Factors Affecting Inverted Repeat Stimulation of Recombination and Deletion in *Saccharomyces cerevisiae*

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ABSTRACT

Inverted DNA repeats are an at-risk motif for genetic instability that can induce both deletions and recombination in yeast. We investigated the role of the length of inverted repeats and size of the DNA separating the repeats for deletion and recombination. Stimulation of both deletion and recombination was directly related to the size of inverted repeats and inversely related to the size of intervening spacers. A perfect palindrome, formed by two 1.0-kb *URA3*-inverted repeats, increased intra- and interchromosomal recombination in the adjacent region 2,400-fold and 17,000-fold, respectively. The presence of a strong origin of replication in the spacer reduced both rates of deletion and recombination. These results support a model in which the stimulation of deletion and recombination by inverted repeats is initiated by a secondary structure formed between single-stranded DNA of inverted repeats during replication.

MANY genomes are known to contain hotspots for spontaneous and induced genetic changes. Inverted DNA repeats (IRs) were the first example of a motif, rather than a specific sequence, having a profound effect on genetic stability. It was discovered that long (485 bp and 1515 bp) palindromic sequences (perfect head-to-head IRs) are deleted at extremely high rates in *Escherichia coli* (Collins 1981; Collins *et al.* 1982; see also review in Leach 1994). While short (less than 20 bp) palindromes and IRs separated by a unique spacer region are usually much more stable, they can be associated with the breakpoints of deletion mutations such as observed in *E. coli* (Albertini *et al.* 1982; Glickman and Ripley 1984) and in mammalian cells (see review in Meuth 1989). Distantly separated long (more than 1 kb) IRs, as found in bacterial transposons Tn*10* and Tn*5*, are also prone to deletion in bacteria (Ross *et al.* 1979; Berg *et al.* 1981; Egner and Berg 1981; Foster *et al.* 1981).

The mechanism of IR-stimulated deletion formation is generally acknowledged to involve an interaction between IRs (for reviews see Erlich 1989; Leach 1994). The initial step involves the formation of a stem-like (or a hairpin-like) structure in a single-strand DNA (ssDNA) of the IRs, possibly during replication. Deletions could then arise by various pathways. The hairpin could be cleaved by a structure-specific nuclease and the surrounding DNA could be end-joined, resulting in a deletion (DasGupta *et al.* 1987; Erlich 1989; Leach 1994; Akgun *et al.* 1997). Alternatively, the hairpin could block DNA synthesis, leading to the slippage of DNA polymerase between fortuitous short repeats that might be present near the base of the stem (Berg *et al.* 1981; Collins 1981; Egner and Berg 1981; Foster *et al.* 1981; Figure 1). In support of the latter mechanism, a stem structure formed *in vitro* blocks DNA synthesis (Huang and Hearst 1980; Huang *et al.* 1981; Kaguni and Clayton 1982; LaDuca *et al.* 1983) and causes its own bypass through replication slippage (Canceill and Ehrlich 1996).

Several results in bacteria support the view that IR-stimulated deletions arise by an interaction between IRs. First, the rate of IR-deletion is directly related to the repeat size (DasGupta *et al.* 1987; Williams and Muller 1987; Peeters *et al.* 1988; Weston-Hafer and Berg 1989; Sinden *et al.* 1991) and inversely related to the distance (spacer) between the repeats (Sinden *et al.* 1991; Chalker *et al.* 1993). Second, the IR-deletion rate is increased when there is a high likelihood of unwinding and forming a stable secondary structure due to the nucleotide composition of the IR and/or spacer (Sinden *et al.* 1991; Chalker *et al.* 1993). Third, cruciform structures formed by inverted repeats were detected *in vivo* using a psoralen crosslinking assay (Zheng and Sinden 1988; Zheng *et al.* 1991; Sinden *et al.* 1991). Support for interaction of IRs in ssDNA leading to deletions comes from *in vivo* evidence of extreme instability of IRs in ssDNA. This was obtained with the single-stranded fd bacteriophage (Herrmann *et al.*

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1978) or M13-based replicons (Janniere and Ehrlich 1987; d'Alencon *et al.* 1994) and with bacterial conjugation, during which the transferred DNA is single stranded (Berg *et al.* 1983; Syvanen *et al.* 1986). Dependence of the palindrome deletion rate on orientation relative to replication origin was found to be in agreement with IR-stimulated replication slippage occurring predominantly in the lagging strand (Trinh and Sinden 1991; Rosche *et al.* 1995), which is likely to have many single-stranded regions. Inverted repeats in bacteria also stimulate more complex rearrangements, such as formation of inverted (Bi and Liu 1996; Lin *et al.* 1997) and direct (Pinder *et al.* 1997) dimers of plasmids. These can be explained by a template switching mechanism similar to replication slippage.

