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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*

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H2A.F/Z histones are conserved variants that diverged from major H2A proteins early in evolution, suggesting they perform an important function distinct from major H2A proteins. Antisera specific for hv1, the H2A.F/Z variant of the ciliated protozoan *Tetrahymena thermophila*, cross-react with proteins from *Saccharomyces cerevisiae*. However, no H2A.F/Z variant has been reported in this budding yeast species. We sought to distinguish among three explanations for these observations: (i) that *S. cerevisiae* has an undiscovered H2A.F/Z variant, (ii) that the major *S. cerevisiae* H2A proteins are functionally equivalent to H2A.F/Z variants, or (iii) that the conserved epitope is found on a non-H2A molecule. Repeated attempts to clone an *S. cerevisiae* hv1 homolog only resulted in the cloning of the known H2A genes *yHTA1* and *yHTA2*. To test for functional relatedness, we attempted to rescue strains lacking the yeast H2A genes with either the *Tetrahymena* major H2A genes (*tHTA1* or *tHTA2*) or the gene (*tHTA3*) encoding hv1. Although they differ considerably in sequence from the yeast H2A genes, the major *Tetrahymena* H2A genes can provide the essential functions of H2A in yeast cells, the first such case of trans-species complementation of histone function. The *Tetrahymena* H2A genes confer a cold-sensitive phenotype. Although expressed at high levels and transported to the nucleus, hv1 cannot replace yeast H2A proteins. Proteins from *S. cerevisiae* strains lacking yeast H2A genes fail to cross-react with anti-hv1 antibodies. These studies make it likely that *S. cerevisiae* differs from most other eukaryotes in that it does not have an H2A.F/Z homolog. A hypothesis is presented relating the absence of H2A.F/Z in *S. cerevisiae* to its function in other organisms.

In all eukaryotes, core histones form an octamer containing two molecules each of the histones H2A, H2B, H3, and H4, around which is wrapped 146 bp of DNA in about 1.75 turns (53). Although core histones are extremely conserved in evolution, nonallelic variants have been identified for every class of histones (56). In the yeast *Saccharomyces cerevisiae*, nonallelic variants of H2A and H2B appear to be functionally redundant, since either of the two genes for each histone type can be made nonfunctional with little or no phenotypic effect (20, 28, 41). However, studies of the evolutionarily conserved H2A.F/Z-type variants hv1 in *Tetrahymena thermophila* (2, 4, 5, 51, 54, 55), H2AvD in *Drosophila melanogaster* (50–52), and Pht1 in the fission yeast *Schizosaccharomyces pombe* (11) argue strongly that, in some organisms, histone H2A variants have distinct functions. During vegetative growth of *Tetrahymena* cells, hv1 is found in the transcriptionally active macronucleus, but not in the transcriptionally inactive micronucleus (2, 4). A polyclonal, monospecific antiserum (α-hv1-gel) against two-dimensional gel electrophoresis-purified hv1 stained small dots (presumably ribosomal gene-containing chromatin) in nucleoli of mammalian cells (5). Also, α-hv1-gel antiserum has been shown to stain a subset of loci in *Drosophila* polytene chromosomes that are active, were active, or will be active during the third larval instar or prepupal stage of development (14), and *H2AvD* has been shown to be an essential gene in *D. melanogaster* (50). Most recently, hv1 has been shown to appear in the micronucleus early in the sexual process of conjugation, when a brief period of micronuclear transcription is detected (47). These studies suggest that hv1, as well as proteins antigenically related to it in mammalian cells and in *D. melanogaster*, are preferentially associated with transcriptionally active or potentially active (competent, poised, or activated) chromatin.

Acid extracts of nuclei from representatives of the animal (mouse), plant (wheat), and fungal (*S. cerevisiae*) kingdoms all contain proteins with H2A-like electrophoretic mobility that cross-react with α-hv1-gel antiserum (4) and H2A.F/Z proteins or genes have been identified in *T. thermophila* (4, 55), *S. pombe* (11), sea urchins (15), *D. melanogaster* (52), chickens (22), and humans (8). Evolutionary analyses (48, 51) indicate that the H2A.F/Z variants diverged from the major H2As early in the evolution of eukaryotes and have evolved even more slowly than the major H2As. Taken together, these studies argue strongly that the H2A.F/Z variants perform an important function in most, if not all, eukaryotes that is distinct from that of the major H2A proteins.

In light of the fact that organisms that evolved before (*T. thermophila*), contemporaneously with (*S. pombe*), and after (*D. melanogaster*, sea urchins, chickens, and humans) *S. cerevisiae* have an H2A.F/Z variant, it is quite surprising that no such variant has been reported in this heavily studied budding yeast species. Also, as noted above, an anti-hv1 antibody that fails to react with the major H2A histones of *T. thermophila* cross-reacts with acid-soluble proteins isolated from *S. cerevisiae* nuclei (5). There are three possible explanations for the failure to identify an H2A.F/Z variant in the budding yeast *S. cerevisiae* other than the fact that this organism actually lacks this evolutionarily conserved variant. First, it is possible that *S. cerevisiae* has an undiscovered H2A.F/Z variant. Second, it is possible that the major *S. cerevisiae* H2A proteins are functionally equivalent to H2A.F/Z variants. This possibility is es-

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pecially attractive, since H2A.F/Z variants have been associated with transcriptionally competent genes and it has been reported that the entire yeast genome is in a DNase-sensitive, transcriptionally competent state (34). Third, it is possible that the conserved epitope is the functionally important feature of H2A.F/Z variants and is found on a non-H2A molecule in *S. cerevisiae*. Since these alternatives have important implications for understanding the function of H2A.F/Z variants as well as for the use of *S. cerevisiae* as a model for studying chromatin structure and function, we have attempted to distinguish among them. We tried repeatedly to clone an hv1-like variant from *S. cerevisiae* by using *Tetrahymena* hv1 cDNA as a probe and succeeded only in recloning the major H2A genes. To determine whether the yeast H2A proteins are functionally related to the major H2A proteins of other organisms or to the variants associated with transcriptionally competent genes, we replaced yeast H2A gene coding sequences with the coding sequences of the *Tetrahymena* major H2A genes or those of the hv1 gene. Although both major *Tetrahymena* H2A proteins and hv1 could be efficiently expressed in yeast cells, only the major *Tetrahymena* H2As could functionally replace yeast H2A proteins. Finally, we show that strains lacking the major yeast H2A genes fail to react with anti-hv1 antiserum. These studies argue that *S. cerevisiae* lacks an H2A.F/Z homolog. We discuss the implications of this finding and propose a model for the function of H2A.F/Z variants based on the composition of yeast chromatin and the composition of transcriptionally competent chromatin in other organisms.

