Mutations in *POL1* Increase the Mitotic Instability of Tandem Inverted Repeats in *Saccharomyces cerevisiae*

Barbara Ruskin*,[1] and Gerald R. Fink*,†

*Whitehead Institute for Biomedical Research and †Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

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ABSTRACT

Tandem inverted repeats (TIRs or hairpins) of 30 and 80 base-pair unit lengths are unstable mitotically in yeast (*Saccharomyces cerevisiae*). TIR instability results from deletions that remove part or all of the presumed hairpin structure from the chromosome. At least one deletion endpoint is always at or near the base of the hairpin, and almost all of the repaired junctions occur within short direct sequence repeats of 4 to 9 base pairs. The frequency of this event, which we call "hairpin excision," is influenced by chromosomal position, length of the inverted repeats, and the distance separating the repeat units; increasing the distance between the inverted repeats as little as 25 base pairs increases their chromosomal stability. The frequency of excision is not affected by representative *rad* mutations, but is influenced by mutations in certain genes affecting DNA synthesis. In particular, mutations in *POL1/CDC17*, the gene that encodes the large subunit of DNA polymerase I, increase the frequency of hairpin deletions significantly, implicating this protein in the normal maintainance of genomic TIRs.

TANDEM inverted repeats (TIRs) in DNA have unique affects on gene expression and gene stability that probably reflect their ability to form transient stem loop (hairpin) structures by intramolecular base pairing within a single DNA strand. Short TIRs of less than 21 base pairs (bp) placed in the 5′ untranslated leader of a gene can inhibit expression of downstream sequences (BAIM *et al.* 1985; PELLETIER and SONENBERG 1985; KOZAK 1986; CIGAN, PABICH and DONAHUE 1988). In yeast, this block occurs at the translational level, presumably because the TIR forms a hairpin in the mRNA that interferes with the binding or scanning of ribosomes (BAIM *et al.* 1985; PELLETIER and SONENBERG 1985; ABASTADO *et al.* 1991).

TIRs also have idiosyncratic genetic consequences. PETES and coworkers showed that, in yeast, a heteroduplex containing a short hairpin in *HIS4* or *LEU2* undergoes high levels of postmeiotic segregation events suggesting that hairpins are repaired less frequently than nonhairpin heteroduplex control strands (NAG, WHITE and PETES 1989). The effect of TIRs on both mismatch repair and translational inhibition correlates with the ability of the inserted TIR to form stable secondary structures: mutations that reduce the base pairing capacity of the TIR reduce or abolish inhibition, and compensatory base changes restore the effects.

Long TIRs (LTIRs) are unstable in bacteria (COLLINS 1980; COLLINS, VOLCKAERT and NEVERS 1982); plasmids carrying LTIRs are recovered having lost part or all of the inverted repeat. LTIRs in bacteria show increased stability if the elements of the repeat are separated (WARREN and GREEN 1985). Systematic studies show that the length of the inverted repeat (WESTON-HAFER and BERG 1991), the sequences at the base of the hairpin stem, and the rate of cruciform formation all influence LTIR instability in bacteria (SINDEN *et al.* 1991). The mitotic consequences of LTIRs in yeast have not been closely examined. GORDENIN and coworkers have reported that bacterial transposon Tn5, which has 1.5-kilobase (kb) terminal (but not tandem) inverted repeats, when inserted into the yeast *LYS2* gene is recombined out (GORDENIN *et al.* 1988). More recently they have found that mutations in DNA polymerase genes and certain *rad* genes, normally associated with the homologous recombination machinery, affect the frequency of Tn5 excision in yeast (GORDENIN *et al.* 1992).

We have found that synthetic LTIRs (30 and 80 bp-unit repeats, or "hairpins") inserted into yeast chromosomes by transformation are unstable mitotically. LTIR instability results from deletions that remove part or all of the presumed hairpin structure, with at least one deletion endpoint at or near the base of the hairpin. Most of the repaired junctions occur within short direct sequence repeats of 4–9 bp. The frequency of this event, which we call "hairpin excision," is influenced by chromosomal position, length of the inverted repeats, and the distance separating the repeat units. Hairpin excision is not affected by repre-

[1] Current address: City College, City University of New York, Department of Biology, 138th and Convent Ave., New York, New York 10031.

TABLE 1

Yeast strains used

Strain[a]	Genotype
BR131	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨BalI/pBR146⟩ at *HIS3*
BR132	*MATα ura3::HP80 his3Δ200 leu2Δ1*
BR150	*MATα ura3::HP30 his3Δ200 leu2Δ1*
BR177	*MATa his4::HP80 ura3-52 leu2Δ1 trp1Δ63*
BR179	*MATa rad52::LEU2 ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BR180	*MATa rad52::LEU2 ura3::HP80* ⟨BamHI/*his4-260,39*⟩ at *HIS4*
BR182	*MATa ura3::HP80* ⟨BamHI/*his4-260,39*⟩ at *HIS4*
BR190	*MATα ura3::HP80 his4::HP80 leu2Δ1 lys1*
BR195	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨HpaI/pBR129⟩ at *LEU2*
BR196	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨HpaI/pBR135⟩ at *LEU2*
BR197	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨HpaI/pBR136⟩ at *LEU2*
BR198	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨HpaI/pBR137⟩ at *LEU2*
BR200	*MATa ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BR201	*MATα his3Δ200 leu2Δ1 ura3-52* ⟨HpaI/pBR139⟩ at *LEU2*
BR202	*MATa rad6Δ ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BR203	*MATa rad10Δ ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BR204	*MATa ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63* ⟨YEp13-2μ-*LEU2*⟩
BR205	*MATa rad1Δ rad52::LEU ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BR206	*MATa ura3::HP80 his4::HP80 leu2Δ1 lys1*
BR208	*MATa rad1Δ rad52::LEU2 ura3::HP80* ⟨BamHI/*his4-260,39*⟩ at *HIS4*
BR212	*MATa rad52::TRP1 ura3::HP80 his4-260 trp1*
BR213	*MATa rad1Δ rad52::TRP1 ura3::HP80 his4-260 trp1*
BR214	*MATα rad52::TRP1 ura3::HP80 his4-280 trp1 lys1,2*
BR215	*MATα rad1Δ rad52:TRP1 ura3::HP80 his4-280 trp1 lys1,2*
BR24-4A	*MATα his3Δ200 leu2Δ1*
BRx24-6D	*MATα his3Δ200 leu2Δ1 ura3-52*
BRx65-8A	*MATα ura3::HP80 leu2Δ1 lys1*
BRx80-9D	*MATα ura3::HP80 his4::HP80 leu2Δ1*
BRx80-11B	*MATa ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
BRx82-1A	*MATa ura3::HP80 his4-260 trp1*
BRx84-7D	*MATα ura3::HP80 his4-280 trp1 lys1 or lys2-8*
BRx112-5D	*MATa ura3::HP80 leu2* ⟨BamHI/*his4-260,39*⟩ at *HIS4*
BRx121-1D	*MATα cdc17-2 ura3::HP80 his4::HP80 leu2Δ1 lys1*
BRx217-8B	*MATa cdc17-1 ura3::HP80 his4::HP80 leu2Δ1 lys1*
BRx218-4D	*MATa pol1-1 ura3::HP80 his4::HP80 leu2Δ1 trp1Δ63*
L4046	*MATa ura3-52 leu2Δ1 trp1Δ63*
L4386	*MATα ura3Δ301 leu2Δ1 lys2*
BRx89	*MATa/α ura3::HP80/ura3-52 his4-280/his4-260 lys1/+ trp1/+ leu2Δ1/+*
BRx226	*MATa/α BRx80-11B/BRx80-9D*
BRx227	*MATa/α BRx80-11B/L4386*
BRx233	*MATa/α BRx80-11B/217-1C*
BRx235	*MATa/α BRx218-4D/BRx80-9D*
BRx244	*MATa/α BRx82-1A/BRx84-7D*
BRx245	*MATa/α BR212/BR214*
BRx246	*MATa/α BR213/BR215*

[a] All strains constructed in this study.

sentative *rad* mutations, but is influenced by mutations in certain genes affecting DNA synthesis. In particular, mutations in *POL1/CDC17*, the gene encoding the large subunit of DNA polymerase I, increase the frequency of hairpin deletions significantly.

