

# The *Schizosaccharomyces pombe* *hst4*<sup>+</sup> Gene Is a *SIR2* Homologue with Silencing and Centromeric Functions

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Although silencing is a significant form of transcriptional regulation, the functional and mechanistic limits of its conservation have not yet been established. We have identified the *Schizosaccharomyces pombe hst4*<sup>+</sup> gene as a member of the *SIR2/HST* silencing gene family that is defined in organisms ranging from bacteria to humans. *hst4*Δ mutants grow more slowly than wild-type cells and have abnormal morphology and fragmented DNA. Mutant strains show decreased silencing of reporter genes at both telomeres and centromeres. *hst4*<sup>+</sup> appears to be important for centromere function as well because mutants have elevated chromosome-loss rates and are sensitive to a microtubule-destabilizing drug. Consistent with a role in chromatin structure, Hst4p localizes to the nucleus and appears concentrated in the nucleolus. *hst4*Δ mutant phenotypes, including growth and silencing phenotypes, are similar to those of the *Saccharomyces cerevisiae* *HSTs*, and at a molecular level, *hst4*<sup>+</sup> is most similar to *HST4*. Furthermore, *hst4*<sup>+</sup> is a functional homologue of *S. cerevisiae* *HST3* and *HST4* in that overexpression of *hst4*<sup>+</sup> rescues the temperature-sensitivity and telomeric silencing defects of an *hst3*Δ *hst4*Δ double mutant. These results together demonstrate that a *SIR*-like silencing mechanism is conserved in the distantly related yeasts and is likely to be found in other organisms from prokaryotes to mammals.

## INTRODUCTION

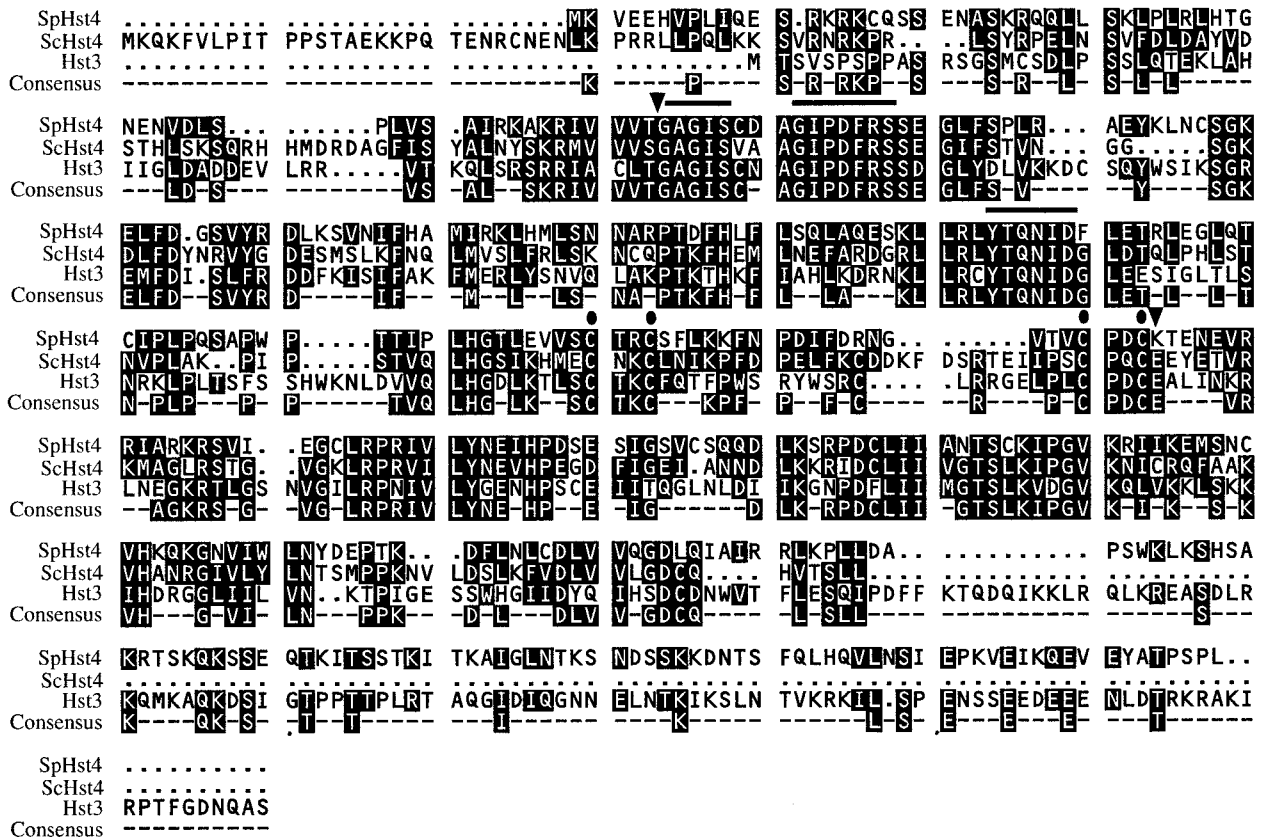
Transcriptional silencing is a general mechanism for regulating the genome that can occur through alterations of large regions of chromatin structure. This phenomenon has been studied extensively in *Drosophila melanogaster*, in which placement of a gene adjacent to heterochromatic regions can result in its variegated expression, and in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Weiler and Wakimoto, 1995). In *S. cerevisiae*, at least three loci are known to be silenced: the silent mating-type loci, the telomeres, and the rDNA repeats (reviewed by Loo and Rine, 1995; Sherman and Pillus, 1997; Lowell and Pillus, 1998). These silenced regions are inaccessible to DNA-modifying enzymes and have a special chromatin structure that is also inaccessible to transcriptional machinery (Nasmyth, 1982; Gottschling, 1992; Singh and Klar, 1992; Kyrion *et al.*, 1993;

Loo and Rine, 1994; Fritze *et al.*, 1997; Smith and Boeke, 1997; Singh *et al.*, 1998). Several *cis*- and *trans*-acting factors are required for transcriptional silencing in *S. cerevisiae*, including the four Silent Information Regulator (*SIR*) genes (Loo and Rine, 1995). *SIR2* is unique among the *SIR* genes in that it is required for silencing at all three loci (Shore *et al.*, 1984; Ivy *et al.*, 1986; Aparicio *et al.*, 1991; Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; reviewed by Sherman and Pillus, 1997). It has been proposed that *SIR2* participates in regulating the level of histone deacetylation and thus the structural compactness of chromatin at these loci (Braunstein *et al.*, 1993), although it has not been established if this regulation is direct. Indeed, histones can be modified not only through acetylation but also by means of methylation, phosphorylation, ubiquitination, and ADP ribosylation (Van Holde, 1989; Wolffe, 1992). Thus, it is possible that *SIR2*'s role may be catalytic or may regulate one or more of these modifications.

A family of *SIR2*-related *HST* (Homologues of *SIR2*) genes has been identified in organisms ranging from bacteria to humans, including four additional genes in *S. cerevisiae* (Chen and Clark-Walker, 1994; Brachmann *et al.*, 1995; Der-

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**Figure 1.** Alignment of full-length *S. pombe* Hst4 protein with full-length *S. cerevisiae* Hst3p and Hst4p. (●) Conserved cysteine residues that form a putative zinc finger; (▼) boundaries of the core domain. Solid bold lines mark conserved sequence motifs diagnostic of the *SIR2* gene family: GAG(I/V)SxxxG(I/V)PDFRS and (Y/I)IQNID. Sequence motif residues in parentheses have additional variations in published sequences, and further differences are observed in incomplete database sequences. Identical and similar residues are shaded. The consensus line lists residues that are identical in at least two of the three proteins. The homology of *hst4*<sup>+</sup> and *HST3* extends through the C-terminal tail of *HST3*. The alignment was performed with the use of the PILEUP program of the Genetics Computer Group.

byshire *et al.*, 1996; Yahiaoui *et al.*, 1996; Tsang and Escalante-Semerena, 1998; Zemzoumi *et al.*, 1998; Frye, 1999; Perez-Martin *et al.*, 1999). Members of this gene family share between 26 and 63% identity over the full lengths of their ORFs and contain a central core domain of ~155 amino acids (Figure 1, bounded by arrowheads) where identity is as high as 89% (Brachmann *et al.*, 1995). The core domain contains two conserved sequence motifs of unknown function (Figure 1, solid line) and two pairs of cysteines (Figure 1, dots) that may form a noncanonical zinc finger. The core domain and each of the conserved sequence motifs and cysteines are essential for Sir2p silencing activity (Sherman *et al.*, 1999). Furthermore, the core domain specifies a conserved silencing function, because a chimeric protein substituting the core domain of a human homologue within Sir2p functions in transcriptional silencing in *S. cerevisiae* (Sherman *et al.*, 1999).

The *HSTs* are divided into three subfamilies based on the overall sequence identities of their encoded proteins (Brachmann *et al.*, 1995). The first contains *S. cerevisiae*, *Kluyveromyces lactis*, and *Candida albicans* *SIR2* homologues and *HST1* from *S. cerevisiae* (Chen and Clark-Walker, 1994; Brachmann *et al.*, 1995; Derbyshire *et al.*, 1996; Perez-Martin *et al.*, 1999).

The second contains *HST3* and *HST4* of *S. cerevisiae* as well as *hst4*<sup>+</sup>, the *S. pombe* homologue described here. The third subfamily contains *HST2* and homologues from many other organisms. *Homo sapiens* has at least five homologues (Brachmann *et al.*, 1995; Frye, 1999). Members from the first and second subfamilies have demonstrated roles in transcriptional silencing. As noted above, *SIR2* is required at each of the silenced loci in *S. cerevisiae*, and *sir2Δ* phenotypes can be partially rescued by overexpressed *HST1* or *K. lactis* or *C. albicans* *SIR2* homologues (Chen and Clark-Walker, 1994; Brachmann *et al.*, 1995; Derbyshire *et al.*, 1996; Perez-Martin *et al.*, 1999). Mutants in the second subfamily also have silencing defects. Although neither the *hst3Δ* nor the *hst4Δ* single mutant has a strong phenotype individually, an *hst3Δ hst4Δ* double mutant has a variety of defects, including loss of telomeric silencing. The double mutant is also temperature sensitive for growth, is hypersensitive to UV radiation in combination with a *rad9Δ* mutant, and displays defects in chromosome segregation (Brachmann *et al.*, 1995). Thus, in addition to their role in transcriptional silencing, *HST3* and *HST4* contribute to cell cycle control, DNA damage, and genomic stability.

Here we report the identification of a *S. pombe* SIR2 homologue, thereby extending the SIR2 family to fission yeast. In *S. pombe* as in *S. cerevisiae*, genes placed adjacent to the silent mating-type loci (Thon and Klar, 1992; Thon *et al.*, 1994) or telomeres (Nimmo *et al.*, 1994) are silenced. Reporter genes are also silenced when placed within the relatively complex centromeres (Allshire *et al.*, 1994, 1995), a regulation that is not found in budding yeast. Many silencing genes have been discovered in *S. pombe*, predominantly through screens focused on regulation of the silent mating loci. These genes include *clr1*<sup>+</sup>, *clr2*<sup>+</sup>, *clr3*<sup>+</sup>, *clr4*<sup>+</sup>, *clr6*<sup>+</sup>, *rik1*<sup>+</sup>, and *swi6*<sup>+</sup>, a homologue of the *D. melanogaster* heterochromatin protein HP1 (Egel *et al.*, 1989; Lorentz *et al.*, 1992, 1994; Thon and Klar, 1992; Ekwall and Ruusala, 1994; Thon *et al.*, 1994; Grewal *et al.*, 1998). Swi6 protein localizes to all three silenced loci in *S. pombe* and is important for transcriptional silencing at all three sites (Lorentz *et al.*, 1992, 1994; Allshire *et al.*, 1995; Ekwall *et al.*, 1995; Grewal and Klar, 1997). Four of the silencing genes, *rik1*<sup>+</sup>, *swi6*<sup>+</sup>, *clr4*<sup>+</sup>, and *clr6*<sup>+</sup>, are also important for chromosome segregation (Allshire *et al.*, 1995; Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). Furthermore, all but *clr6*<sup>+</sup> have been demonstrated to repress meiotic recombination at the mating loci (Egel *et al.*, 1989; Klar and Bonaduce, 1991; Lorentz *et al.*, 1992; Thon and Klar, 1992; Thon *et al.*, 1994). Although extensive genetic screens have been carried out to identify additional silencing genes in fission yeast, no homologues of the SIR silencing genes have been previously identified.