MATERIALS AND METHODS

Construction of plasmids for yeast *HTA1* and *HTA2* replacement. Clones containing the yeast *HTA1* and *HTA2* genes were obtained unexpectedly in unsuccessful attempts to isolate the yeast hv1 homolog from a λgt11 library consisting of mechanically sheared genomic DNA cloned into the *Eco*RI site after addition of *Eco*RI linkers, with *Tetrahymena* hv1 cDNA (55) as a probe. The inserts (4.2 kb for *HTA1* and 3.8 kb for *HTA2*) from the phage clones were excised with *Eco*RI and were subcloned into the *Eco*RI sites of the plasmid vector pBluescript KS(+) (Stratagene) to produce clones pXL1 and pXL5, respectively. The coding regions were subcloned, and their sequences were found to be identical to previously obtained sequences (12). A 1.8-kb *Sac*I-*Eco*RI fragment (note that the *Eco*RI site is derived from the added linker and hence does not naturally exist in the yeast genome) from pXL1 was subcloned into *Sac*I-*Eco*RI sites on a new pBluescript KS(+) vector to yield clone pXL2. Because the *Hin*dIII site within the *HTA1* coding sequence is a convenient site at which to insert a selectable marker gene, pXL2 was then cut with *Eco*RV and *Xho*I and blunt end religated to remove the *Hin*dIII site in the polylinker. The resulting plasmid is called pXL3. Plasmid pDG82, obtained from Christopher Sommer, was constructed by blunt-end ligation of the 3.8-kb *Bam*HI-*Bgl*II fragment of pNKY51 (1) into the *Xba*I site of pUC18. It contains duplicated *hisG* genes of *Salmonella* cells flanking the yeast *URA3* gene (the "gene-blaster" construct). The 3.8-kb *Bam*HI fragment of pDG82 was blunt end ligated into the *Hin*dIII site of pXL3, giving rise to pXL4, from which a 5.6-kb *Sac*I-*Eco*RI fragment can be cut out for one-step gene replacement transformation (40) to knock out the *HTA1* gene. To knock out *HTA2*, the same 3.8-kb *Bam*HI fragment from pDG82 was blunt end ligated into the *Msc*I (*Bal*I) site in the *HTA2* coding region on plasmid pXL5 to obtain the clone pXL6, from which a 5.4-kb *Hpa*I fragment can be released for knocking out the *HTA2* gene.

Knockouts of yeast *HTA1* and *HTA2*. *S. cerevisiae* SS328 (*MATα his3Δ200 lys2-801^a ade2-101^o ura3-52 GAL suc2*) and SS330 (*MAT***a** *his3Δ200 tyr1 ade2-101^o ura3-52 GAL suc2*), obtained from Eric Phizicky (39), were used as starting strains from which *HTA1* and *HTA2* genes were to be knocked out. The experimental strategy is outlined in Fig. 1.

The 5.6-kb *Sac*I-*Eco*RI fragment from pXL4 was used to knock out *HTA1*. URA$^+$ transformants were selected, grown nonselectively on YPD medium, and then streaked onto 5-fluoroorotic acid (5-FOA) plates to select for the loss of the *URA3* gene so that the same marker could be used again for subsequent manipulations. The 5-FOA-resistant cells were then transformed with a centromere-containing plasmid, pXL90, which has a wild-type *HTA1* gene and *HIS3* as the selectable marker. pXL90 was constructed by ligation of the 2.4-kb *Eco*RI fragment of the *HTA1* gene from plasmid pJC102 (obtained from Michael Grunstein [43]) into the *Eco*RI site of plasmid pAB622, obtained from Fred Sherman's laboratory (also known as pRS313 and originally developed by Sikorski and Hieter [45]). Because pXL90 can now provide the single H2A gene required for viability, the chromosomal *HTA2* gene can be knocked out with the 5.4-kb *Hpa*I

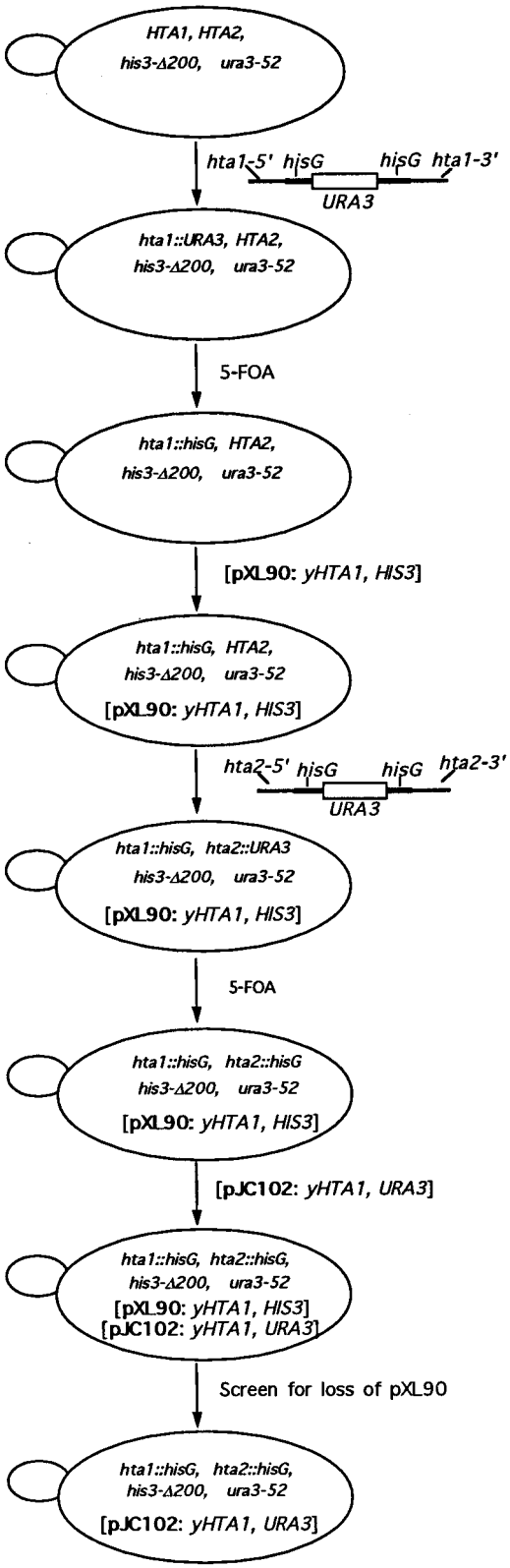

FIG. 1. Schematic illustration of the construction of *HTA1* plus *HTA2* knockout strains.

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description or genotype	Source or reference
Plasmids		
pJC102	Wild type *yHTA1* on a YCP (*URA3*)	43
pAB622	YCP (*HIS3*), also known as pRS313	45
pXL87	*tHTA1* on pAB622	This report
pXL88	*tHTA2* on pAB622	This report
pXL89	*tHTA3* on pAB622	This report
pXL90	*yHTA1* on pAB622	This report
Strains		
SS328	*MAT*α *his3*Δ*200 lys2-801*^{a} *ade2-101*^{o} *ura3-52 GAL suc2*	
SS330	*MAT***a** *his3*Δ*200 tyr1 ade2-101*^{o} *ura3-52 GAL suc2*	
XLY1	{pJC102} *hta1 hta2*; otherwise isogenic to SS328	
XLY2	{pJC102} *hta1 hta2*; otherwise isogenic to SS330	
XLY3	pXL90 in XLY1	
XLY4	pXL87 in XLY1	
XLY5	pXL88 in XLY1	
XLY6	pXL89 in XLY1	
XLY7	pAB622 in XLY1	
XLY13	pXL90 in XLY2	
XLY14	pXL87 in XLY2	
XLY15	pXL88 in XLY2	
XLY16	pXL89 in XLY2	
XLY17	pAB622 in XLY2	
XLY3f-5f	Derivatives of XLY3-5 (after loss of pJC102)	
XLY13f-15f	Derivatives of XLY13-15 (after loss of pJC102)	

fragment from pXL6 and selected on uracil drop-out plates. Again these URA$^+$ transformants were subsequently selected with 5-FOA to obtain cells that had excised the *URA3* gene. To allow later 5-FOA selection against plasmids containing the yeast H2A gene, pXL90 was then replaced by transforming pJC102 into the cells and then screening for loss of the *HIS3*-containing plasmid (pXL90) with replica plating procedures (35). We designated these cells as XLY1 and XLY2 for derivatives of SS328 and SS330, respectively. The genotypes of these strains and their derivatives are listed in Table 1.