Deletions of IRs also occur in eukaryotes and they have been examined extensively in the yeast *Saccharomyces cerevisiae*. Replication is involved in IR-stimulated deletions, since mutations in DNA polymerases (DNA-Pol) α and δ increase deletions of the 1.5-kb Tn*5* IRs separated by a long (2.7 kb) spacer and deletions of palindromes or quasipalindromes (IRs separated by very short distance; Gordenin *et al.* 1992, 1993; Ruskin and Fink 1993). The strongest effect was observed for deletions of the Tn*5* long IRs. The deletion rate was elevated as much as 1000-fold by mutations in DNA-Polδ (Gordenin *et al.* 1992, 1993), demonstrating that even relatively stable IRs have a large potential to undergo rearrangement. Genomes of higher eukaryotes contain many imperfect and nearly perfect IRs (Wilson and Thomas 1974; Cech and Hearst 1975; Deininger and Schmid 1976; Bell and Hardman 1977; Deumling 1978; Hardman *et al.* 1979; Houck *et al.* 1979; Krayev *et al.* 1980; Biezunski 1981a,b; Russell and Mann 1986; Schroth and Ho 1995) which could be a source of IR-stimulated genome instability. Frequent deletions of very long perfect palindromes were recently described in mice (Collick *et al.* 1996; Akgun *et al.* 1997). Based on the location of the breakpoints, many of the deletions can be explained by a replication slippage mechanism. However, an alternative mechanism is needed to account for the class of deletions that do not remove the center of a palindrome. Such a class was observed for one of the constructs studied (Akgun *et al.* 1997).

In yeast, IRs not only stimulate deletions but also increase homologous recombination in adjacent regions. We found that IRs elevate both interchromosomal (allelic) recombination (Gordenin *et al.* 1993) and intrachromosomal recombination between homologous or diverged direct repeats (Tran *et al.* 1997). This recombinagenic effect appears to be a general feature of IR motifs, since in yeast the terminal repeats of bacterial transposon Tn*5* and inverted *URA3* genes increase recombination in an adjacent sequence (Gordenin *et al.* 1993; Tran *et al.* 1997). Elevated intrachromosomal recombination of the sequences within the IRs was recently demonstrated for the IRs of a long (15 kb) palindromic transgene in mice (Akgun *et al.* 1997).

We proposed (Gordenin *et al.* 1993; Tran *et al.* 1997) that arrest of the replication complex at the basal part of the stem formed by IRs in ss-DNA could initiate IR-associated recombination as well as deletions. Homologous recombination could be an alternative to replication slippage when DNA elongation is blocked by a noncanonical DNA structure (Figure 1). Arrest of the replication complex can lead to recombination in several different ways: (i) It was suggested (Bierne and Michel 1994; Kuzminov 1995) and recently demonstrated in *E. coli* (Michel *et al.* 1997) that impaired DNA strand elongation can lead to formation of a double-strand break (DSB). DSBs can also occur as a result of cleavage by structure-specific endonucleases as proposed by Leach (1994). (ii) The stalled 3′ end of the nascent strand could separate from the template, forming a 3′-tail that could invade homologous double-stranded DNA (ds-DNA). This is reminiscent of the model suggested for bypass of a DNA lesion by means of recombination-dependent replication in bacteriophage T4 (Formosa and Alberts 1986). (iii) A single-stranded region that can occur near the secondary structure formed by IRs in the template strand could initiate a search for homology as proposed in Tran *et al.* (1997).

Homologous recombination stimulated by IRs can lead to a much larger variety of genome rearrangements than simple deletion of IRs, especially in genomes containing many repeats. Therefore, it is important to know the types of changes that can be stimulated by IRs and what types of IRs are prone to deletion and recombination. In this paper we investigate the mechanisms of IR-stimulated deletion and recombination and the potential for various IR motifs in yeast to cause such rearrangements. Among the factors examined are the location of an IR within a replicon, the size of an IR and the distance between IRs. In support of the replication model (Figure 1) we found that both deletions and recombination are more frequent as the length of repeat is increased and/or when the spacer is decreased. By varying these factors a hyperrecombinagenic IR-structure that increases recombination as much as 17,000-fold was identified. Even relatively small IRs have the potential to be at-risk motifs for recombination when DNA replication is altered, as shown with a DNA polymerase δ (DNA-Polδ) mutant *pol3-t*.

