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Increase in Ty1 cDNA Recombination in Yeast *sir4* Mutant Strains at High Temperature

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ABSTRACT

Transposition of the Ty1 element of the yeast *Saccharomyces cerevisiae* is temperature sensitive. We have identified a null allele of the silent information regulator gene *SIR4* as a host mutant that allows for transposition at high temperature. We show that the apparent increase in transposition activity in *sir4* mutant strains at high temperature is dependent on the *RAD52* gene and is thus likely resulting from an increase in Ty1 cDNA recombination, rather than in IN-mediated integration. General cellular recombination is not increased at high temperature, suggesting that the increase in recombination at high temperature in *sir4* mutants is specific for Ty1 cDNA. Additionally, this high-temperature Ty1 recombination was found to be dependent on functional Sir2p and Sir3p. We speculate that the increase in recombination seen in *sir4* mutants at high temperature may be due to changes in chromatin structure or Ty1 interactions with chromosomal structures resulting in higher recombination rates.

GENOME integrity is critical to the viability of organisms. Both the organization of genetic elements within the genome and the actual code provided by the base order are important to the carefully regulated output of functional enzymes, structural proteins, and nucleic acids. There are many processes that work against genomic stability, which are necessarily highly regulated to protect the integrity of the genome. One example is transposable elements. The insertion of a transposable element can lead to a selective advantage brought on by a change in gene product or gene regulation. Conversely, the proliferation of these elements in the genome of an organism can lead to several problems, including the disruption of essential genes or removal of a portion of the genome by recombination between two integrated elements. The instability brought on by the presence of these transposable elements, therefore, must be regulated by the organism to minimize the damaging effects.

The yeast *Saccharomyces cerevisiae* is host to several families of Ty transposable elements. Ty1 consists of two long terminal repeats (LTRs) flanking a region of DNA that encodes the structural proteins and enzymes necessary for replication and integration (Voytas and Boeke 2002). The open reading frames (ORFs) encode Gag and Pol, functionally similar to Gag and Pol of retroviruses. Gag encodes structural proteins and Pol encodes the enzymes integrase (IN), reverse transcriptase (RT), and protease (PR). Transposition begins with the transcription and translation of the element by the host cell machinery. The Gag and Pol regions are translated as polyproteins, which are subsequently processed by PR. Virus-like particles (VLPs) are likely assembled from unprocessed Gag and Gag-Pol and enclose the Ty1 mRNA, primer, PR, RT, and IN. Within these VLPs, reverse transcription takes place, synthesizing the cDNA that is then integrated into the host genome via an IN-mediated integration reaction. Alternatively, cDNA sequences resulting from reverse transcription may be recombined with chromosomal Ty1 sequences in a *RAD52*-dependent recombination/repair process. The life cycle of a retrotransposon does not include an infectious extracellular stage comparable to that of retroviruses.

In most common lab strains of *S. cerevisiae*, ~30 copies of the Ty1 element are present. Studies have shown that Ty1 RNA is present as 0.1–0.8% of the total RNA, yet processed Ty1 proteins are difficult to detect when uninduced (Curcio and Garfinkel 1991a). Transposition events, therefore, normally occur at very low levels, $\sim 10^{-5}$–10^{-7} events per generation (Curcio and Garfinkel 1991b). Overexpression of a plasmid Ty1 element by induction with a galactose promoter (*GAL1*), however, shows a dramatic increase in the appearance of transposition events (10,000–28,000-fold higher) with compara-

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tively little increase in Ty1 RNA and protein levels (220- to 225-fold higher; Curcio and Garfinkel 1992). Galactose-induced Ty1 transposition, therefore, overcomes the "transpositional dormancy" that normally characterizes endogenous Ty1 elements and does so by improving the efficiency of Ty1 RNA and protein use, rather than by simply increasing the amounts of these products. This indicates that the transpositional dormancy of endogenous Ty1 elements is most likely regulated by post-translational mechanisms.

There are numerous examples of endogenous host genes involved at multiple levels to limit transpositional activity. Transcriptional expression of endogenous elements has been shown to vary over a 50-fold range, and Ty1 elements located within the rDNA region are silenced (Bryk *et al.* 1997; Morillon *et al.* 2002). Activation of the invasive-filamentous pathway in diploid cells induces Ty1 transcription (Morillon *et al.* 2000). Protein processing has been shown to increase with galactose induction of Ty1, suggesting that PR may not be adequately activated without GAL induction (Curcio and Garfinkel 1992). The nucleotide excision repair/transcription factor IIH (NER/TFIIH) complex inhibits Ty1 cDNA accumulation and *FUS3* destabilizes VLP-associated proteins (Lee *et al.* 1998; Curcio and Garfinkel 1999; Conte and Curcio 2000). Chromatin assembly factor I and histone regulatory proteins maintain a chromatin structure that prevents integration of Ty1 cDNA and mutations in the *RAD52* epistasis group demonstrate increased transposition and a marked increase in the level of Ty1 cDNA (Qian *et al.* 1998; Rattray *et al.* 2000). The nature of these interactions indicates that cellular processes affecting repair and differentiation control levels of transposition in response to environmental signals (Curcio and Garfinkel 1999).

Indeed, Ty1 transposition is regulated by changes in the temperature of the environment. Transposition is optimal at ~24–28°. Increases in temperature to 33–35° decrease transposition to undetectable levels (Paquin and Williamson 1984; Lawler *et al.* 2002). We have previously shown that at high temperatures the Ty1 polyproteins are not cleaved, indicating that PR is no longer active (Lawler *et al.* 2002). In this study, we describe a genetic screen used to identify mutations in host genes that allow transposition at high temperature. Following chemical mutagenesis, yeast cells were plated and colonies subsequently identified for transposition at 37°. Several mutant strains were isolated, which demonstrate higher transposition levels than the isogenic wild-type strain at temperatures >34°. We have named the mutants *htt*, for high-temperature transposition. In this study we describe the cloning and identification of one of the mutants as *sir4*. *SIR4* is a silent information regulator gene implicated in many cellular processes, including cell aging and chromatin silencing, and has previously been shown to regulate integration of the Ty5 retroelement of yeast (Zhu *et al.* 1999; Gartenberg 2000; Gasser and Cockell 2001; Xie *et al.* 2001). The results presented here are the first implication of *SIR4* regulation of Ty1.

MATERIALS AND METHODS

Yeast strains and plasmids: The yeast strains and plasmids used in this study are given in Table 1. Yeast strains were maintained according to standard methods, and media prepared as previously described (Rose *et al.* 1990).

Mutagenesis screening: YH23 cells containing plasmid pX3 were mutagenized with ethyl methanesulfonate (EMS), plated onto SC-Ura-Trp medium, and grown at 22° for 5 days. Colonies were then replica plated to galactose medium prewarmed to 36° and incubated for 3 days. Cells were then replica plated to YPD plates, incubated 1 day at 36°, and finally were replica plated to SC-Trp medium containing 5-fluoroorotic acid (5-FOA). Medium containing 5-FOA inhibits the growth of Ura^+ cells, so that only cells that have lost the Ty1 plasmid will grow (Boeke *et al.* 1984).

***TRP1* strain construction:** The analysis of transposition in spores of mutant crosses was found to be complicated by the genetics of the *TRP1* locus. Strain YPH645 contains a wild-type *TRP1* locus. The *trp1Δ1* mutation, which is present in our mutant strains, removes a portion of the upstream activating sequence (UAS) of the *GAL3* gene, an inducer of the galactose catabolic pathway. As a result, the level of galactose-induced transposition in *trp1Δ1* strains is slightly reduced. At high temperature, the difference in galactose induction between *TRP1* and *trp1Δ1* strains is even more pronounced, such that the level of transposition in *TRP1* stains is significantly greater than that in strains harboring *trp1Δ1*. To make the high-temperature phenotype stronger, the *trp1Δ1* locus in each of the *htt* mutant strains was replaced with the wild-type *TRP1* sequence. The Trp^+ strains JKc1005 and JKc1015 were constructed by transforming strains JKc125 and YH8, respectively, with a 2697-bp PCR fragment derived from a *TRP1* strain. The fragment contains the deleted *TRP1* region, ~700 bp 5′ flanking sequence, and 545 bp 3′ flanking sequence. PCR was used to confirm that the *TRP1* locus was properly regenerated. Primer sequences for PCR were 5′-TTTTAAAGCGGCTGCTTGAG-3′ (forward primer) and 5′-AAGGCAGATCAAACTTTCGC-3′ (reverse primer).

