Instability of a Plasmid-Borne Inverted Repeat in *Saccharomyces cerevisiae*

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ABSTRACT

Inverted repeated DNA sequences are common in both prokaryotes and eukaryotes. We found that a plasmid-borne 94 base-pair inverted repeat (a perfect palindrome of 47 bp) containing a poly GT sequence is unstable in *S. cerevisiae*, with a minimal deletion frequency of about 10^{-4}/mitotic division. Ten independent deletions had identical end points. Sequence analysis indicated that all deletions were the result of a DNA polymerase slippage event (or a recombination event) involving a 5-bp repeat (5′ CGACG 3′) that flanked the inverted repeat. The deletion rate and the types of deletions were unaffected by the *rad52* mutation. Strains with the *pms1* mutation had a 10-fold elevated frequency of instability of the inverted repeat. The types of sequence alterations observed in the *pms1* background, however, were different than those seen in either the wild-type or *rad52* genetic backgrounds.

INVERTED repeated DNA sequences (palindromic repeats) are common in the genomes of both prokaryotes and eukaryotes. In single-stranded DNA molecules these repeats are capable of self-pairing, forming "hairpin" structures. In supercoiled plasmids *in vitro*, these sequences sometimes pair in a cruciform configuration (GELLERT, O'DEA and MIZUUCHI 1983; GIERER 1966; LILLEY 1980). Several types of experiments suggest that, in some cases, hairpin structures and cruciforms may be biologically relevant. In *Saccharomyces cerevisiae*, the properties of hairpin structures formed as a consequence of heteroduplex formation between a strand without a palindromic insertion and one with a palindromic insertion have been examined (NAG, WHITE and PETES 1989; NAG and PETES 1991). We found that such structures are poorly recognized by the mismatch repair system. Palindromic insertions of 14 bp (7 bp on each side) were sufficient to confer this property. It has also been observed that efficient recognition of the bacteriophage N4 promoter requires formation of a hairpin structure (GLUCKSMANN *et al.* 1992). A third, less direct, argument that hairpin (and/or cruciforms) form *in vivo* is at the observed instability of these sequences in prokaryotes and eukaryotes (reviewed by EHRLICH 1989; RIPLEY 1990).

Most studies of the stability of inverted repeats have been done in *Escherichia coli*. Such studies indicate that inverted repeats of even a few base pairs can stimulate deletion formation (ALBERTINI *et al.* 1982; GLICKMAN and RIPLEY 1984; SCHAAPER, DANFORTH and GLICKMAN 1986). These deletions often do not represent the precise excision of the inverted repeat, but involve small direct repeats flanking the inverted repeat. DASGUPTA *et al.* (1987) found that the frequency of deletions stimulated by palindromes was strongly affected by its location; the same palindrome stimulated deletion formation at different locations 10–10,000-fold. At least part of this effect is likely to reflect the presence or absence of direct repeats in flanking DNA sequences. A systematic analysis of the effects of the length of palindrome on deletion formation in pBR322 in *E. coli* was done by WESTON-HAFER and BERG (1991). These workers found that a 32-bp palindrome stimulated deletion formation (between flanking four base repeats) 100-fold more effectively than a 26-bp repeat at the same location. The deletion frequency for the 32-bp repeat was about 1×10^{-4}.

There is little information concerning the stability of palindromic sequences in eukaryotes. Sequences of spontaneous mutations at the *aprt* locus of hamster cells suggest inverted repeats may stimulate deletion formation (NALBANTOGLU *et al.* 1986). In *S. cerevisiae*, the excision of a Tn*5* element inserted within the *LYS2* gene was examined (GORDENIN *et al.* 1988, 1992). This element contains two inverted repeats of 1.5 kb separated by 6 kb, and is flanked by 9-bp direct repeats. This element underwent infrequent excision events (frequency of 10^{-8}), and most of these excision events involved short direct repeats flanking the large inverted repeats. These excision events were attributed to DNA polymerase slippage because several mutations affecting DNA polymerase increased the rate of excision.