MATERIALS AND METHODS

Strains and plasmids: All strains used in this study (Table 1) are isogenic and are derived from *pol3-t*-DM-*MATα lys2-Tn5-13 ura3-x leu2-2 trp1-Δ1* (Gordenin *et al.* 1992). *POL* and *pol3-t* strains containing insertions of bacterial transposon Tn*5*, quasipalindrome InsH and nonpalindromic insertion InsE in the *Bam*HI-*Xho*I region of *LYS2* have been described previously (Gordenin *et al.* 1993). The plasmids pACYC184::Tn*5* and pLL12::Tn*5-13* (Gordenin *et al.* 1988, 1993) with insertion of

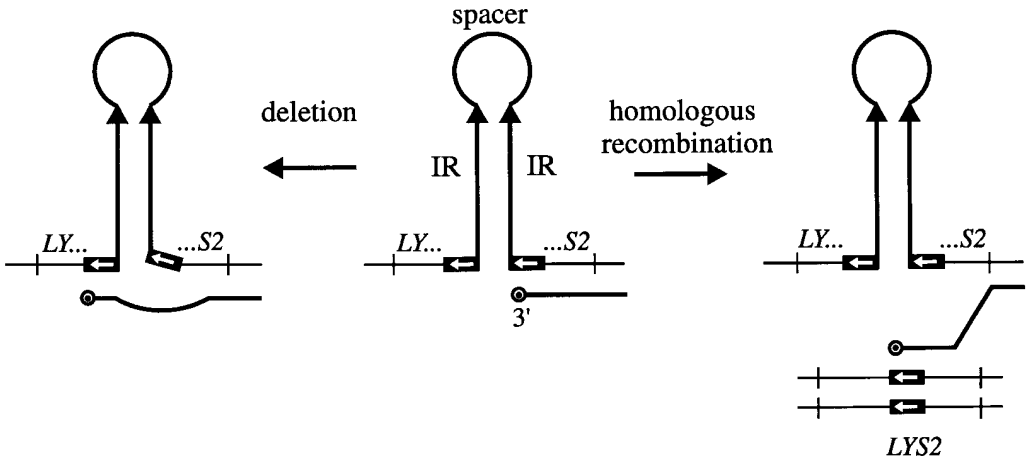

Figure 1.—Replication model for IR-generated genomic rearrangements (based on Gordenin *et al.* 1993). Presented is a strand of the *LYS2* gene with an IR-insert undergoing replication. During replication of the IR sequence (long arrows), single-stranded regions of DNA could give rise to a stem-like secondary structure, thereby inhibiting extension of the 3′-end. This could lead to deletion or recombination. Deletion pathway: replication slippage between short direct repeats (arrows inside black boxes) at the base of secondary structure leads to deletion of the IR (precise excision). Recombination pathway: the stalled replication generates recombinagenic single-stranded ends which can initiate recombination with homologous *LYS2* sequence. If recombination involves identical sequences in sister chromatids it will not result in rearrangements. If a repeat in the same or another chromosome is involved, recombination could lead to deletions and translocations.