Library transformation and cloning: Yeast strain JKc1005 was transformed with a p366 LEU2 CEN library obtained from American Type Culture Collection, Rockville, Maryland (Schiestl and Gietz 1989). Approximately 1.7×10^5 transformants were selected on SC-Leu and screened for restoration of mating. For each transformation plate, a lawn consisting of a *MAT***a** *his1* strain was replica plated to YPD, followed by a replica plating of a transformation plate to the same YPD plate. The YPD plates were incubated at 30° for 5 hr, allowing sufficient time for mating to occur. Cells were then replica plated to minimal media and incubated at 30° until colonies appeared. Positive colonies from the original transformation plates were tested for the loss of high-temperature transposition.

Sequencing: The *LEU2* library plasmid was rescued into *Escherichia coli* strain MC1666 (*pyrF trpC LeuB*) and sequenced using primer JK048 (5′-CACTATCGACTACGCGATCA-3′). To determine the location of the cloned DNA, the resulting sequence was compared to the yeast genome using BLASTN on the Saccharomyces Genome Database website (http://genome-www.stanford.edu/Saccharomyces/). To identify the mutant *sir4* allele, genomic DNA was isolated from strain JKc1003. The *sir4* locus was amplified by PCR in two ~2-kb halves using primers JK125/JK126 (5′-AAAAAAGGAAGCTTCAACCCAC-3′/5′-TCAGTTAGGCTATCATTATCTGAAGA-3′) and JK127/

TABLE 1

Yeast strains and plasmids

Strain	Genotype	Plasmid	Strain background	Source/parent strain
YH23	*MAT*α Δ *ura3-167 his3Δ200 leu2Δ1 trp1Δ1*	pX3	GRF167	Jef Boeke/YH8
JB721	*MAT***a** *his3Δ200 ura3-167*			
JKc125	*MAT*α *ura3-167 his3Δ200 leu2Δ1 trp1Δ1 sir4-119*	pGTy1H3m*his3AI*	GRF167	This study
YPH645	*MAT***a** *ura3-52 his3Δ200*	none	S288C	Philip Heiter
JKc1005	*MAT*α *ura3-167 his3Δ200 leu2Δ1 sir4-119*	pGTy1H3m*his3AI*	GRF167	This study
JKc1015	*MAT*α *ura3-167 his3Δ200 leu2Δ1*	pGTy1H3m*his3AI*	GRF167	This study
JKc1065	*MAT***a** *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir3Δ::kanMX4 sir4Δ::HIS3*	pX3	GRF167	Jeff Smith/JS331 × JS3A-89
JKc1066	*MAT*α *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir2Δ::HIS3*	pX3	GRF167	Jeff Smith/JS218 A, B
JKc1067	*MAT*α *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir3Δ::HIS3*	pX3	GRF167	Jeff Smith/JS244 A, B
JKc1068	*MAT***a** *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir2Δ::kanMX4 sir4Δ::HIS3*	pX3	GRF167	Jeff Smith/JS346
JKc1072	*MAT*α *ade2-1 can1-100 his 3-11,15 leu2-3 trp1-1 ura3-1*	pGTy1H3m*his3AI*	W303	Alan Myers/W303-1B
JKc1075	W303-1B *ADE2 lys2Δ sir4::LEU2*	pGTy1H3m*his3AI*	W303	Jasper Rine/ JRY4582
JKc1159	JKc1015 *fus3::kanMX4*	pGTy1H3m*his3AI*	GRF167	This study
JKc1160	JKc1005 *fus3::kanMX4*	pGTy1H3m*his3AI*	GRF167	This study
JKc1161	JKc1015 *spl2::kanMX4*	pGTy1H3m*his3AI*	GRF167	This study
JKc1162	JKc1005 *spl2::kanMX4*	pGTy1H3m*his3AI*	GRF167	This study
JKc1163	*MAT*α *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15*	pX3	GRF167	Jeff Smith/JS209
JKc1167	JKc1005 *rad52::LEU2*	pGTy1H3m*his3AI*	GRF167	This study
JKc1168	JKc1015 *rad52::LEU2*	pGTy1H3m*his3AI*	GRF167	This study
JKc1190	*MAT*α *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir4Δ::HIS3*	pX3	GRF167	Jeff Smith/JS329
JKc1195	JKc1005	pJEF1105	GRF167	This study
JKc1196	JKc1015	pJEF1105	GRF167	This study
JKc1223	JKc1005 *hmr::LEU2*	pGTy1H3m*his3AI*	GRF167	This study
JKc1241	JKc1015 *hmr::kanMX4*	pGTy1H3m*his3AI*	GRF167	This study

Plasmid name	Features	Source
pX3	*TRP1*-marked Gal-Ty1, 2μ *URA3*	
pGTy1H3m*his3AI*	*his3mAI*-marked Gal-Ty1, 2μ *URA3*	Curcio and Garfinkel (1991b)
pSM20	*rad52::LEU2*	L. Prakash and S. Prakash
pJEF1105	*neo*-marked Gal-Ty1, 2μ *URA3*	Boeke *et al.* (1988)
pCS1	*FUS3, LEU2* 2μ	This study
pJK592	*LEU2 HIS3* CEN	This study

128 (5′-CCTTTCAATAAAAGTGAAAGCAAACC-3′/5′-AGAAA AACAGGGTACACTTCGTTAC-3′). The resulting PCR products were cloned into the vector pCR2.1-TOPO vector by TOPO cloning (Invitrogen, Carlsbad, CA). DNA was prepared from resulting clones and the two halves of the *sir4-119* allele were sequenced using the following primers: JK125, JK111 (5′-ACT CATTTTTATCAGGAG-3′); JK112 (5′-TAACATCAAAGAAG ATCG-3′); JK113 (5′-GAATCCCACATTGATTCG-3′); JK127, JK114 (5′-AGCCAATTTTTTGGAAAC-3′); and JK115 (5′-ACC TTATTGAACAAGGGA-3′).

***rad52* strain construction:** Plasmid pSM20, containing the *rad52::LEU2* allele, was digested with *Bam*HI; digestion was confirmed by gel electrophoresis, yielding two bands: 5 and 4.6 kb. The remaining digest was transformed into yeast (Schiestl and Gietz 1989), and cells were plated to SC-Leu medium to select for integration of the disrupted *rad52* allele. Isolated transformants were confirmed by UV-induced growth sensitivity as compared to the isogenic *RAD52* parent.

***FUS3* plasmid construction and *spl2::kanMX4* and *fus3::kanMX4* strain construction:** Plasmid pCS1 was constructed by cloning an ~1 kbp *Not*I/*Hin*dIII fragment containing *FUS3* from pJL70 (kindly provided by Joe Lawler) into the same sites of pRS425 (Sikorski and Hieter 1989). The *spl2::kanMX4* and *fus3::kanMX4* alleles were PCR amplified using genomic DNA from Research Genetics (Birmingham, AL) strains 1964 and 3042, respectively. The primer pair used for *spl2::kanMX4* was JK123/JK124 (5′-TTCCTACCCCAATGATGGTT-3′/5′-GTGGCGGTCATCGA AGAT-3′) and the primer pair used for *fus3::kanMX4* was JK121/ JK122 (5′-CGTTCAAAAGAACATACATAAGGA-3′/5′-CACAAG ACAAAAGAAGGGGTAG-3′). The resulting PCR product from each strain was then transformed into strains JKc1005 and JKc1015. Following transformation, the cells were washed in

water and then resuspended in 1 ml YPD medium. Cells were grown for ~8 hr, at 30°, with shaking, to allow for expression of the *kanMX4* gene. Cells were then plated to YPD containing 75 ug/ml G418. Replacement of the wild-type allele was confirmed by PCR of genomic DNA from G418-resistant colonies.