Construction of plasmids containing *Tetrahymena* H2A genes. To allow the correct expression of *Tetrahymena* H2A genes in yeast cells, we constructed plasmids containing the *Tetrahymena* H2A gene coding sequences flanked by the 5′ and 3′ regulatory sequences of the yeast *HTA1* gene. For convenience of manipulation, the *Eco*RI fragment from pJC102 containing the yeast *HTA1* gene was subcloned onto plasmid pBluescript KS(+) (Stratagene) to obtain a clone, pKSHTA1. Oligonucleotide-directed mutagenesis was used to introduce a *Cla*I site (underlined below) on both sides of the *HTA1* coding sequence (oligonucleotide primer [Oligo] 1204 for 5′-*Cla*I = CAAATTTCATCGATATAAAA TATAAA**ATG**; Oligo 2205 for 3′-*Cla*I = GAATTA**TAA**GATCGATTCTGG TAT [initiation and termination codons are in boldface type]) to obtain clone pXL81. The *Cla*I site downstream of the coding sequence is *dam*^{s}; hence we transformed pXL81 into a *dam*$^-$ *Escherichia coli* strain, reisolated the plasmid, digested it with *Cla*I to eliminate the smaller *HTA1* coding region, and religated the remainder of the plasmid (pXL82) so that it could serve as a vector for constructs containing PCR-amplified *Tetrahymena* H2A coding regions (described below).

The *Tetrahymena HTA1* gene in pXL53 (32) was amplified with primers 2263 (*GAAATCGAT*AAAAGCAAA**ATG**TCAAC) flanking the 5′ coding region and 3264 (CAAGAATAGATCGATTTTGG) flanking the 3′ coding region, cleaved with *Cla*I, and ligated into the *Cla*I site on pXL82 to obtain pXL83.

In *T. thermophila*, TAA/G codes for glutamine (6, 21, 24, 29, 37). Therefore, the *Tetrahymena HTA2* gene in pXL46 (32) was first mutagenized to change codon 28 from TAA_{Gln} to CAA_{Gln} by using Oligo 2285 (GCTGGTCTTCAAT TCCCCGTTGG) to give pXL84. The *H2A2* coding sequence was then PCR amplified from pXL84 with the primers 3287 (*GGATCGAT*AATAATAACG **ATG**) and 2288 (TAAT*ATCGAT*TAA**TCA**ATGTTC), cleaved with *Cla*I, and ligated into the *Cla*I site of pXL82 to get pXL85.

For the hv1 gene, the 435-bp *Nar*I-*Bgl*II fragment containing the second intron in the hv1 genomic clone (51) was replaced with the 350-bp *Nar*I-*Bgl*II fragment of the cloned cDNA fragment (55) to remove the second intron of 85 bp to give phv1. The TAA codon at position 37 was changed to CAA by oligonucleotide-directed mutagenesis with Oligo 3259 (CTGGGAATTGTAAACCAGC). The resulting clone (phv1-CAA) was then PCR amplified with Oligo 1262 (*GGATC GATATAAAATAT*AAA**ATG**GCTGGCGGAAAAGGC) and Oligo 1261 (*GGA TCG*ATGTACATTACTAC**TC**) as primers, cleaved with *Cla*I, and then ligated into the *Cla*I site of plasmid pXL82 to get pXL86.

All of the constructs described above (pXL83 for H2A1, pXL85 for H2A2, and pXL86 for hv1) were restriction mapped to check the orientation of the coding sequences. For those clones with correct orientations, the coding regions and the junction regions with yeast *HTA1* regulatory sequences were checked by sequencing. The *Eco*RI fragments from these constructs, containing the yeast *HTA1* promoter, a *Tetrahymena* H2A coding region, and the yeast *HTA1* terminator, were then subcloned into the *Eco*RI site of a yeast centromere plasmid (YCP) vector, pAB622, giving rise to pXL87 for H2A1, pXL88 for H2A2, and pXL89 for hv1. A control plasmid (pXL90) containing the wild-type yeast *HTA1* gene was constructed by ligation of the *Eco*RI fragment from pXL81 into the *Eco*RI site of pAB622. The characteristics of these plasmids are listed in Table 1.

Isolation of yeast and *Tetrahymena* proteins and Western blotting (immunoblotting). Yeast cells were grown in complete or appropriate selective medium (−HIS−URA or −HIS) to late log phase. Total yeast proteins were isolated by lysis of cells in 0.4 N NaOH–1.7% β-mercaptoethanol. Histones were prepared by acid extraction of nuclei isolated from yeast cells containing wild-type H2A genes (strain 177, obtained from Elizabeth Grayhack, Department of Biochemistry, University of Rochester, and originally called JHPY20-2CA as obtained from Tom Stevens, Institute of Molecular Biology, University of Oregon; this strain is *MAT***a** *his3*Δ200 *leu 2-3,112 ura3-52 pep4*Δ::*URA3*) or from strain XLY4 or XLY4f by a method developed by Sobel and Allis (46). Whole proteins or histones were precipitated by 10% trichloroacetic acid, washed in acetone, and solubilized in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-Cl [pH 6.8], 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01 mg of bromophenol blue per ml). Total yeast proteins were run on an SDS-polyacrylamide gel electrophoresis (PAGE [15% polyacrylamide]) gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) with a semidry transfer unit (Gelman Biotrans, Ann Arbor, Mich.) in 48 mM Tris–39 mM glycine–0.08% SDS–20% methanol. Blots were stained with Ponceau-S solution (1:10 dilution with 1% acetic acid of 2% Ponceau-S in 30% trichloroacetic acid) and photographed to obtain a visual record of the proteins in each lane.

Tetrahymena cells were grown in SPP medium (super proteose peptone [1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% Sequestrene]) to about 2×10^5 cells per ml. To extract whole-cell proteins, about 10^6 cells were spun down and washed once in 10 mM Tris-HCl (pH 7.5). Cells were resuspended in 50 μl of 10 mM Tris-HCl (pH 7.5) and lysed by addition of an equal volume (50 μl) of 2× sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01 mg of bromophenol blue per ml). Different amounts of proteins were loaded on the gel for quantitation purposes. *Tetrahymena* histones were prepared from isolated nuclei by standard procedures (3).

For immunostaining, blots were blocked in 1% bovine serum albumin (BSA) in TBS (150 mM NaCl, 50 mM Tris-Cl [pH 8.1]) for 1 h, followed by incubation in primary antisera (1:200 dilution of α-H2A and/or 1:10,000 dilution of α-hv1-HPLC) with 1% BSA–0.05% Tween 20 in TBS. Both antisera were described previously (47). For most of the studies described here, α-hv1-HPLC was used instead of the α-hv1-gel antiserum previously used to detect hv1 epitopes in yeast cells (4) because it has a much higher titer. GARB (biotinylated-goat anti-rabbit immunoglobulin G; Zymed, South San Francisco, Calif.) was used as a secondary antibody, and horseradish peroxidase-conjugated streptavidin (Zymed) was used to enhance the signal. Developing was done with horseradish peroxidase color development reagent (Bio-Rad, Richmond, Calif.) as specified by the manufacturer.

To address the question of α-hv1 antiserum cross-reactivity to the major yeast histones, conditions from the earlier study (4) were duplicated. The gel and blot were prepared as described above, except the transfer was to nitrocellulose in Towbin electroblotting buffer (49). Blocking was done in 3% BSA–150 mM NaCl–10 mM Tris-HCl (pH 7.4) with incubation of primary antibody in the same buffer with 10% normal goat serum. The α-hv1-gel antiserum was used at a 1:200 dilution with incubation overnight at 4°C. The secondary antibody was GARB, and the blot was developed with 4CN Plus chromogenic reagent (DuPont, NEN, Boston, Mass.).