In this study, we examine the stability of a 94-bp palindromic insertion (47-bp inverted repeats) inserted within the β-galactosidase gene of a plasmid propagated in yeast. Each repeat is internally repetitious, containing a 29-bp poly GT tract. Poly GT

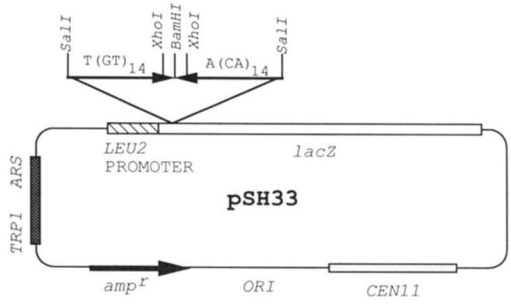

FIGURE 1.—Structure of the plasmid pSH33. This plasmid, derived from pHT259 (TU and CASADABAN 1990), was used to assay deletion of the poly GT palindromic repeats. The deletions were monitored by examining β-galactosidase expression in yeast carrying the plasmid. Deletion or alteration of the palindromic repeats that restored the correct reading frame appeared as blue colonies on plates containing X-gal.

tracts are extremely common in eukaryotes and are known to frequently undergo changes in tract length in yeast (HENDERSON and PETES 1992). Below, we show: (1) a poly GT-containing palindromic sequence is unstable with a mimimal deletion rate of about 10^{-4} events per division; (2) all deletions sequenced (10 of 10) involve an interaction between two flanking 5-bp repeats of the sequence 5′ CGACG; (3) the rate and type of deletion are unaffected by a *rad52* mutation; and (4) a mutation in the *PMS1* gene (affecting mismatch repair) increases the instability of the palindromic insertion, but does not stimulate interactions between the 5-bp CGACG repeats.

MATERIALS AND METHODS

Media and growth conditions: Media for yeast growth were prepared as described by SHERMAN, FINK and HICKS (1983). X-Gal plates for yeast were prepared as described by ROSE and BOTSTEIN (1983). Yeast strains were grown at 30°.

E. coli strains were grown at 37° in LB broth or in X-gal medium (MANIATIS, FRITISCH and SAMBROOK 1982). *E. coli* strain DH5α (BRL) was used for cloning and plasmid rescues, and was handled following standard molecular techniques (MANIATIS, FRITISCH and SAMBROOK 1982).

Plasmid and yeast strain constructions: The plasmid pSH33 was constructed by insertion of two copies of the oligonucleotide GATCGTCGACA$(TG)_{14}$TACTCGA (HENDERSON and PETES 1992) arranged in a head-to-head orientation into the *Bam*HI site of the plasmid pHT259 (TU and CASADABAN 1990). The plasmid pHT259 contains the *LEU2* promoter and the first 12 codons of the yeast Leu2 protein fused to the eighth codon of the *E. coli* β-galactosidase gene. In addition, the plasmid contains an amp^R gene, *CEN11*, ARS, and *TRP1* sequences (Figure 1). The resulting plasmid was transformed into *E. coli*. Since the *LEU2* promoter functions in both yeast and *E. coli*, yeast transformants and *E. coli* transformants containing pHT259 form blue colonies on X-gal plates. Since the insertion of the palindromic sequences in pSH33 results in a frameshift mutation in the β-galactosidase gene, yeast or *E. coli* transformants containing pSH33 form white colonies on X-gal plates. The structure of the insertion in pSH33 was confirmed by DNA sequence analysis.

All three yeast strains used in this study were derived from the strain PD3 (*α trp1 ade6 ura3 his4-Sal tyr7-1 arg4-17*), which was derived by transformation from the strain AS4 (WHITE *et al.* 1992). All three strains are isogenic except for changes introduced by transformation. The strain PD3:33 was constructed by transforming PD3 with pSH33, selecting Trp^+ transformants. The strain SH52 is isogenic with PD3 except for a *leu2* mutation and an insertion of the wild-type *LEU2* gene into the *RAD52* gene, creating a *rad52* mutation (HENDERSON and PETES 1992). The strain SH52:33 was constructed by transforming SH52 with pSH33. The strain DNY95 was constructed by D. NAG (University of North Carolina) and is isogenic with PD3 except for two changes. It is wild type at the *HIS4* locus, and it contains a null mutation of the *PMS1* gene. The *pms1* mutation was inserted using the plasmid pJH523 obtained from J. HABER (Brandeis University). The strain DNY95:33 was constructed by transforming DNY95 with pSH33.