MATERIALS AND METHODS

Strains: The yeast strains used in this study are listed in Table 1. Hairpin alleles: Haploid yeast strains containing the *ura3::HP30* (BR150) or *ura3::HP80* alleles (BR132) were derived by gene replacement (ROTHSTEIN 1983). Strain BRx24-4A (Ura^+ Leu^-) was cotransformed with Yep13 (*LEU2*, 2 μm) and the 1.2-kb *Hin*dIII fragment from pBR81 or pBR54, respectively. Leu^+ transformants were screened for a Ura^- phenotype by replica-plating to synthetic complete medium (SC) containing 5-fluoroorotic acid (5-FOA; BOEKE *et al.* 1987). A *his4::HP80* strain was constructed by two-step gene replacement (BOEKE *et al.* 1987). Strain L4046 (His^+, Ura^-) was transformed to Ura^+ with *Xho*I-digested pBR110, grown nonselectively and screened for His^-, Ura^- segregants by replica-plating to SC media lacking histidine and uracil. The resulting *his4::HP80* mutant (BR177) was crossed to a *ura3::HP80* strain (BRx65-8A) and the diploid sporulated to obtain the canonical double hairpin-containing strains BRx80-11B and BRx80-9D. The structures of all putative hairpin-containing strains

were verified by Southern blot analyses. Strain BR131 is BRx24-6D (His$^-$, Leu$^-$, Ura$^-$) transformed to His$^+$ by transformation with pBR46 cleaved at the unique *Bal*I site at position 740 of the *HIS3* gene (GenBank). Strains BR195–198 and 201 are BRx24-6D transformed to Leu$^+$ with pBR129, 135–137 and 139, respectively, each cleaved at the unique *Hpa*I site at position 239 of *LEU2* (GenBank).

POL1 isogenic strains: Three different *pol1* temperature-sensitive mutants were individually crossed to either BRx80-11B or BRx80-9D (*ura3::HP80, his4::HP80*), and *pol1* mutant spores containing hairpins at both *URA3* and *HIS4* were backcrossed. Isogenic wild-type and mutant pairs were then constructed by gene replacement using the wild-type copy of *POL1* on an integrating plasmid. BR190 is BRx121-1D (*cdc17-2*) transformed to Ura$^+$ with *Bst*XI-digested pCM57 (plasmid kindly provided by G. LUCCHINI), a YIp5 derivative containing the wild-type *POL1* gene (PIZZAGALLI *et al.* 1988), counter-selected on 5-FOA medium for loss of the *URA3*-marked plasmid, and screened for temperature-resistant colonies that still showed high Ura$^+$ reversion frequencies. BR200 was derived from BRx218-4D (*pol1-1*), and BR211 from BRx217–8B (*cdc17-1*) by the same procedures.

rad deletion mutants: BR179 is BRx80-11B transformed to Leu$^+$ with the *Bam*HI fragment of pSM20 (SCHILD *et al.* 1983), containing a *LEU2*-marked disruption of the *RAD52* gene. BR205 is BRx80-11B cotransformed with *Bam*HI-digested pSM20 and *Bam*HI-digested pDMradΔ1; Leu$^+$ transformants (*rad52::LEU2*) were screened for UV sensitivity (*radΔ1*). Similarly, BR212–215 were constructed using a *Bam*HI fragment from pSM21, containing a *TRP1*-marked *RAD52* disruption (SCHILD *et al.* 1983), and transforming BRx82-1A (*MATa*) or BRx84-7D (*MATα*) in the presence (BR213 and BR215) or absence (BR212 and BR214) of *Bam*HI-digested pDMradΔ1. BR202 and 203 are BRx80-11B transformed to Ura$^+$ with *Bam*HI-digested pR671 (*rad6Δ::hisG-URA3-hisG*) or *Eco*RI-*Bgl*II-digested pR10.25 (*rad10Δ::hisG-URA3-hisG*; both plasmids kindly provided by L. PRAKASH) and then counter-selected on 5-FOA medium for loss of the *URA3* marker flanked by direct *hisG* repeats (ALANI, CAO and KLECKNER 1987).

Plasmid constructions: Plasmid pBR81 was constructed by partial digestion of YEp24 (BOTSTEIN *et al.* 1979) with *Pst*I and ligation in the presence of excess 27 bp linker sequences obtained by gel purification of pUC19 digested with *Pst*I and *Asp*718. Insert number was determined by sequencing of double-stranded mini-prep DNA using the Pharmacia T7 Sequencing kit (28–3380–01) according to specifications of the manufacturer. Plasmid pBR43 was constructed from YIp5 (BOTSTEIN *et al.* 1979) in a similar manner except that the unit insert used to make the palindrome was a GC-rich, 75-bp *Xba*I-*Pst*I fragment: 5′-CTAGAGTC$(G)_2$ C$(G)_3$ C$(G)_3$ C$(G)_5$ CAC$(G)_6$ CGC$(G)_2$C$(G)_3$ C$(G)_4$ C$(G)_5$ CAC$(G)_6$ CGC$(G)_3$ ACCTGCA-3′ (ABRAMS, MURDAUGH and LERMAN 1990). Plasmid pBR54 was constructed by partial digestion of YEp24 with *Pst*I, ligation to the 142-bpr *Pst*I-*Xba*I-*Pst*I palindrome isolated from pBR43, and screening for inserts at the *Pst*I site in the *URA3* 5′ leader. Plasmids pBR46 and pBR129 contain the 2-kb *Sma*I-*Sal*I fragment from pBR43 inserted into the polylinker of integrative vector pRS303 (*HIS3*) or pRS305 (*LEU2*), respectively (SIKORSKI and HIETER 1989), digested with *Sma*I and *Sal*I. Plasmids pBR135–139 were made by first destroying the *Xba*I site in the polylinker of pBR129 by Klenow treatment, digesting the resulting plasmid at the unique *Xba*I site within the palindrome, filling in the 5′ overhangs with Klenow, and ligating in the presence of either: no DNA (pBR135), excess λ DNA digested with *Alu*I (pBR139 = 25 bp, pBR136 = 60 bp), or pBR322-*Msp*I markers (New England Biolabs) treated with Klenow fragment (pBR137 = 218 bp). The insert sizes in pBR136 and pBR139 were determined by restriction digest analyses on high-percentage agarose gels.

HP80 was inserted into the *HIS4* leader at a *Dra*I site created by changing a C to an A residue at position 1312 by oligonucleotide-directed mutagenesis of plasmid ϕDH38, which is the 1.1-kb *Pvu*II-*Sal*I fragment of *HIS4* in M13 mp18 (HEKMATPANAH and YOUNG 1991). Mutagenesis was accomplished with a 60-mer (see below) and the Amersham oligonucleotide-directed mutagenesis kit version 2 (RPN.1523) according to specifications of the manufacturer. Plasmid pBR107 is the *HIS4* 1.1 kB *Eco*RI-*Sal*I fragment containing the new *Dra*I site cloned into the *Eco*RI-*Sal*I sites of pUC18, in which the *Pst*I site in the polylinker was destroyed by digestion with T4 DNA polymerase. The new *Dra*I site of pBR107 was converted to a *Pst*I site by partial *Dra*I digestion and *Pst*I linker ligation, and the 150-bp *Pst*I palindromic fragment isolated from pBR54 was inserted into the *Pst*I site to create pBR109. Plasmid pBR110 is the 1.1-kb *Eco*RI-*Sal*I fragment of pBR109 cloned into *Eco*RI and *Sal*I-digested YIp5. pDMradΔ1 is pD618 (*rad1::URA3* kindly provided by L. PRAKASH) with a deletion of *URA3* on a 1.1kB *Hin*dIII fragment (provided by D. MILLER).

Transformation and DNA manipulation techniques: Yeast transformations were carried out by the lithium acetate method (ITO *et al.* 1983) using 50 μg of sheared calf thymus DNA per transformation as carrier. Yeast transformants were selected by plating on appropriate selective media. *Escherichia coli* transformations were performed by either the calcium chloride method (MANDEL and HIGA 1970) or the method of Hanahan (1985). Plasmid DNA from *E. coli* was obtained by the alkaline lysis method, and restriction endonuclease analysis and agarose gel electrophoresis were performed as described in MANIATIS, FRITSCH and SAMBROOK (1982). Yeast DNA was prepared as described in BOEKE *et al.* (1985).

PCR amplification: Each polymerase chain reaction (PCR) consisted of approximately 8×10^7 cell-equivalents of genomic DNA template, and 0.4 μg (50 picomole) of each oligonucleotide primer using the "GeneAmp" kit of Perkin Elmer Cetus. Optimal amplification conditions were determined empirically. Twenty cycles of: 1-min (1′) denaturation at 94°, 1′ annealing at 65° and 1′ elongation at 72°, followed by another 15 cycles with the elongation period extended to 3′, were performed in an Ericomp thermocycler (San Diego, California). PCR amplification products were separated by electrophoresis through 2.5% NuSieve low melting point agarose gels (American Bioanalytical) containing 5 μg/ml ethidium bromide in Tris-borate-EDTA buffer. When necessary, products were purified from gel slices by electroelution into 3 M sodium acetate followed by ethanol precipitation.