This study describes the cloning and phenotypic characterization of the *S. pombe* *hst4*<sup>+</sup> gene. *S. pombe* *hst4*Δ mutants have phenotypes pointing to defects in chromatin structure and function. *hst4*<sup>+</sup> is not an essential gene, but mutants display growth and morphological defects as well as defects in transcriptional silencing, centromeric function, and genomic stability. Thus, members of the SIR2/HST subfamilies are likely to be similar in both molecular detail and organismal function.

## MATERIALS AND METHODS

### Yeast Strains and Culture Conditions

Genotypes of the strains used are presented in Table 1. *S. pombe* strains were grown in yeast extract (YE), yeast extract plus supplements (YES), or Edinburgh Minimal Medium (EMM) (Moreno *et al.*, 1991). Malt extract plates were used for sporulation (BIO 101, Vista, CA). 5-FOA plates are EMM supplemented with 1 g/l 5-FOA (Toronto Research Chemicals, North York, Ontario, Canada). For scoring of sectored colonies, YE medium was supplemented with 12 mg/l adenine. *S. pombe* cells were grown at 32°C on plates and at 30°C in liquid culture unless otherwise noted. Crosses were carried out by mixing fresh cultures on malt extract plates and either selecting for diploids after 18 h or sporulating for 3 d before dissecting tetrads. Transformations were performed with the use of a lithium acetate protocol (Moreno *et al.*, 1991), with the following modifications. A 50-ml culture was grown to an optical density ( $A_{600}$ ) of ~0.6. The cells were resuspended in 10 ml of 0.1 M lithium acetate and incubated at 32°C for 45 min before resuspending in 0.5 ml of 0.1 M lithium acetate. After adding DNA and polyethylene glycol, the cells were incubated at 32°C for 40 min followed by 20 min at 45°C. Cells were resuspended in 100 μl of water and plated on EMM plates supplemented with appropriate amino acids.

*S. cerevisiae* were grown in YPD or minimal medium lacking the appropriate nutrient for plasmid selection (Sherman, 1991). 5-FOA plates were made by adding 1 g/l 5-FOA to the supplemented

minimal medium. Cells were grown at 30°C unless otherwise specified. Transformations were carried out with the use of a lithium acetate protocol (Schiestl and Gietz, 1989). The *GAL10* galactose inducible promoter was induced in 2% galactose. These cultures were then plated onto rich or minimal plates with 2% galactose as the carbon source.

### Oligonucleotide Primers Used in This Study

Primers (5'–3') used in this study were as follows: JB708, RTCDATRT-TYTGNGTRTA; JB710, GGNRTNCCNGAYTTYMG; 5'*EagI*, ATATACTGCAGATGGGCGCCGTAAGTGGAGGAGCACGTC; 3'*XhoI*, CCGTGAAGAGGAATCGTG; T7, TAATACGACTCACTAT-AGGG; promoter, AAAGTGCAGCTACGTAATTTTGGAAATTC; 5'*start*, ACCCGTACCGTCAATCAC; 5'*us*, GTTACTATCCCATA-ACGT; *his3A*, GTAAGAAGAAAAGCGATCGATGAG; 5'*hst4-ura4*, GGATTTTAAATATATTTTATTAAGGCACTCAACTTTTTGGATTT-AAGAAATCCAAATTTACGTAGCTTAGTAAGCTTAGCTACAAA-TCCCACTG; 3'*hst4-ura4*, CAGGCATTATTTAAACCTCATTTGGGG-TAAGAAGGATAAATGATATTTTAAACATGGTTATTGGAAGC-AAGCTTGTGATATTGACGAAAC; *ko check*, GCAATTAACCAA-CGCGAC; and *ura4B*, GATTGGTGTGGAACAGA.

### hst4<sup>+</sup> Identification and Plasmid Construction

We used degenerate primers JB708 and JB710 and a touchdown molecular amplification protocol as described (Brachmann *et al.*, 1995) to probe genomic DNA and genomic and cDNA libraries. We obtained evidence for multiple members of the HST family in *S. pombe* (Freeman-Cook and Pillus, unpublished results) and focused our analysis on an ~250-base pair (bp) fragment from a *S. pombe* cDNA library (a generous gift of F. LaCroutte, Centre de Genetique Moleculaire, Gif-sur-Yvette, France). The band was purified, labeled, and used to probe a genomic library (Weaver *et al.*, 1993). A full-length clone was isolated, and the gene was cloned from the library vector into Bluescript pKS<sup>+</sup> (Stratagene, La Jolla, CA) and pUC19 (New England Biolabs, Beverly, MA) in two steps: a 1.7-kilobase (kb) *EcoRI* fragment containing the 3' end of the gene was added to a 1.3-kb *PstI/EcoRI* fragment containing the promoter and the 5' end of the gene (pLP554 in pKS<sup>+</sup> and pLP553 in pUC19). These clones contain a 125-bp intron in the 5' end of the gene after nucleotide 193 that was removed with the use of a molecular amplification strategy while simultaneously creating an *EagI* site directly downstream of the start codon to facilitate epitope tagging. Primers 5'*EagI* and 3'*XhoI* were used to amplify a spliced version of the 5' end of the gene from a cDNA library. This 0.5-kb molecular amplification product was sequenced, digested with *PstI/BglII*, and cloned into pLP554 at *PstI/BglII* to create pLP591. This clone has the 5' intron and the promoter deleted. A three-amino acid insertion (GGR) was created directly after the starting methionine to create the *EagI* restriction site. The genetic map position of *hst4*<sup>+</sup> was determined by probing an ordered *S. pombe* cosmid filter (Hoheisel *et al.*, 1993).

### Epitope Tagging and Expression

*hst4*<sup>+</sup> was epitope tagged by inserting triple HA- (influenza virus hemagglutinin, from pLP8 [Wilson *et al.*, 1984]) or protein A (proA) (from pLP580 [Aitchison *et al.*, 1995]) tags as *NotI* fragments into the *EagI* site of pLP591 to create pLP607 or pLP744, respectively.

Plasmids for *hst4*<sup>+</sup> expression in *S. cerevisiae* were constructed with the use of pLP548 in which the *GAL1/GAL10* divergent promoter was cloned into pRS313 (Sikorski and Hieter, 1989) as a 0.7-kb *EcoRI/BamHI* fragment. *SmaI/EcoRV* fragments from pLP607 and pLP744, containing the entire *hst4*<sup>+</sup> ORF, were inserted into the *EcoRV* site of pLP548. The resulting plasmids have N-terminal HA- (pLP610) and proA- (pLP748 and pLP749) tagged *hst4*<sup>+</sup> under the control of the *GAL10* inducible promoter.

**Table 1.** Yeast strains used in this study

Strain	<i>ura4</i> <sup>+</sup> or <i>his3</i> <sup>+</sup> insertion	<i>hst</i> locus	<i>mat</i>	Auxotrophic markers/Ch16 <sup>a</sup>	Plasmid	Source
<i>S. pombe</i> strains						
LPY3277		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		This study
LPY3278		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		This study
LPY3279			<i>h</i> <sup>-</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		This study
LPY3280			<i>h</i> <sup>+</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		This study
LPY3537			<i>h</i> <sup>-</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i> Ch16 <i>ade6-216 m23::ura4::TEL</i>		This study
LPY3551		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i> Ch16 <i>ade6-216 m23::ura4::TEL</i>		This study
LPY3552			<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i> Ch16 <i>ade6-216 m23::ura4::TEL</i>		This study
LPY3555			<i>h</i> <sup>-</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i> Ch16 <i>ade6-216 m23::ura4::TEL</i>		This study
LPY3556		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i> Ch16 <i>ade6-216 m23::ura4::TEL</i>		This study
LPY3562		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3563			<i>h</i> <sup>+</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3564	<i>otr1R(SphI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3565	<i>otr1R(SphI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3566	<i>otr1R(SphI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3570	<i>otr1R(SphI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3590	<i>his3::TEL1L</i>		<i>h</i> <sup>90</sup>	<i>ade6-216 his3-D1 leu1-32 ura4-D18</i> <i>otr1R(SphI)::ade6</i> <sup>+</sup>		This study
LPY3591	<i>his3::TEL1L</i>	<i>hst4Δ::ura4</i> <sup>+</sup>	<i>h</i> <sup>90</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		This study
LPY3977	<i>cnt1/TM1(NcoI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 leu1-32 ura4</i>		This study
LPY3978	<i>cnt1/TM1(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6-216 his3-D1 leu1-32 ura4</i>		This study
LPY3979			<i>h</i> <sup>-</sup>	<i>ade6-216 his3-D1 leu1-32 ura4</i>		This study
LPY3980		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6-210 arg3-D4 leu1-32 ura4</i>		This study
LPY3991	<i>imr1R(NcoI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 leu1-32 ura4</i>		This study
LPY3992		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6-216 leu1-32 ura4</i>		This study
LPY3993			<i>h</i> <sup>+</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4</i>		This study
LPY3994	<i>imr1R(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>-</sup>	<i>ade6-210 his3-D1 leu1-32 ura4</i>		This study
LPY4011		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>	pSK248	This study
LPY4012		<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>	pLP1093	This study
LPY4019			<i>h</i> <sup>+</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>	pSK248	This study
LPY4020			<i>h</i> <sup>+</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>	pLP1093	This study
LPY4379	<i>cnt3/TM3(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18</i> <i>cnt1/TM1(NcoI)::ade6</i> <sup>+</sup>	pSK248	This study
LPY4380	<i>cnt3/TM3(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18</i> <i>cnt1/TM1(NcoI)::ade6</i> <sup>+</sup>	pLP1093	This study
LPY4381	<i>cnt3/TM3(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>-</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4-D18</i> <i>cnt1/TM1(NcoI)::ade6</i> <sup>+</sup>	pSK248	This study
LPY4384	<i>cnt3/TM3(NcoI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4-D18</i> <i>cnt1/TM1(NcoI)::ade6</i> <sup>+</sup>	pSK248	This study
LPY4385	<i>cnt3/TM3(NcoI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>+</sup>	<i>ade6 arg3-D4 his3-D1 leu1-32 ura4-D18</i> <i>cnt1/TM1(NcoI)::ade6</i> <sup>+</sup>	pLP1093	This study
LPY4387	<i>cnt1/TM1(NcoI)::ura4</i> <sup>+</sup>	<i>hst4Δ::his3</i> <sup>+</sup>	<i>h</i> <sup>-</sup>	<i>ade6 arg3-D4 leu1-32 ura4</i>		This study
FY336	<i>cnt1/TM1(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6-210 leu1-32 ura4-DS/E</i>		Allshire lab
FY498	<i>imr1R(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>+</sup>	<i>ade6-210 leu1-32 ura4-DS/E</i>		Allshire lab
FY691	<i>imr1R(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>A</sup>	<i>ade6-210 leu1-32 ura4-DS/E clr2-E22</i>		Allshire lab
FY695	<i>imr1R(NcoI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>A</sup>	<i>ade6-210 leu1-32 ura4-DS/E clr4-S5</i>		Allshire lab
FY973	<i>otr1R(SphI)::ura4</i> <sup>+</sup>		<i>h</i> <sup>-</sup>	<i>ade6-210 his1-102 leu1-32 ura4-DS/E</i>		Allshire lab
FY1180			<i>h</i> <sup>+</sup>	<i>ade6-210 leu1-32 ura4-D18</i> <i>otr1R(SphI)::ade6</i> <sup>+</sup>		Allshire lab
FY1648			<i>h</i> <sup>-</sup>	<i>ade6-216 arg3-D4 his3-D1 leu1-32 ura4-D18</i>		Allshire lab
FY1863	<i>his3::TEL1L</i>		<i>h</i> <sup>90</sup>	<i>ade6-210 his3-D1 leu1-32 ura4-D18</i> <i>otr1R(SphI)::ade6</i> <sup>+</sup>		Allshire lab
<i>S. cerevisiae</i> strains						
LPY4361	<i>ADH4::URA3-TEL</i>	<i>hst3Δ3::TRP1</i> <i>hst4Δ1::TRP1</i>	<i>a</i>	<i>his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0371	This study
LPY4364	<i>ADH4::URA3-TEL</i>	<i>hst3Δ3::TRP1</i> <i>hst4Δ1::TRP1</i>	<i>a</i>	<i>his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0610	This study
LPY4366	<i>ADH4::URA3-TEL</i>	<i>hst3Δ3::TRP1</i> <i>hst4Δ1::TRP1</i>	<i>a</i>	<i>his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0748	This study
LPY4367	<i>ADH4::URA3-TEL</i>	<i>hst3Δ3::TRP1</i> <i>hst4Δ1::TRP1</i>	<i>a</i>	<i>his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0749	This study
LPY4377	<i>ADH4::URA3-TEL</i>		<i>a</i>	<i>his3Δ200 leu2Δ::TRP1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0548	This study
LPY4378	<i>ADH4::URA3-TEL</i>	<i>hst3Δ3::TRP1</i> <i>hst4Δ1::TRP1</i>	<i>a</i>	<i>his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52</i>	pLP0548	This study