Tn*5* were used to change the length of IRs or spacer between them. (Nucleotide sequences and detailed descriptions of all plasmids constructed in this study are available upon request.) The length of the Tn*5* IRs (1534 bp) was gradually decreased. The *URA3* fragment flanked by inverted polylinkers from the pUC4K-IXX plasmid (Barany 1985) was cloned between either *Hpa*I, *Ehe*I, *Xho*I, *Eco47*III, *Hin*dIII and *Bgl*II sites located symmetrically at a distance of 185, 323, 485, 705, 1115, or 1515 bp, respectively, from the external ends of the Tn*5* (Figure 2 and Table 1). (Note: the size of the *URA3* fragment varied between 1112 and 1206 bp, depending on the polylinker sites used for construction.) Those inserts were used to study the effect of IR size on deletion and recombination. The insert with 323-bp IRs and 1.2-kb *URA3* spacer, used as a control in set experiments, was designated InsL. A set of plasmids with constant length of 1515-bp inverted repeats of Tn*5* and varying spacer was constructed as follows. At the first step, the central *Bgl*II-*Bgl*II fragment of Tn*5* was replaced by the 7233-bp *Bam*HI-*Bam*HI fragment from bacteriophage lambda DNA. Subsequently, the *URA3* gene (1.2 kb) was cloned into the *Eco*RI site of lambda DNA resulting in 1515-bp inverted repeats separated by 8.5-kb spacer construction. The 8.5-kb spacer was shortened in two subsequent steps. First, the *Bgl*II-*Bgl*II 3103-bp fragment with the flanking 550-bp was deleted (the size of flanking deletion was confirmed by sequencing) which generated the 4.8-kb spacer. Second, the *Bsp*MII-*Pma*CI (1483 bp) deletion in the sequence of lambda DNA produced the 3.3-kb spacer. To estimate the effect of a replication origin on IR stability, the 1.5-kb fragment containing the *TRP1* gene and the strong origin of replication ARS1 was cloned between 1515-bp and 185-bp inverted repeats using *Bgl*II and *Hpa*I sites inside IS*50* repeats. As a control, 1515- and 185-bp inverted repeats, separated by the same fragment of *TRP*1 gene but with an inactivated origin of replication, were constructed. For this purpose we used the mutated ARS1 from the plasmid Yrp12S9 (Celniker *et al.* 1984) which contains a 20-bp deletion in the A-domain completely destroying an ARS function. U-strains and OR-strains (Table 1) with *URA3* or *TRP1*-marked derivatives of the Tn*5* were created by one-step replacement of the Tn*5-13* insert in the *pol3-t*-DM strain. Strains F5 and F60, containing 323-bp IRs with a 58-bp spacer, were created as follows. An *Xca*I-*Hin*dIII deletion inside the *URA3* gene separating the 323-bp inverted repeats derived from Tn*5* led to shrinkage of the distance between inverted repeats from 1.1 kb to 58 bp. The resulting quasipalindrome (named as InsQ) was recloned in centromere plasmid pFL39 (Bonneaud *et al.* 1991) and subsequently, the generated plasmid was introduced into the U8 and U28 strains. Transformants were replica plated on media containing 5-fluoro-orotic acid (5-FOA; Boeke *et al.* 1984), and *ura3* mutants that resulted from a gene conversion transfer of the 323-bp quasipalindrome from the centromere plasmid into the chromosomal *LYS2* gene were selected. In several cases the *lys2* allele with the Tn*5* insert was modified by replacement transformation in the strains carrying a *lys2* duplication (see below). All replacements were confirmed by Southern analysis.

The *URA3*-based inserts in the *LYS2* gene were developed from constructs described in Tran *et al.* (1997; Figure 3). The *ADE2* gene (2241 bp) from plasmid pASZ11 (Stotz and Linder 1990) was inserted between *ura3* repeats (containing a *Nco*I frameshift mutation) located in inverted (LIR) or direct orientation (DIR) or behind a single *ura3-Nco*I gene (SOLO). These constructs were placed in both orientations into the *Xho*I site of *LYS2* on the plasmid and then transferred into the chromosomal *LYS2* gene by a gene disruption procedure (Rothstein 1983), either into the Lys^+ derivative of *POL*-DM strain, yielding T105, IR28, IS27 strains, or into the *POL* and *pol3-t* strains already carrying a *lys2* duplication (see below). All strains with *URA3* based inserts carried the *ade2 Eco*RI-*Stu*I deletion allele and complete deletion of the *URA3* gene (Gordenin *et al.* 1993). The P1 strain carrying an insertion of the *URA3*-palindrome was made by transformation of the *POL*-DM strain with a ligation mix that contained two fragments: (i) 5′ *LYS2*+*URA3* fragment—the 5′ region (*Hpa*I to *Xho*I sites) of *LYS2* fused (*Xho*I/*Nsi*I) to the *Nsi*I-*Bgl*II region of *URA3*, and (ii) *URA3*+3′ *LYS2* fragment—the same region of *URA3* fused (*Xho*I/*Nsi*I) to the *Xho*I site of the 3′ region (*Xho*I to *Bst*EII sites) of *LYS2*. The P30 strain was created by replacement of the *ura3-Nco*I*ADE2* insertion in strain IR28 with a single copy of the *URA3* gene. The structure of all