Measurement of the rate of loss or alteration of the palindromic insertion: As described above, the palindromic insertion in the coding sequence of the β-galactosidase gene in pSH33 is 94 bp in length and, therefore, results in a frameshift mutation. Yeast or *E. coli* strains containing this plasmid form white colonies on X-gal plates. The frequency of loss of this insertion or an alteration in the length of the insertion to restore the correct reading frame can be measured by monitoring the frequency of blue colonies (HENDERSON and PETES 1992). As described below, when the plasmids isolated from blue colonies were examined by DNA sequence analysis, all plasmids contained a deletion or alteration of the palindromic insertion that restored the correct reading frame. To measure the rate of appearance of blue colonies, we grew cultures of the various strains in medium lacking tryptophan (to force retention of the plasmid). The cultures were then diluted into rich growth medium (YPD) to a concentration of 100 cells/ml; 0.1-ml aliquots of each diluted culture were seeded into a 96-well microtiter dish, and incubated at 30° for 22–26 hr. The contents of each well were harvested by centrifugation, resuspended in 1 ml of sterile water, and plated on several X-gal plates; these plates lacked leucine and threonine (to derepress the *LEU2* promoter), and tryptophan. Plates were incubated for 10 days to allow for complete expression of the β-galactosidase phenotype to develop. Rates of formation of blue colonies for strains PD3:33 and SH52:33 were determined using the proportion of cultures that had no blue colonies in a fluctuation analysis as described by LURIA and DELBRUCK (1943) and as modified by LEA and COULSON (1949). Since the strain DNY95:33 had many blue colonies per culture, the method of the median was used to obtain rate estimates (LEA and COULSON 1949).

Plasmid rescue and DNA sequence analysis: Sequence analysis was done on plasmids rescued from yeast and transformed into *E. coli*as described by HOFFMAN and WINSTON (1987). For DNA sequence analysis, double-stranded sequencing templates were prepared by the method of KRAFT *et al.* (1988). The primer (5′GATGTGCTGCAAGGCG 3′) was used to sequence the mutant derivatives in conjunction with the USB Sequenase™ kit according to the protocols provided by the manufacturer.

RESULTS

Instability of plasmid-borne palindromic GT tracts: The plasmid pSH33 (Figure 1) contains a 94-bp palindromic insertion (47 bp on each side) in the coding sequences of the β-galactosidase gene; approximately half of this insertion consists of poly GT sequences. Since the insertion creates a frameshift in the β-galactosidase gene, yeast strains containing the

TABLE 1

Stability of a palindromic repeat inserted upstream of the β-galactosidase gene

Strain	Genotype	No. of cultures	Avg. number of cells/culture	Mean number of blue colonies per culture on X-gal plates[a]	Rate $(\times 10^{-4})^2$	Avg. rate $(\times 10^{-4})$
PD3:33	*RAD52 PMS1*	19	12360	1.1	0.97 ± 0.3	0.9
		16	2520	0.21	0.83 ± 0.4	
SH52:33	*rad52 PMS1*	20	1720	0.16	0.93 ± 0.5	0.9
		19	3290	0.31	0.94 ± 0.4	
DNY95:33	*RAD52 pms1*	20	7050	7.2	10 ± 2.5	11
		19	3580	4.6	12 ± 4	

As described in the text, deletions or alterations of the palindromic insertion were detected by formation of blue colonies on X-gal plates (indicating production of functional beta-galactosidase).