Transposition assays: For patch assays using plasmids pGTy1H3m*his3AI* or pX3, strains were grown as ~12 × 12-mm patches on SC-Ura medium to maintain plasmid selection. Cells were then replica plated to galactose medium and incubated at the appropriate temperature for 24–48 hr to induce transposition. Following galactose induction patches were printed to SC-His medium for pGTy1H3m*his3AI* assays or to YPD medium, followed by replica plating to 5-FOA and finally to SC-Trp for pX3 assays. For quantitative assays using plasmid pGTy1H3m*his3AI*, strains were initially grown as ~12 × 12-mm patches on SC-Ura medium to maintain plasmid selection. Cells were then replica plated to galactose medium and incubated at the appropriate temperature for 44–48 hr to induce transposition. Following galactose induction patches were printed to SC-His medium. The cells remaining on the galactose plates were transferred to 10 ml sterile water (dilution 1). A total of 50 μl of this dilution was transferred to 5 ml sterile water (dilution 2). A total of 100 μl of dilution 1 was plated to SC-His medium, except for temperatures >32°, in which case cells in dilution 1 were pelleted, resuspended in ~200 μl water, and plated to SC-His. A total of 50 μl of dilution 2 was plated to YPD. Following incubation at 30° (YPD 2 days; SC-His 4 days) resulting colonies were counted. Transposition frequency was calculated by dividing the number of colonies on SC-Hisby the total number of cells plated on SC-His, as determined by the colony number on YPD, factoring in the dilution and original volume of dilution 1 plated.

Cell homogenates: The cell growth procedure was based on a protocol previously described (Merkulov *et al.* 1996). Specifically, cultures were grown at either 22° or 37°. The starting density was A_{600} of ~0.2 and cells were collected when the density reached an A_{600} of ~2 (~12 hr at 37° and ~36 hr at 22°). Aliquots (1.5 ml) of the culture were collected by centrifugation, resuspended in 40 μl of buffer B [10 mm *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)/KOH, pH 8.0, 15 mm KCl, 5 mm EDTA], and frozen at −75°. Aliquots were thawed on ice and the total volume was brought to 200 μl with buffer B. Cold glass beads were added to the meniscus. A total of 40 μl of 100% trichloroacetic acid (TCA) was added and the samples were vortexed at top speed for 4 min. Samples were placed immediately on ice, and 1 ml ice-cold 5% TCA was added. Samples were spun for 20 min, 14,000 × *g*, in the cold. The liquid was aspirated and the pellet was resuspended in 1 ml cold water. Samples were spun for 10 min as before and the supernatant was aspirated. Proteins were extracted by resuspending the pellet in 150 μl sample buffer (6% SDS, 0.5 m Tris base) and incubating at 50° for 10 min. Samples were spun for 1 min (14,000 × *g*) and the supernatant was removed to a fresh tube. The extraction process was repeated and the supernatants were pooled. One-third volume of a solution of 0.25 m DTT, 50% glycerol, and 0.2% bromphenol blue was added and samples were spun (14,000 × *g*) for 3 min. The supernatant was transferred to a fresh tube.

VLP preparation: Cells were grown and lysed as described (Eichinger and Boeke 1988). Extract (7.5 ml) was loaded onto a 70/30/20 (5/5/15 ml) sucrose step gradient and centrifuged for 180 min at 28,000 rpm in a Sorvall AH629 swinging bucket rotor. The remaining extract was saved for use as whole-cell extract. Fractions were collected by puncturing the bottom of the tube and collecting 1.2-ml fractions. To pellet VLPs, peak fractions 4, 5, and 6 were pooled, diluted to 11 ml with buffer B, and pelleted for 1 hr, at 35,000 rpm in a Sorvall A1256 fixed-angle rotor. The pellet was resuspended in 150 μl buffer B.

Immunoblotting: Whole-cell extracts and purified VLPs were mixed with an equal volume of 2× sample loading buffer (20% v/v glycerol, 0.125 m Tris-Cl pH 6.8, 5% w/v SDS, 10% v/v 14 m β-mercaptoethanol, 0.2% w/v bromphenol blue) and boiled (3 min) prior to loading on 10% (Gag blots) or 7.5% (Pol blots) SDS gel. Gels were transferred to nitrocellulose (for Gag blots) or polyvinyl difluoride (PVDF) membrane (for Pol blots) in Tris-glycine buffer containing 10% methanol at 24 V for 1 hr. Membranes were blocked in PBS containing 5% nonfat dried milk. Membranes were then probed with antibody as described (Merkulov *et al.* 2001). Antibody binding was detected using the appropriate secondary antibody followed by ECL (nitrocellulose) or ECL-Plus (PVDF) reagent and exposed to X-ray film. Anti-Gag (anti-VLP polyclonal serum R2-F) and anti-IN (8B-11 monoclonal antibody) are described elsewhere (Eichinger and Boeke 1988; Monokian *et al.* 1994).

cDNA Southern blotting: Cells were grown as described (Eichinger and Boeke 1988), except that galactose induction was at various temperatures. Cells from 10 ml of galactose-induced culture were frozen and genomic DNA was isolated as described (Keeney and Boeke 1994). A total of 10 ul of DNA was digested in a 40-ul reaction containing 1 ul RNase (10 mg/ml). Samples were electrophoretically separated (0.75% agarose gel), transferred to Gene Screen, and hybridized to a 520-bp *Xho*I-*Hin*dIII *neo* probe from pJEF1105 labeled with the Amersham (Arlington Heights, IL) Bioscience Rediprime II random prime labeling system. The membrane was washed twice for 5 min in 2× SSC at room temperature and once for 30 min in 2× SSC, 0.1% SDS at 65°, and exposed on the Kodak Image Station 2000R using the rad PADD (high signal capture).

Recombination assays: The plasmid pJK592 (*LEU2*, *HIS3*, *CEN*) was constructed by cloning a 1145-bp *Pst*I/*Bam*HI *HIS3* fragment from pJJ217 into the same sites of pRS415 (Sikorski and Hieter 1989; Jones and Prakash 1990). Undigested plasmid or plasmid digested at the *Nde*I site within the *HIS3* ORF was transformed as described (Schiestl and Gietz 1989). For cotransformation of linear DNA, a 345-bp PCR product containing *HIS3* sequences flanking the *Nde*I cut site was generated using oligonucleotides JK177 (5′-CAGAAAGCCCTAG TAAAGCGT-3′) and JK178 (5′-TCCAAACCTTTTTACTCC ACG-3′) on *Bam*HI-digested pJJ217 plasmid template DNA. Transformants were selected on SC-leucine medium. Integrity of the *HIS3* ORF was assessed by replica plating to SC-histidine medium.

RESULTS

Screening for *htt* mutants: A genetic screen was used to identify mutant genes that can restore Ty1 transposition at high temperature. Yeast cells containing a galactose-inducible Ty1 element on plasmid pX3 were mutagenized with EMS. Following mutagenesis, yeast cells were screened for transposition at 36° as described in materials and methods. Colonies that appeared to have increased transposition over nonmutagenized control colonies were selected for further analysis. Corresponding colonies on the original glucose plates were reassayed for transposition and were also screened for integration of the Ty1 plasmid as identified by growth on selective medium without galactose induction. The Ty1 plasmid was removed from positive clones and replaced with fresh plasmid. Replacement of the Ty plas-

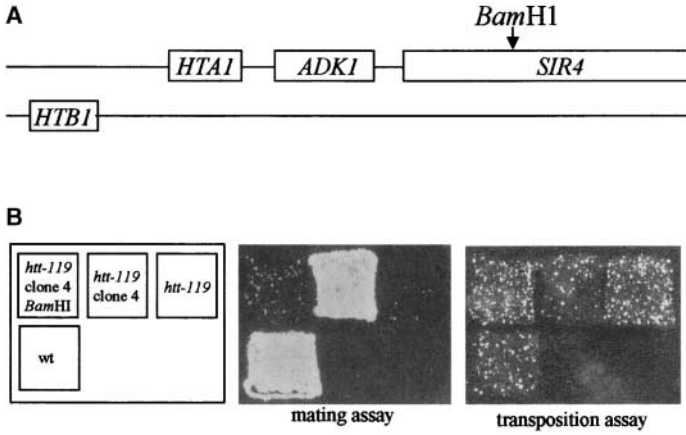


FIGURE 1.—(A) Region of chromosome IV contained on the rescued *LEU2* library plasmid. Complete ORFs contained on the library clone are given by gene name. The approximate location of the unique *Bam*HI site within the *SIR4* ORF is shown. (B) Mating and transposition assays identify *htt-119* as *sir4*. A schematic indicating the strains used is shown on the left. The middle shows a mating assay and the right side is a high-temperature transposition assay, using plasmid pGTy1H3m*his3AI*. Clone 4 in the *htt-119* strain rescues mating and negates the *htt* phenotype (middle patch, top row). Disruption of the *SIR4* ORF by filling in the *Bam*HI site (indicated in A) reduces mating and restores the *htt* phenotype (left-hand patch, top row).

mid present during the mutagenesis with a new plasmid assured us that the mutation of interest was present in the host genome and not on the Ty plasmid. Following a secondary screening, four potential clones remained that consistently demonstrated significant levels of transposition at 35.5°–36° as compared to the wild-type strain. We have named the putative mutant loci *htt*, for high-temperature transposition.