For direct sequencing of PCR products, electroeluted fragments were used as template for asymmetric PCR amplifications performed as above except the concentration of the antisense oligonucleotide was reduced 100-fold. Asymmetric PCR products were purified by phenol-chloroform extraction followed by two ammonium acetate-ethanol precipitations using 20-μg glycogen as carrier. Pellets were washed in 75% ethanol, dried and resuspended in 10-μl water. The resulting DNA, predominantly single-stranded, was sequenced using the sense oligonucleotide with a T7-deaza sequencing kit from Pharmacia (27-1683-01), according to specifications of the manufacturer. Sequencing reactions were separated by electrophoresis through 5% dena-

turing polyacrylamide gels and visualized by autoradiography.

Oligonucleotides: All oligonucleotides were synthesized and purified by Research Genetics (Huntsville, Alabama). The 60-mer used for site-directed mutagenesis of the *HIS4* leader was: 5′-CGGC$(A)_4$CCATTATTCAG$(A)_8(T)_6$AAAC TATTGTATTACTATTACACAGCG-3′. The *URA3* and *HIS4* leader-specific oligonucleotides used for PCR analyses were:

URA3 sense oligo (89–113): 5′-CAGAAGGAAGAAC-GAAGGAAGGAGC-3′

URA3 antisense oligo (289–263): 5′-GCTTGGCAGCAA-CAGGACTAGGATG-3′

HIS4 sense oligo (1116–1131): 5′-GCTAAACCGAT-GCACAGTGACTCACG-3′

*HIS4*antisenseoligo(1437–1412):5′-TCAGGCTCGAGC-CATCC$(A)_4$GTACC-3′

Reversion assays: Strains were grown in triplicate. Five-milliliter cultures of each strain were grown to saturation in synthetic complete medium (SC; 1–3 days), harvested by centrifugation and resuspended in an equal volume of water. Appropriate dilutions were made into water for plating onto nonselective SC media for viable cell, and SC-histidine or SC-uracil selective media for His^+ or Ura^+ revertant counts, respectively. When measuring reversion from an integrated plasmid, the viable cell count was measured on SC medium selecting for the plasmid marker, and reversion counted on SC medium selecting for both the plasmid marker and hairpin loss. Temperature-sensitive strains and wild-type controls were grown, and reversion frequencies measured at 23°; all others assays were at 30°. The number of revertants was scored from days 3–10 after plating, and the average reversion frequencies and standard deviations determined for between 4–10 independent cultures at days 4–6, when the number of viable cells on nonselective medium remained constant (all petites had grown). Calculated reversion frequencies represent minimum estimates because occasional jackpot cultures (greater than two standard deviations from the mean) were not included into the final calculation.

Media and genetic analysis: Yeast media and culture conditions were as described by SHERMAN, FINK and LAWRENCE (1979). Sporulation medium contained 1% potassium acetate. Bacterial media were made as described by DAVIS, BOTSTEIN and ROTH (1980).

RESULTS

Tandem inverted repeats (hairpins) are mitotically unstable: Haploid yeast strains containing 30-bp (BR150, *ura3::HP30*) or 80-bp (BR132, *ura3::HP80*) hairpins in the 5′ leader of *URA3* were tested for growth on complete medium (YPD) and synthetic complete medium lacking uracil (SC-ura). Neither insertion causes a growth defect on complete medium or synthetic complete + uracil (SC + ura), but both insertions cause a uracil requirement on SC-ura (Figure 1). Strains harboring the *ura3::HP30* allele have a slightly leaky Ura^- phenotype (-/+), exhibiting slow background growth on SC-ura medium after extended periods of incubation. In contrast, the *ura3::HP80* allele has a tight Ura^- phenotype. The Ura^- phenotypes caused by hairpin insertions are unstable: after about 3 days of growth at 30° on SC-ura medium, Ura^+ colonies arise as papillants from

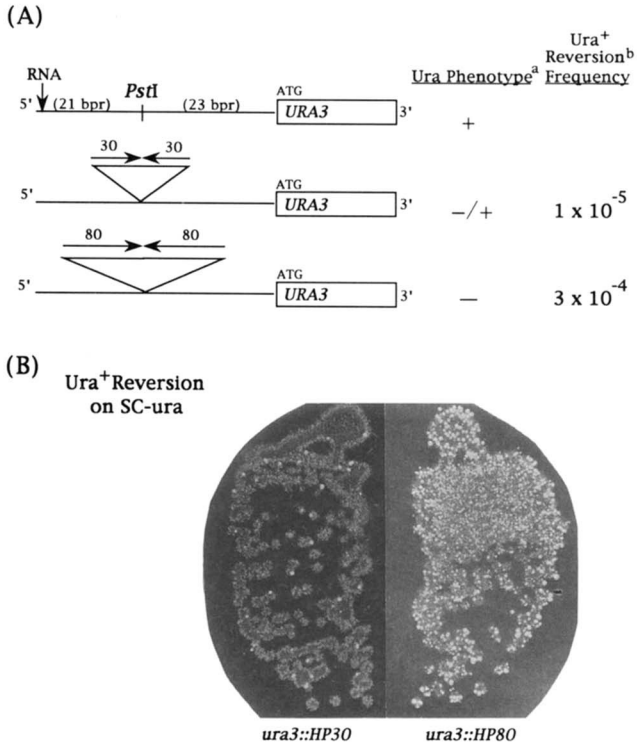

FIGURE 1.—Structure and properties of *URA3* hairpin strains. (A) Left: The *URA3*, *ura3::HP30* and *ura3::HP80* alleles are diagrammed schematically. The relative distances between the *Pst*I hairpin insertion site and the transcription (vertical arrow) and translation (ATG) initiation sites are indicated in base pairs (bp). Right: Growth phenotypes in the absence of uracil. [a] Ura phenotype on day 5 at 30°; leaky Ura^- phenotype indicated by -/+. [b] Average number of Ura^+ colonies/total number viable cells determined for at least six independent cultures on days 4–6 at 30°. (B) Papillation of Ura^+ revertants from strains BR150 (*ura3::HP30*) and BR132 (*ura3::HP80*) photographed after 6 days on a SC-ura plate at 30°.

the background of Ura^- cells (Figure 1B). Approximately one in 10^5 cells containing HP30, and three in 10^4 cells with the GC-rich HP80 revert to Ura^+ in the BRx24-4A genetic background (Figure 1A) as determined by quantitative reversion assays (MATERIALS AND METHODS). Ura^+ phenotypes and reversion frequencies were unaffected by varying the temperature between 18° and 36° (data not shown). This reversion frequency is so high that it is impossible to isolate a pure colony containing the *ura3::HP80* allele (see subsequent section).

Hairpin revertants are caused by deletions between small direct repeats: The structure of the *URA3* region in *ura3::HP30* Ura^+ papillae was first investigated by Southern analysis of DNA isolated from independent revertants. A diagnostic test for the *ura3::HP30* hairpin allele is the ability to cleave the 1.2-kb genomic *Hin*dIII fragment carrying the *URA3* gene with *Asp*718, the restriction site at the center of the LTIR. We digested genomic DNA samples from BRX24-4A (Ura^+), BR150 (*ura3::HP30*), and 12 independent Ura^+ revertants, with *Hin*dIII and *Asp*718.

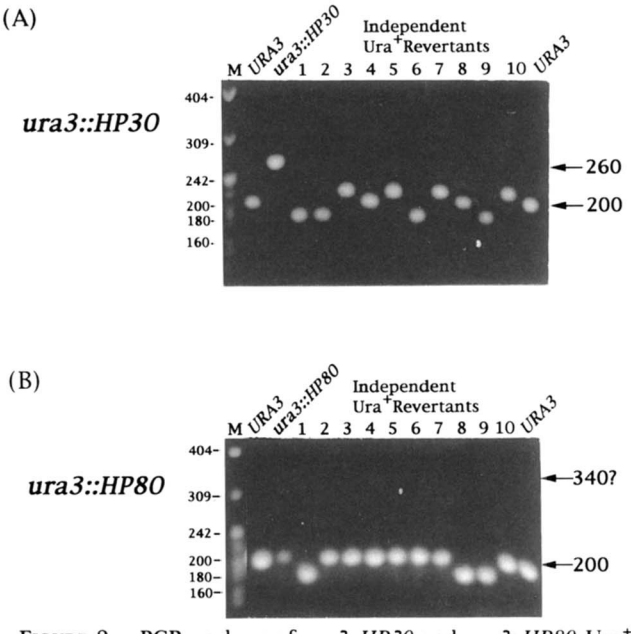

FIGURE 2.—PCR analyses of *ura3::HP30* and *ura3::HP80* Ura$^+$ revertants. (A) PCR amplification products from isogenic strains BRx24-4A (*URA3*), BR150 (*ura3::HP30*), and 10 independent Ura$^+$ revertants from BR150. The predicted size of the PCR products from *URA3* (200) and *ura3::HP30* (260) are in base pairs (bp) and indicated by arrows on the right. The size of pBR322-*Msp*I digested DNA markers (M) are in bp on the left. (B) PCR amplification products from isogenic strains BRx24-4A (*URA3*), BR132 (*ura3::HP80*) and 10 independent Ura$^+$ revertants are shown as described in (A). The expected position of the *ura3::HP80* PCR product of 340 bp is indicated by an arrow and labeled (340?) on the right.