Indirect immunofluorescence with α-hv1 antiserum. Yeast cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.3]) for 2 h at room temperature with shaking. After the cells had been washed with a buffer containing 100 mM K_3PO_4 (pH 7.5)–1.2 M sorbitol, cell walls were removed by digestion with yeast lytic enzyme (ICN Biochemicals, Cleveland, Ohio) at 10 U/ml for 30 min at 37°C with gentle shaking. Spheroplast suspensions were spotted onto polylysine-treated slides, dried, and made permeable with 0.2% Triton X-100 in PBS for 5 min. Blocking was done with NGS/PBS (10% normal goat serum in PBS) for 30 min at room temperature, followed by incubation with α-hv1-HPLC at 1:200, 1:400, and 1:800 dilutions with NGS/PBS in a moist chamber for 2 h at room temperature. A 1:160 dilution of GARF (fluorescein

isothiocyanate-conjugated goat anti-rabbit immunoglobulin G [Sigma, St. Louis, Mo.]) in NGS/PBS was used as a secondary antibody. Cells were stained with the DNA-specific dye 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) at 0.01 μg/ml in PBS and mounted with 90% glycerol in PBS in the presence of 100 mg of an antibleaching agent, 1,4-diazobicyclo-{2,2,2,}-octane (DABCO; Aldrich Chemical), per ml.

Quantitative mating assay. A total of 10^6 SS328-derived cells were mixed with 10^7 SS330 cells (39) and then filtered onto a 0.45-μm-pore-size, 25-mm-diameter nitrocellulose filter (type HA; Millipore). Filters were placed on the surface of YPD plates and incubated for 5 h at 30°C. Cells were resuspended in 5 ml of H_2O and vortexed vigorously to disperse clumps. One hundred microliters of 10^{-1} and 10^{-2} dilutions were plated on plates lacking Tyr and then replica plated onto plates lacking both Tyr and Lys to screen for diploids (**a**/α cells) and on −Tyr plates to score for **a** and **a**/α cells. Mating efficiency was calculated as number of colonies on −Tyr−Lys plates divided by the number of colonies on −Tyr plates.

Other procedures. All cloning, sequencing, oligonucleotide-directed mutagenesis, and Southern blottings were done essentially as described in reference 7.

Yeast transformation was done either by the lithium acetate (LiAc) method (42) or by electroporation with an Electro Cell Manipulator 600 (BTX, San Diego, Calif.) according to protocols provided by the manufacturer.

RESULTS

Failure to clone an hv1 homolog from *S. cerevisiae*. We previously cloned the *Drosophila* H2A.F/Z homolog by using an hv1 cDNA probe (52). We tried repeatedly to clone an hv1-like variant from *S. cerevisiae* (expected because α-hv1 antiserum reacts with a yeast histone) by using the same strategy and succeeded only in recloning the major H2A genes or cloning non-H2A sequences with very small, nonsense regions of homology. These results, coupled with the fact that yeast histones and their genes have been intensely studied without report of another H2A-like gene or protein, make it unlikely that an undiscovered H2A.F/Z homolog exists in *S. cerevisiae*.

Construction of yeast strains for plasmid shuffle experiments. We next sought to determine whether the known histone genes in *S. cerevisiae* were functionally related to the major H2A genes of *T. thermophila* or to hv1, the H2A.F/Z variant. Wild-type *S. cerevisiae* cells contain two H2A genes, *HTA1* and *HTA2* (12). These will be referred to as *yHTA1* and *yHTA2* to distinguish them from the *Tetrahymena* genes, which are similarly named. Grunstein and colleagues have demonstrated that yeast cells with either copy of the H2A genes are viable (43). Although a haploid yeast strain containing the frameshift mutations *hta1-1* and *hta2-1* and plasmid pJC102 (*URA3 ARS1 CEN3 HTA1*) was made available to us by M. Grunstein (43), we chose to construct knockouts of the H2A genes to eliminate possible reversions and to create the same mutations in strains of both mating types to carry out mating analyses. Analysis of the function of *Tetrahymena* H2A proteins in at least two different yeast strains also should eliminate the possibility of strain-specific effects.

Figure 1 illustrates the procedures used for the gene disruption experiments. Briefly, the *yHTA1* gene was disrupted first. A plasmid containing the wild-type *yHTA1* gene was then transformed into the cell to provide H2A functions when *yHTA2* was subsequently knocked out. The plasmid was then replaced by a *URA3*-containing plasmid, pJC102, for the purpose of doing a plasmid shuffle experiment.

***Tetrahymena* major H2A genes can replace yeast H2A genes.** To test if *Tetrahymena* major H2A genes can replace yeast H2A genes by plasmid shuffle experiments (Fig. 2), we first created stage I cells by transforming a *HIS3* tester plasmid containing either the *Tetrahymena tHTA1* (pXL87) or *tHTA2* (pXL88) gene into yeast strain XLY1 to obtain yeast strains XLY4 and XLY5, respectively. We also used a plasmid containing the wild-type yeast gene *yHTA1* (pXL90) as a positive control (strain XLY3) and the plasmid vector (pAB622) as a negative control (strain XLY7). Cells containing both the $URA3^+$ plasmid pJC102 with the yeast *HTA1* gene and each of the $HIS3^+$ tester plasmids grow on medium lacking both uracil and histidine as expected. To obtain stage II cells that have lost pJC102, we treated them with 5-FOA, which kills cells that are $URA3^+$. We obtained colonies with all constructs except the one containing only vector sequences. Growth dependence of the 5-FOA-resistant cells (called XLY3f, XLY4f, and XLY5f, corresponding to XLY3, XLY4, and XLY5, respectively, after the loss of plasmid pJC102) on the presence of the $HIS3^+$ tester plasmids was demonstrated by replica plating of cells grown on plates containing histidine onto plates not containing histidine. No cells were obtained that grow with histidine but do not grow without it (data not shown). Loss of the $HIS3^+$ tester plasmid from stage I cells containing both plasmids was readily observed (data not shown). This argues that the growth of cells at stage II depends on the presence of the tester plasmids. To demonstrate the absence of wild-type yeast histone H2A proteins from the 5-FOA-selected strains transformed with *Tetrahymena HTA1* or *HTA2*, total proteins from cells at stages I and II were extracted and analyzed by SDS-PAGE. Western blots clearly demonstrated H2A proteins with the mobility of *Tetrahymena* H2A1 (Fig. 3A, lane 4) or *Tetrahymena* H2A2 (Fig. 3A, lane 6) as the sole H2A protein species in XLY4f (*tHTA1*) and XLY5f (*tHTA2*). No staining of yeast H2A was detectable. Therefore, cells are viable after complete replacement of yeast H2A genes with either of the two *Tetrahymena* major H2A genes. Interestingly, only a slightly lower growth rate was observed at 30°C for *Tetrahymena HTA1*- or *HTA2*-transformed strains (XLY4f and XLY5f) compared with that of the positive control (XLY3f [Table 2]). No significant differences in growth were observed for stage I cells (Table 2) containing both yeast and *Tetrahymena* H2A genes. The ability to mate also seems unaffected in yeast strains containing *Tetrahymena* major H2A genes either in the presence or absence of the yeast H2A gene (Table 2).