<sup>a</sup> The *ura4* allele, when not designated, is either *DS/E* or *D18*; the *ade6* allele, when not designated, is either 210 or 216.

For expression studies in *S. pombe* with the endogenous *hst4*<sup>+</sup> promoter, primers T7 and promoter were used to amplify a 1-kb fragment from pLP554, which was then cut with *Pst*I and inserted into the *Pst*I site of pLP744 to create pLP935 (proA). A *Sac*I fragment from pLP935 was then cloned into the *S. pombe* LEU2 vector pSK248 (Stone *et al.*, 1993) to create pLP1093 for the proA tag.

### Null Mutant Construction

The *hst4Δ::his3*<sup>+</sup> null mutant was created by replacing the core domain of *hst4*<sup>+</sup> from amino acids 75–162 with a 1.9-kb fragment containing the *his3*<sup>+</sup> gene. To use the 5' *Eco*RI site of *hst4*<sup>+</sup> to insert *his3*<sup>+</sup>, we deleted the 3' *Eco*RI site of *hst4*<sup>+</sup>. A *Pst*I/*Cla*I fragment with the *Cla*I site filled with VENT polymerase (New England Biolabs) prepared from pLP553 was ligated into Bluescript pKS<sup>+</sup> at *Pst*I/*Xho*I with the *Xho*I site filled to create pLP1077. This plasmid was digested with *Eco*RI/*Xho*I and filled in, and a 1.9-kb filled-in *Bgl*III *his3*<sup>+</sup> fragment from pAF1 (Ohi *et al.*, 1996) was inserted to create pLP862. Approximately 2 μg of a 3.3-kb *Bsa*AI fragment from pLP862 was transformed into LPY3170. Among candidate *his*<sup>+</sup> transformants, the correct disruption was verified by molecular amplification with the use of primers 5'start, 3'*Xho*I, 5'us, and *his3A* and confirmed by Southern blot analysis. The *hst4Δ::ura4*<sup>+</sup> complete null mutant, replacing the entire ORF with a 1.8-kb *ura4*<sup>+</sup> gene fragment, was created with the use of primers 5'*hst4-ura4* and 3'*hst4-ura4*. Approximately 3 μg of the molecular amplification product was transformed into LPY3277, and colonies were selected that could grow on medium lacking uracil but could no longer grow on medium lacking histidine. The null mutant was confirmed by molecular amplification with the use of primers 5'us, *his3A*, *ko* check, and *ura4B*. The two null alleles were created with different markers to facilitate silencing assays with either a *ura4*<sup>+</sup> or a *his3*<sup>+</sup> reporter gene. Both alleles have the same growth and morphology phenotypes (our unpublished results), and thus both appear to be complete-loss-of-function alleles.

### Sequence Analysis

Alignments were generated with the use of the PILEUP program (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI) with homologue sequences obtained from GenBank. Pairwise comparisons were performed with the use of the BESTFIT program from the Genetics Computer Group. Default settings were used for PILEUP, BESTFIT, and BLAST analysis. Database searches were performed with the use of the National Center for Biotechnology Information BLAST network service ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and the Sanger Center *S. pombe* database search service ([www.sanger.ac.uk](http://www.sanger.ac.uk)). The *hst4*<sup>+</sup> nucleotide sequence has been submitted to GenBank under accession number AF173939.

### Cytological Techniques

*S. pombe* cells were prepared for DAPI staining by fixing 1 ml of a saturated culture in 1 ml of 30% methanol (MeOH)/70% acetone for at least 20 min at –20°C. Cells were rehydrated with 5-min washes in 100 μl of 75, 50, and 25% MeOH in PBS. Cells were then resuspended in 100 μl of PBS, and 6 μl of cells was mixed with 1 μl of DAPI (0.25 μg/ml; Boehringer Mannheim, Indianapolis, IN) on a slide immediately before viewing. For immunofluorescence, 30 ml of cells was grown to an optical density of 0.3–0.5, centrifuged, and resuspended in 10 ml of MeOH at –20°C for 15 min. Cells were then washed in 1 ml of 60% MeOH/40% PBS, washed in 1 ml of PBS, and resuspended in 1 ml of PBS plus 1.2 M sorbitol with 0.25 mg/ml zymolyase (70,000 U/g, ICN, Costa Mesa, CA) at 37°C for 30 min. Cells were then resuspended in 1 ml of 1% Triton X-100 in PBS for 1 min, washed three times in 1 ml of PBS, and stored in 500 μl of PBS plus 1% BSA plus 100 mM lysine-HCl (PBSBL) at 4°C overnight. Multiple-well slides for immunofluorescence were prepared by adding 10 μl of 0.1% poly-L-lysine to each well for 30 min, washing

six times with 10 μl of water, and air drying. Ten microliters of cell suspension was added to each well for 30 min. After aspirating the remaining liquid, the cells were dried onto the slide. All antibodies were diluted in PBSBL. Primary antibody incubation was at room temperature for 16 h in a moist chamber (1:2000 dilution of rabbit immunoglobulin G [Sigma, St. Louis, MO] for proA or 1:100 dilution of anti-Nop1 D77 [Aris and Blobel, 1988]). Cells were then washed six times in PBSBL, followed by the addition of secondary antibody for 20 h at room temperature (1:200 fluorescein-conjugated donkey anti-rabbit antibody [Amersham, Piscataway, NJ] or 1:300 Texas Red-conjugated goat anti-mouse antibody [Jackson ImmunoResearch, West Grove, PA]). Cells were then washed four times with 10 μl of PBSBL, incubated in 10 μl of 0.25 μg/ml DAPI for 5 min, and washed six times with 10 μl of PBSBL. The cells were then dried on the slide and were mounted with Citifluor (Ted Pella, Redding, CA). Staining was visualized with a Leica (Heidelberg, Germany) DMRXA microscope with a Cooke (Tonawanda, NY) Sensicam charge-coupled device camera. Images were captured and manipulated with the use of SlideBook software (Intelligent Imaging Innovations, Denver, CO).

### Pedigree Analysis

Individual cells from populations grown to stationary phase in liquid YES were micromanipulated onto YES plates and grown at 32°C. Three classes of cells were identified for micromanipulation: wild-type cells, *hst4Δ* cells that appeared to be normal in cell size, and *hst4Δ* cells that were at least three times as long as wild-type cells. These cells were then monitored at intervals to determine subsequent divisions. The number of cells examined reflects the number that could be readily micromanipulated and monitored on three plates.

### UV Sensitivity Assay

Saturated cultures from a series of fivefold dilutions in a 96-well plate were plated onto YES plates with the use of a pin replicator. The plates were treated with 80 J/m<sup>2</sup> UV irradiation with the use of a Stratelinker (Stratagene) immediately after plating and again after 12 h. Control plates were left untreated. Plates were placed in a dark box to prevent photoreactivation and incubated at 30°C.

### Silencing Assays

Assays were performed to evaluate the expression level of reporter genes placed at seven silenced loci: within *cnt1*, *cnt3*, *otr1*, and *imr1*, at the telomere of the linear minichromosome Ch16, at the endogenous telomere of chromosome I, and adjacent to the silent mating loci (Thon and Klar, 1992; Allshire *et al.*, 1994, 1995; Nimmo *et al.*, 1994, 1998). Saturated cultures from a series of fivefold dilutions were plated onto YES, supplemented EMM, and 5-FOA plates. Plates were incubated at 32°C or room temperature (~20°C) until full growth was achieved (3–10 d). Plates were incubated for extended periods to ensure that any differences seen were not due simply to the slow growth of the *hst4Δ* mutant strains.

### Minichromosome-Loss Assay

LPY3552 and its *hst4Δ* derivative, LPY3551, containing the linear minichromosome Ch16 (Nimmo *et al.*, 1994) were used to determine chromosome stability. Ch16 carries the *ade6-216* mutation that complements the *ade6-210* allele present at the chromosomal locus. Loss of the minichromosome in an *ade6-210* background results in red color formation on YE plates supplemented with minimal adenine (12 mg/l). The frequency of chromosome loss is calculated from the frequency of half-sector colonies, as described by Allshire *et al.* (1995). Three separate isolates were examined for the wild-type strain and four isolates were examined for the *hst4Δ* strain, for a total of 14,529 and 18,059 colonies, respectively. The chromosome-loss experiments were all performed at 30°C, although it has been

**Table 2.** *S. pombe* Hst4 protein is most similar to *S. cerevisiae* Hst4p

	<i>S. pombe</i> Hst4 BLAST E-value	<i>S. pombe</i> Hst4 BESTFIT (gaps) <sup>a</sup>	<i>S. pombe</i> Hst4 BESTFIT core <sup>b</sup>
Hst4p	$4 \times 10^{-59}$	49/59% 285 aa (6)	55/62%
Hst3p	$1 \times 10^{-41}$	40/50% 271 aa (5)	49/62%
Hst1p	$4 \times 10^{-15}$	39/50% 127 aa (4)	40/51%
Sir2p	$5 \times 10^{-13}$	36/51% 110 aa (3)	36/50%
Hst2p	$3 \times 10^{-8}$	34/43% 101 aa (3)	36/41%

<sup>a</sup> Percent identity/similarity of *S. pombe* Hst4 protein to the *S. cerevisiae* proteins over the indicated lengths; aa, amino acids.