[a] Mean number of mutants per culture and the derived estimates of rates were calculated by fluctuation analysis (LURIA and DELBRUCK 1943; LEA and COULSON 1949) for strains PD3:33 and SH52:33. For strain DNY95:33, these parameters were calculated using the method of the median (LEA and COULSON 1949).

insertion form white colonies on X-gal plates. In a wild-type strain (PD3:33) containing pSH33, we observed blue derivatives occurring at a rate of about 10^{-4}/mitotic division (Table 1). This rate estimate was made by performing a fluctuation analysis on a number of independent cultures (LURIA and DELBRUCK 1943; LEA and COULSON 1949), as described in MATERIALS AND METHODS.

To determine whether strains that formed blue colonies on X-gal plates containing plasmids with an alteration in the palindromic DNA sequences, we rescued plasmids derived from 10 independent blue derivatives into *E. coli* and sequenced them. All 10 independent clones had the same deletion (Figure 2). The bold type indicates the region deleted. The end points of the deletion have the five base repeat 5′CGACG. Interestingly, although this repeat occurs four times near the palindromic insertion, all deletions involve the repeats I and III. Presumably, we did not detect deletions involving repeats I and IV, or repeats II and III, since such deletions would not restore wild-type β-galactosidase activity; thus, our observed deletion rate (10^{-4}/mitotic division) is a minimal estimate. Deletions between II and IV, however, would have generated an in-frame β-galactosidase gene, but were not detected. Explanations for the apparent preference for the I-III interaction to the II-IV interactions will be described in the DISCUSSION.

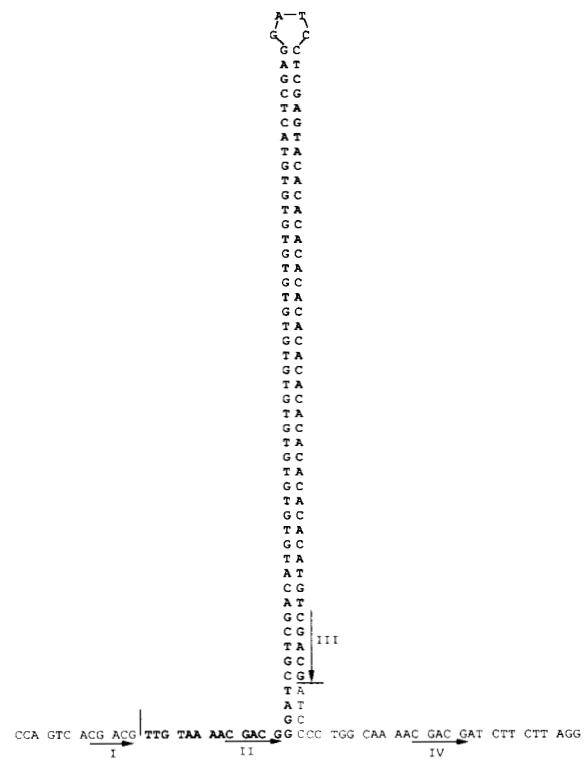

FIGURE 2.—Putative hairpin structure formed during deletion production in pSH33. All plasmids sequenced from PD3:33 and SH52:33 contained the same deletion. The deleted sequences are shown in bold face type, with lines indicating the end points of the deletion. The end points are a direct repeat of the sequence 5′ CGACG 3′. This repeat occurs four times in this region and each is identified by an arrow and a Roman numeral. The depiction of bases as triplets indicates the correct reading frame for the β-galactosidase gene. Deletions occurring between I and III, or II and IV would be in-frame with respect to the β-galactosidase gene, whereas deletions involving I and IV, or II and III would be out-of-frame.

Deletion of plasmid-borne palindromes is unaffected by the *rad52* mutation: The *RAD52* gene is required for most types of mitotic and meiotic recombination in yeast (reviewed by PETES, MALONE and SYMINGTON 1991). To examine the effects of this gene product on deletions of the palindromic insertion, we transformed pSH33 into a strain containing a deletion of *RAD52* (SH52; HENDERSON and PETES 1992). The observed rate of colonies that were blue on X-gal in this strain was identical to that observed with the wild-type strain (Table 1). This result suggests that the rate of loss of plasmid-borne palindromic sequences is independent of the *RAD52* gene product.