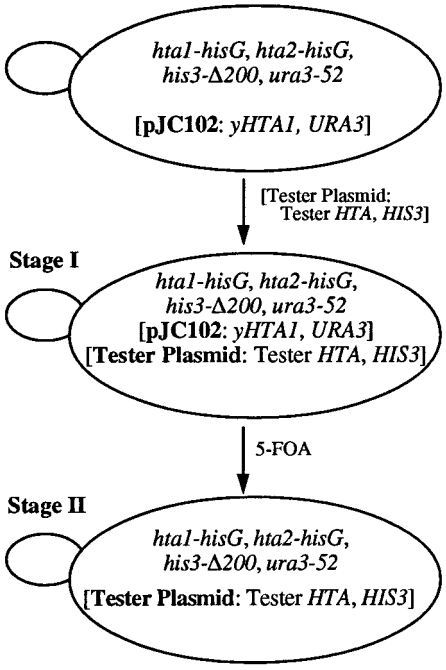

FIG. 2. Illustration of the plasmid shuffle experiment. Tester plasmid refers to either pXL87 (*tHTA1*), pXL88 (*tHTA2*), pXL89 (*tHTA3*), pXL90 (*yHTA1*), or pAB622 (vector). Stage I refers to cells containing both pJC102 and a tester plasmid; stage II refers to cells with only the tester plasmid.

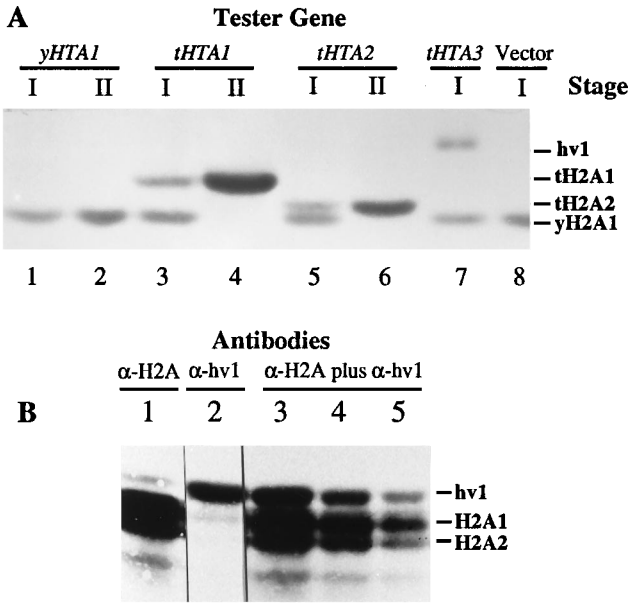

FIG. 3. Western blot analysis of yeast cells transformed with *Tetrahymena* H2A genes. (A) Total yeast proteins were run on an SDS-PAGE (15% polyacrylamide) gel. The blot was probed with a combination of α-hv1-HPLC (1:10,000) and α-H2A (1:200). Lanes 1, 3, 5, 7, and 8 are extracts of stage I cells (XLY3, XLY4, XLY5, XLY6, and XLY7, respectively), each of which contains plasmid pJC102 (*yHTA1*) and a tester plasmid: lane 1, pXL90 (*yHTA1*, a positive control); lane 3, pXL87 (*tHTA1*); lane 5, pXL88 (*tHTA2*); lane 7, pXL89 (*tHTA3*); lane 8, pAB622 (vector only as a negative control). Lanes 2, 4, and 6 are the extracts of stage II cells (XLY3f, XLY4f, and XLY5f, respectively), corresponding to XLY3, XLY4, and XLY5 after the elimination of plasmid pJC102. (B) Total *Tetrahymena* proteins were run on an SDS-PAGE (15% polyacrylamide) gel to control for antibody specificity when probed with α-H2A (lane 1) or α-hv1-HPLC (lane 2) as well as to estimate visually the relative staining intensities of hv1 and H2A proteins when probed with the combination of the two antisera (lanes 3 to 5) used under the conditions described for panel A. Lanes 1 to 5 contain 9, 9, 9, 6, and 3 μl, respectively, of *Tetrahymena* extracts.

***Tetrahymena* H2A1 and H2A2 transformants confer a recessive cold-sensitive phenotype.** To determine whether *Tetrahymena* H2A genes have any effect on growth of yeast cells, the ability of transformed yeast cells to grow on plates was examined at various temperatures (Fig. 4). No significant differences were observed for cells at the two-plasmid stage (i.e., XLY3, XLY4, XLY5, and XLY7 [data not shown]). However, when either the *tHTA1* or *tHTA2* gene alone provides the H2A functions in yeast cells (XLY4f or XLY5f [Fig. 4]), the cells grow much more slowly at a lower temperature (11°C [Fig. 4C]). These slowly growing colonies remain small even after 3 weeks of incubation in a moist chamber. This phenotype is recessive, because cells containing both yeast *HTA1* (pJC102) and either *Tetrahymena HTA1* (pXL87) or *Tetrahymena HTA2* (pXL88), i.e., XLY4 or XLY5, give rise to colonies with sizes similar to that of the positive control (XLY3) at low temperatures.

These cold-sensitive mutants can be rescued by putting back plasmid pJC102 containing the wild-type *yHTA1* gene (data not shown).

The gene encoding hv1 (*tHTA3*) cannot replace yeast H2A genes. Similar experiments were carried out for the *tHTA3* gene encoding the minor histone H2A, hv1. Tester plasmid pXL89 was transformed into XLY1 to obtain strain XLY6. When XLY6 was grown in medium lacking histidine at various temperatures (16, 30, and 37°C) and then plated onto 5-FOA plates at the same temperatures, no colonies were isolated. Figure 5 illustrates that XLY3 (containing *yHTA1*), XLY4 (containing *tHTA1*), and XLY5 (containing *tHTA2*) can lose plasmid pJC102 to generate 5-FOA-resistant colonies, whereas XLY6 (containing *tHTA3*) and XLY7 (containing only the pAB622 vector) fail to produce URA^- colonies. This suggests that the hv1 coding sequences cannot functionally replace yeast H2A coding sequences even when they are expressed under the control of the *yHTA1* 5′ and 3′ flanking sequences.

***tHTA3* is expressed efficiently in yeast cells, and hv1 is localized to nuclei.** There are several possible explanations for the failure of *HTA3* to function in yeast cells. hv1 may be unstable in yeast cells and hence may be either rapidly degraded or truncated, as demonstrated for the expression of the *Xenopus* H5 gene in yeast cells, which is properly transcribed but whose protein product is only two-thirds the size of native H5 (44). However, hv1 is detectable in large amounts on Western blots of proteins isolated from hv1-transformed cells at stage I (Fig. 3A, lane 7), and no mobility difference is detectable when these proteins are electrophoresed adjacent to bona fide *Tetrahymena* hv1 (data not shown). It is also unlikely that the amount of hv1 protein accumulated in the cell is inadequate for normal H2A function, since the intensity of antibody staining argues that hv1 (lane 7) is expressed at levels similar to those of H2A1 (lane 3) when assayed with a combination of antisera specific for *Tetrahymena* H2A and hv1. On a separate gel, *Tetrahymena* total protein was stained with α-H2A (Fig. 3B, lane 1) or α-hv1-HPLC (Fig. 3B, lane 2) to control for the specificity of the two antisera and to visually assess the quantitation of the H2A proteins in *Tetrahymena* when probed with a combination of the two antisera (Fig. 3B, lanes 3 to 5). Since hv1 constitutes about one-fifth of the total H2A proteins in *T. thermophila* (2), the relative staining intensities of hv1 and H2A1 proteins by the mixture of antibodies are close to the relative amounts of the two proteins if we assume that H2A1 and H2A2 are present in about equal amounts and hence that the H2A1 protein stains twice as intensely as hv1 protein in total *Tetrahymena* histones. Hence hv1 and H2A1 genes from *Tetrahymena* are not expressed at markedly different levels in yeast cells, making it unlikely that the failure of hv1 to rescue yeast *HTA* gene deletions is due to insufficient hv1 expression.