TABLE 1

Strains used in this study

Strains	Non-IR inserts in *LYS2* (size, in bp)	IR inserts in *LYS2*			Integrated *lys2* allele[d]	Genetic events examined[e]
		IRs[b]	Size of spacer[c] (bp)	Size of IRs (bp)		
POL[a]-DM	—	Tn*5*	2750	1535	—	N/A
pol3-t-DM	—	Tn*5*	2750	1535	—	N/A
k27-*POL*-DM	InsE (61)	—	—	—	—	N/A
k27-*pol3-t*-DM	InsE (61)	—	—	—	—	N/A
k10-*POL*-DM	—	InsH	9	69	—	N/A
k10-*pol3-t*-DM	—	InsH	9	69	—	N/A
U16-*pol3-t*	—	[Tn*5*]	1206 (*URA3*)	1515	—	Deletion
U19-*pol3-t*	—	[Tn*5*]	1166 (*URA3*)	1115	—	Deletion
U22-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	705	—	Deletion
U25-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	485	—	Deletion
U28-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	323	—	Deletion
U31-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	185	—	Deletion
U49-*pol3-t*	—	[Tn*5*]	8457 (*URA3*)	1515	—	Deletion
U46-*pol3-t*	—	[Tn*5*]	4804 (*URA3*)	1515	—	Deletion
U43-*pol3-t*	—	[Tn*5*]	3321 (*URA3*)	1515	—	Deletion
OR21-*pol3-t*	—	[Tn*5*]	1526 (*TRP1*ARS$^-$)	1515	—	Deletion
OR29-*pol3-t*	—	[Tn*5*]	1546 (*TRP1*ARS$^+$)	1515	—	Deletion
F5-*POL* F60-*pol3-t*	—	InsQ	58	323	–	Deletion
HE1-*POL* HE120-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE7-*POL* HE128-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE500-*POL* HE512-*pol3-t*	—	InsH	9	69	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE25-*POL* HE144-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*5′* (*URA3*)	INTRA
HE31-*POL* HE150-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*5′* (*URA3*)	INTRA
HE36-*POL* HE156-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*3′* (*URA3*)	INTRA
HE42-*POL* HE162-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*3′* (*URA3*)	INTRA
HE49-*POL* HE170-*pol3-t*	—	Tn*5*	2750	1535	*lys2-8* (*URA3*)	INTRA
HE53-*POL* HE178-*pol3-t*	InsE (61)	—	—	—	*lys2-8* (*URA3*)	INTRA
HE59-*POL* HE180-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*3′* (*LEU2*)	INTER
HE65-*POL* HE186-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER
HE507-*POL* HE523-*pol3-t*	—	InsH	9	69	*lys2*-Δ*3′* (*LEU2*)	INTER
HE82-*POL* HE212-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*5′* (*URA3*)	INTER
HE87-*POL* HE209-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*5′* (*URA3*)	INTER
HE89-*POL* HE239-*pol3-t*	—	Tn*5*	2750	1535	*lys2*-Δ*3′* (*URA3*)	INTER
HE93-*POL* HE246-*pol3-t*	InsE (61)	—	—	—	*lys2*-Δ*3′* (*URA3*)	INTER
HE95-*POL* HE241-*pol3-t*	—	Tn*5*	2750	1535	*lys2-8* (*URA3*)	INTER
HE98-*POL* HE236-*pol3-t*	InsE (61)	—	—	—	*lys2-8* (*URA3*)	INTER
HE362-*POL* HE395-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	323	*lys2*-Δ*3′* (*LEU2*)	INTER
F78-*POL* F45-*pol3-t*	—	InsQ	58	323	*lys2*-Δ*3′* (*LEU2*)	INTER
HE367-*POL* HE401-*pol3-t*	—	[Tn*5*]	1206 (*URA3*)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA

TABLE 1

Continued

Strains	Non-IR inserts in *LYS2* (size, in bp)	IR inserts in *LYS2*			Integrated *lys2* allele[d]	Genetic events examined[e]
		IRs[b]	Size of spacer[c] (bp)	Size of IRs (bp)		
HE370-*POL* HE404-*pol3-t*	—	[Tn*5*]	1166 (*URA3*)	1195	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE374-*POL* HE407-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	705	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE376-*POL* HE410-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	485	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE379-*POL* HE413-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	323	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE381-*POL* HE416-*pol3-t*	—	[Tn*5*]	1112 (*URA3*)	185	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE419-*POL* HE437-*pol3-t*	—	[Tn*5*]	8457 (*URA3*)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE422-*POL* HE440-*pol3-t*	—	[Tn*5*]	4804 (*URA3*)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE427-*POL* HE444-*pol3-t*	—	[Tn*5*]	3321 (*URA3*)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE250-*POL* HE301-*pol3-t*	—	[Tn*5*]	1526 (*TRP1*ARS$^-$)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE265-*POL* HE313-*pol3-t*	—	[Tn*5*]	1546 (*TRP1*ARS$^+$)	1515	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE607-*POL* HE620-*pol3-t*	—	[Tn*5*]	1526 (*TRP1*ARS$^-$)	185	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE611-*POL* HE623-*pol3-t*	—	[Tn*5*]	1546 (*TRP1*ARS$^+$)	185	*lys2*-Δ*3′* (*LEU2*)	INTRA
F72-*POL* F67-*pol3-t*	—	InsQ	58	323	*lys2*-Δ*3′* (*LEU2*)	INTRA
T105-*POL*	—	*URA3*-LIR	2456 (ADE2)	1020	—	N/A
IS27-*POL*	*URA3*-SOLO-A (3510)	—	—	—	—	N/A
IR28-*POL*	*URA3*-DIR-A (4753)	—	—	—	—	N/A
P1*POL*	—	*URA3*-PAL	—	1020	—	N/A
P30-*POL*	*URA3* (1257)	—	—	—	—	N/A
E231-*POL* HE474-*pol3-t*	—	*URA3*-LIR	2456 (ADE2)	1020	*lys2*-Δ*3′* (*LEU2*)	INTRA
E251-*POL* HE476-*pol3-t*	*URA3*-SOLO-A (3510)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE460-*POL* HE478-*pol3-t*	*URA3*-DIR-A (4753)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE819-*POL* HE826-*pol3-t*	*URA3*-SOLO-B (3510)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
HE825-*POL* HE832-*pol3-t*	*URA3*-DIR-B (4753)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
P100-*POL*	—	*URA3*-PAL	—	1020	*lys2*-Δ*3′* (*LEU2*)	INTRA
P300-*POL*	*URA3* (1255)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTRA
E161-*POL* HE486-*pol3-t*	—	*URA3*-LIR	2456 (ADE2)	1020	*lys2*-Δ*3′* (*LEU2*)	INTER
HE181-*POL* HE488-*pol3-t*	*URA3*-SOLO-A (3510)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER
HE467-*POL* HE490-*pol3-t*	*URA3*-DIR-A (4753)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER
HE802-*POL* HE810-*pol3-t*	*URA3*-SOLO-B (3510)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER
HE804-*POL* HE814-*pol3-t*	*URA3*-DIR-B (4753)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER
P150-*POL*	—	*URA3*-PAL	—	1020	*lys2*-Δ*3′* (*LEU2*)	INTER
P350-*POL*	*URA3* (1255)	—	—	—	*lys2*-Δ*3′* (*LEU2*)	INTER

[a] *POL*, strains with wild-type alleles of DNA polymerase genes [same as POL$^+$ strains in Gordenin *et al.* (1992, 1993)].

[b] [Tn*5*], inverted repeats containing parts of the Tn*5* (see Figure 2).

[c] The genetic marker described in parentheses is contained in the spacer.

[d] Structure of the integrated *lys2* allele is shown in Figure 4. The marker indicated in brackets determines the locus where second *lys2* allele was integrated (see *Experimental system* in results).

[e] N/A, nonapplicable. These are the initial strains used for integration mutant *lys2* alleles; "Deletion" indicates precise excision of whole insertion and one of the flanking direct repeats resulting in Lys$^+$ revertants (see Figure 6); INTRA, intrachromosomal recombination; INTER, interchromosomal recombination.