The mutants were crossed with an otherwise isogenic *MAT***a** strain (JB721) to determine if the *htt* mutations were recessive or dominant and contained within a single-gene locus. Unfortunately, the strains were found to have a very poor sporulation frequency. The mutant strains were therefore crossed to the nonisogenic strain YPH645, and at least six complete tetrads were obtained for each mutant. All of the mutants were confirmed to be recessive, single-gene mutations and showed comparable levels of high-temperature transposition.

Cloning of *htt119 (sir4)*: During tetrad analysis, the *htt119* mutant was found to have a mating defect that segregated with the *htt* phenotype. Thus, the mutant gene was cloned via complementation of the mating defect by transformation of a wild-type *LEU2* genomic library. Of seven putative positive clones, loss of the *LEU2* library plasmid resulted in mating deficiency and restoration of the *htt* phenotype in all seven clones, indicating that a gene located on the library plasmid was likely responsible for restoration of mating and for repression of the high-temperature transposition phenotype. Rescue of the library plasmid and retransformation into the original *htt-119* strain revealed that the restoration of mating and repression of the high-temperature transposition phenotype were indeed associated with the presence of the plasmid and not a chromosomal entity. Each of the rescued plasmids had a similar restriction enzyme digest profile (data not shown).

Sequencing of one of the rescued plasmids revealed genomic sequence from chromosome IV containing five ORFs (Figure 1A). *SIR4* was the most likely candidate gene because *sir4* mutants are known to be defective in mating (HERSKOWITZ *et al.* 1992). A unique *Bam*HI site in the plasmid located within the *SIR4*-coding region was filled in to create a frameshift mutation, and the resulting plasmid was transformed into the *htt119* strain. The high-temperature transposition phenotype was maintained (and mating was not restored), indicating that *sir4* is indeed the mutated host gene responsible for the high-temperature transposition phenotype in *htt-119* (Figure 1B). We refer to our mutant allele as *sir4-119*.

Genomic PCR products obtained from two halves of the *sir4-119* allele were cloned and sequenced. Sequencing of two independent clones revealed a nonsense mutation of CAA to TAA (GLN to STOP) at amino acid 148 of the 1359 residues in the *SIR4* protein. The *sir4-119* allele is therefore essentially a null allele.

Transposition activity is increased in *sir4* mutant strains at high temperature: Transposition was quantitated in two distinct strain backgrounds containing *sir4* mutant alleles, using plasmid pGTy1H3m*his3AI*, a galactose-inducible element containing a reverse-oriented intron-disrupted *his3* marker gene (Figure 2; CURCIO and GARFINKEL 1991b). The intron is spliced following transcription, and subsequent reverse transcription generates Ty1 cDNA containing a functional *HIS3* marker gene. The results are shown in Figure 3. Transposition in *sir4* strains at high temperature is increased ~30-fold over that in wild-type strains. Two distinct *sir4* null alleles, *sir4*Δ and *sir4-119*, showed high-temperature transposition in two strain backgrounds, indicating that the effect is not strain or allele dependent. As shown previously, the GRF167 strain has a higher rate of transposition than W303 (LAWLER *et al.* 2002). Interestingly, in the W303 strain background, the *sir4* mutant demonstrates increased levels of transposition at all temperatures.

Processing of Ty1 proteins in *sir4* strains: During galactose induction, Ty1 produces a 49-kD Gag protein and lesser amounts of a 199-kD Gag-Pol fusion protein. The fusion protein contains the Gag structural protein,

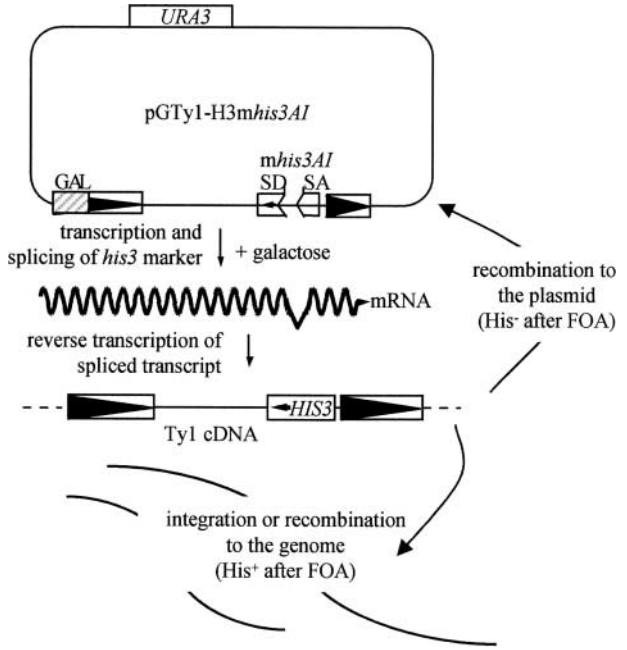


FIGURE 2.—Schematic of the transposition assay used to quantitate transposition. The pGTy1-H3m*his3AI* plasmid, containing the m*his3AI* marker gene, is diagrammed at the top (CURCIO and GARFINKEL 1991b). Solid triangles represent LTR sequences. The *HIS3* gene is inserted into Ty1 in the reverse orientation of Ty1 transcription. Inserted into the *HIS3* coding region is an artificial intron (AI) with splice donor (SD) and acceptor (SA) sites in the same transcriptional orientation as Ty1; this gene is termed m*his3AI*. The m*his3AI* gene carries a *HIS3* promoter but produces an inactive protein due to the inserted intron. Induction of Ty1 transcription in galactose-containing medium produces a Ty1 mRNA containing an intron, which is subsequently spliced out. This message now contains, at the 3′ end, anti-sense message for *HIS3*. If this message is successfully reverse transcribed and integrated into the genome, a functional *HIS3* gene is formed. IN-mediated integration of Ty1 cDNA into the host genome results in a true transposition event. Alternatively, the Ty1 cDNA can be homologously recombined, in a *RAD52*-dependent process, to *HIS3* or Ty1 sequences on the plasmid or in the host genome.

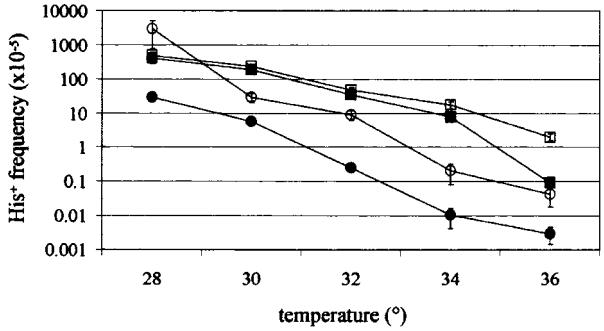


FIGURE 3.—*sir4* mutant strains show increased transposition at high temperature. Wild-type and *sir4* mutant strains containing a galactose-inducible Ty1 element on plasmid pGTy1m*his3AI* were quantitated for His^+ cell formation following galactose induction at the indicated temperatures. Points represent the average His^+ frequency of three patches and error bars indicate the standard deviation. Strains are as follows: GRF167, solid squares (JKc1015); GRF167 *sir4-119*, open squares (JKc1005); W303, solid circles (JKc1072); W303 *sir4::LEU2*, open circles (JKc 1075). Error bars are not visible for some points, as the bars are smaller than the symbols.