To determine whether the same class of events was observed in the *rad52* strain SH52:33, we rescued and sequenced plasmid DNA from ten independent blue derivative strains. All ten of the sequenced clones

contained the same deletion that was seen in the wild-type strain. Therefore, the deletion of the inverted repeats is *RAD52*-independent. This result indicates that either the palindromic sequences are deleted by a mechanism that does not involve recombination, or the deletions occur as a consequence of a *RAD52*-independent mode of recombination.

A mutation in a gene affecting mismatch repair (*PMS1*) increases the instability of poly GT-containing palindromes: The *PMS1* gene of *Saccharomyces cerevisiae* encodes a protein that is homologous to *mutL* of *Salmonella* (KRAMER *et al.* 1989). Yeast strains with the *pms1* mutation have phenotypes consistent with a defect in mismatch repair (mutator activity, high rate of postmeiotic segregation, etc.) (WILLIAMSON, GAME and FOGEL, 1985). Since we found previously that *pms1* strains had an elevated rate of tract stability for nonpalindromic poly GT tracts (M. STRAND and T. PETES, unpublished results), we examined the effects of this mutation on palindromic poly GT insertions. The plasmid pSH33 was transformed into the strain DNY95 (*pms1* derivative isogenic to strains PD3 and SH52 except for changes introduced by transformation). The resulting strain DNY95:33 had a 10-fold increased rate of formation of blue colonies on X-gal plates (Table 1).

To determine if this higher rate was a consequence of a higher rate of deletion of the palindromic insertion, we rescued and sequenced plasmids from 10 independent blue derivatives. None of these plasmids contained the deletion diagramed in Figure 2. All 10 contained 2-bp insertions. In eight of the plasmids, a single GT was added to the poly GT tract nearest the *LEU2* promoter; in the remaining two transformants, a single GT was added to the promoter-distal poly GT tract. These insertions restore β-galactosidase activity because the resulting insertion is 96 bp in length and, therefore, in-frame. We conclude that the increased instability of the poly GT-containing palindrome observed in the *pms1* strain is not a consequence of an increased rate of deletion, but reflects an increased rate of internal changes within the repeated poly GT tracts.

DISCUSSION

In this study, we show that: 1) poly GT-containing hairpins are unstable in a yeast genome, with a minimal deletion rate of about 10^{-4}; 2) the deletion end points suggest an interaction between a unique pair of five base repeats of the sequence 5′ CGACG 3′; 3) the rate and character of the deletion events is unchanged in a *rad52* strain; and 4) deletions of the *PMS1* gene decrease the stability of the palindromic poly GT tract by decreasing the stability of the individual poly GT tracts, rather than by increasing the frequency of palindrome deletion.

One unexplained feature of the data is the preference for interactions between repeats I and III, rather than repeats II and IV (Figure 2). A model of secondary structure that might explain this preference is shown in Figure 3. In this model, a second small hairpin is formed near the base of the major hairpin. In this configuration, repeat II is within the stem of a hairpin, and not accessible for a pairing reaction that would lead to deletion formation. If this structure is favored over that shown in Figure 2, it would explain the preponderance of the deletion formed by the I-III interaction. We cannot exclude the alternative possibility that an interaction between repeats II and IV would not lead to functional β-galactosidase and, therefore, such events would not be detected in our system. Since the expected alteration is in-frame and is in a region that would not be expected to affect the activity of the fusion protein, this possibility is somewhat unlikely.