Histone proteins must be transported into nuclei in order to assemble with DNA to form nucleosomes. Heterologous his-

TABLE 2. Growth rate and mating ability of yeast transformants at stages I and II

Strain and stage	Gene(s) or plasmid	Doubling time (min)[a]	Mating efficiency[b]
Stage I			
XLY3	*yHTA1 yHTA1*	125	1.00
XLY4	*yHTA1 tHTA1*	151	0.97
XLY5	*yHTA1 tHTA2*	125	0.97
XLY6	*yHTA1 tHTA3*	143	1.06
XLY7	*yHTA1* pAB622	150	0.90
Stage II			
XLY3f	*yHTA1*	158	1.00
XLY4f	*tHTA1*	188	0.96
XLY5f	*tHTA2*	167	0.91

[a] For stage I, cells were grown in medium lacking both uracil and histidine. The optical density at 600 nm (OD_{600}) was determined every 60 min. Log (OD_{600}) was plotted against time to generate a linear curve, and the doubling time was calculated as $T = \log 2/\text{slope}$. For stage II, doubling times were obtained as described for stage I cells, except that cells were grown in histidine dropout medium and OD_{600} was determined every 70 min.

[b] Quantitative mating was done as described in Materials and Methods. Relative mating efficiency was obtained by using the mating efficiency of the wild-type control (XLY3) as a reference.

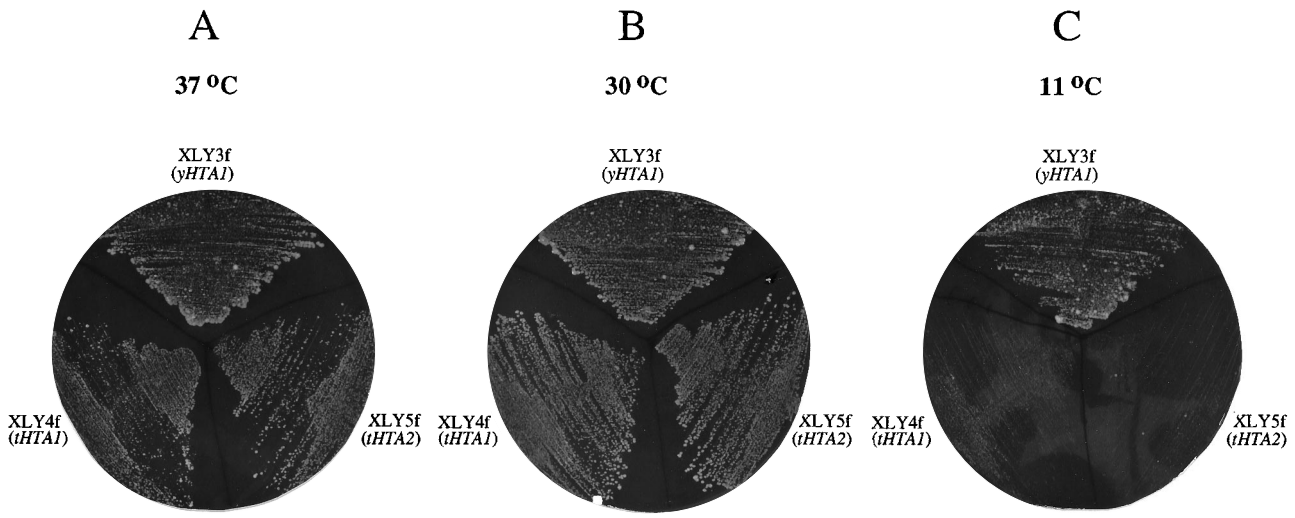

FIG. 4. Growth of yeast transformants on plates at different temperatures. Stage II cells were streaked onto −HIS plates, grown at 37°C for 4 days (A), 30°C for 4 days (B), or 11°C for 12 days (C).

tones may not carry the necessary signals for correct nuclear targeting and hence may fail to carry out the normal functions. In situ immunofluorescence staining with α-hv1-HPLC shows clearly that hv1 accumulates in nuclei of (stage I) yeast cells containing plasmids expressing both the *yHTA1* and *tHTA3* genes (Fig. 6). Thus, the failure of hv1 to rescue yeast *HTA* gene deletions is not due to an inability of hv1 to be transported into yeast nuclei.

Anti-hv1 antiserum reacts with the yeast major H2A proteins. If yeast cells lack an hv1 homolog, the question arises as to why, in our earlier studies, we found that α-hv1-gel antiserum stains yeast histones (4). The simplest explanation of this observation is that the major yeast histones cross-react with the α-hv1-gel serum. To determine if that is the case, histones extracted from nuclei of yeast strains lacking any yeast *HTA* gene or containing the yeast *HTA1* gene were analyzed with Western blots probed with the α-hv1-gel antiserum. As seen in Fig. 7, the wild-type yeast strain and strain XLY4, which contains the *yHTA1* gene, show a band at the yeast H2A position on the blot, while strain XLY4f, which completely lacks endogenous yeast *HTA* genes (containing only the *Tetrahymena HTA1* gene), does not show staining. These observations indicate that the apparent hv1 homology originally suggested by the reactivity of yeast histones with α-hv1-gel serum (4) was due to cross-reactivity with one or both of the major yeast H2A proteins.

Sequence comparison of yeast and *Tetrahymena* H2A proteins. The protein sequences of yeast H2A1 and H2A2 (12)

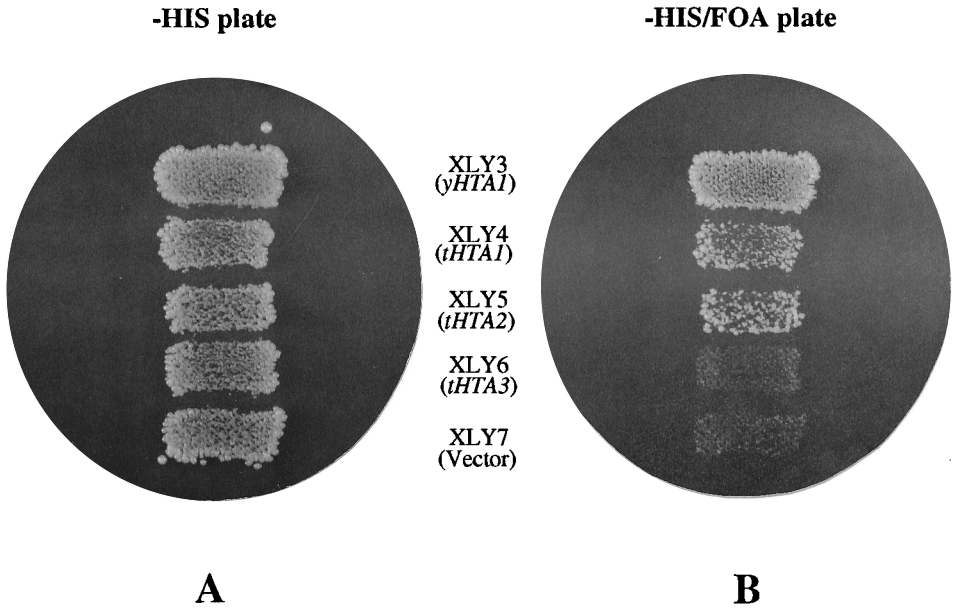

FIG. 5. *Tetrahymena HTA3* gene cannot replace yeast H2A genes. Cells grown in −HIS medium were plated onto −HIS plates and incubated for about 20 h to allow the loss of plasmid pJC102 (*URA3 yHTA1*). These cells were then replica plated onto −HIS–5-FOA plates to select for cells that have lost plasmid pJC102. XLY3 (*yHTA1*), XLY4 (*tHTA1*), and XLY5 (*tHTA2*) can produce 5-FOA-resistant colonies, whereas XLY6 (*tHTA3*) and XLY7 (pAB622) cannot.