All 12 Ura$^+$ revertants had lost the diagnostic *Asp*718 site within the loop of the hairpin (data not shown), showing that the appearance of Ura$^+$ revertants is due to DNA rearrangements.

The DNA rearrangements of the *ura3::HP30* and *HP80* revertants were characterized by polymerase chain reaction (PCR) amplification of the 5′ leader region and direct DNA sequence analyses of the PCR products (Figure 2; MATERIALS AND METHODS). PCR amplifications of the wild-type *URA3* gene (BRx24-4A) and of the *ura3::HP30* allele (BR150) yield the expected fragment sizes, a 200-bp fragment for wild-type *URA3* and a 260-bp PCR product for the *ura3::HP30* allele (Figure 2A). Amplification of DNA samples from 10 independent Ura$^+$ revertants reveals that the PCR products are all smaller than that from the starting *ura3::HP30* strain, and are either the same size as, smaller than, or larger than the 200-bp PCR product of the wild-type *URA3* gene. A similar analysis of 10 independent Ura$^+$ revertants from *ura3::HP80* (Figure 2B) reveals two classes of products, those the same size as, and those smaller than the 200-bp PCR product from *URA3* without the LTIR. Interestingly, when genomic DNA from *ura3::HP80* (BR132) is amplified, no PCR product is obtained at normal levels or of the predicted size of 340 bp. Sometimes a smaller discrete fragment is produced in trace amounts (Figure 2B, lane *ura3::HP80*). The failure to obtain the predicted PCR product suggests that HP80 forms a stable structure in isolated genomic DNA that blocks TAQ polymerase under the conditions used for PCR amplification. Because the frequency of HP80 reversion is so high (Figure 1), the variable appearance of subquantitative and smaller HP80 PCR products probably results from amplification of DNA from the few cells that have rearranged the hairpin during growth of the yeast culture (see also Figure 5A, *his4::HP80*; MATERIALS AND METHODS).

The PCR products (Figure 2) were purified and used as templates for asymmetric PCR amplification to produce single-stranded DNA for direct DNA sequence analyses (MATERIALS AND METHODS). All classes of Ura$^+$ revertants represent deletion events that arise from either precise or imprecise excision of the hairpin structures from the chromosome (Figure 3). Class I Ura$^+$ revertants (2/12 *ura3::HP30* and 7/10 *ura3::HP80*) have undergone a precise excision of the hairpin that regenerates a wild-type *URA3* gene. This precise excision could result from an event between the repeated *Pst*I sites flanking the hairpin (within 9-bp direct repeats). Class II Ura$^+$ revertants (5/12 *ura3::HP30*) have undergone an imprecise excision of the hairpin that leaves 12–15 bp of one side of the LTIR within the *URA3* leader (14-bp insertion). This imprecise excision could result from an event between the tetranucleotide repeat AGGA found within and at the base of the HP30 hairpin (Figure 3, shaded boxes). Class III Ura$^+$ revertants (5/12 *ura3::HP30* and 3/10 *ura3::HP80*) have undergone an imprecise excision that deletes the hairpin along with the entire upstream 5′ leader of the *URA3* gene (17-bp deletion). This excision could occur by an event between CTGCA repeats at the beginning of the *URA3* leader and at the base of the hairpin, within the *Pst*I insertion site (Figure 3, shaded boxes). In summary, the Ura$^+$ revertants obtained from both hairpin-containing *URA3* alleles represent recombination events between short direct repeats that either partially or completely remove the hairpin. Furthermore, at least one excision endpoint occurs at the base of the hairpin.

Selection for Ura$^+$ revertants imposes considerable constraints on the spectrum of events that can be recovered. Deletions that extend 5′ into the transcriptional regulatory sites or 3′ into the translation initiation site and coding sequences would be Ura$^-$ and fail to be detected. These Ura$^-$ segregants would differ from the parental *ura3::HP30* or *ura3::HP80* strains because they would fail to revert (papillate) to Ura$^+$. A screen for such spontaneous nonpapillators yielded two independent isolates, both from the *ura3::HP30* background (average recovery frequency of 4×10^{-4}). PCR amplification and DNA sequence

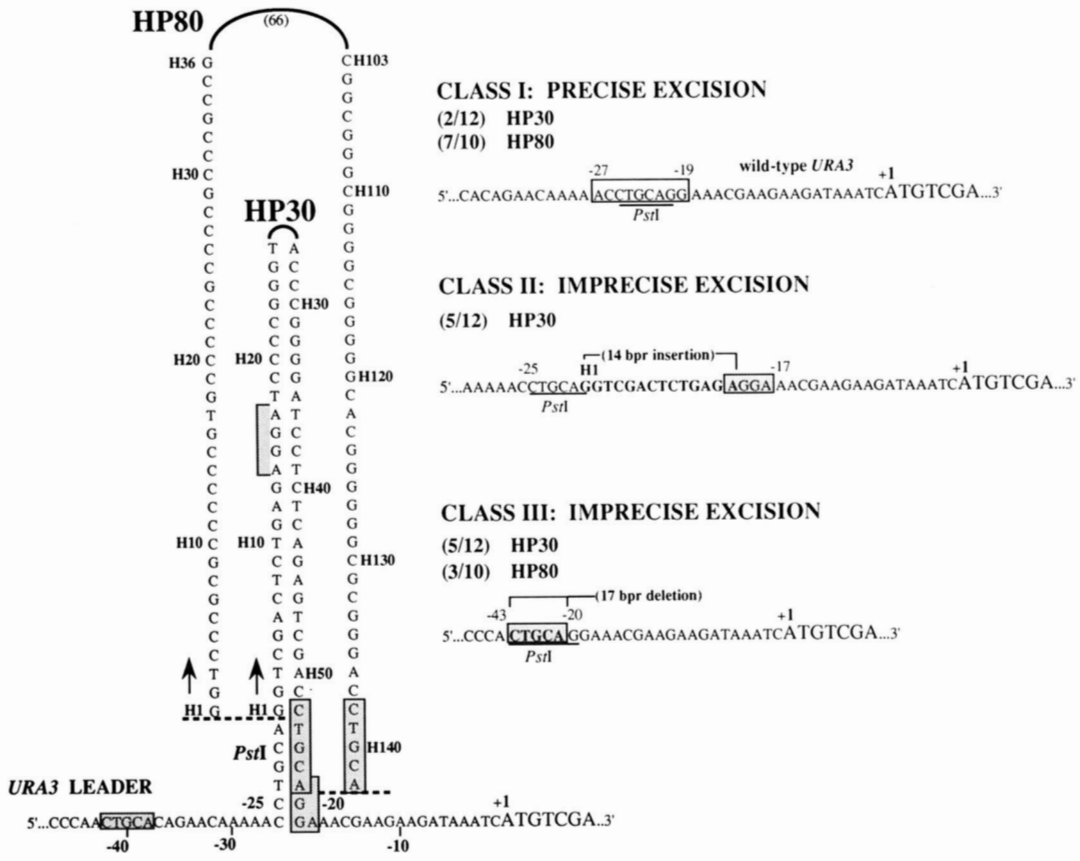

FIGURE 3.—Sequences of *ura3::HP30* and *ura3::HP80* Ura$^+$ revertants. (Left) The DNA sequence of the *URA3* leader is drawn horizontally, with the HP30 and HP80 inverted repeats drawn as hairpins in the *Pst*I site (CTGCAG −20 to −25). The bases comprising HP30 and HP80 are numbered (H1 to H54, or H1 to H142, respectively), 5′ to 3′ from the *Pst*I insertion site. The *URA3* leader is numbered relative to the +1 ATG initiator codon. Direct repeats in shaded boxes correspond to recombination junctions shown on the right. (Right) DNA sequences of the Ura$^+$ revertants amplified in Figure 2, with data from an additional two independent *ura3::HP30* revertants included. The number of revertants obtained/number sequenced for each hairpin in each class is in parentheses. Direct repeats at recombination junctions are boxed within each revertant sequence. Class II junctions (AGGA): HP30 14 to 17/*URA3* −21 to −18. Class III junctions (CTGCA): *URA3* −42 to −38/HP30 52 to 56, or HP80 138 to 142. *URA3* leader insertions are shown in bold type. The size of deletions of, or insertions into, the *URA3* leader after hairpin excision are in parentheses, and their positions indicated by brackets.

analyses (Figure 4) revealed that the stable Ura$^-$ mutants represent a Class III (deletion) event that removes the hairpin and all wild-type leader sequence downstream from the hairpin, including the first two bases of the ATG translation initiation codon. The deletions occur between perfect 5-bp direct repeats (Figure 4, GTCGA) that are within 8-bp direct repeats containing a single mismatch. We have named this deletion *ura3Δ301*. Surprisingly, all Class III deletion events that remove either HP30 (54-bp insert) or HP80 (140-bp insert) sequences delete the same number of nucleotides (17–21 bp) of adjacent 5′ or 3′ wild-type leader sequence (Figures 3 and 4). No larger deletions causing stable Ura$^-$ phenotypes were found, although there are numerous other short direct repeats within the body of the *URA3* gene. The consistency in the amount of wild-type leader sequence deleted in Class III revertants suggests that the hairpin excision event is somehow constrained by the distance between the short direct repeats and not by the absolute length of the inverted repeat that is deleted.

