of organisms. Proteins encoded by H3.3 genes of birds, mammals (38), and *Drosophila* (11) are identical to each other but differ at four residues from the major, replication-dependent H3 histones in these organisms. Because H3 histones evolve very slowly, it was not clear whether the small differences in protein sequence between replication and replacement H3 histones are indicative of functional variation at the protein level or are simply neutral polymorphisms that arose after gene duplication. Phylogenetic analysis of 73 H3 histones (34) indicated that the H3.3 replacement variants of animals, plants, and *Tetrahymena* evolved independently. This suggested that it is the replication independence (constitutive synthesis), not the particular protein sequence, of these variants that is important in the function of H3 replacement variants. One way to test this hypothesis would be to exchange the coding regions of replication-dependent and -independent H3 genes. Unfortunately, this experiment is impractical in higher eukaryotes, where homologous recombination is rare and histone genes are repeated.

In *Tetrahymena*, the replacement variant hv2 differs from the major H3 at a remarkable 16 residues. In this study, using newly developed mass transformation and gene replacement techniques (6, 12, 13), we demonstrated that the *HHT3* gene encoding the replacement H3 variant hv2 or the *HHT2* gene encoding the major H3 can be knocked out completely in *Tetrahymena*. Surprisingly, when cells lacking hv2 were starved, mRNA derived from the *HHT2* gene, which is normally not

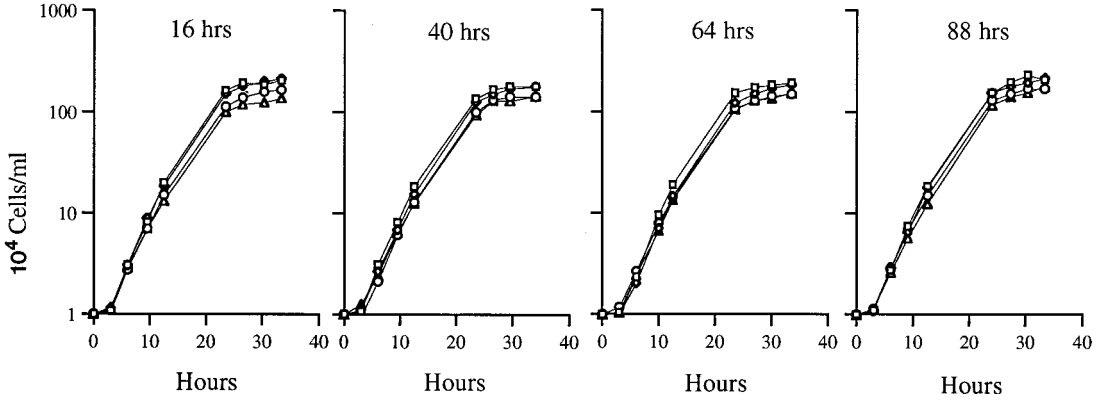

FIG. 7. Wild-type (WT) (□), Δ*HHF1* (◇), and Δ*HHT3* cells recover equally well from various periods of starvation. Cells were starved in 10 mM Tris (pH 7.5) at 30°C for the indicated times and then were transferred to SPP medium at a starting density of 10^4 cells/ml. Δ*HHT3*-1 (○) and Δ*HHT3*-2 (△) are two independent *HHT3* knockouts. Δ*HHF1* is an *HHF1* knockout strain used as a control.

expressed, now was easily detectable. Since these *HHT3* knockout cells grew well and survived prolonged periods of starvation as well as wild-type cells did, it seems clear that in *Tetrahymena*, the specific protein sequence found in hv2 is not essential for the function of this H3 replacement variant. This result is consistent with the evolutionary analysis by Thatcher et al. (34), showing that the *Tetrahymena* hv2 is more closely related to the *Tetrahymena* major H3 histone than to the similarly regulated H3.3 histones of higher eukaryotes. Because replacement H3 variants have been found in diverse organisms (animals, plants, and protists), it seems likely that all eukaryotes need a constitutively expressed H3 gene (and possibly other constitutively expressed core histone genes as well). We suggest that organisms that appear to lack a distinct replacement H3 gene probably have a constitutively expressed promoter (as well as a replication-dependent one) associated with one or more of the major H3 genes. In this scenario, replacement genes can arise when a gene containing both a constitutive and an S-phase promoter duplicates, allowing one of the two genes to accumulate mutations that inactivate its S-phase promoter.