<sup>b</sup> Percent identity/similarity in the core domain from the GAGIS motif to the YIQNID motif (see Figure 1).

noted in other studies that chromosome loss is further elevated at decreased temperatures (Ekwall *et al.*, 1996). The results reported here likely represent a conservative estimate of the severity of the chromosome-loss phenotype.

## RESULTS

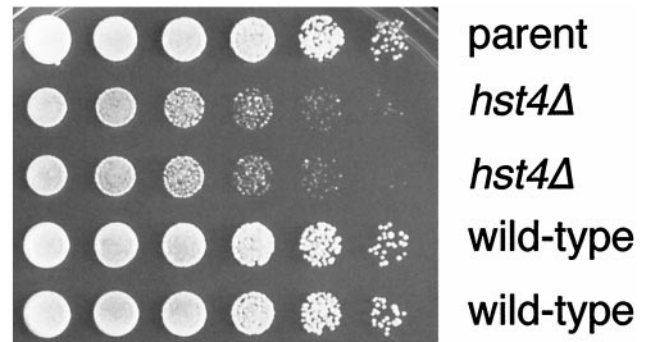
### Identification of a *S. pombe* Homologue of SIR2

We used degenerate primers designed from two highly conserved sequence motifs (Brachmann *et al.*, 1995) to amplify a 250-bp fragment from a *S. pombe* cDNA library that was then used to isolate a full-length genomic clone of the gene that we have named *hst4*<sup>+</sup>.

*hst4*<sup>+</sup> encodes a 416-amino acid predicted ORF that maps to chromosome I between *ras1*<sup>+</sup> and *cdc3*<sup>+</sup> (see MATERIALS AND METHODS). *hst4*<sup>+</sup> contains both sequence motifs and the two pairs of cysteines characteristic of the HSTs (Figure 1). Comparison of cDNA and genomic sequences revealed that *hst4*<sup>+</sup> contains a single 125-bp intron that splits the first of the conserved sequence motifs. Table 2 shows expect (E) values from BLAST analysis for *S. pombe* Hst4p relative to Sir2p and each of the other *S. cerevisiae* homologues. The table also shows percent identity and similarity from BESTFIT analysis of full-length *S. pombe* Hst4p to each of the homologues as well as a comparison of the core domains. The sequence analysis reveals that *hst4*<sup>+</sup> is a member of the subfamily represented by *HST3* and *HST4* and is most closely related to *HST4*. Figure 1 shows an alignment of the full-length *S. pombe* Hst4p with *S. cerevisiae* Hst3p and Hst4p, illustrating the high level of similarity present throughout the proteins.

### *hst4*Δ Mutants Have Multiple Growth and Morphological Defects

To better understand the function of *hst4*<sup>+</sup>, we created heterozygous null alleles in diploid cells by replacing a large portion of the core domain containing both conserved sequence motifs with the *his3*<sup>+</sup> gene or by replacing the entire ORF with *ura4*<sup>+</sup>. The region of the core deleted in the *his3*<sup>+</sup> allele is essential for *SIR2* function in *S. cerevisiae* (Sherman *et al.*, 1999). After sporulating the heterozygous diploids, tetrads were dissected and germinated to reveal four viable spore products. Thus, *hst4*<sup>+</sup> is not an essential gene. When

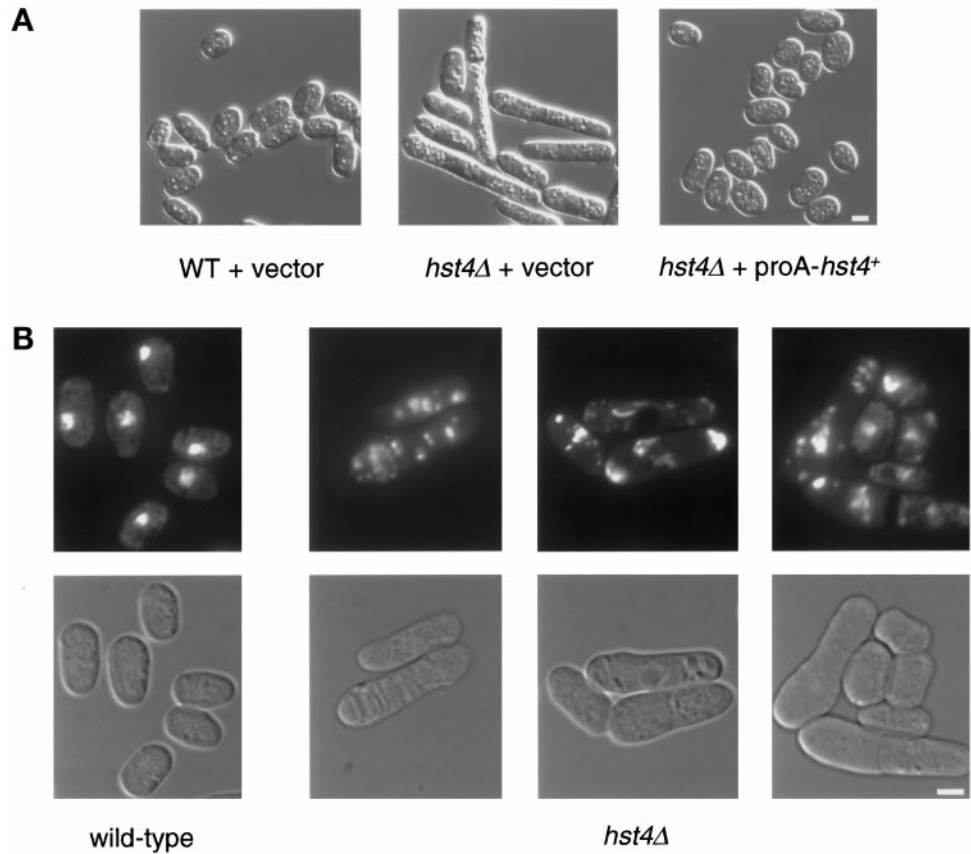


**Figure 2.** *S. pombe hst4*Δ mutants exhibit growth defects. A wild-type parent (FY1648) and four sister spore products from an *hst4*Δ heterozygous diploid (LPY3277 and LPY3278 are the *hst4*Δ strains, LPY3279 and LPY3280 are the isogenic wild-type strains) were grown to saturation in rich (YES) liquid medium at 30°C. Fivefold serial dilutions were plated onto YES plates that were incubated at 14, 16, 20, 25, 30, 32, 35, and 37°C (32°C plate is shown).

*hst4*Δ mutant and wild-type cells were compared by flow cytometry, both strains looked comparable, indicating that there were no major defects in cell cycle progression (our unpublished results). Although viable, *hst4*Δ haploid cells have growth defects. The two *hst4*Δ spores produced smaller colonies than the wild-type spores. Figure 2 shows a dilution assay in which fivefold dilutions of four spore products from the same tetrad were plated onto rich medium at 32°C. The two null strains grew to the same dilution as their wild-type sister strains, but the colony size was much smaller. This phenotype was observed at all temperatures tested (14–37°C) (Figure 2 and our unpublished results). After prolonged growth, mutant and wild-type colonies eventually reached the same size.

Morphological defects in *hst4*Δ cells were present in logarithmically growing populations. Ordinarily, *S. pombe* cells elongate throughout the cell cycle, doubling their size before cytokinesis. In *hst4*Δ mutant cultures, a subset of cells was significantly longer than normal, and this subpopulation became more prevalent as cultures reached saturation. In a saturated wild-type culture grown in rich medium, cells arrest with G<sub>2</sub> DNA content but small cell size (Costello *et al.*, 1986). In *hst4*Δ mutants, 33% (558 of 1670) of the cells were at least twice as long as saturated wild-type cells and reached lengths up to seven times that of wild-type cells. In contrast, only 5% (64 of 1412) of wild-type cells were twice as long as average wild-type length (Figure 3A). This phenotype was specifically attributable to the null allele because expression of a proA-tagged *hst4*<sup>+</sup> plasmid construct restored wild-type morphology (Figure 3A).

To determine if the long cells were viable, both wild-type and short and long cells from a saturated mutant culture were micromanipulated to follow their subsequent divisions individually. Of 78 normal wild-type cells micromanipulated, 60 (77%) grew to form colonies. The remainder failed to divide or divided at most twice after micromanipulation. In contrast, of 66 apparently normal short *hst4*Δ mutant cells, only 29 (44%) produced colonies. Of 66 long mutant cells, only 3 (5%) continued division to form a colony. Thus, even normal-appearing mutant cells have decreased viability, and



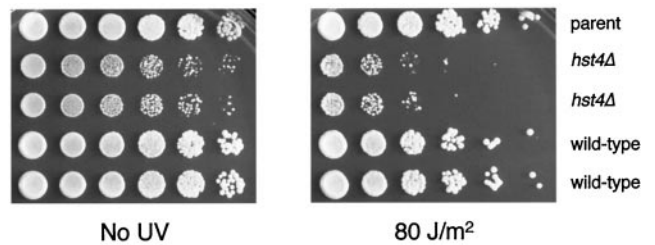
**Figure 3.** (A) *S. pombe hst4Δ* mutants have morphological defects. A wild-type strain containing the pSK248 vector (LPY4019) and an isogenic *hst4Δ* mutant strain containing either the same vector (LPY4011) or N-terminally proA-tagged *hst4<sup>+</sup>* under the control of its endogenous promoter (LPY4012) were grown to saturation in liquid YES medium at 30°C. The cells were photographed with the use of Nomarski optics. Bar, 5 μm. (B) *hst4Δ* mutant cells have fragmented chromatin. A wild-type strain (LPY3280) and an isogenic *hst4Δ* strain (LPY3278) were grown to saturation at 30°C in liquid YES medium, fixed, and stained with DAPI to visualize their chromatin. The bottom panels show Nomarski images of the DAPI-stained cells seen in the top panels. Bar, 5 μm.

the abnormally long cells are not viable in most instances. This decreased viability probably contributes to the modest sporulation and germination defects of *hst4Δ* null mutants that we have observed (our unpublished results).

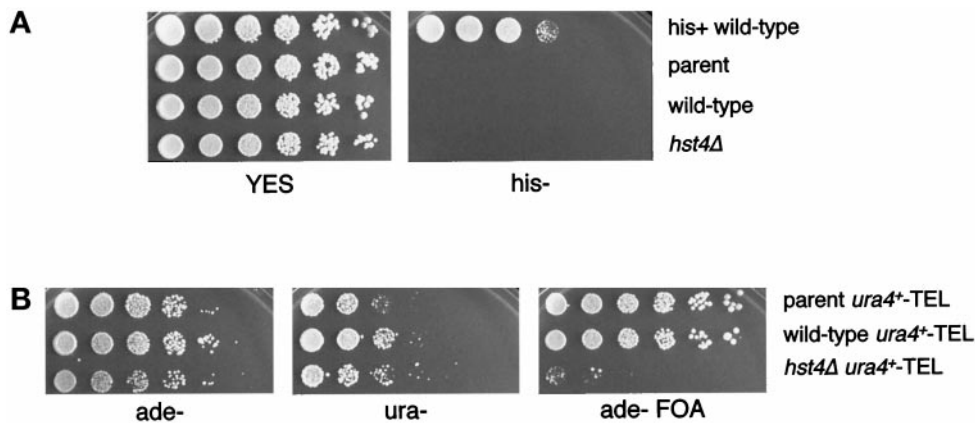
Examination of chromatin in the long *hst4Δ* mutant cells with the use of DAPI staining revealed striking differences from wild-type cells (Figure 3B). At saturation, cells normally arrest with duplicated DNA that appears as a compact sphere in the center of the small cells (Costello *et al.*, 1986). In contrast, the DNA of the long mutant cells appeared to be fragmented. There was often more than one focus of DAPI-stained material, and it was frequently dispersed in the cell (Figure 3B). The fragmented DNA was observed in 17% (225 of 1322) of the mutant cells. Fragmented chromatin may result from one of several situations. For example, the chromosomes could be intact in a fragmented nucleus, or the chromatin could be fragmented in an otherwise intact nucleus. To evaluate these possibilities, we followed the nuclear envelope in cells in which the nuclear pore component Cut11p was tagged with Green Fluorescent Protein (West *et al.*, 1998). In cells in which the DNA had begun to fragment, the nuclear envelope remained intact and relatively normal. However, in some cells, the nuclear envelope became exaggerated in size as fragmentation became more severe. Thus, it is possible that changes in both nuclear and chromatin integrity contributed to the fragmentation visible in the DAPI-stained cells. The long cells also had aberrant septa, with many cells having multiple, wide, or off-center septa or

deposits of septal material that did not appear to bisect the cell (our unpublished results).