Hairpin removal does not occur by homologous recombination: The fact that short direct repeat sequences (4–9 bp) are found at the recombination junctions of all *URA3* hairpin revertants suggests that homology is involved in the excision event. To examine whether the homologous recombination machinery mediates hairpin excision, we asked whether mutations known to impair homologous recombination in yeast decrease Ura$^+$ reversion frequencies. To study the effects of *rad1* and *rad52* mutations on hairpin excision and mitotic homologous recombination, we deleted *RAD52* or both *RAD1* and *RAD52* from haploid *ura3::HP80* strains carrying an intrachromosomal duplication of *his4* heteroalleles (*his4–260, 39*; JACKSON and FINK 1981). Ura$^+$ and His$^+$ reversion frequencies were measured in parallel to determine the effect of the single or double *rad* mutations on hairpin excision at *URA3*, and on mitotic homologous recombination between the two mutations within the

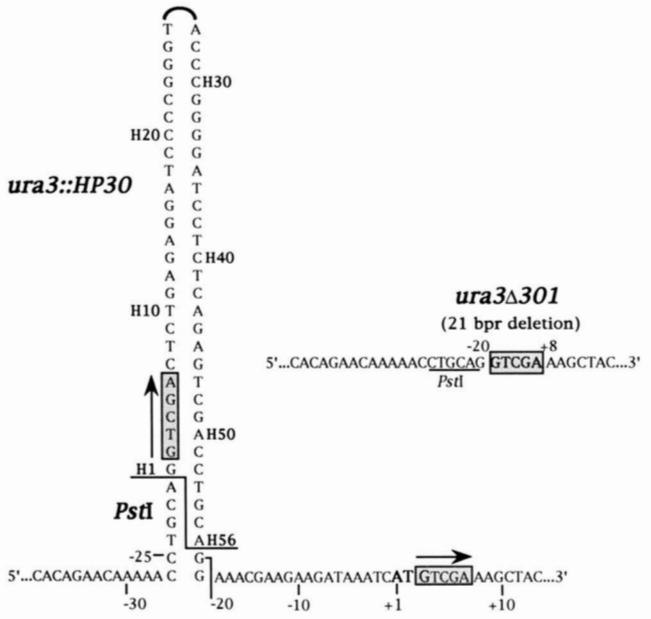

FIGURE 4.—DNA sequence of *ura3Δ301*. (Left) The DNA sequence of *ura3::HP30* presented as described in Figure 3. Direct repeats in shaded boxes indicated by arrows refer to those found at the recombination junction on the right. (Right) Structure of the *ura3Δ301* mutation, a stable Ura⁻ revertant of BR150 (*ura3::HP30*) lacking the *URA3* 5′ leader downstream of the hairpin including the ATG initiator codon. The direct repeats at the recombination junction are in the shaded box (GTCGA): HP30 2 to 6/*URA3* +3 to +7, as numbered on the left. The resulting *URA3* deletion size is indicated in parentheses.

TABLE 2

Effect of *RAD52* and *RAD1,52* deletions on hairpin excision at *URA3* and homologous recombination at *HIS4*

Strain	Genotype[b]	Mean frequency of recombinants (±SD)[a] per 10^5 viable cells: Ura⁺	His⁺
BR182	*RAD⁺*	6.4 (±0.68)	7.3 (±2.9)
BR180	*rad52::LEU2*	6.5 (±1.5)	1.6 (±0.33)
BR208	*rad52::LEU2, rad1Δ*	9.4 (±2.1)	0.87 (±0.32)
BRx244	$\frac{RAD^+}{RAD^+}$	6.5 (±3.0)	7.9 (±5.8)
BRx245	$\frac{rad52::LEU2}{rad52::LEU2}$	5.8 (±3.0)	8.6 (±4.5)
BRx246	$\frac{rad1\Delta}{rad1\Delta}, \frac{rad52::LEU2}{rad52::LEU2}$	11 (±7.9)	0.17 (±0.10)

[a] Standard deviations.
[b] All haploid strains are *ura3::HP80*, ⟨*his4-260,39*⟩.
All diploid strains are $\frac{ura3::HP80}{ura3::HP80}, \frac{his4\text{-}260}{his4\text{-}280}$.

his4 duplication. The *rad52* deletion has no effect on the frequency of hairpin excision at *URA3*, though recombination at *HIS4* is reduced about 4.5-fold (Table 2, BR182 and BR180). In the *rad52,rad1* double mutant (BR208), hairpin excision remains unaffected while recombination at *HIS4* is diminished

TABLE 3

UV treatment of *ura3::HP80* diploids

Strain	Genotype	45″ UV:	Mean frequency of recombinants per 10^5 viable cells: Ura⁺ −	Ura⁺ +	His⁺ −	His⁺ +
BRx226	$\frac{ura3::HP80}{ura3::HP80}$		18	16	—	—
BRx227	$\frac{ura3::HP80}{ura3\Delta301}$		6.5	5.5	—	—
BRx89	$\frac{ura3::HP80}{ura3\text{-}52}, \frac{his4\text{-}280}{his4\text{-}260}$		97[a]	96	6.1	210

[a] The 15-fold difference between Ura⁺ frequency in BRx227 and BRx89 probably reflects gene conversion events between the hairpin-containing leader in *ura3::HP80* and the normal leader sequences present in *ura3-52*.

over eightfold. We also tested diploid hairpin strains for *rad* mutation effects, and found that diploid strains homozygous for the *rad52,rad1* double mutations are dramatically impaired (46-fold) in homologous recombination between *his4* interchromosomal heteroalleles (*his4–260/his4–280*), but have normal hairpin excision frequencies at *URA3* (Table 2, BRx244 and BRx246). The fact that deletions of RAD52 and RAD1, gene products known to be required for homologous recombination, fail to decrease the frequency of hairpin excision in both haploid and diploid strains suggests that the mechanism of DNA hairpin excision differs from that of homologous recombination.

UV irradiation, a treatment known to stimulate homologous recombination events, fails to increase the frequency of hairpin excision. Ura⁺ reversion frequencies were measured in diploid strains homozygous (BRx226) or heterozygous (BRx227) for the *ura3::HP80* allele either with or without UV irradiation (Table 3). Treatment of hairpin-containing strains with UV had no effect on Ura⁺ reversion frequencies in either homo- or heterozygous *ura3::HP80* diploids. Mitotic interchromosomal recombination between the *his4* heteroalleles (*his4–260/his4–280*), a control for UV-stimulated homologous recombination, was stimulated 34-fold (BRx89). Ura⁺ reversion in *ura3::HP80* homozygous diploids is about twofold higher than in heterozygous diploids with a single hairpin, showing that homozygous hairpins are deleted independently.

Finally, excision of the *ura3::HP80* hairpin is not stimulated during meiosis as are most homologous recombination events. Homozygous *ura3::HP80* diploids induced to undergo meiosis in 1% potassium acetate give frequencies of Ura⁺ reversion similar to those of the unsporulated mitotic diploids (data not shown). Moreover, the *ura3* hairpin alleles do not