as well as the enzymes PR, IN, and RT/RNaseH. PR subsequently processes both the Gag and Gag-Pol proteins. We have previously shown that processing of the Gag-Pol polyprotein is defective at high temperature (LAWLER *et al.* 2002); therefore we looked at Ty1 processing in our *sir4-119* strain. Cell homogenates from wild-type (JKc1015) and *sir4-119* mutant (JKc1005) galactose-induced cells grown at 22° and 37° were immunoblotted using monoclonal antibody 8B-11 to the integrase protein. This anti-IN monoclonal antibody detects processed IN (Pol-p71; apparent molecular mass, ~90 kD) as well as the full-length Gag-Pol-p199 polyprotein and any processing intermediates (Figure 4). As expected, the amount of Ty1 protein detected at high temperature is greatly reduced as compared to permissive temperature (Figure 4A, lanes 1 and 2 *vs.* lanes 3 and 4). In comparison to the *SIR4* strain, cell homogenates derived from the *sir4-119* strain at high temperature show increased levels of mature IN protein (Figure 4A, lane 3 *vs.* lane 4). We also looked at production and processing of integrase by immunoblotting sucrose gradient-purified VLPs, as well as whole-cell extract (Figure 4B). In whole-cell extract at high temperature, the amount of IN protein detected in the *sir4-119* strain is slightly greater than that found in the wild-type strain (Figure 4B, lane 6 *vs.* lane 8). The Gag-Pol polyprotein is not detected in the whole-cell extracts. In VLPs purified from cells induced at high temperature, the amount of Gag-Pol polyprotein and higher-molecular-weight intermediates in the *sir4-119* VLPs are reduced as compared to wild type, while the amount of the lower-molecular-weight intermediates is increased (Figure 4B, lane 10 *vs.* lane 12). The mature IN protein in the *sir4-119* mutant strain at high temperature is slightly increased as compared to wild type in whole-cell extracts, and is less so in purified VLPs. Although protein processing is dramatically reduced at high temperature, these results suggest that both production and processing of the Ty1 Gag-Pol polyprotein are slightly increased in the *sir4-119* strain, as compared to wild type, at high temperature.

High-temperature transposition is not due to altered expression of specific protein kinases: Microarray analysis has been done of the expression comparison between *SIR4* and a *sir4* null mutant (WYRICK *et al.* 1999; http://staffa.wi.mit.edu/cgi-bin/young_public/factor.cgi?gene=SIR4&s=2). In a *sir4* deletion strain, the expression of the protein kinase *FUS3* is reduced 60-fold. *FUS3* is an interesting possibility for implication in high-temperature transposition because studies have shown that *FUS3* is involved in the regulation of transpositional dormancy through the destabilization of Ty1 proteins (CONTE *et al.* 1998; CONTE and CURCIO 2000). The high-temperature

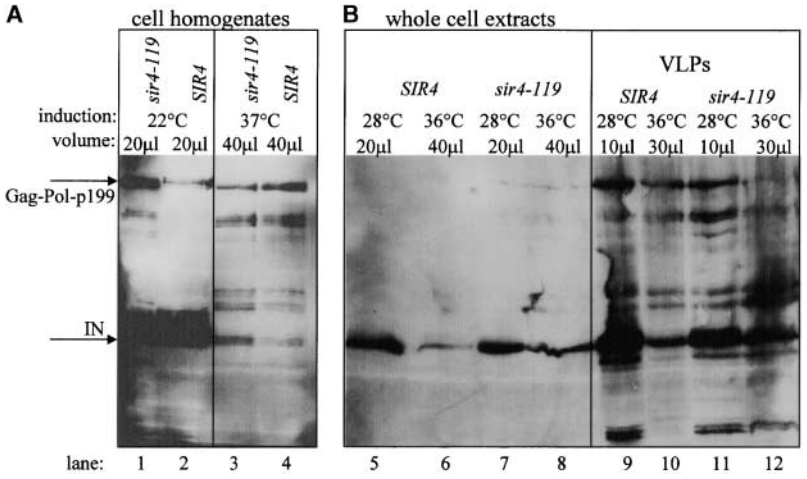


FIGURE 4.—Processing of Ty1 Pol proteins in wild-type and *sir4* strains. (A) Immunoblots of cellular homogenates from galactose-induced yeast cultures (JKc1015 and JKc1005) probed with anti-IN. The *SIR4* genotype of the strain used is indicated above the lanes and induction refers to the temperature of galactose induction. Cultures were normalized by cell density and the same volume of cells was processed for each sample. Volume indicates what volume of the final protein sample was loaded onto the gel. Note that increased volume was loaded from 36° samples to get a visible signal. The lanes are numbered at the bottom. The Gag-Pol-p199 polyprotein and IN (Pol-p71) are indicated. (B) As in A, using samples from whole-cell extract and purified VLPs.

transposition phenotype could be a result of reduced *FUS3* expression in a *sir4* mutant, resulting in stabilization of Ty1 proteins. If so, deletion of *fus3* would be expected to yield the high-temperature transposition phenotype. Conversely, overexpression of Fus3p would be expected to suppress the htt phenotype. *fus3* and *fus3 sir4-119* mutant strains were generated by direct replacement of the *fus3* ORF with *kanMX4* in our *sir4-119* strain and the isogenic wild type. *fus3* deletion does not confer the htt phenotype, nor does the deletion of *fus3* in the *sir4-119* strain enhance the phenotype. For overexpression, a high-copy plasmid containing the *FUS3* gene was transformed into isogenic wild-type and *sir4-119* strains. This plasmid had no effect on the high-temperature transposition phenotype (data not shown).

Microarray data also revealed that expression of *SPL2*, which encodes a protein with similarity to cyclin-dependent kinase inhibitors, is increased 100-fold in the absence of *sir4*. If high-temperature transposition is due to Sir4p-mediated upregulation of this gene, then deletion of *SPL2* would be expected to suppress high-temperature transposition. Isogenic *spl2* and *spl2 sir4-119* strains were generated by direct replacement of the *spl2* ORF with *kanMX4*. The high-temperature phenotype is maintained in the *spl2 sir4-119* strains and *spl2* deletion alone has no effect on transposition as compared to the wild-type control (data not shown). These results indicate that neither *fus3* nor *spl2* is involved in the *sir4*-mediated increase in transposition activity at high temperature.

Ty1 cDNA production is not increased by high temperature or *sir4* deletion: We have previously shown that the level of cDNA production decreases as temperature increases (LAWLER *et al.* 2002). We therefore assessed the level of Ty1 cDNA in whole-cell extracts of galactose-induced wild-type and *sir4* mutant cells at increasing temperatures (Figure 5). The decrease in Ty1 cDNA with increasing temperature is the same in wild-type and *sir4* strains. Thus, the increase in transposition in *sir4* mutant strains is not due to an increase in Ty1 cDNA production.

Increased transposition at high temperature in *sir4* mutants is *RAD52* dependent: The process of transposition creates free DNA ends, in the form of Ty1 cDNAs, which are normally bound by integrase enzyme and subsequently integrated into the chromosome. However, ends not properly bound by integrase are likely to be dealt with by the cellular recombination pathways by being degraded or integrated into chromosomal DNA. In yeast, homologous recombination (HR) is the

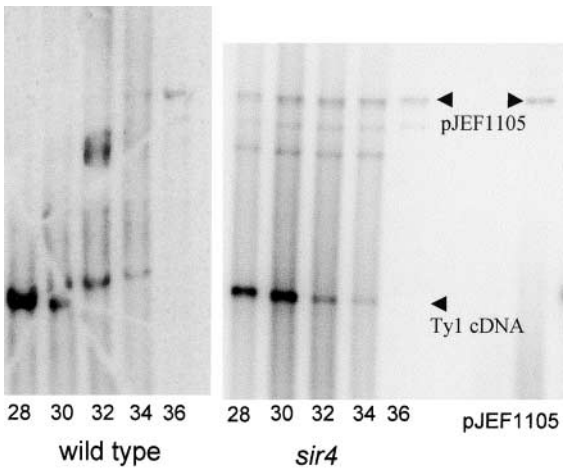


FIGURE 5.—Ty1 cDNA synthesis as a function of temperature. Wild-type (JKc1196, Table 1) and mutant (JKc1195, Table 1) cells containing a galactose-inducible Ty1 element were grown at the indicated temperatures. Southern blot analysis of extracted DNA indicates that production of the 3.5-kbp Ty1 cDNA product decreases as temperature increases and is undetectable at 36° in both the wild-type and sir4 mutant strains. Nucleic acids were extracted, digested with *Eco*RI, RNase treated, electrophoretically separated (1% agarose gel), and transferred. The numbers below the lanes indicate the temperature (°) at which cells were galactose induced. The lane labeled pJEF1105 contains *Eco*RI-digested Ty1 plasmid DNA, indicated by the larger ~10.0-kbp band. The membrane was probed with a ^{32}P-labeled *neo* cDNA probe.