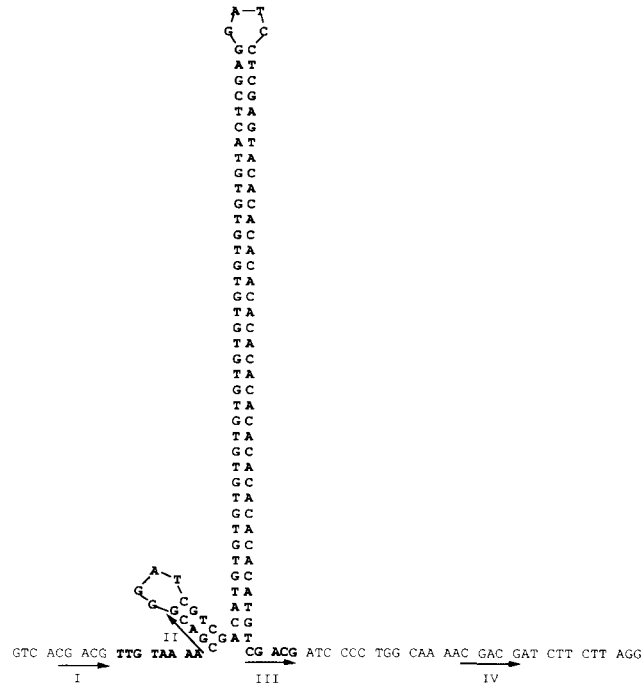

FIGURE 3.—Alternative hairpin structure of the palindromic insertion in pSH33. In this configuration, repeat II is within a stem of a small hairpin and is inaccessible for interaction with another repeat. As in Figure 2, deleted sequences are shown in bold face type, and the 5′ CGACG repeats are indicated by numbered arrows.

The deletion events observed in wild-type yeast strains appear similar to results obtained in *E. coli*. In *E. coli*, plasmid-borne small (20–200 bp) palindromic sequences are found to be unstable and deletion end points are small (4–6 bp) direct repeats (reviewed by EHRLICH 1989; RIPLEY 1990). In general, this instability is attributed to DNA polymerase slippage (STREISINGER *et al.* 1966) rather than recombination since the frequency of these events is relatively unaffected by *recA* and since similar events are observed during *in vitro* DNA replication. A model to explain the deletions by DNA polymerase slippage is shown

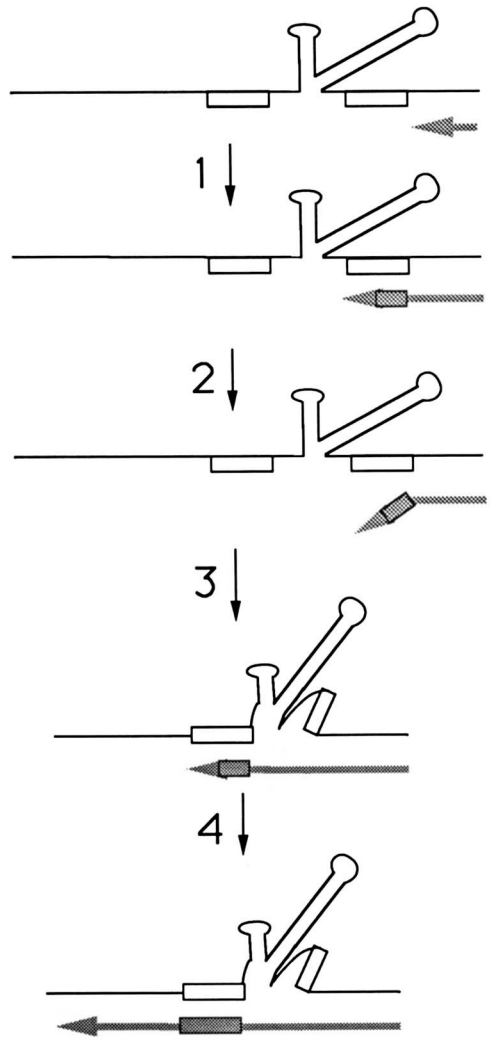

FIGURE 4.—Model for deletion of palindromic sequences during DNA replication. In this figure, the template DNA strand is indicated by thin lines and the elongating DNA strand is shown as a thick line with a terminal arrow. Rectangles indicate repeats I and III of Figure 2. During replication, the region containing the palindromic insertion becomes transiently single-stranded and forms hairpin structures. The elongating strand replicates repeat I (step 1), and then partially dissociates from the template (step 2). The growing strand reassociates with repeat III (step 3) and continues replication (step 4). If the resulting structure does not undergo mismatch repair, the expected products after a second round of replication would be one chromosome with a deletion and another that retains the palindromic insertion.