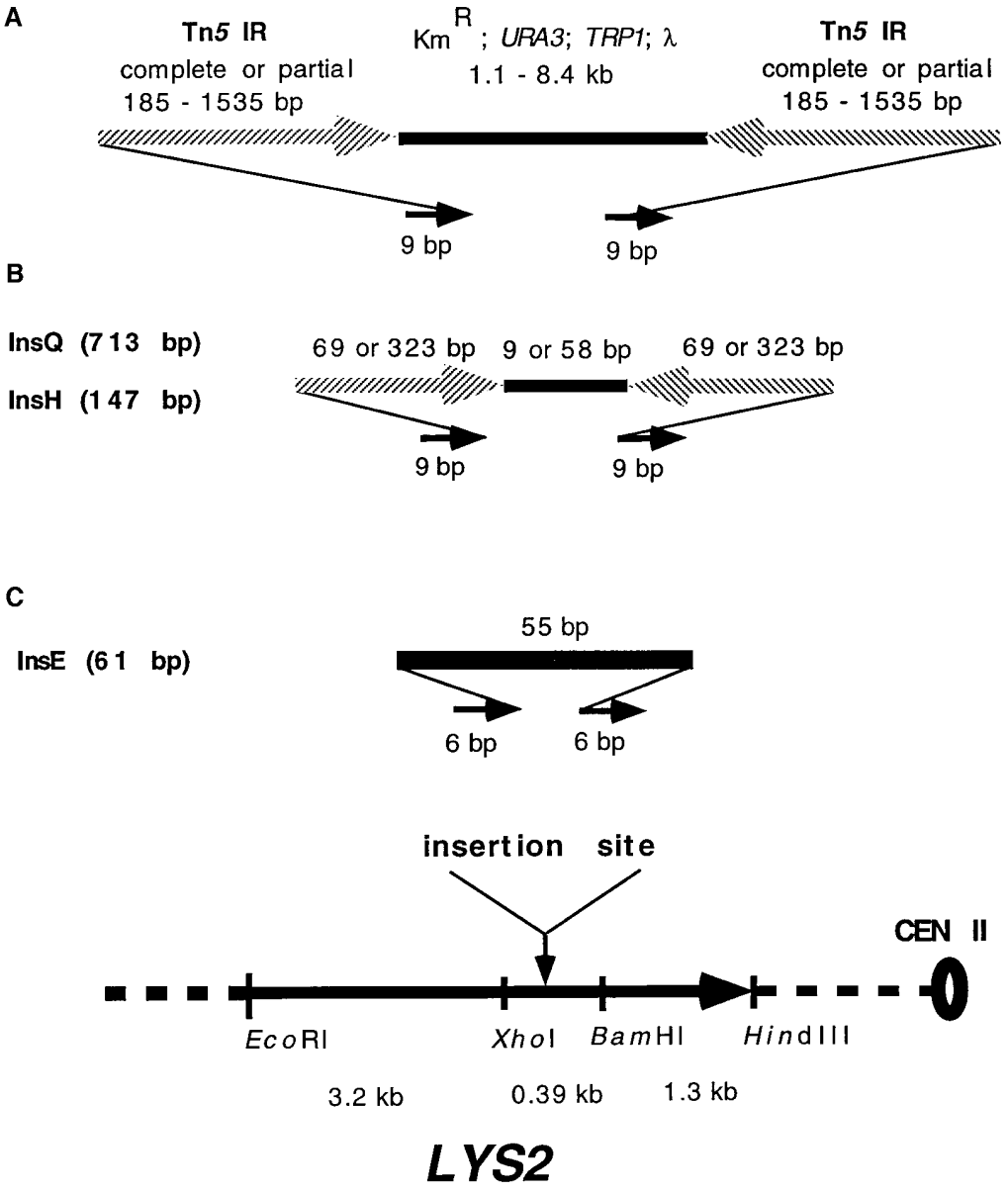

Figure 2.—Tn*5*-based inserts in the yeast *LYS2* gene. All inserts are located at the same site in the *Bam*HI-*Xho*I region of *LYS2* (Gordenin *et al.* 1993). The position of the restriction sites and distances between them in *LYS2* are shown, along with the orientation of *LYS2* on chromosome *II* (arrowhead shows *LYS2* transcription direction). The inverted repeats (gray) and flanking short direct repeats (black) are indicated as arrows; the spacer between the IRs is shown as a thick black line. (A) IRs with a long spacer. The Tn*5*-derivatives are flanked by the duplicated 9-bp sequence of *LYS2*. The size of the long inverted repeats in different constructs varied from 185–1535 bp. The inverted repeats of the Tn5 are separated by an internal spacer containing the Km^R gene (2.7 kb; the initial Tn*5* sequence), the *S. cerevisiae TRP1* gene (1.5 kb), the *S. cerevisiae URA3* gene (1.1 kb) or the *URA3* gene containing fragments of bacteriophage lambda DNA of various sizes (up to 7.3 kb). Three series of Tn*5*-based inverted repeats were constructed in this study: (i) different size IRs (185, 323, 485, 705, 1115, 1515 bp) with a constant *URA3* spacer (1.2 kb) (The 323-bp IR with a *URA3* spacer was referred to as InsL.); (ii) constant size IRs (1515 bp) separated by different spacers (3.3, 4.8, 8.5 kb) that contained the *URA3* gene and fragments of bacteriophage lambda DNA; and (iii) IRs (1515 and 185 bp) separated by the *TRP1* gene (1.5 kb) containing a strong origin of replication ARS1 or a mutated ARS1. (B) IRs with a short spacer. The IRs are InsH, with a 69-bp IR and a 9-bp spacer, and InsQ, with a 323-bp IR and a 58-bp spacer. Both inserts are flanked by 9-bp direct repeats. (C) Nonpalindromic insert InsE. This insert resulted from an imprecise deletion of Tn*5*(*URA3*), leaving a small piece of the external end of the left Tn*5*-IR (Gordenin *et al.* 1993). InsE is 61-bp long and contains 6-bp flanking direct repeats.