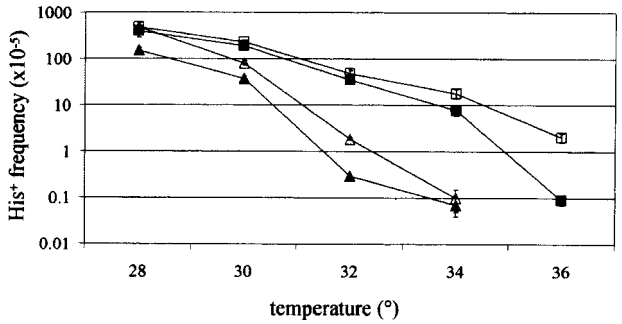


FIGURE 6.—The high-temperature transposition phenotype is *RAD52* dependent. Wild type, *rad52*, and *sir4* mutant strains containing a galactose-inducible Ty1 element on plasmid pGTy1H3m*his3AI* were quantitated for frequency of His^+ cell formation following galactose induction at the indicated temperatures. Points represent the average frequency of His^+ cell formation of three patches, and error bars indicate the standard deviation. Strains are as follows: wild type, solid squares (JKc1015 *SIR4 RAD52*); *sir4*, open squares (JKc1005 *sir4-119 RAD52*); *rad52*, solid triangles (JKc1168 *SIR4 rad52*); *sir4 rad52*, open triangles (JKc1167 *sir4-119 rad52*). The frequency of His^+ cell formation in the *rad52* mutant strains at 36° was below the detection level of the assay. Error bars are not visible for some points, as the bars are smaller than the symbols.

predominant mechanism, so it is likely that Ty1 cDNA is homologously recombined. Thus, the assay we used to quantitate transposition can generate His^+ papillae by one of three mechanisms: IN-mediated integration into the yeast genome, recombination with *his3AI* or Ty sequences on the plasmid, or recombination with Ty or *HIS3* sequences in the host genome (Figure 2). We refer to these collectively as "His^+ events." Recombination of cDNA to the plasmid or the genome requires the recombination/repair gene *RAD52* (PETES *et al.* 1991; SHARON *et al.* 1994). To determine whether the increase in His^+ events seen at high temperature results from true integration-mediated transposition or recombination, *sir4* mutant strains in which *rad52* was also disrupted were tested for transposition using the assay described in Figure 3. His^+ events in *rad52* mutant strains result from transposition, while His^+ events seen in wild-type *RAD52* strains are a combination of transposition and recombination. The results, shown in Figure 6, reveal that the high-temperature transposition phenotype is abolished at high temperature in *rad52* mutant strains; *e.g.*, the frequency of His^+ events in *SIR4 rad52* and *sir4 rad52* strains is the same at 34°. This result indicates that the high-temperature transposition phenotype seen in our *sir4* strain at high temperature is due to an increase in homologous recombination rather than to an increase in true transposition events. It is also notable that as temperature increases, the frequency of His^+ events drops more rapidly in *rad52* strains as compared to *RAD52* strains, indicating that the percentage of events due to recombination increases as temperature increases. Interestingly, a comparison of transposition in *rad52 vs. rad52 sir4* strains reveals that the number of His^+ events is significantly higher in the *sir4* mutant at permissive temperature through 32°. At this temperature, His^+ cell formation in the *sir4 rad52* strain is approximately sixfold increased as compared to that in *SIR4 rad52*, indicating that *SIR4* may regulate transposition at permissive temperatures.

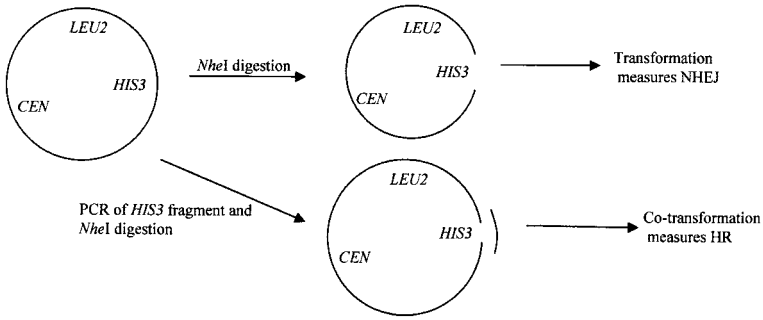
Sir4p is known to play a direct role in transposition of the Ty5 element of yeast. In *sir4* mutant strains, the number of Ty5-mediated events increases 10-fold, with >90% of these events resulting from plasmid recombination (ZOU *et al.* 1996, 1999; XIE *et al.* 2001). Thus, we tested whether the *HIS3* marker was being transferred to the plasmid or the genome. Individual His^+ papillae resulting from galactose induction at various temperatures were plated onto rich media to allow for loss of the plasmid and then printed to 5-FOA to select for growth of cells that had lost the *URA* plasmid (BOEKE *et al.* 1984). Finally, the cells were plated to selective medium lacking histidine. Only cells in which the *HIS3* marker has been transferred to the genome can grow (Figure 2). Thus, we can determine what percentage of His^+ events are recombination of *HIS3*-containing cDNA to the plasmid. At the nonpermissive temperature of 36°, the percentage of events resulting from plasmid recombination increases significantly in both strains (Table 2). Both wild-type and *sir4-119* strains show very little plasmid recombination at 26°, the optimal temperature for transposition (LAWLER *et al.* 2002). The actual

TABLE 2

Percentage of His^+ events resulting from plasmid recombination or genomic events in wild-type and *sir4-119* strains

	26°		30°		34°		36°	
Event type:	Genomic	Plasmid	Genomic	Plasmid	Genomic	Plasmid	Genomic	Plasmid
% of wild-type events[a] (JKc1015)	99	1	91	7	100	0	67	23
% of *sir4-119* events[a] (Jkc1005)	98	2	91	7	94	6	55	37

[a] The percentages of genomic and plasmid events do not always add up to 100%, as there are occasional papillae that grow weakly on 5-FOA; these events were not scored.



recombination mechanism	strain, temperature (°)	ratio of transformation efficiency: digested plasmid/undigested plasmid	normalized to *SIR4*, 30°
HR (undigested plasmid+ *HIS3* PCR fragment)	*SIR4*, 30	15.54 (4.11)	1
	sir4, 30	11.7 (4.41)	0.75
	SIR4, 36	36.14 (10.63)	2.3
	sir4, 36	18.35 (6.24)	1.2
NHEJ (undigested plasmid alone)	*SIR4*, 30	1.1 (0.6)	1
	sir4, 30	0.04 (0.02)	0.03
	SIR4, 36	0.61 (0.05)	0.6
	sir4, 36	0.05 (0.02)	0.05

FIGURE 7.—Effects of temperature and *sir4* deletion on recombination. A plasmid transformation assay into *SIR4* (JKc1015) and *sir4* (JKc1005) strains (lacking a Ty1 plasmid) was used to assess HR and NHEJ. To assess NHEJ linearized plasmid was transformed, and to assess HR the linearized plasmid was cotransformed with a PCR product homologous to sequences flanking the gap. HR and NHEJ were measured in wild-type and *sir4* strains at 30° and 36° and are reported as the ratio of the transformation efficiency (CFUs per microgram of DNA transformed) of *Nde*I-linearized plasmid to uncut plasmid. The standard deviations derived from three distinct transformation experiments are given in parentheses.

number of plasmid and genomic events from the two strains at each temperature were compared using Fisher's exact test. None of the comparisons were significant, indicating that deletion of *sir4* does not change the ratio of plasmid to genomic events at a given temperature (data not shown).