in Figure 4. In this model, the inverted repeats fold into a hairpin structure that brings the direct repeats closer together. After replicating one repeat, the newly synthesized strand is able to mispair with the nearby repeat and continue replication, thereby deleting the intervening sequences. Alternatively, the loss of the palindrome could result from unequal recombination between repeats I and III. Although this model cannot be ruled out, we favor the slippage model because of the high frequency and the *RAD52*-independence of the events. In addition, if unequal crossovers between direct repeats of 5 bp were very common, one would expect that many mutations in *S. cerevisiae* would be deletions. Very few spontaneous mutations represent this type of change (PETES, MALONE and SYMINGTON 1991).

In previous studies, GORDENIN *et al.* (1988, 1992) measured the frequency of excision of the palindromic Tn*5* element from within the yeast *LYS2* gene. This estimated excision rate (10^{-8}–10^{-9}) was 4–5 orders of magnitude lower than that observed for the short palindromic insertion in our studies (10^{-4}). This difference could reflect either the difference in the behavior of plasmid-borne *vs.* chromosome-borne insertions or an effect of the structure of the palindrome. The second of these two possibilities is more likely since unpublished results from two yeast groups (B. RUSKIN and G. FINK; D. GORDENIN, E. PERKINS and M. RESNICK) indicates that small (60–100 bp) palindromic insertions in the chromosome are excised at frequencies of 10^{-4}–10^{-6}. In addition, GORDENIN *et al.* (1988) found a frequency of 10^{-8} for excision of a plasmid-borne copy of Tn*5* in yeast. Thus, it is apparently easier to form the hairpin structure as an intermediate for excision of the palindrome for a relatively small perfect palindrome than for large inverted repeats that are separated by nonpalindromic DNA. Although we have found no instability of palindromes 4–26 bp in length inserted within the *HIS4* gene (D. NAG and T. PETES unpublished data), we have not investigated the properties of longer palindromes at the same position.

The effect of several mutations on the excision of palindromes has been examined. In our studies and those of B. RUSKIN and G. FINK (unpublished results), the excision rate is unaffected by the *RAD52* gene product. The frequency of excision of Tn*5* in yeast is elevated by mutations in either DNA polymerases I and III (GORDENIN *et al.* 1992); mutations in DNA polymerase I also increase the excision rates of palindromes from the nontranslated regions of *HIS4* and *URA3* (B. RUSKIN and G. FINK, unpublished results). Since strains of the *rad52 pol3* genotype had a much lower level of Tn*5* excision than strains that had only the *pol3* mutation, GORDENIN *et al.* (1992) suggested that the *RAD52* gene product might have a role in palindrome excision. Our results (as well as those of B. RUSKIN and G. FINK) indicate that *RAD52* is not required for the excision of perfect palindromes. The lack of an effect of *RAD52* and the observed effects of mutations affecting DNA polymerase are most consistent with the hypothesis that the palindromes are lost as a consequence of DNA polymerase slippage.

In a study of Tn*10* excision in *E. coli*, LUNDBLAD and KLECKNER (1985) found that strains carrying a *mutL* mutation had 10- to 50-fold elevated levels of precise excision. In yeast strains containing a mutation in the MutL homolog *PMS1*, we did not observe a substantial elevation in the levels of palindrome exci-

sion. Aside from the obvious possibility of species-specific mechanisms for palindrome excision, this difference could also reflect the different structures of the insertions used in the two studies, short palindromic repeats in our study *vs.* the nontandemly arranged long inverted repeats of Tn*10*.

In summary, we have shown that a 94-bp plasmid-borne palindromic insertion is unstable. The deletion rate of the insertion is about 10^{-4}, and that deletion rate is unaffected by *RAD52*. Our results and those of others indicate that these deletions are likely to be a consequence of DNA polymerase slippage.

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