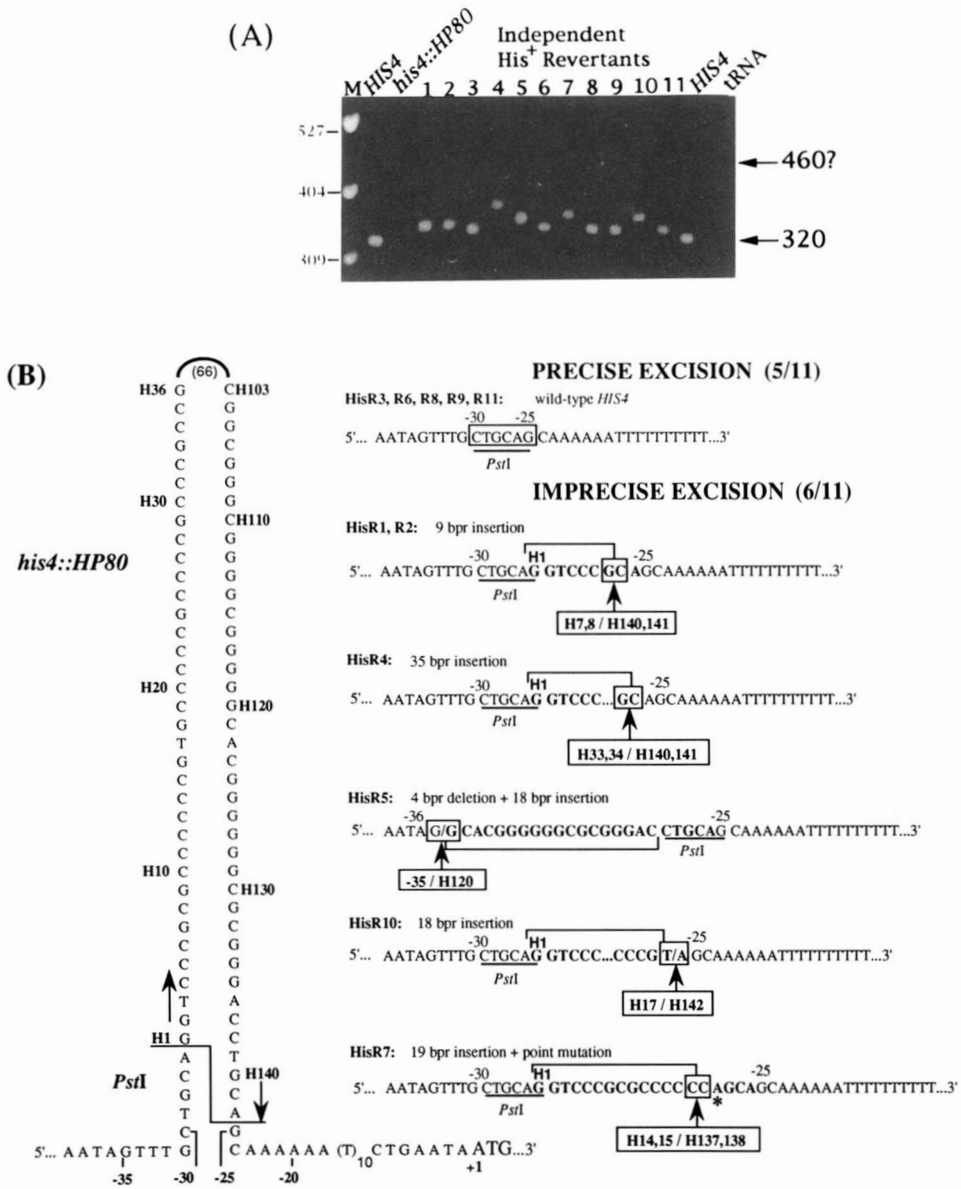

FIGURE 5.—PCR and sequence analyses of *his4::HP80* His$^+$ revertants. (A) PCR amplification products from isogenic strains L4046 (*HIS4*), BR177 (*his4::HP80*) and 11 independent His$^+$ revertants from BR177. The size of pBR322-*Msp*I digested DNA markers (M) are in bp on the left. The size of the PCR products from *HIS4* (320) and the expected product from *his4::HP80* (460?) are in bp and indicated by arrows on the right. As seen for *ura3::HP80* (Fig. 2B), the HP80 TIR cannot be amplified from the *HIS4* locus (*his4::HP80*). Amplification with tRNA as a template (tRNA) yields no product. (B; left) The DNA sequence of the *HIS4* leader in strain BR177 is drawn horizontally, with the HP80 inverted repeats drawn as a hairpin in the *Pst*I site (boxed CTGCAG; −30 to −25). The bases comprising the hairpin are numbered (H1 to H142) 5′ to 3′ from the *Pst*I insertion site. The *HIS4* leader is numbered relative to the +1 ATG initiator codon. (B; right) DNA sequences of the His$^+$ revertants amplified in (A). The number of revertants obtained/number sequenced in each class is in parentheses. Direct repeats at recombination junctions are boxed within each revertant sequence. The sequence junctions are numbered as in the hairpin diagram (left) and are indicated in boxes below. TIR-derived insertion sequences are shown in bold type, and insertion sizes are indicated in bp; *HIS4* leader deletion sizes are in bp. The starred A residue in HisR7 represents the mismatch (T to A) next to the recombination junction.

Hairpin excision from *URA3* at different chromosomal positions and with increasing distance between inverted repeats

Strain	Genotype[b]	Locus	Plasmid	Mean frequency of Ura$^+$ recombinants per 10^6 viable cells (±SD)[a]	
BR132	*ura3::HP80*	*URA3*	No	300	
BR131	*ura3:HP80*	*HIS3*	pBR46	1400	
BR195	*ura3::HP80*	*LEU2*	pBR129	12 (±2.0)	Fold decrease
BR196	*ura3::HP82*	*LEU2*	pBR135	38 (±9.0)	≡1
BR201	*ura3::HP82* ***+25i***	*LEU2*	pBR139	3.0 (±1.0)	13
BR197	*ura3::HP82* ***+60i***	*LEU2*	pBR136	0.84 (±0.15)	45
BR198	*ura3::HP82* ***+218i***	*LEU2*	pBR137	0.95 (±0.11)	40

[a] Standard deviations.
[b] *ura3::HP82****+25i*** has a 25-bp, ***+60i*** a 60-bp, and ***+218i*** a 218-bp insert separating the HP82 inverted repeats.

show unusual meiotic segregation: in more than 200 meioses examined, both *ura3::HP30* and *ura3::HP80* segregated 2:2 in meiotic tetrads.

A tandem inverted repeat at *HIS4* is also mitotically unstable: The HP80 hairpin was inserted into the *HIS4* leader between the RNA start sites and the ATG initiator codon (MATERIALS AND METHODS) to test whether the *his4::HP80* construct shows properties similar to *ura3::HP80*. We characterized hairpin excision events from the *HIS4* locus by PCR amplifi-

cation (Figure 5A) and sequencing of DNA from His$^+$ revertants (MATERIALS AND METHODS). The recombination junctions from 11 independent revertants are illustrated in Figure 5B. Similar to the Ura$^+$ revertants, many of the His$^+$ revertants result from precise hairpin excision between the *Pst*I 6-bp short repeats (5/11). However, the remainder of the His$^+$ revertants display a wider spectrum of partial excisions, primarily between dinucleotide repeats (5/11). One isolate retained part of the hairpin and also removed 4 bp of upstream *HIS4* leader (Figure 5B, HisR5).

In His$^+$ revertant 7 (HisR7), sequences at the recombination junction have been mutated. The revertant DNA sequence results from recombination between a CC dinucleotide repeat followed by a T to A transversion at the adjacent base (Figure 5B, HisR7, starred A). To ensure that the mutation did not arise by the procedures used to analyze the DNA, we repeated the amplification and sequencing of HisR7 DNA and obtained the same sequence as in the first experiment. The alteration of DNA sequence in this revertant suggests that hairpin excision is resolved by DNA replication, either during the normal DNA replication process, or by DNA gap repair (reviewed in SANCAR and SANCAR 1988).

The frequency of hairpin excision depends upon chromosomal position and the proximity of the inverted repeats: We asked whether chromosomal position generally affects the frequency of hairpin excision by integrating the *ura3::HP80* gene on a marked plasmid into the *HIS3* (BR131), or the *LEU2* (BR195) genes. At *HIS3*, Ura$^+$ reversion frequencies of *ura3::HP80* are about two orders of magnitude higher than from a similar plasmid integrated at the *LEU2* locus (Table 4; MATERIALS AND METHODS). The *ura3::HP80* hairpin (without plasmid sequences) is excised from *URA3* at a frequency intermediate between the *LEU2* and *HIS3* locations. Thus, the HP80 hairpin surrounded by the same local sequences is excised at extremely different frequencies when inserted into different chromosomal locations.

We examined the effect of increasing the distance between the inverted repeat sequences by inserting random pieces of λ phage or pBR322 DNA into the *Xba*I restriction site at the center of the *ura3::HP80* hairpin. Each plasmid construct was integrated at the *LEU2* locus, and the frequencies of Ura$^+$ reversion events from the marked plasmids were quantified. The control construct (BR196) has an 82-bp perfect inverted repeat (*ura3::HP82*; MATERIALS AND METHODS). Addition of an extra two bases in the hairpin stem stimulates the Ura$^+$ reversion frequency threefold (BR195 *vs.* BR196, Table 4). As the inverted repeats are separated by about 25 bp, the reversion frequency drops 12-fold (BR201), and when the distance is increased to 60 bp, the frequency drops 45-fold (BR197). No further stabilization is observed by separating the inverted repeats from 60–218 bp (BR198). Therefore, separation of the inverted repeats by as little as 25–60 bp increases their chromosomal stability by more than an order of magnitude. Whether these inverted repeats can be completely stabilized by separating them further remains to be established.