In *S. cerevisiae*, an *hst3Δ hst4Δ rad9Δ* triple mutant is hypersensitive to UV irradiation (Brachmann *et al.*, 1995). To determine if the *S. pombe* mutant was UV sensitive, wild-type and mutant cells were treated with a range of UV irradiation. A series of fivefold dilutions of cells from four spore products of a parental ditype tetrad were plated on



**Figure 4.** The *S. pombe hst4Δ* mutant is sensitive to UV irradiation. Fivefold serial dilutions of a wild-type parent (FY1648) and four sister spore products from an *hst4Δ* heterozygous diploid (LPY3277 and LPY3278 are the *hst4Δ* strains, LPY3279 and LPY3280 are the isogenic wild-type strains) were grown to saturation in liquid YES medium at 30°C. Serial fivefold dilutions were plated onto YES plates and either left untreated or UV irradiated at 80 J/m<sup>2</sup> with the use of a Stratalinker (Stratagene) immediately after plating and again after 12 h.



**Figure 5.** *S. pombe hst4Δ* mutants have telomeric silencing defects. (A) *hst4Δ* cells maintain normal silencing of an *his3*<sup>+</sup> gene located at the left telomere of chromosome I. Fivefold serial dilutions of an *his*<sup>+</sup> wild-type control (FY1180), the parent telomere-marked strain (FY1863), and wild-type (LPY3590) and *hst4Δ* (LPY3591) telomere-marked strains from the same tetrad were plated onto YES medium or EMM lacking histidine and grown at 32°C. The presence of the reporter gene was confirmed by molecular amplification (our unpublished results). (B) *hst4Δ* mutants derepress a *ura4*<sup>+</sup> gene

located at the telomere of the Ch16 minichromosome. Fivefold serial dilutions of the parent strain (LPY3537) and wild-type (LPY3556) strains from the same tetrad were plated onto EMM plates lacking adenine (*ade*<sup>-</sup>) to show the number of cells plated that contain the Ch16 minichromosome, onto EMM plates lacking uracil (*ura*<sup>-</sup>) to show the low levels of *ura4*<sup>+</sup> expressed from the telomeric locus, and onto 5-FOA EMM plates lacking adenine (*ade*<sup>-</sup> FOA), which assay the expression levels of the *ura4*<sup>+</sup> reporter gene. Note that all strains assayed are *ura4*<sup>-</sup> at the chromosomal locus (see Table 1).

rich medium and treated with 80 J/m<sup>2</sup> UV immediately after plating and again after 12 h (Figure 4). At this intermediate level of radiation, there is little to no sensitivity of the parent or wild-type spores compared with the untreated control. However, there was a modest but reproducible UV hypersensitivity seen in the two mutant spore products.

When wild-type cells are treated with UV radiation, they arrest with an elongated morphology as part of the DNA-damage checkpoint (Saka *et al.*, 1997). We examined wild-type and mutant cells that had been treated with UV. The irradiated *hst4Δ* cells arrested comparably to wild-type cells. Therefore, the mutant's UV sensitivity does not appear to be due to a defect in the checkpoint response but rather to some other mechanism.

### *hst4*<sup>+</sup> Is Required for Silencing at the Telomeres and Centromeres

Transcriptional silencing has been described in *S. pombe* at three loci: the telomeres (Nimmo *et al.*, 1994), the silent mating loci (Thon and Klar, 1992; Thon *et al.*, 1994), and the centromeres (Allshire *et al.*, 1994, 1995). Genes placed within or adjacent to these loci are transcriptionally repressed. To determine whether *hst4*<sup>+</sup> plays a role in transcriptional silencing, we crossed the *hst4Δ* mutants into strains containing reporter genes at each of the three loci.

Although a majority of known silencing genes in both *S. pombe* and *S. cerevisiae* were identified based on their phenotypes at the silent mating loci, this did not appear to be the primary target for *hst4*<sup>+</sup> function. *hst4Δ* mutants had a very modest, if any, effect on silencing the mating loci. Furthermore, the mutant strains did not exhibit the haploid sporulation phenotype typical of other *S. pombe* silencing mutants (Lorentz *et al.*, 1992; Thon and Klar, 1992; Thon *et al.*, 1994; Grewal *et al.*, 1998; our unpublished results).

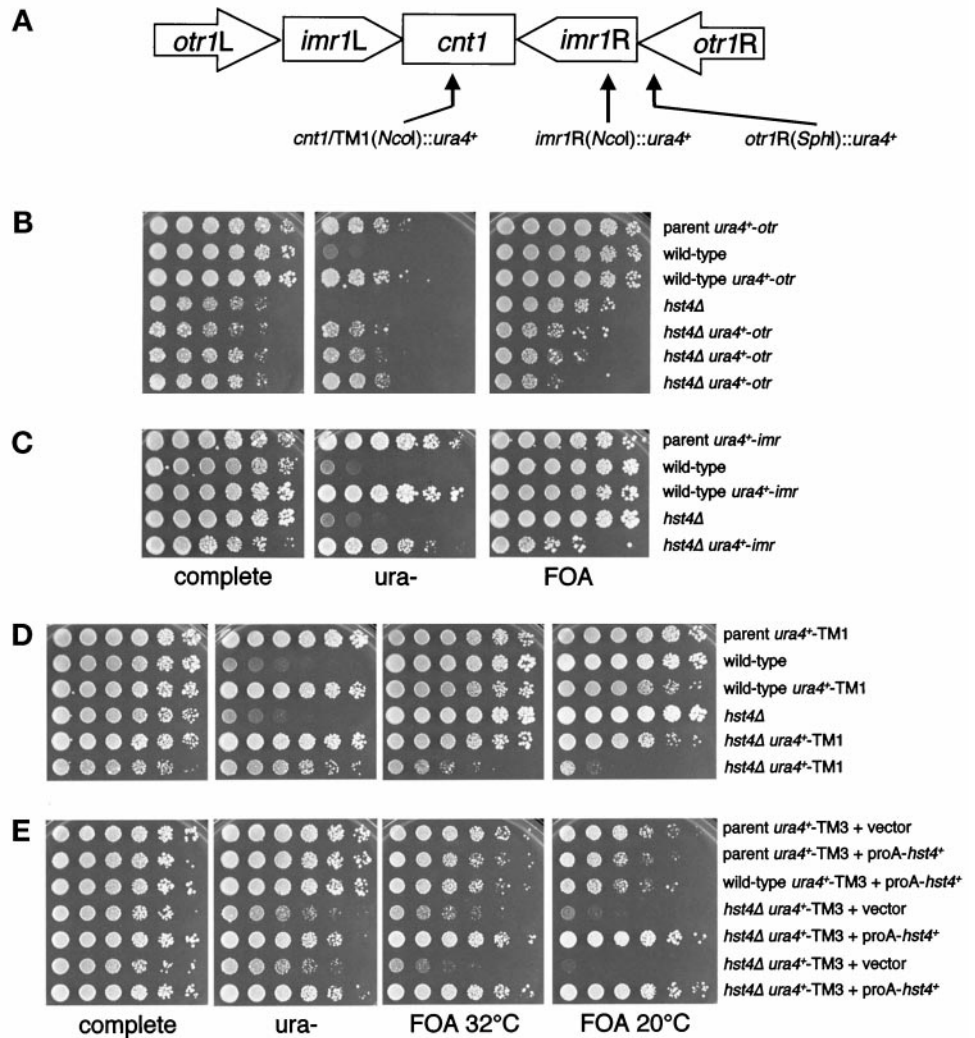
Telomeric silencing was monitored in two ways: at a natural telomere and with the use of a minichromosome construct. In the natural context, expression of a *his3*<sup>+</sup> reporter gene adjacent to the left telomere of chromosome I was evaluated. In wild-type strains, this reporter is com-

pletely silenced and cells are unable to grow on medium lacking histidine (Nimmo *et al.*, 1998). To assess whether derepression of this reporter occurred in an *hst4Δ* mutant, we crossed the *hst4Δ* mutant into the marked strain. The null mutant was unable to restore growth on medium lacking histidine and thus did not derepress this reporter gene (Figure 5A). The second assay for telomeric silencing uses a minichromosome that is marked at the telomere with *ura4*<sup>+</sup>. In this construct, both the reporter gene and the telomeric context of the minichromosome are distinct from the *his3*<sup>+</sup> marked natural telomere (Nimmo *et al.*, 1994, 1998). We crossed the minichromosome into an *hst4Δ* strain and plated a series of fivefold dilutions onto various media: plates lacking adenine to select for the minichromosome and to control for the number of cells plated, plates lacking uracil to evaluate the low levels of *ura4*<sup>+</sup> expressed from the telomeric locus, and 5-FOA plates to assess expression of the *ura4*<sup>+</sup> gene from the telomere of the minichromosome (Figure 5B). Because 5-FOA is toxic to cells expressing the *ura4*<sup>+</sup> gene product (Boeke *et al.*, 1984; Allshire *et al.*, 1994), cells expressing *ura4*<sup>+</sup> are unable to grow on medium containing this compound. In a wild-type background, the *ura4*<sup>+</sup> gene is epigenetically silenced. That is, the strains are able to form colonies on medium lacking uracil, but they are also able to form colonies on medium containing 5-FOA. Thus, in a wild-type population, some cells express the *ura4*<sup>+</sup> reporter gene and some repress its transcription. In this assay, the *hst4Δ* mutant cells had a 25- to 625-fold growth defect on 5-FOA (Figure 5B), demonstrating increased expression of the normally silenced reporter gene.