In *Tetrahymena*, two H3 genes (*HHT2* and *HHT1*) encode the same major H3 protein. Therefore, it is not surprising that *HHT2* can be completely replaced. The other major H3 gene, *HHT1*, also has been knocked out and has been found not to be essential for the vegetative growth of *Tetrahymena* (43a). However, in starved cells lacking the *HHT3* gene, only *HHT2* mRNA becomes easily detectable; little *HHT1* mRNA was detected on the same blot. It is unlikely that this is due to the sequence similarity between the *HHT3* and *HHT2* genes, because the DNA sequence of *HHT3* is 86.2% identical to *HHT1* and 86.4% identical to *HHT2* within the coding region. Since transcriptional regulation is the major mechanism regulating mRNA abundance in *Tetrahymena* (32), the difference observed here is probably due to differences in their promoters. The *HHT2* promoter may still have the potential to be constitutively expressed, while the *HHT1* promoter may not. Our analyses of the upstream regions of the *HHT2* gene (up to position −230) and the *HHT3* gene (up to position −640) revealed the presence of a common sequence (GGAGAT), which could be related to constitutive expression. More detailed studies of these sequence elements by DNA-mediated transformation or with a newly developed in vitro transcription system for ciliate genes (23) may help answer this question.

It has been shown previously that different genes in a histone multigene family can compensate for the lack or inactivation of one of its members. In yeast, the expression of one of the H2A-H2B gene pairs (*HTA1*/*HTB1*) increased to compensate for the disruption of the other pair (*HTA2*/*HTB2*) (28). Takami et al. (33) reported that targeted disruption of an H3-IV/ H3-V gene pair in higher eukaryotes caused increased expression of the remaining H3 genes in the chicken DT40 cell line. Therefore, this type of regulation may be a general phenomenon in eukaryotes. However, our observation that the *HHT2* gene is up-regulated in nongrowing cells lacking hv2 is the first demonstration of compensation between a replication and a replacement histone variant or between core histone variants containing such a high degree (16 of 135 residues) of sequence divergence.

The studies described here argue strongly that constitutive expression, not the particular primary sequence, is the main requirement for the function of the H3 replacement variant in *Tetrahymena*. Also, our finding that only the *HHT2* gene (and not the *HHT1* gene) is strongly expressed during starvation in the absence of the gene encoding hv2 and that *HHT2* (but not *HHT1*) appears to become essential in the absence of the hv2 argues that constitutive expression of an H3 gene is essential. However, we still do not know the precise function of H3 replacement variants. It should be noted that the replacement H3 variant hv2 is specific to the transcriptionally active macronucleus and is the only H3 that is synthesized constitutively in wild-type cells. Transcription is the only function of which we are aware that is macronucleus specific, constitutive, and essential. We hypothesize, therefore, that the function of hv2 (and of H3.3 gene products in multicellular eukaryotes) is to replace H3 proteins that turn over during transcription. A similar hypothesis for the function of an H3 replacement variant in plants has been proposed (37) based on the more rapid turnover of acetylated forms of the variant, suggesting that this function might be universal. We have shown previously that the ratio of H3 mRNA to H4 mRNAs (from the *HHF1* and *HHF2* genes) in growing and starved *Tetrahymena* remains relatively constant (3) and that small amounts of H2A mRNAs are also present in starved cells (24). Thus, it seems likely that all of the core histones are being turned over in nongrowing *Tetrahymena* cells, suggesting that entire nucleosomes and not specifically the H3 component turns over in association with transcription. If this hypothesis is true, all of the core histone multigene families must have at least one member whose expression is either completely constitutive (i.e., a replacement variant) or both S-phase regulated and constitutive (i.e., partially replication dependent). We are currently trying to test this hypothesis by replacing the wild-type *Tetrahymena HHT3* with an epitope-tagged *HHT3* gene, which should allow determination of whether hv2-containing nucleosomes isolated from cells starved for increasing times become enriched in genes that are specifically transcribed in starved cells.

Our results argue strongly that it is the constitutive synthesis and not the particular amino acid sequence of hv2 that is important, consistent with the previous finding that the H3 replacement variants of multicellular animals, multicellular plants, and *Tetrahymena* arose independently in each of these phylogenetic lineages. These observations imply that the amino acid differences that distinguish H3.3 variants from major H3 histones are neutral replacements, without functional consequences. It should be noted, however, that *Drosophila* and vertebrate H3.3 variants are distinguished from their major H3 histones by four identical replacements and that the H3.3 variants of *T. thermophila* and *T. pyriformis* differ from the major *Tetrahymena* H3 histones (which are identical in the two species) by identical replacements at 13 positions. Thus, although the replacement function of these variants may be paramount, it is still possible that their particular sequences result in small, selectable differences that are important in the course of multiple generations throughout evolutionary time but cannot be detected in laboratory assays.

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