Homologous recombination mechanisms are not increased by high temperature or the absence of Sir4p: The two major DNA repair pathways are HR and nonhomologous end joining (NHEJ). In yeast, HR is much more efficient, such that in wild-type cells, the large majority of chromosomal breaks are repaired using this pathway (LEE *et al.* 1999). We have shown above that the increase in His^+ events in a *sir4* mutant at high temperature is *RAD52* dependent, and therefore most of these events are likely the result of homologous recombination of Ty1 cDNAs with plasmid and genomic targets, rather than integration. This increase could be due to an induction of the general cellular homologous recombination pathway by high temperature, rather than a direct result of the absence of Sir4p on the fate of Ty1 cDNAs. However, Rattray and Symington have previously demonstrated that homologous recombination of an integrated chromosomal sequence does not vary with temperature (RATTRAY and SYMINGTON 1995). It is also possible that the increase in recombination of Ty1 cDNAs is an indirect effect of the absence of Sir4p, as cellular recombination mechanisms are known to be influenced by cell ploidy. In yeast, mating type is determined by the presence of a mating-type cassette at the active *MAT* locus of chromosome III. The right and left arms of chromosome III contain cassettes, *HMR* and *HML*, containing regulatory genes for the **a** and α mating types, respectively, which are kept silenced by a Sir4p-containing chromatin complex. In *sir4* mutant strains, *HMR* and *HML* are unsilenced, thus allowing expression of both **a** and α mating factors, and resulting in a pseudodiploid state. Homologous recombination of induced chromosomal breaks is slightly increased in *MAT***a**/α diploids as compared to *MAT***a**/**a** diploids (LEE *et al.* 1999; CLIKEMAN *et al.* 2001). Thus, it is possible that the increase in His^+ events seen at high temperature in *sir4* strains is a result of an increase in homologous recombination due to the induced "pseudodiploid state" in *sir4* mutants. We used a plasmid transformation assay to measure HR and NHEJ activity in wild-type and *sir4* strains at 30 and 36° (TSUKAMOTO *et al.* 1997; BOULTON and JACKSON 1998). A CEN plasmid (pJK592) containing the selectable markers *LEU2* and *HIS3* was transformed into wild-type and *sir4* strains at 30° and 36°, selecting for Leu^+ colonies. Both yeast strains harbor the *his3*Δ*200* deletion, such that very little homology exists between the *HIS3* plasmid sequence and the genome (107 bp 5′ and 28 bp 3′ of plasmid *HIS3* sequences are present at the *his3*Δ*200* chromosomal locus). An outline of the experimental design and the results are shown in Figure 7. To assess NHEJ, the plasmid was digested with the enzyme *Nde*I, which cuts within the *HIS3* coding sequence, and then transformed. As expected, *sir4* strains were deficient in NHEJ, at both 30° and 36° (TSUKAMOTO *et al.* 1997; ASTROM *et al.* 1999). To assess HR, *Nde*I-digested plasmid was cotransformed with a PCR-generated *HIS3* fragment that spans the *Nde*I cut site. The HR assay also includes NHEJ activity, but the

A

strain	locus expression: *HML* (α)	*MAT* (α)	*HMR* (a)	mating type	# His⁺ events at 36° (x10⁻⁵)	fold-↑
SIR4 ■	-	+	-	α	0.3 (.09)	1
sir4 □	+	+	+	a/α	4.3 (1.5)	14.3
SIR4 hmr ◆	-	+	-	α	1.3 (0.6)	4.3
sir4 hmr ◇	+	+	-	α	1.5 (0.6)	5

B

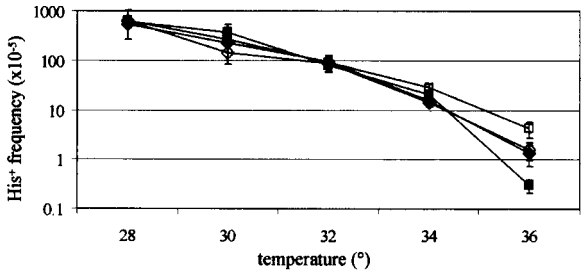


FIGURE 8.—The effect of mating-type control on high-temperature His$^+$ cell formation. Wild-type, *sir4*, and *hmr* mutant strains containing a galactose-inducible Ty1 element on plasmid pGTy1H3m*his3AI* were quantitated for frequency of His$^+$ cell formation following galactose induction at the indicated temperatures. (A) Table of locus expression, phenotypic mating type, and His$^+$ cell formation at 36°. The numbers in parentheses are the standard deviations. The fold increase (far-right column) is normalized to wild type (*SIR4*). Strains and symbols are as follows: wild type, solid squares (JKc1015 *SIR4*); *sir4*, open squares (JKc1005 *sir4-119*); *hmr*, solid diamonds (JKc1241 *SIR4 hmr::kanMX4*); *sir4 hmr*, open diamonds (JKc1223 *sir4-119 hmr::kanMX4*). (B) Graphical representation of His$^+$ formation at a range of temperatures. Strain symbols are as noted above. Points represent the average transposition of three patches, and error bars indicate the standard deviation.

level of HR activity is minimally 15-fold that of NHEJ, such that the contribution of NHEJ is not significant. As expected, HR is much more efficient than NHEJ in both wild-type and *sir4* mutant strains. HR is modestly increased (~2-fold) at high temperature in the wild-type strain, but not the *sir4* strain. Thus, neither cell-type nor temperature induction of the general cellular HR pathway in *sir4* strains is responsible for the high-temperature phenotype. It is notable that transformation efficiency is increased at least twofold in both wild-type and mutant strains at 36° (data not shown).

Effect of *MAT* on the frequency of high-temperature His$^+$ events: To directly assess the effect of *sir4* deletion-induced pseudodiploidy on high-temperature His$^+$ cell formation, wild-type and *sir4 MAT*α strains deleted for *HMR* were quantitated for His$^+$ cell formation at high

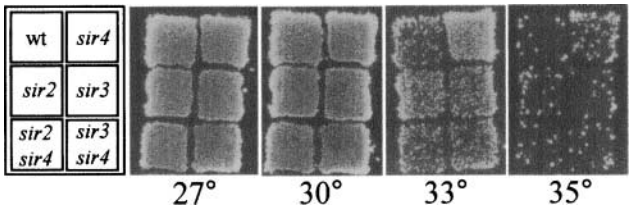


FIGURE 9.—The *sir4* high-temperature transposition phenotype is dependent on both *sir2* and *sir3*. Apparent transposition in a panel of *sir* mutant strains was assayed at a range of temperatures using plasmid pX3. The relevant phenotype of each strain is shown on the schematic grid to the left, and the temperature of galactose induction is indicated at the bottom. The increased activity in the *sir4* mutant strain is seen at 33° and 35°. Strains are as follows: wt (JKc1163), *sir4* (JKc1190), *sir2* (JKc1066), *sir3* (JKc1067), *sir2 sir4* (JKc1068), and *sir3 sir4* (JKc1065).

temperature. The deletion of the **a**-factor-containing *HMR* locus removes the pseudodiploid state, and these strains regain the ability to mate (Figure 8A). In this particular assay, His$^+$ events occurring in the *sir4* mutant at 36° were 14.3-fold higher than those in wild type (Figure 8A, far right column). In a *sir4 hmr*Δ mutant, His$^+$ events were increased only 5-fold above those in wild type, suggesting that removal of the pseudodiploid state decreases the high-temperature recombination activity. Given that the *sir4* mutant had no effect on recombination of a non-Ty1 substrate (Figure 7), this result suggests that the recombination activity may be specific for Ty1 cDNA. Interestingly, a *SIR4 hmr* strain, which we expected to be comparable to wild type, showed a 4.5-fold increase in His$^+$ events as compared to the wild type *SIR4 HMR* strain. This surprising result indicates that the *HMR* locus alone inhibits high-temperature His$^+$ cell formation.

Increased recombination is dependent on the Sir complex: Sir4p complexes with other proteins during its normal role in silencing. We tested a panel of double knockouts of *sir4* with *sir2* or *sir3*, other genes in the *SIR* family. Since the *sir* genes are disrupted with *HIS3*, transposition was assayed using plasmid pX3, which contains a *TRP1*-marked galactose-inducible Ty element. Neither the *sir2* nor the *sir3* mutants displayed the high-temperature transposition phenotype (Figure 9). Additionally, knocking out either of these genes in conjunction with *sir4* eliminates the high-temperature phenotype. Therefore, increased recombination in the absence of Sir4p requires Sir2p and Sir3p. In this assay, the plasmid marker is functionally expressed (without galactose induction) and therefore the plasmid must be eliminated to assess transposition. Thus, all Trp$^+$ events are due to genomic recombination or IN-mediated transposition. The fact that an increase in Trp$^+$ events at high temperature is seen in the *sir4* mutant strain is additional evidence that a significant number of the recombination events occurring at high temperature are targeted to the genome.