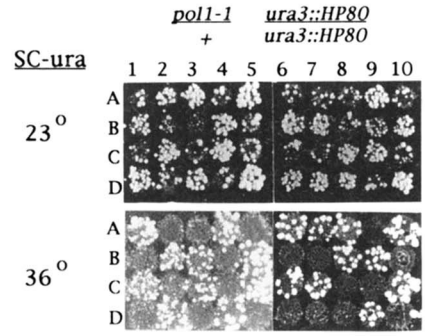

FIGURE 6.—Tetrad analysis of Ura$^+$ reversion in spores from *pol1–1/POL1* diploids homozygous for *ura3::HP80*. Spores from BRx235 (*pol1–1/POL1, ura3::HP80/ura3::HP80*) were grown on complete medium at 23° and replica-plated to SC-ura plates at either 23° (permissive; top) or 36° (nonpermissive; bottom) and photographed after 3 days. Tetrads are numbered above, and spores indicated by letters on the side. Any apparent temperature effect among the ts$^+$ spores is only a difference in growth rates of the starting cells and revertant colonies (hence they look bigger), and not the number of revertant colonies/total cells that one determines by quantitative reversion assays.

Hairpin removal is stimulated by mutations in DNA polymerase I but not by most *RAD* mutations: *POL1* (*CDC17*) is an essential gene in yeast (CARSON and HARTWELL 1985; JOHNSON *et al.* 1985), which encodes the DNA polymerase I catalytic subunit responsible for discontinuous DNA synthesis. We first noticed a dramatic increase in hairpin deletion frequencies from *pol1* mutants when we replica-plated the spores from a dissection of a diploid, homozygous for *ura3::HP80* and heterozygous for *pol1–1* (PIZZAGALLI *et al.* 1988), onto SC-ura selective medium at permissive (23°; Figure 6) or semipermissive (30°; data not shown) temperatures. High-frequency Ura$^+$ reversion segregates 2:2 in crosses and is always linked to the temperature-sensitivity of the *pol1–1* mutation (Figure 6). The same effect is seen when another *POL1* mutant allele, *cdc17–2* (CARSON and HARTWELL 1985; HARTWELL and SMITH 1985), segregates 2:2 among spores from a *ura3::HP80* homozygous diploid replica-plated to selective media (data not shown). Quantitative reversion assays performed on sister spores from these crosses revealed up to 30-fold increases in *ura3::HP80* excision frequencies in mutant strains (data not shown).

To verify this result, we constructed isogenic *POL1* and *pol1* strains containing the hairpin constructions and assayed the reversion frequencies of these strains (MATERIALS AND METHODS). The results of quantita-

TABLE 5

Frequency of hairpin loss from *URA3* and *HIS4* in isogenic *RAD* mutants

Strain	Genotype[b]	Mean frequency of recombinants (±SD)[a] per 10^6 viable cells: Ura$^+$	His$^+$
BRx80-11B	*RAD*$^+$	28 (±1)	0.26 (±0.07)
BR202	*rad6Δ*	28 (±5)	0.22 (±0.08)
BR203	*rad10Δ*	26 (±3)	0.14 (±0.02)
BR204[c]	*RAD*$^+$*::LEU2*	120 (±29)	0.22 (±0.09)
BR179	*rad52::LEU2*	120 (±16)	0.18 (±0.04)
BR205	*rad1Δ, rad52::LEU2*	140 (±18)	0.38 (±0.08)

[a] Standard deviations.
[b] All strains are *ura3::HP80, his4::HP80*, and isogenic.
[c] BR204 is BRx80-11B with YEp13 (*LEU2*, 2-μm plasmid) maintained by leucine starvation. Reversion assays for BR204 and the other *LEU2*-marked deletion strains (BR179 and BR205) were performed in SC media lacking leucine.

TABLE 6

Frequency of hairpin loss from *URA3* and *HIS4* in DNA polymerase I mutants

Strain[b]	Genotype[c]	Mean frequency of recombinants (±SD)[a] per 10^6 viable cells: Ura$^+$	Fold increase	His$^+$	Fold increase
BRx121-1D	*pol1-1*	63 (±10)	11	3.0 (±0.6)	14
BR190	*POL1*	5.8 (±0.3)	1	0.22 (±0.15)	1
BRx218-4D	*cdc17-2*	9.1 (±1.0)	3	2.2 (±1.5)	8
BR200	*CDC17*	2.9 (±0.3)	1	0.29 (±0.03)	1
BRx217-8B	*cdc17-1*	17 (±8.3)	1	0.44 (±0.17)	1
BR211	*CDC17*	12 (±2.0)	1	0.51 (±0.25)	1

[a] Standard deviations.
[b] Strains are listed as isogenic pairs.
[c] All strains are *ura3::HP80, his4::HP80.*

tive Ura$^+$ and His$^+$ reversion assays in isogenic *pol1* mutant and wild-type strains are presented in Table 6. The *pol1-1* and *cdc17-2* alleles of *POL1* increase the frequency of HP80 excision from both *URA3* and *HIS4* from three- to 14-fold in otherwise isogenic strains. The phenotypically more severe mutant allele, *cdc17-1*, does not significantly change hairpin excision frequencies (Table 6).

Other temperature sensitive (ts) mutations in enzymes related to DNA synthesis do not show this dramatic increase in hairpin reversion. No consistent effect on hairpin excision from both the *URA3* and *HIS4* loci could be found with DNA replication mutants *cdc2, 7, 8, 9* and *21*. Although some spores and some crosses showed increases or decreases in hairpin reversion frequencies as compared with the *Cdc*$^+$ controls, these differences did not segregate consistently with the cdc/ts phenotypes.

We also tested mutations representative of the three general epistasis groups of *RAD* (radiation-sensitive) genes (Haynes and Kunz 1981) to see whether they affect HP80 hairpin excision from *URA3* and *HIS4*; many of these genes are required for DNA repair, and mutations in some stimulate mitotic recombination. Hairpin excision frequencies were quantified in isogenic strains with null mutations of *rad6, rad10, rad52*, or *rad1, 52* (Table 5; MATERIALS AND METHODS). None of the DNA repair mutations had a significant effect on HP80 deletion frequencies from either *URA3* or *HIS4*. We also tested haploids obtained from two successive crosses of the double hairpin strain BRx80-11B to *rad50* or *rad3* point mutants, with no obvious difference in hairpin excision frequencies between normal and mutant nonisogenic strains (data not shown).

DISCUSSION

We have inserted long tandem inverted repeats (LTIRs) with 30 (HP30) or 80 (HP80) bp unit repeats into the 5′ leaders of the yeast *URA3* and *HIS4* genes. These structures, which can potentially form hairpins by intramolecular base-pairing, are removed from the chromosome at frequencies depending on their size (Figure 1) and location (Tables 4 and 5). The mitotic instability of HP80 is diminished by increasing the distance between the repeat units by only 25–60 bp (Table 4). Hairpin deletions preferentially occur between short direct repeats of 2–9 bp, with at least one deletion endpoint located at the base of the hairpin (Figures 3 and 5B), and are unaffected by mutations or conditions that decrease or increase homologous recombination in yeast (Tables 2 and 3). We conclude that the mechanism of hairpin deletion in yeast differs from that of homologous recombination between long identical sequences located in tandem, on homologous chromosomes or dispersed on different chromosomes.

The HP80 hairpin is excised from the *HIS4* leader at 100- to over 600-fold lower frequencies than the same hairpin at *URA3* (Table 5, BRx80-11B and BR204; Table 6). There are several possible explanations for this difference. It could be that excision frequencies are dependent on the size of the short direct repeats that flank the LTIR. Precise excision of the hairpin from *ura3::HP80* occurs within a 9-bp direct repeat, whereas there is only a 6-bp direct repeat at the base of the *his4::HP80* hairpin (Figures 3 and 5B). While this may be a factor, there is no simple, direct correlation between flanking repeat length and hairpin excision frequency. For example, deletions more frequently occur between 4- and 5-bp flanking repeats (Class II and III) than between 9-bp repeats (Class I) in the 12 independent *ura3::HP30* revertants analyzed (Figure 3). Alternatively, the different frequencies could reflect some aspect of the different chromosomal environments into which the hairpins have been inserted (as in Table 4). For example, different levels of transcriptional activity in the two regions might influence the frequency of hairpin removal if the nontranscribed DNA strand can occa-

sionally form a hairpin while RNA polymerase copies its homologue. Stable RNAs (detected by Northern blot and primer extension analyses) are made from both *ura3::HP80* and *his4::HP80* genes, demonstrating that the hairpins inhibit gene expression posttranscriptionally (data not shown). Although much more *URA3* than *HIS4* mRNA can be detected in cells with or without hairpin inserts, whether this reflects actual transcription rates is unknown. An additional potential factor is that the proximity of the hairpin to an origin of replication might affect its excision frequency.