Like previously characterized *S. pombe* silencing mutants, an *hst4Δ* strain displayed centromeric silencing defects that varied depending on the location of the reporter gene within the centromeric repeats. *S. pombe* centromeres consist of a central domain that is flanked by large inverted repeats (Chikashige *et al.*, 1989; Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Murakami *et al.*, 1991; Takahashi *et al.*, 1992) (Figure 6A). We examined silencing



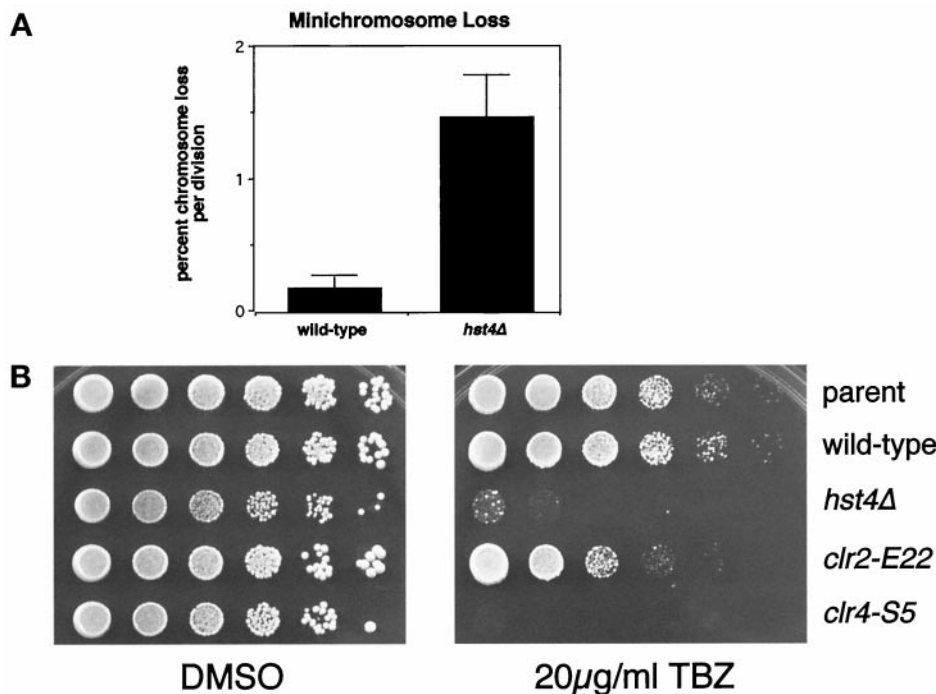
**Figure 6.** *S. pombe* *hst4*<sup>+</sup> is required for centromeric silencing. (A) Schematic representation of *S. pombe* centromere 1, which contains a central domain flanked by the *imr* and *otr* inverted repeats. (B) *hst4*Δ mutants derepress the *otr* locus. Fivefold dilutions were plated onto EMM plates as a growth control (complete), onto EMM plates lacking uracil (*ura*<sup>-</sup>), and onto 5-FOA plates (FOA) to assay silencing of the *ura4*<sup>+</sup> reporter gene. Shown are the marked parent strain, four sister spore products plated at 32°C, and two additional isolates of marked *hst4*Δ strains. Strains in order of plating are: FY973, LPY3563, LPY3564, LPY3562, LPY3565, LPY3566, and LPY3570. The derepression seen was the same when plates were grown at 20°C (our unpublished results). (C) *hst4*<sup>+</sup> is required for silencing within the *imr* repeat. As above, the parent strain, the marked and unmarked wild-type strains, and the marked and unmarked *hst4*Δ strains were plated onto EMM plates as a growth control (complete), onto EMM plates lacking uracil (*ura*<sup>-</sup>), and onto 5-FOA plates (FOA). Strains in order of plating are: FY498, LPY3993, LPY3994, LPY3992, and LPY3991. The derepression phenotype was the same when plates were grown at 20°C. (D) *hst4*<sup>+</sup> is also required for silencing within the central domain of *cen1* (TM1). The variable 5-FOA sensitivity seen at this locus is shown on the 5-FOA plate, where one isolate of the marked *hst4*Δ showed no sensitivity yet a second isolate had an ~25-fold growth defect on 5-FOA at 32°C. When the plates were grown at 20°C rather than 32°C, the 5-FOA sensitivity increased to 625-fold in the marked *hst4*Δ strain. Strains in order of plating are: FY336, LPY3979, LPY3978, LPY3980, LPY3977, and LPY4387. (E) The *hst4*Δ mutant also shows temperature-dependent derepression within the central domain of *cen3*. The marked *hst4*Δ strain was ~25-fold more 5-FOA sensitive than the unmarked strain at 32°C and ~625-fold more sensitive at 20°C. This sensitivity at both temperatures was rescued by expression of N-terminally proA-tagged *hst4*<sup>+</sup>. Strains in order of plating are: LPY4379, LPY4380, LPY4381, LPY4384, and LPY4385. Note that, as in Figure 5B, all strains assayed in panels B–E are *ura4*<sup>-</sup> at the chromosomal locus (see Table 1).



of a *ura4*<sup>+</sup> reporter gene placed within the central domain of centromere 1 (*cen1*) and centromere 3 (*cen3*), the *imr* repeat of *cen1*, and the *otr* repeat of *cen1*. In a wild-type background, *ura4*<sup>+</sup> inserted at any of these locations is epigenetically silenced, so the strains are able to form colonies on both 5-FOA and medium lacking uracil (Allshire *et al.*, 1994, 1995).

An *hst4*Δ mutant strain exhibited variable growth defects of up to 625-fold on 5-FOA medium when a *ura4*<sup>+</sup> gene was located within the *otr* repeat of *cen1* (Figure 6B). Similar growth defects were seen when the reporter gene was located within the *imr* repeat or the central domain of *cen1* (TM1) (Figure 6, C and D). The growth defect on 5-FOA seen at TM1 was temperature dependent; when plates were grown at 20°C, the defect increased to 625-

fold (Figure 6D). However, the derepression was variable. Figure 6D shows an example of a strain that shows strong derepression and a separate isolate that has apparently wild-type levels of silencing. There was also dramatic and temperature-dependent 5-FOA sensitivity seen when the reporter was present in the central domain of *cen3* (TM3). At 32°C, there was variable 5-FOA sensitivity similar to that seen at the other loci. At room temperature, however, the 5-FOA sensitivity was almost complete (Figure 6E). Wild-type cells grew well on 5-FOA at room temperature, but the mutant strains showed very little growth. This silencing defect was rescued by expression of the proA-tagged *hst4*<sup>+</sup> plasmid construct (Figure 6E). Thus, the *hst4*Δ mutant exhibits silencing defects at telomeres and centromeres.



**Figure 7.** (A) *hst4Δ* mutants have increased chromosome loss. Wild-type (LPY3552) and *hst4Δ* (LPY3551) strains containing the Ch16 minichromosome were grown to saturation in liquid YES at 30°C and plated onto YE plates supplemented with 12 mg/l adenine. The percent chromosome loss per division was calculated from the number of half-red colonies divided by the number of white colonies plus half-red colonies. For the *hst4Δ* strain, a total of 18,059 colonies were scored in four separate experiments. For the wild-type strain, a total of 14,529 colonies were scored in three experiments. (B) *hst4Δ* mutants are TBZ sensitive. Five-fold dilutions of a parent strain (LPY3102), a wild-type strain (LPY3279), and an isogenic *hst4Δ* mutant strain (LPY3277), as well as a TBZ-insensitive *clr2* mutant (FY691) and a TBZ-sensitive *clr4* mutant (FY695), were plated onto YES plates with DMSO as a growth control and YES plates with 20 μg/ml TBZ.

### Increased Chromosome Loss in *hst4Δ* Mutants

Because the *hst4Δ* mutants had decreased viability associated with fragmented DNA and centromeric silencing defects, it seemed possible that the *hst4Δ* phenotypes might result from aberrant chromatin structure, particularly at the centromeres. At least four previously characterized genes known to be important for centromeric silencing are also important for chromosome maintenance and thus centromeric function. *clr4*, *rik1*, *swi6*, and *clr6* mutants are reported to have chromosome-loss rates from 9- to >100-fold greater than wild-type (Allshire *et al.*, 1995; Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). To determine if the centromeric silencing defect was associated with a defect in centromeric function in the *hst4Δ* mutant, we assayed maintenance of the Ch16 minichromosome using colony color. This minichromosome construct contains the *ade6-216* allele that complements the chromosomal *ade6-210* allele present in the mutant and wild-type strains. Cells that maintain the minichromosome are *ade+* and give rise to white colonies. Loss of the minichromosome results in red colony color. When the chromosome-loss event occurs in the first cell division, colonies arise that are half white and half red. To examine chromosome maintenance in the *hst4Δ* strain, the *hst4Δ* mutation was crossed into a strain containing the Ch16 minichromosome. The *hst4Δ* mutant lost the minichromosome in 1.5% of cell divisions, a rate that was eightfold higher than in the wild-type strain (Figure 7A). Thus, *hst4Δ* cells are defective in chromosome maintenance as well as silencing.

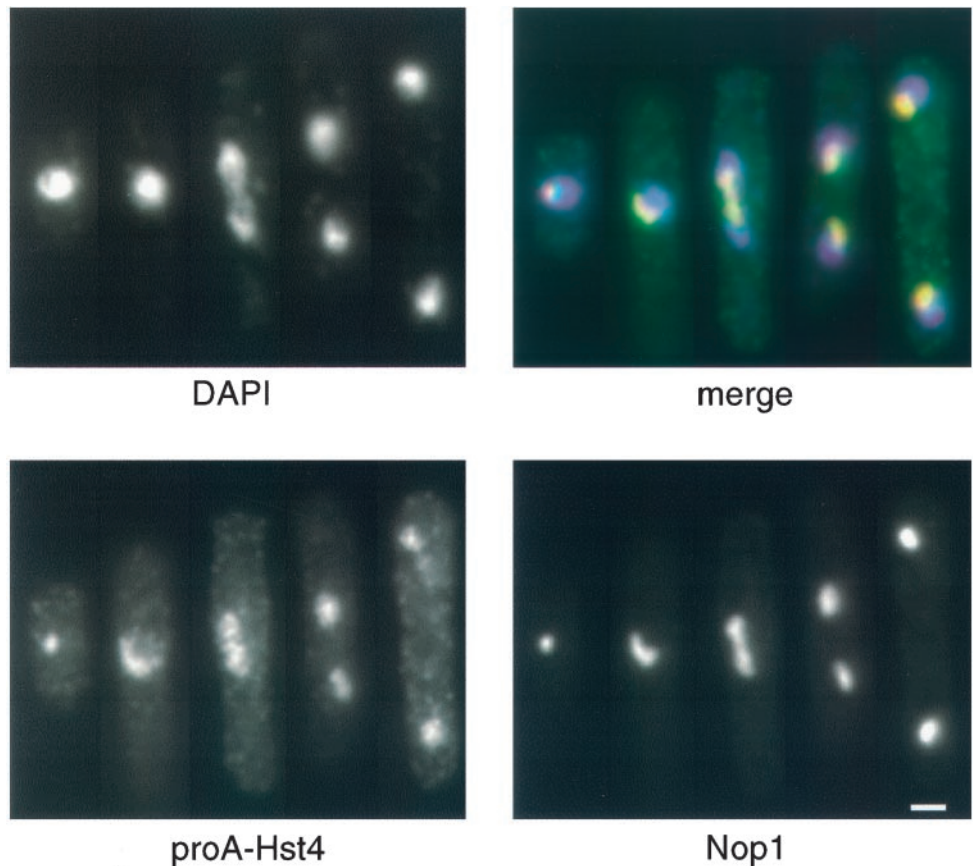
Genes that function in regulating centromeric chromatin structure might be predicted to affect the interaction of microtubules with the kinetochore during mitosis. In fact, at least three of the four previously identified *S. pombe* genes that are required for chromosome maintenance are sensitive to the microtubule-destabilizing drug thiabendazole (TBZ)

and have genetic interactions with the gene encoding  $\beta$ -tubulin, *nda3+* (Ekwall *et al.*, 1996). To determine if *hst4+* may function in a similar way, we assayed TBZ sensitivity in the *hst4Δ* mutant and compared it with the previously established sensitivity of a *clr4* mutant and insensitivity of a *clr2* mutant and wild-type strains. The parent and wild-type strains and the *clr2* mutant strain grew well, as expected. However, the *hst4Δ* mutant was sensitive to TBZ, similar to the *clr4* mutant control strain (Figure 7B). When TBZ-treated cells were examined microscopically, the arrested *hst4Δ* and wild-type cells had the same morphology. Thus, TBZ sensitivity of the *hst4Δ* mutant may reflect either a potential role in mitotic spindle-chromosome interactions or a more general structural role in chromatin.