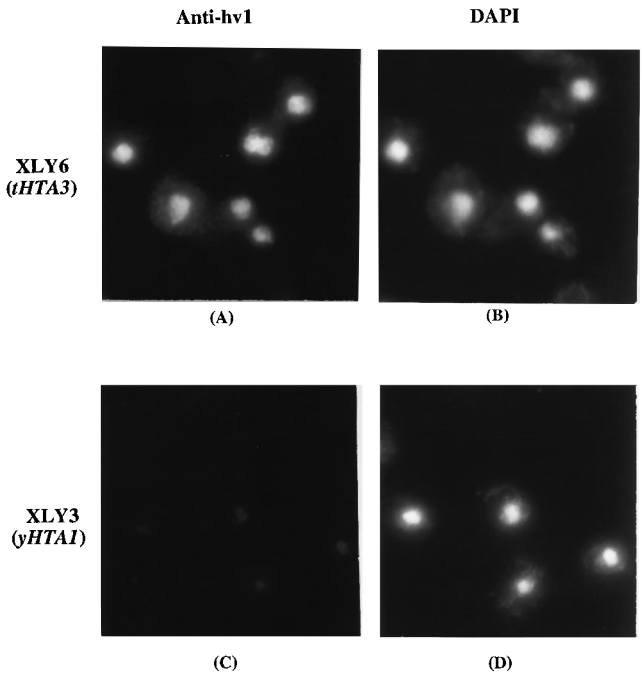

FIG. 6. Immunocytochemistry showing that hv1 protein can be transported into yeast nuclei. (A) Fluorescein-labeled antibody staining shows the localization of hv1 proteins in stage I XLY6 (*tHTA3*) cells containing *tHTA3* and *yHTA1* plasmids. (B and D) DAPI stain indicates the location of the yeast nuclei. (C) XLY3 control (*yHTA1*) cells show faint fluorescein staining, probably because of weak cross-reactivities of α-hv1 antiserum with yeast H2A1 proteins at this dilution (1:800).

and *Tetrahymena* H2A1, H2A2 (32), and hv1 (4, 51, 55) are aligned and compared in Fig. 8. Aside from the divergent N and C termini, there are significant differences in the 102-amino-acid central core regions of these proteins. The yeast H2A1 and H2A2 proteins are identical in this region. The *Tetrahymena* H2A1 and H2A2 proteins differ from each other by only 1 amino acid in the central core, whereas they differ from yeast H2A proteins by 26 (H2A1) or 25 (H2A2) amino acids. The hv1 protein, however, differs from the yeast H2A proteins by 40 amino acids within this region of 102 residues. Ten (or 9) residues of these 40 differences between yeast H2A proteins and hv1 are shared by the *Tetrahymena* major H2A proteins and therefore are not likely to be responsible for the inability of hv1 to function in yeast cells. The remaining 30 amino acids in hv1 are completely different from those of both yeast H2A proteins and both *Tetrahymena* major H2A proteins. The hv1 protein also has an insertion after the 20th amino acid in the central core and a deletion at the 80th position (Fig. 8). At present, we do not know if these differences or the divergent N- and C-terminal regions account for the functional differences between hv1 and the major H2A proteins. Experiments with chimeric fusion proteins between the major H2A and minor hv1 proteins should answer this question.

DISCUSSION

Histone proteins can function in a heterologous system. Although core histone genes are conserved during evolution, sequence divergence among different species is nonetheless observed for every histone, especially for the less-conserved H2A and H2B proteins. Primary sequence differences may be derived from neutral mutations, in which case histone proteins from different species should be functionally interchangeable. It is also possible that some of the differences actually reflect species-specific fine-tuning of the interactions between a particular histone and other nucleosome core histones or other proteins that interact with the nucleosomes. In vitro reconstitution experiments with histone molecules from diverse species have given contradictory results. Some authors seem to argue that heterologous histones can be associated into regular nucleosome particles with indistinguishable properties (25, 36). Others, however, observed different properties for the hybrid nucleosomes reconstituted with histones from different species (17, 30, 31, 38). We demonstrate in this report that the major histone H2A genes from *T. thermophila*, when placed under the control of the regulatory elements of a yeast H2A gene, can be expressed and the heterologous histones are functional in yeast cells. To our knowledge, this is the first published demonstration that histone proteins can function in vivo in a heterologous system.

The protein sequences of yeast H2A1 and H2A2 (12) and *Tetrahymena* H2A1, H2A2 (32), and hv1 (4, 51, 55) are aligned and compared in Fig. 8. H2A proteins in different organisms frequently have divergent N and C termini. However, in addition to having the expected divergent termini, there also are significant differences in the 102-amino-acid central core regions, which are much more highly conserved in the major H2A proteins of different organisms. In the core region, the yeast H2A1 and H2A2 proteins are identical, while the *Tetrahymena* H2A1 and H2A2 proteins differ from each other by only one amino acid. They differ from yeast H2A proteins by 26 (H2A1) or 25 (H2A2) amino acids. The fact that yeast cells containing only *Tetrahymena* major H2A proteins show nearly normal growth and mating argues strongly that most of the amino acid substitutions that distinguish the major H2A proteins of different organisms are neutral mutations, with little effect on nucleosome structure or function in vivo.

Cold-sensitive histone mutations. While this work was in progress, another study (23) described mutations in the *yHTA1* gene created in vitro by random PCR mutagenesis that, like our *tHTA* transformants, also resulted in a cold-sensitive phenotype. To our knowledge these mutants and the ones described here demonstrate the first reported conditional mutations in histones. Interestingly, none of the in vitro mutations are at sites that differ between the yeast and *Tetrahymena* major H2A proteins. Thus, the yeast H2A mutations and the strains containing only *Tetrahymena HTA* genes should provide different starting points for identifying suppressors of the cold-

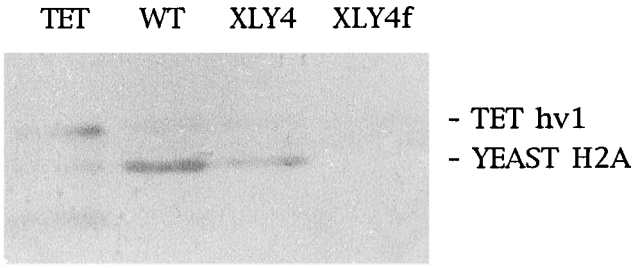

FIG. 7. Western blot analysis of yeast cells containing or lacking yeast H2A. Histones were run on an SDS-PAGE (15% polyacrylamide) gel, immunoblotted, and probed with α-hv1-gel antiserum. Lanes: 1, *Tetrahymena* histone; 2, histone from wild-type (WT) yeast cells with endogenous *HTA* genes present; 3, histones from XLY4 containing plasmids carrying *yHTA1* and *tHTA1* genes; 4, histones from XLY4f, with only the plasmid for *tHTA1*. The positions on the blot of *Tetrahymena* (TET) hv1 and yeast H2A proteins are indicated.