LTIR instability in prokaryotes is a well-known phenomenon. Although relatively short TIRs (16–33 bp inverted repeats) can be replicated in *E. coli*, longer perfect TIRs of 75 bp to 1.5 kb are very unstable (reviewed in EHRLICH 1989). The classical studies on LTIR instability used 1- to 3-kb inverted repeats inserted into the Ap^R gene of plasmid pBR322 (COLLINS 1980; COLLINS, VOLCKAERT and NEVERS 1982). Ampicillin-resistant, rearranged plasmids had either the center of symmetry (hairpin loop) or the entire LTIR excised from the plasmid. All sequenced rearrangements could be explained by recombination between short direct repeats of 4–6 bp. Plasmid stability is regained in *E. coli* by increasing the distance between the inverted repeats (WARREN and GREEN 1985).

Excision of transposons (Tn) from the bacterial chromosome also involves recombination between short direct repeats either flanking or within long (but not tandem) inverted repeats, and is independent of the host homologous recombination system (reviewed in BERG 1989; GALAS and CHANDLER 1989; KLECKNER 1989). Excision of Tn10 sometimes leaves behind a 23-bp TIR flanked by 9-bp direct repeats (FOSTER *et al.* 1981). These Tn10 remnants are excised at high frequencies that depend on chromosomal position and the length of the inverted repeats. Mutations in *PolA*, the bacterial homolog of the yeast *POLI* gene, increase the frequency of this excision event (LUNDBLAD and KLECKNER 1985).

The behavior of LTIR structures in yeast shows many similarities to that described for LTIRs in *E. coli*. Most but not all hairpin deletion events in yeast also occur by recombination between short direct repeats (Figures 3, 4 and 5B). Furthermore, separation of the chromosomal inverted repeats by as little as 25–60 bp greatly stabilizes them in yeast (Table 4 and see below). No further stabilization is observed by separating the inverted repeats from 60–218 bp. Yeast LTIRs are removed from the yeast chromosome by a mechanism independent of the normal homologous recombination machinery (Tables 2 and 5), and LTIR deletion frequencies also depend on chromosomal position (Table 4). As with the LTIRs in *E. coli*, so in yeast the length of the LTIR influences its stability: the addition of only 2 bp to the stem of *ura3::HP80* (*ura3::HP82*) increases hairpin excision from the same chromosomal position threefold (Table 4). That the GC-rich HP80 is excised at about 30-fold higher frequencies than HP30 from the *URA3* gene probably reflects the increased stability of the longer HP80 as well as the different base composition of the two sequences.

LTIR instability is proposed to result from the ability of PolI to switch DNA templates during replication (STREISINGER *et al.* 1966; reviewed in EHRLICH 1989). While copying one strand of a DNA duplex, PolI can precociously slip, or switch template strands between short stretches of sequence homology. Replication slippage, or template switching events can create deletions or insertions, and explains various genomic rearrangements between short direct repeats seen *in vitro* (KUNKEL *et al.* 1989 and references therein; PAPANICOLAOU and RIPLEY 1989), and *in vivo* in *E. coli* (FARABAUGH *et al.* 1978; EDLUND and NORMARK 1981; RIPLEY and GLICKMAN 1982; SCHAAPER, DANFORTH and GLICKMAN 1986; SINDEN *et al.* 1991), yeast (GIROUX *et al.* 1988; LEE *et al.* 1988; GORDENIN *et al.* 1992), and mammals, where several complex chromosomal rearrangements associated with genetic defects occur in the vicinity of LTIRs (reviewed in MEUTH 1989).

Template switching is thought to be stimulated by conditions that stall polymerase, which might then allow the nascent DNA strand to be extruded from the replication complex and anneal to a distal region with short sequence homology. Because TIRs can stall a number of DNA polymerases (BACKMAN and YANOFSKY 1978; KAGUNI and CLAYTON 1982; WEAVER and DEPAMPHILIS 1984), including yeast POLI (yPOLI; BADARACCO *et al.* 1985), they could stimulate template switching between short direct repeats brought into proximity by hairpin formation. As a replication fork passes through the LTIR, the "leading" strand is replicated by DNA PolIII, and the "lagging" strand is primed with short RNA oligonucleotides by the DNA PolI/DNA primase complex before PolI can initiate DNA synthesis. During the brief window of time that the lagging strand template is essentially single-stranded, stable hairpin formation could be favored. Stalling of PolI when it encounters the stable secondary structure allows template switching between short direct sequence repeats close to the arrested replication complex.

This template switching model is consistent with our data on the sequence of hairpin revertants and the increased frequency of hairpin revertants in *pol1* mutant strains. Most of the hairpin deletions we obtained occur by recombination between short direct repeats. Moreover, at least one deletion endpoint

always mapped at or near the base of the hairpin. Most importantly, mutations in yeast DNA polymerase I (*POL1/CDC17*) increase the frequency of hairpin loss from mitotically growing, isogenic cells three- to 14-fold (Table 6). The *pol1–1* and *cdc17–2* "slow stop" mutations complete the cell cycle before arresting at the next round of DNA synthesis upon temperature shift (LUCCHINI *et al.* 1990), and map to a region in the amino terminal portion of the protein (LUCCHINI *et al.* 1988) responsible for the association of DNA PolI with the primase complex (PIZZAGALLI *et al.* 1988; LUCCHINI *et al.* 1990). Our results can be interpreted to mean that dissociation of PolI from DNA primase increases the frequency of hairpin deletion *in vivo*. This interpretation is consistent with *in vitro* replication studies using purified yPolI and yPolI-DNA primase complex (KUNKEL *et al.* 1989). Association of yPolI with DNA primase *in vitro* did not increase general replication fidelity: both enzymes produced deletions, over half of which could be explained by replication slippage between short direct repeats of 3–9 bp, with the frequency of template misalignment events increasing with the length of the direct repeats. More importantly, replication with yPolI in the absence but not the presence of DNA primase produced complex deletions whose endpoints clustered around the base of a potential hairpin structure. The deletions could all be explained by yPolI stalling at the hairpin structure, looping around and copying the newly replicated strand, and then switching templates between short direct repeats, thereby deleting the intervening region of DNA.

We found that mutations in DNA PolIII (*CDC2*; BOULET *et al.* 1989; SITNEY, BUDD and CAMPBELL 1989) do not affect the frequency of hairpin excision. Mutations in *CDC2*, which shares homology to the mammalian polymerase that performs leading strand DNA synthesis, might increase the stability of LTIRs by slowing down replication. However, neither the *cdc2–1* (L. Hartwell) or the *tex1* (GORDENIN *et al.* 1991) mutations significantly affected hairpin excision in our experiments (data not shown). This result is somewhat surprising because *tex1* was originally isolated as a ts mutation that stimulates Tn5 excision from the yeast *LYS2* gene 100-fold (GORDENIN *et al.* 1991), and was later found to be allelic to *POL3/CDC2* (renamed *pol3-t*; GORDENIN *et al.* 1992). That the *tex1* mutation does not stimulate hairpin loss in our studies probably reflects the mechanistic difference between loss of Tn5, which has 1.5-kb terminal inverted repeats separated by 2.7 kb, and that of the 30- and 80-bp LTIRs studied here. Another difference between the two systems is that increased Tn5 excision is *RAD52*-dependent, whereas HP30 and HP80 excision is not (Tables 2 and 5). Unpublished results of S. HENDERSON and T. PETES also indicate that a poly-GT hairpin (47-bp unit repeats) is excised at high frequencies from a plasmid in yeast by a *RAD52*-independent mechanism (personal communication).

An alternative to the polymerase stalling model is one that invokes a specific DNA repair system that recognizes hairpins. The repair system could induce unique components to function with normal components of the replication machinery. Transcription of *CDC8* (ELLEDGE and DAVIS 1987), *CDC9* (PETERSON *et al.* 1985) and possibly *POLI/CDC17* (JOHNSTON *et al.* 1987; but see ELLEDGE and DAVIS 1987) is induced by DNA damage, and thus these gene products may play a role in certain types of DNA repair as well as in normal DNA replication. This hypothetical hairpin repair system would be different from those described for other types of DNA damage. The system would have to be independent of *RAD1, 3, 6, 9, 10, 50* and *52*, because mutations in these genes do not reduce hairpin excision frequencies (Tables 2 and 5, and data not shown). Moreover, hairpin deletion is unaffected by UV treatment (Table 3), which activates DNA repair functions necessary to remove bulky lesions from DNA (reviewed in SANCAR and SANCAR 1988). This hypothetical system is unlikely to be inducible because we cannot detect any trans-acting DNA repair activity induced by LTIRs: hairpins at *URA3* and *HIS4* revert independently from one another (Table 3 and data not shown). The two models need not be exclusive; hairpins could be excised by both the replication machinery and a specific repair pathway. A search for mutations that abolish or reduce hairpin reversion could reveal genes in the hypothetical repair pathway. Additionally, mutations that affect hairpin excision frequencies could identify activities like helicases or topoisomerases that may participate in hairpin formation *in vivo*. Identifying new mutants that modulate hairpin excision frequencies might also help elucidate the effect of chromosomal environment on hairpin deletion and other more complex genome rearrangements.

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