### *S. pombe* Hst4 Is a Nuclear Protein Enriched in the Nucleolus

To determine its subcellular localization, we generated epitope-tagged constructs of Hst4p. A proA tag was inserted into the N terminus of Hst4, directly after the initiating methionine, and the proA-Hst4 protein was expressed from a plasmid under the control of the endogenous *hst4+* promoter. This tagged construct appeared fully functional in that it was able to rescue the long-cell phenotype and the TM3 centromeric derepression of the *hst4Δ* mutant (Figures 3A and 6E). Indirect immunofluorescence of cells expressing this proA-Hst4 construct demonstrated that Hst4p was nuclear and was concentrated in a condensed spot within the nucleus. Colocalization of proA-Hst4 and Nop1, a nucleolar antigen (Aris and Blobel, 1988), identified this subnuclear staining as nucleolar (Figure 8). The nucleolar concentration was observed in cells from all stages of the cell cycle, and clear colocalization was seen in 90% (159 of 177) of cells examined.

**Figure 8.** *S. pombe* Hst4p localizes to the nucleolus throughout the cell cycle. A wild-type strain containing N-terminally tagged proA-*hst4*<sup>+</sup> under the control of its endogenous promoter (LPY4020) was fixed and stained with DAPI to visualize DNA (top left), with rabbit immunoglobulin G to visualize proA-*hst4*<sup>+</sup> (bottom left), and with anti-Nop1p antibodies to stain the nucleolus (bottom right). A merged image of the five cells in different stages of the cell cycle is shown at top right. The DAPI staining is in blue, the proA-Hst4 staining is in green, and the Nop1p staining is in red. In the merged image, the overlap between the proA-Hst4p and Nop1p signals is yellow. The speckled cytoplasmic staining present in the proA-*hst4*<sup>+</sup> panel is nonspecific in that it was also seen with secondary antibody alone in wild-type cells not expressing the proA-*hst4*<sup>+</sup> construct (our unpublished results). Bar, 2  $\mu$ m.



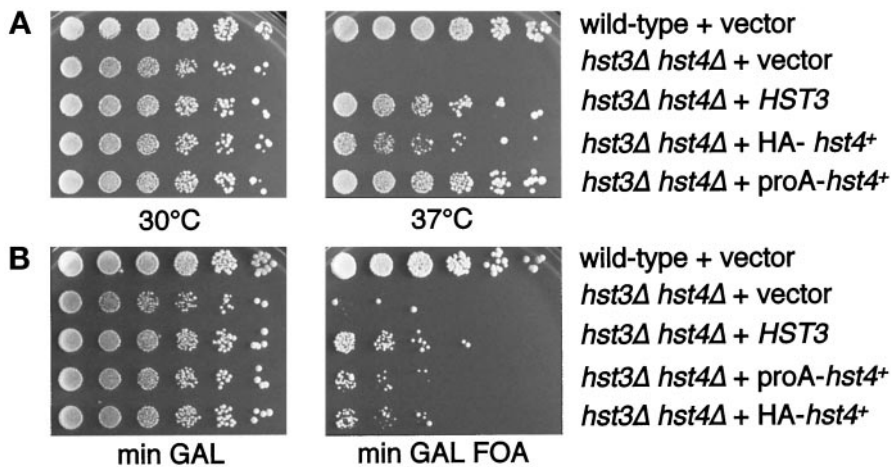
***S. pombe hst4*<sup>+</sup> Is a Functional Homologue of *S. cerevisiae HST3* and *HST4***

Because members of the *SIR2* gene family share a high degree of sequence similarity, it was of interest to determine the extent of their functional conservation. As detailed above, *hst4*<sup>+</sup> shares molecular and phenotypic similarities with *HST3* and *HST4* from *S. cerevisiae*. Therefore, we asked if *hst4*<sup>+</sup> could rescue the phenotypes of a *S. cerevisiae sir2Δ* mutant or an *hst3Δ hst4Δ* double mutant. Overexpression of *hst4*<sup>+</sup> was unable to rescue the silencing phenotypes of a *S. cerevisiae sir2Δ* mutant. This result was not necessarily surprising because a positive result demands both cross-species and cross-subfamily complementation, and it was previously shown that overexpressed *HST3* cannot rescue *sir2Δ* mutant phenotypes (Brachmann *et al.*, 1995). We next asked whether *hst4*<sup>+</sup> could complement *hst3Δ hst4Δ* double mutant phenotypes, because these three genes are most closely related. Although *hst3Δ* and *hst4Δ* mutants do not independently have strong defects, the double mutant displays pleiotropic phenotypes, including temperature sensitivity for growth at 37°C, decreased chromosome stability, and hypersensitivity to UV irradiation in combination with a *rad9Δ* mutant (Brachmann *et al.*, 1995). Furthermore, *HST3* and *HST4* are required for telomeric silencing, because reporter genes placed at the telomere are derepressed in a double mutant (Brachmann *et al.*, 1995). To determine if *hst4*<sup>+</sup> could complement these phenotypes, we transformed

an *hst3Δ hst4Δ* double mutant with N-terminally epitope-tagged HA-*hst4*<sup>+</sup> or proA-*hst4*<sup>+</sup> overexpressed under the control of a galactose-inducible promoter.

*hst4*<sup>+</sup> complemented the temperature-sensitive growth phenotype of the *hst3Δ hst4Δ* double mutant. Figure 9A shows a wild-type strain and an *hst3Δ hst4Δ* double mutant transformed with vector, *HST3*, HA-*hst4*<sup>+</sup>, or proA-*hst4*<sup>+</sup>. The wild-type strain grew equally well at 30 and 37°C. The double mutant containing vector alone grew at 30°C but was unable to grow at 37°C. The *HST3* control transformant complemented this temperature sensitivity, as expected. Both tagged versions of *hst4*<sup>+</sup> were able to rescue the *hst3Δ hst4Δ* temperature-sensitive phenotype, demonstrating functional conservation between *S. pombe* and *S. cerevisiae HST* genes.

*hst4*<sup>+</sup> was also able to relieve the telomeric silencing defect of the double mutant (Figure 9B). A wild-type strain containing a telomeric *URA3* reporter gene grew on medium containing 5-FOA, indicative of the transcriptional silencing of the *URA3* gene. An *hst3Δ hst4Δ* double mutant with the same reporter was unable to grow on 5-FOA, and this derepression of *URA3* was complemented by *HST3*. The telomeric derepression was also partially rescued by expression of *hst4*<sup>+</sup> (Figure 9B). *hst4*<sup>+</sup> could not, however, complement the UV hypersensitivity of an *hst3Δ hst4Δ rad9Δ* triple mutant (our unpublished results). Thus, because *hst4*<sup>+</sup> rescued the temperature-sensitivity and telomeric silencing defects of the *hst3Δ hst4Δ* mutant,



**Figure 9.** *S. pombe hst4<sup>+</sup>* is a functional homologue of *S. cerevisiae HST3* and *HST4*. (A) *S. pombe hst4<sup>+</sup>* complements the temperature-sensitive phenotype of an *hst3Δ hst4Δ* double mutant. Shown are serial five-fold dilutions plated at 30 or 37°C onto rich galactose plates to induce expression of the HA- or proA-tagged *hst4<sup>+</sup>*, which are under the control of the *GAL10* promoter. Strains in order of plating are: LPY4377, LPY4378, LPY4361, LPY4364, and LPY4366. (B) *S. pombe hst4<sup>+</sup>* is also able to partially rescue the telomeric silencing defect of the *hst3Δ hst4Δ* double mutant. Shown are five-fold dilutions plated onto minimal GAL plates and minimal GAL 5-FOA plates. Strains in order of plating are: LPY4377, LPY4378, LPY4361, LPY4367, and LPY4364.

partial function of these genes has been conserved in the two yeasts.

## DISCUSSION

The fission yeast *S. pombe* and the budding yeast *S. cerevisiae* are only distantly related, yet much has been learned through analysis of both their comparable and contrasting ways of accomplishing such essential processes as DNA replication and cell cycle control (for review, see Forsburg, 1999). Likewise, many parallels can be drawn between silencing in the two yeasts. In both, the silent mating-type loci and the telomeres are transcriptionally silenced (Gottschling *et al.*, 1990; Aparicio *et al.*, 1991; Thon and Klar, 1992; Nimmo *et al.*, 1994; Thon *et al.*, 1994). In contrast, *S. pombe*'s larger and more complex centromeres are also silenced (Chikashige *et al.*, 1989; Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Murakami *et al.*, 1991; Takahashi *et al.*, 1992; Steiner *et al.*, 1993; Allshire *et al.*, 1994, 1995). Even though the two organisms share at least two silenced loci, to date no homologues of the *S. cerevisiae SIR* silencing proteins have been reported in *S. pombe*.

Here we have described the identification of a *S. pombe* silencing gene homologue that is a member of the *SIR2* silencing gene family. *S. pombe hst4<sup>+</sup>* is most closely related to the *S. cerevisiae* genes *HST3* and *HST4*. Like these homologues, it plays a role in chromatin structure. Indeed, *hst4<sup>+</sup>* is required for transcriptional silencing, chromatin integrity, and genomic stability. However, it is distinguished from *SIR2* and other *HSTs* by its apparent role in centromeric function. These studies thus functionally extend the *SIR2* gene family to *S. pombe* and demonstrate that similar silencing mechanisms may exist in the two distantly related yeasts.

### *hst4<sup>+</sup>* Is Closely Related to the *SIR2* Gene Family Both Molecularly and Functionally

*S. pombe hst4Δ* mutants are phenotypically similar to *S. cerevisiae hst3Δ hst4Δ* double mutants. *S. pombe hst4Δ* mutants accumulate large cells that are inviable. The abnormally long *hst4Δ* mutant cells are reminiscent of those seen

in several cell-division-cycle (*cdc*) mutants in which cell growth continues after nuclear division arrests (see Nurse *et al.*, 1976; Kelly *et al.*, 1993; Maiorano *et al.*, 1996; Gould *et al.*, 1998). If the mutant cells experience irreparable alteration of their chromatin that triggers arrest of the nuclear division cycle without halting cell growth, long cells would arise, as observed in many *cdc* mutants. *HST3* and *HST4* in *S. cerevisiae* are redundant genes that are together required for cell cycle progression, telomeric silencing, and chromosome maintenance (Brachmann *et al.*, 1995). Parallel to the *S. pombe hst4Δ* mutant cell cycle defect, *S. cerevisiae hst3Δ hst4Δ* mutants accumulate as large-budded cells that are larger than wild-type cells and have reduced viability (Brachmann *et al.*, 1995). The growth and morphology phenotypes in both yeasts may be related to a role for these *HST* genes in the maintenance of chromatin structure and integrity.