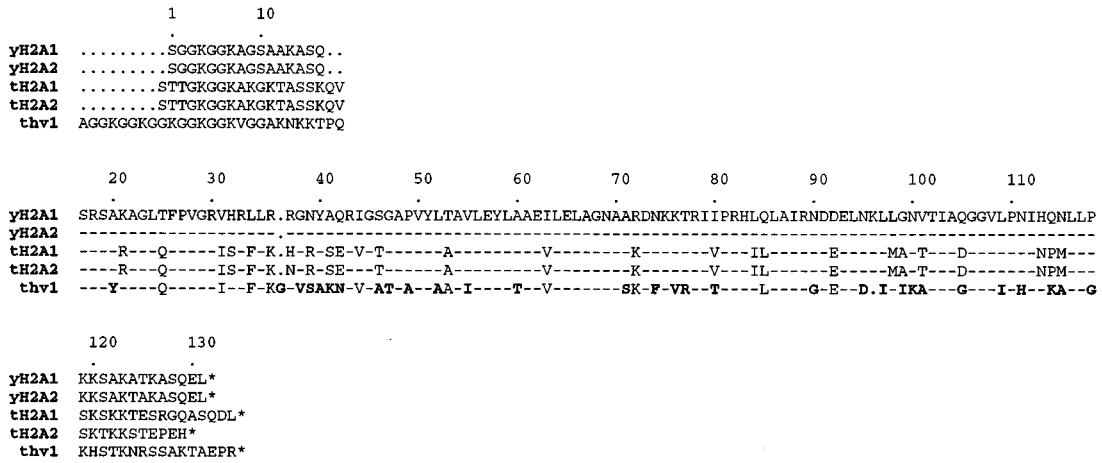

FIG. 8. Sequence compilation of yeast and *Tetrahymena* H2A proteins. Amino acids of the central core region (residues 17 to 118) are aligned. The numbers refer to the yeast H2A1 protein. Dashes indicate amino acids with identity to those of the yeast H2A1. Boldface letters indicate amino acid replacements unique to hv1.

sensitive phenotype which could be novel proteins that interact with H2A in chromatin.

Major and minor histones have distinct functions. Grunstein and colleagues have shown quite elegantly that neither of the two nonallelic variants of yeast H2A is essential for any stage of the life cycle (reviewed in reference 20). This suggests that, in yeast cells, histone subtypes are functionally redundant and that the amino acid differences between them are neutral mutations. It should be noted, however, that the yeast histone subtypes differ by only two amino acids, whereas the differences between histone subtypes in other organisms can be much more profound. Studies of the *Drosophila* H2A.F/Z variant *H2AvD* argue that this gene is essential and that its distinct function cannot be performed by major H2A genes (50), even though the haploid *Drosophila* genome has over 100 copies of the major H2A genes and only a single copy of *H2AvD*. Deletion of the gene encoding a recently described H2A.F/Z variant in the fission yeast *S. pombe* has been shown to result in slow growth, altered colony morphology, increased resistance to heat shock, and decreased fidelity of minichromosome segregation (11). These studies suggest that the H2A.F/Z variant in fission yeast cells performs an important function, although in the absence of parallel studies of the deletion of the major histone genes in this fission yeast species, it is not certain that the function differs from that of the major genes. In studies to be described elsewhere (33), we have shown that deletion of either of the two *Tetrahymena* major H2A genes, which together produce 80 to 90% of the *Tetrahymena* H2A, is without phenotypic effect, while deletion of the gene encoding hv1 is lethal. Here we have demonstrated that when *Tetrahymena* major and minor H2A genes are expressed at similar levels under control of the same yeast H2A gene regulatory elements, only the major H2A genes have nearly complete H2A functions, whereas hv1 fails to rescue yeast H2A deletions. Taken together, these studies and their distinct evolutionary lineages (48, 51) argue strongly that major H2A genes and H2A.F/Z variants have different functions.

Yeast cells lack an H2A.F/Z variant. Our studies also provide a likely answer to the question of whether the two known yeast H2A genes are functionally related to the major H2A genes or to the H2A.F/Z variants of other eukaryotes. In keeping with evolutionary analyses showing that yeast H2A proteins cluster with the major H2A proteins (48, 51), we have shown that *Tetrahymena* major H2A genes, but not the H2A.F/Z-type variant hv1, can function in yeast cells. These studies, coupled with the fact that our attempts to clone a variant H2A gene from a yeast genomic library by using the hv1 gene as a probe repeatedly yielded the major H2A genes but not a variant gene and our demonstration that it is cross-reactivity with the major H2A genes that explains staining of yeast histones with α-hv1 serum, make it likely that yeast cells do not have a gene homologous to the H2A.F/Z genes of most other eukaryotes.

Evolutionary loss of two types of histones in *S. cerevisiae*. The chromatin of most organisms contains a linker histone and an H2A.F/Z variant in addition to the major core histones. The yeast *S. cerevisiae* is the only organism known that lacks both of these types of histones. Organisms that evolved earlier (e.g., *T. thermophila*) and later (e.g., metazoans) than *S. cerevisiae* have both of these histones. The simplest explanation of these observations is that *S. cerevisiae* lost the genes encoding a linker histone and H2A.F/Z rather than that all other organisms gained both types of histone. Since few, if any, organisms (with the possible exception of *S. pombe*, for which no H1 has yet been described) whose histone genes or proteins have been extensively characterized have lost even one of these two proteins, it seems likely that the losses of both histones in *S. cerevisiae* are related evolutionary events.

Absence of H1 but not the presence of H2A.F/Z is a constant feature of transcriptionally competent chromatin. As noted earlier, a number of lines of evidence argue that hv1 and, presumably, H2A.F/Z variants in other organisms are associated with transcriptionally competent chromatin (47). Another feature of transcriptionally active or competent chromatin in vivo in *T. thermophila* (13) and in other organisms (10, 16, 26, 27) is a relative deficiency of linker histone H1. In *S. cerevisiae*, most if not all of the genome is thought to be competent for transcription (34), and this yeast species appears to lack H1 completely (18, 19). Thus, a constant feature of transcriptionally active chromatin in protozoa, fungi, and higher eukaryotes is a relative or complete absence of H1, while H2A.F/Z variants are present in most eukaryotes but not in *S. cerevisiae*.

A hypothesis relating the function of H2A.F/Z variants to their absence in yeast cells. Histone H1 is thought to play a role in higher-order chromatin structure. It is believed that H1 and the amino termini of core histones interact to convert chromatin from a 10-nm fiber into a more compacted 30-nm fiber which is thought to be the transcriptionally inactive form of chromatin (see Chapter 7 in reference 53). In chromatin,

H2A can be cross-linked to histone H1 (9), suggesting that H2A, by interacting with H1, could play a role in the higher-order structure of chromatin. We have argued above that the evolutionary losses of genes encoding H1 and H2A.F/Z in *S. cerevisiae* are likely to be related events. These considerations lead us to suggest that the absence of histone H1 and of H2A variants in *S. cerevisiae* may be indicative of a functional relationship between these two proteins. In organisms that contain H1, variant H2As which are preferentially associated with transcriptionally competent genes could facilitate the removal of H1, resulting in the decondensation of chromatin required for transcription. We propose that the evolutionary loss of H1 in budding yeast cells resulted in the entire genome being competent for transcription, obviating the need for an H2A variant, explaining the coincidental absence of both genes in this organism. Thus, *S. cerevisiae* may lack some of the early steps in the multistep process of gene activation that, in other organisms, lead from H1-containing chromatin that is incompetent for transcription to H1-deficient, transcribed chromatin. In this scenario, a major function of H2A.F/Z variants is to facilitate the removal of H1 during the developmental acquisition of transcriptional competence.

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