DISCUSSION

In a genetic screen to isolate host regulators of transposition, we have cloned a gene mutation conferring a high-temperature transposition phenotype and have conclusively identified the gene as *SIR4*. The cloned mutant allele has a stop mutation at amino acid 148 of 1358 and is thus essentially a null allele. Quantitative assays in two *sir4* mutant strains confirm that a deletion of the *sir4* gene product results in an ~15- to 30-fold increase in apparent transposition over wild-type strains at high temperature. It is notable that in the W303 strain background, which has an inherently lower level of transposition activity, deletion of *sir4* resulted in increased transposition activity at permissive temperature as well.

SIR4 is a silent information regulator gene implicated in many cellular processes including cell aging and chromatin silencing (reviewed in Gartenberg 2000; Gasser and Cockell 2001). *SIR4* acts as a regulator of many genes through the creation of silent chromatin, in complex with *SIR2* and *SIR3*. These three proteins assemble at silencer regions with the aid of *SIR1*, *ORC* (the origin recognition complex), and *RAP1* (Moazed *et al.* 1997). Interestingly, the yeast element Ty5 integrates preferentially to the silenced mating loci and telomeres in yeast, via interaction of Ty5 integrase to Sir4p (Xie *et al.* 2001). Ty1, however, targets to Pol III promoters, not silenced chromatin (Devine and Boeke 1996). It is unknown whether Ty1 integrase interacts directly with Sir4p, or if IN plays any role in Ty1 recombination. Ty1 IN does contain a nuclear localization sequence (NLS), indicating that it could play an active role in chromosomal recombination of Ty1 cDNA, even in the absence of functional VLPs (Moore *et al.* 1998).

In the Ty1 assay system that we used to quantitate transposition, the selectable marker can be transferred to the host genome by IN-mediated recombination or by homologous recombination to Ty1 or marker sequences present in the yeast cell. The latter activity requires the *RAD52* gene product, which is required for homology-dependent recombination in yeast. The increase in apparent transposition in *sir4* strains at high temperature is *rad52* dependent, as assayed using a splicing-dependent *HIS3*-marked Ty1 element, indicating that the increase is due to recombination of *HIS3*-containing cDNA products, rather than to IN-mediated integration of Ty1 cDNA. However, there is a slight but significant increase in true transposition (*RAD52*-independent) events in the *sir4* mutant strain (as compared to wild type) at permissive temperature (Figure 6), suggesting that Sir4p may also play a role in regulating proper integration.

We found that the proportion of recombination of Ty1 cDNA relative to integration increases in both wild-type and *sir4* mutant strains at high temperature (Table 2). Sharon *et al.* have previously shown that a block in true IN-mediated transposition events via a mutation in the IN protein increases the rate of homologous recombination (Sharon *et al.* 1994). Consistent with this observation, the recombination data in this article show that at high temperature, when IN-mediated transposition is blocked (by a processing defect), an underlying recombination pathway is revealed. By an unknown molecular mechanism, the absence of Sir4p increases the relative frequency of recombination, resulting in a high-temperature transposition phenotype. We have shown that the increase in Ty1 recombination is not due to a temperature-induced increase in global recombination, suggesting that the increase in recombination at high temperature may be specific for Ty1 cDNA substrates. We favor three possible mechanisms by which a mutation in *SIR4* could increase recombination at high temperature.

SIR4 inhibits Ty1 processing or destabilizes Ty1 cDNA: We have previously shown that high temperature greatly reduces the production and PR-mediated processing of Ty1 proteins (Lawler *et al.* 2002). In this study, we found that production of processed IN appears to be slightly restored in *sir4* mutant strains. Recombination to Ty sequences requires the generation of at least partial Ty1 cDNA products. In this study, we confirm that the level of Ty1 cDNA decreases rapidly as temperature increases in both wild-type and *sir4* mutant strains. It is possible that partial cDNA products are produced at high temperature, which may not appear as a distinct band by Southern blotting. A slight increase in Gag-Pol polyprotein processing in the absence of Sir4p could, via increased RT activity, result in a slight increase in partial cDNA products, providing sufficient substrate to give the level of increased recombination observed in *sir4* mutant strains. It may be significant that the difference in processing is more notable in whole-cell homogenates or extracts than in purified VLPs, since it is unknown to what extent the VLP structure is needed for the recombination process. Alternatively, the absence of Sir4p could, directly or indirectly, increase the stability of partial Ty1 cDNAs, thus allowing more time for recombination events.

Cellular recombination is induced by a pseudodiploid state resulting from the absence of Sir4p: Both HR and NHEJ are subject to mating-type control in yeast (Lee *et al.* 1999; Clikeman *et al.* 2001). HR-mediated repair of a defined double-strand break is increased in *MAT***a**/α diploid cells, as compared to a homozygous diploid cell (*MAT***a**/*MAT***a**). NHEJ-mediated recombination, however, decreases 10-fold in heterozygous (*MAT***a**/α) diploid cells, as compared to a non-**a**/α diploid (*mat*Δ/*MAT*α) strain (Astrom *et al.* 1999; Lee *et al.* 1999). Sir4p is central to the maintenance of silencing of the *HML* and *HMR* mating-type cassettes, so in the absence of Sir4p, both *MAT***a**- and *MAT*α-factor genes are expressed, resulting in a pseudodiploid, nonmating cellular state. Thus, it is possible that the increase in His^+ events at

high temperature is due to an increase in HR induced by pseudodiploidy. We tested this hypothesis by deletion of the *hmr* mating-type cassette in a *sir4* mutant strain, thus eliminating the expression of **a**-factor genes in the *sir4* mutant (Figure 8). We do indeed see an approximate threefold reduction of the high-temperature transposition phenotype, indicating some mating-type control of this recombination activity. Interestingly, this does not account for all of the high-temperature activity. Quite surprisingly, the frequency of His^+ events at high temperature increased (~4-fold) in the *SIR4 hmr* control strain. Although *HMR* is expected to be silenced in the presence of Sir4p, there may be basal level expression of **a**-factor that affects transposition or recombination activity directly or affects expression of haploid-specific genes that regulate transposition or recombination activity. It is also possible that Sir4p-mediated *MAT* silencing is weakened at high temperature.

Transposition of the Ty1 element is known to be subject to cell-type regulation at permissive temperature. Transcription of Ty1 is decreased ~10-fold in diploids, mediated via a DNA sequence within Ty1 that binds the **a**1/α2 regulatory protein (Errede *et al.* 1985, 1987). Transposition activity is also slightly reduced in diploid (**a**/α) cells, but not in proportion to the decrease in mRNA production (Paquin and Williamson 1986). In the strain used in this study, deletion of *hmr* and *sir4* had no effect on transposition activity at permissive temperature (Figure 8B).

SIR4 maintains a chromatin structure that inhibits Ty1 cDNA recombination: Since deletion of Sir4p increases recombination at high temperature, we should consider how structural changes to chromatin in the absence of Sir4p affect this process, perhaps opening silenced regions to recombination. Many chromosomal structural changes take place in the absence of Sir4p. Sir2p, Sir3p, and Sir4p are considered the structural components of silenced chromatin, and the absence of Sir4p greatly disrupts normal formation of silenced chromatin. Since Sir4p is found mainly in complex with Sir2p and Sir3p, we also assayed for transposition in strains mutated for these genes and in combination with *sir4*Δ. Neither *sir2*Δ nor *sir3*Δ strains demonstrate the high temperature transposition phenotype. The phenotype in *sir4*Δ strains is abolished in combination with either *sir2*Δ or *sir3*Δ. This result suggests that in the absence of Sir4p, the remainder of the Sir complex is mediating the increase in recombination seen at high temperature.

No one model presented here is exclusive of the others. Indeed it is likely that the model by which Sir4p suppresses recombination is complex. Future experiments will endeavor to sort out these possibilities and attempt to determine if the increase in recombination in *sir4* mutants is due to cellular effects brought about by temperature or simply due to the fact that IN-mediated transposition is blocked at high temperature. If recombination increases in the absence of *sir4* because IN-mediated transposition is blocked, then we would expect to see increased recombination of Ty1 elements harboring mutations in the IN protein in *sir4* mutant strains. Future studies will also address the identification of genomic recombination sites in *sir4* mutant strains and the possible interaction of IN with Sir4p.

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