Additional phenotypes of these family members in both yeasts support the idea that the genes are important in the maintenance of chromatin structure. The *S. pombe hst4Δ* mutant (Figure 4) and the *S. cerevisiae hst3Δ hst4Δ* double mutant (in combination with a *rad9Δ* mutation) (Brachmann *et al.*, 1995) are sensitive to UV irradiation. In both yeasts, UV sensitivity has been correlated with mutations in genes thought to play a role in chromatin structure (Kaufman *et al.*, 1997; Grewal *et al.*, 1998). Alteration of chromatin structure in the *hst* mutants could make the DNA more accessible to UV radiation-induced damage. Additionally, the *SIR2* family members in both yeasts, including *hst4<sup>+</sup>*, are required for transcriptional silencing. *S. pombe hst4Δ* mutants (Figures 5 and 6) and *S. cerevisiae hst3Δ hst4Δ* mutants (Brachmann *et al.*, 1995) exhibit decreased silencing, indicating that these proteins may be involved either directly or indirectly in chromatin structure.

Direct evidence that these homologues are important for chromatin structure and integrity comes from chromosome-maintenance assays. *hst4<sup>+</sup>* is important for chromosome maintenance, as demonstrated by the fact that a *S. pombe hst4Δ* strain is at least eight times more likely to missegregate the Ch16 minichromosome per cell division than a wild-type strain. This quantitative effect is almost certainly an underestimate of the chromosome-maintenance defect of the null mutant because, in *S. pombe*, each of the three

chromosomes is essential for viability. Thus, for the assay to detect a chromosome loss, an event must occur that results in loss of the minichromosome and that leaves the remaining chromatin intact, allowing the cell to proceed through several rounds of cell division to form a colony. Because at least 30% of a population of *hst4Δ* cells is aberrantly long and not capable of giving rise to a colony, these clearly defective cells cannot be scored and are thus unrepresented in the chromosome-loss quantitation. Based on the dispersed DNA phenotype seen in many long cells (Figure 3B), it is likely that chromosome-loss events contribute to the loss of viability in the mutant cells. If it were possible to take into account the rate of chromosome loss in these cells, the total loss rate would be expected to be much higher.

We found that overexpression of *hst4<sup>+</sup>* in *S. cerevisiae* rescues a subset of the *hst3Δ hst4Δ* double mutant phenotypes. *hst4<sup>+</sup>* is able to rescue the temperature sensitivity for growth and to partially rescue the telomeric silencing defect of the *S. cerevisiae* mutants. However, *hst4<sup>+</sup>* does not rescue the UV-hypersensitivity phenotype seen in the *S. cerevisiae* mutant. Thus, partial function has been conserved in these two yeasts. The *S. cerevisiae* genome duplicated ~10<sup>8</sup> years ago, long after the *S. cerevisiae* and *S. pombe* lineages split (Wolfe and Shields, 1997). This duplication of the genome may have allowed similar genes to evolve different functions. Although *HST3* and *HST4* lie outside a genomic block that was duplicated between chromosome XV and chromosome IV (Wolfe and Shields, 1997), given their redundant functions and proximity to duplicated blocks it seems possible that they were part of the duplication event and that intermediate genes have undergone subsequent rearrangements. Knowledge of the functions of this subfamily is limited, in part, by the redundancy of the *S. cerevisiae* homologues. In *S. pombe*, *hst4<sup>+</sup>* may fulfill the role of both *S. cerevisiae* genes, thus providing a unique opportunity to learn more about the subfamily.

#### ***hst4<sup>+</sup>* Is Similar to but Distinct from Other *S. pombe* Silencing Genes That Have a Role in Chromatin Structure**

Several genes are already known to be important for transcriptional silencing in *S. pombe*. *clr1<sup>+</sup>*, *clr2<sup>+</sup>*, *clr3<sup>+</sup>*, *clr4<sup>+</sup>*, *clr6<sup>+</sup>*, *rik1<sup>+</sup>*, and *swi6<sup>+</sup>*, like *hst4<sup>+</sup>*, are required for wild-type levels of silencing (Egel *et al.*, 1989; Lorentz *et al.*, 1992, 1994; Thon and Klar, 1992; Ekwall and Ruusala, 1994; Thon *et al.*, 1994; Allshire *et al.*, 1995; Grewal and Klar, 1997; Grewal *et al.*, 1998). These silencing genes fall into two classes based on additional phenotypes. Mutations in the first class include *clr1*, *clr2*, and *clr3*, which disrupt silencing but have no additional phenotypes. Mutations in the second class (*clr4*, *clr6*, *rik1*, and *swi6*) result in increased chromosome loss, linking their centromeric silencing defects with defects in centromeric function (Allshire *et al.*, 1995; Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). It has been proposed that Clr4p, Rik1p, and Swi6p interact directly or indirectly with microtubules at the kinetochore (Ekwall *et al.*, 1996). Mutants in all three genes are sensitive to the microtubule-destabilizing drug TBZ and show genetic interactions with the  $\beta$ -tubulin gene *nda3* (Ekwall *et al.*, 1996). Phenotypic analysis places *hst4<sup>+</sup>* in the second class of silencing genes because it is important for chromosome maintenance and is also sensitive

to TBZ. If these genes are required to maintain proper centromeric chromatin structure, minor alterations in chromatin context could result in silencing defects. More severe alterations could result in kinetochore defects and ultimately chromosome loss and reduced viability.

Several other phenotypes link *hst4<sup>+</sup>* with the second class of silencing genes. Both *clr6* and *hst4Δ* mutants are UV sensitive, a phenotype common in genes controlling the assembly of chromatin (Kaufman *et al.*, 1997; Grewal *et al.*, 1998). Furthermore, Hst4 protein localizes to the nucleolus, like overexpressed Clr4p (Sawin and Nurse, 1996; Ivanova *et al.*, 1998) and *S. cerevisiae* Sir2p (Gotta *et al.*, 1997). Because Hst4p also has diffuse nuclear localization outside the nucleolus, it is possible that it localizes to the silenced centromeres and telomeres as well. In *S. cerevisiae*, reporter genes placed within the rDNA repeats are silenced, and *SIR2* is required for this transcriptional silencing (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997). Reporter genes have not been integrated into *S. pombe* rDNA. However, by analogy to *S. cerevisiae*, it seems likely that this region may be silenced. *hst4<sup>+</sup>* and *clr4<sup>+</sup>* could play a role in silencing the rDNA locus in *S. pombe*.

Although *hst4<sup>+</sup>* shares many phenotypes with the second class of silencing genes in *S. pombe*, it also has distinct phenotypes. In particular, *hst4Δ* mutants have growth and morphology defects not seen in other silencing gene mutants. Furthermore, whereas *clr4*, *rik1*, and *swi6* mutants have chromosomes that lag on the mitotic spindle, severely fragmented DNA like that seen in *hst4Δ* mutants is not seen in *clr4*, *clr6*, *rik1*, or *swi6* mutants (Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). There are several possibilities to explain these similar but distinct phenotypes. Double mutants between *hst4Δ* and *clr1*, *clr2*, *clr3*, *clr4*, *rik1*, and *swi6* do not have dramatically enhanced silencing defects (our unpublished results), supporting the hypothesis that the genes act in the same pathway. However, the different phenotypes seen in each individual mutant suggest that *hst4<sup>+</sup>* function is not fully overlapping with that of the other genes but may converge on some of the same functions. This apparent contradiction may be explained if under certain circumstances or at some loci *hst4<sup>+</sup>* works together with the other silencing genes to maintain proper chromatin structure, whereas in other cases it works in a different pathway or in a different complex. For example, Sir2p is a shared member of multiple complexes that function at distinct loci in *S. cerevisiae* (Gotta *et al.*, 1997; Moazed *et al.*, 1997; San-Segundo and Roeder, 1999; Shou *et al.*, 1999; Straight *et al.*, 1999).

#### **Conserved HST Function from Bacteria to Humans**

The *SIR2* gene family is broadly conserved. Here we provide direct evidence that this gene family is functionally conserved as well, supporting the idea that *SIR*-like silencing mechanisms may be universally conserved. Our study further establishes a distinct role for members of this gene family in centromeric function hinted at by earlier chromosome-loss phenotypes in *S. cerevisiae* mutants (Brachmann *et al.*, 1995). Silencing of centromeric or centromere-like loci occurs in *S. pombe*, *D. melanogaster*, and bacteria, and a link between centromeric silencing and centromeric function also exists (Allshire *et al.*, 1994; Weiler and Wakimoto, 1995; Rodionov *et al.*, 1999). In *S. pombe*, mutations in *clr4*, *clr6*, *rik1*, *swi6*, and *hst4* disrupt centromeric silencing and result

in elevated chromosome-loss rates, indicating that centromeric function is perturbed (Allshire *et al.*, 1995; Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). In *D. melanogaster*, certain *Su-var3(6)* alleles that suppress position-effect variegation seen within centromeric heterochromatin also result in abnormal chromosome segregation (Baksa *et al.*, 1993). Indeed, alleles of *Su-var205*, the gene that encodes the heterochromatin-associated protein HP1, suppress repression of markers within centromeric heterochromatin and are also defective in chromosome segregation (Kellum and Alberts, 1995). Furthermore, the mitotic transmission of an unstable marker chromosome is modified by genes known to affect position-effect-variegation (Wines and Henikoff, 1992). Finally, even bacteria show this link between transcriptional silencing and chromosomal segregation functions. Partition modules on bacterial plasmids have a centromere-like role. They are required for proper segregation of the plasmid into daughter cells after replication. Genes placed within the partitioning module are silenced, and in mutants with decreased silencing, plasmid partitioning is defective (Rodionov *et al.*, 1999). Thus, the link between centromeric silencing and centromeric structure is preserved from prokaryotes to multicellular eukaryotes.

Our study links a member of the *SIR2* gene family with centromeric silencing and centromeric function. *hst4Δ* mutants have growth and morphology defects in addition to phenotypes that suggest a role for the gene in chromatin structure. The mutants have fragmented DNA, silencing defects, and chromosome-maintenance defects. Like other recently identified *S. pombe* silencing genes, *hst4<sup>+</sup>* appears to have a specialized function rather than a generic role at all silenced loci (see Nimmo *et al.*, 1998). It is possible that other *S. pombe* *SIR2* homologues will be found to function at other loci. Indeed, additional *S. pombe* homologues exist that may fulfill these roles (Freeman-Cook and Pillus, unpublished results; Derbyshire and Strathern, personal communication). The phenotypes of *S. pombe hst4Δ* mutants and the cognate *S. cerevisiae* mutants suggest a conserved chromosomal function for specialized *SIR2* homologues in other organisms, including humans.

The extent of divergent functions of the other conserved *SIR2/HST* genes will be important to define. For example, it has been suggested that a *Salmonella typhimurium* homologue may have phosphoribosyltransferase activity (Tsang and Escalante-Semerena, 1998). Indeed, in recent studies, recombinant *S. typhimurium* and *H. sapiens* proteins have been shown to have weak ADP ribosyltransferase activity *in vitro* (Frye, 1999). In *Leishmania major*, a homologue is reported to encode a cytoplasmic and secreted protein that may contribute to antigenicity and ultimately influence the host immune response to this parasite (Zemzoui *et al.*, 1998). In *C. albicans*, a *SIR2* homologue appears to influence switching between filamentous and colonial growth, an epigenetic program that is correlated with pathogenicity (Perez-Martin *et al.*, 1999). Finally, a new role for *SIR2* itself has been established with the observation that *sir2* mutants are defective in the pachytene checkpoint of meiosis (San-Segundo and Roeder, 1999). Although upon initial consideration these observations may seem perplexingly distinct, we consider it likely that an underlying mechanistic similarity exists for *SIR2/HST* gene function. Determining what that function may be, whether it is an enzymatic activity, a

structural role, or a more direct effect on transcriptional regulation, will be critical for ultimately understanding conserved mechanisms of silencing.

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