URA3-based insertions in the *Xho*I site of *LYS2* was confirmed by Southern analysis.

Three mutant *lys2* alleles cloned either into pFL26 or pFL34 integrative plasmids (Bonneaud *et al.* 1991) were used in this study to create intrachromosomal and interchromosomal *lys2* duplications (Figure 4). The *lys2-8* allele derived from the pLL12-*lys2-8* plasmid (Noskov *et al.* 1990) is an *Eco*RI-*Hin*dIII fragment (4795 bp) of the *LYS2* gene containing a missense mutation Ser_{880} to Tyr_{880} due to a C→A base pair change located at position 2639 of the reading frame. The *lys2-*Δ*3′* and *lys2-*

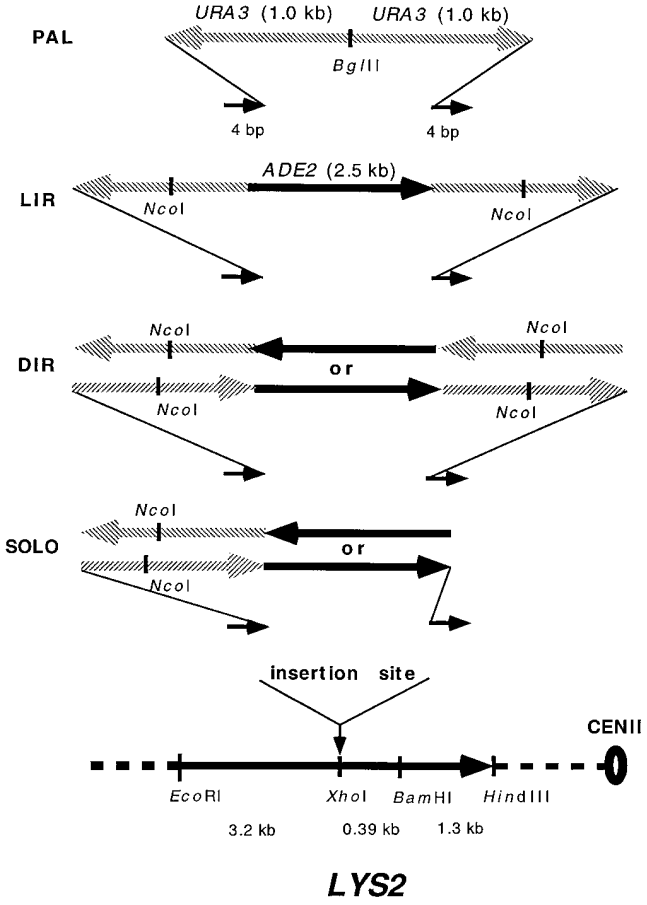

Figure 3.—*URA3*-based inserts in *LYS2*. PAL, a perfect palindrome consisting of *URA3* inverted repeats (gray lines); LIR, long inverted repeats of the *URA3* sequence (gray lines) separated by the *ADE2* gene; DIR, the *ADE2* gene flanked by direct repeats of *URA3*; SOLO, a single copy of both the *URA3* and the *ADE2* genes or just a single copy of *URA3* (not shown). The DIR and SOLO constructs were inserted in both orientations. The *Nco*I site in *URA3* that was mutated in some constructs is indicated. Arrowheads show direction of transcription for the *URA3*, *ADE2*, *LYS2* genes.

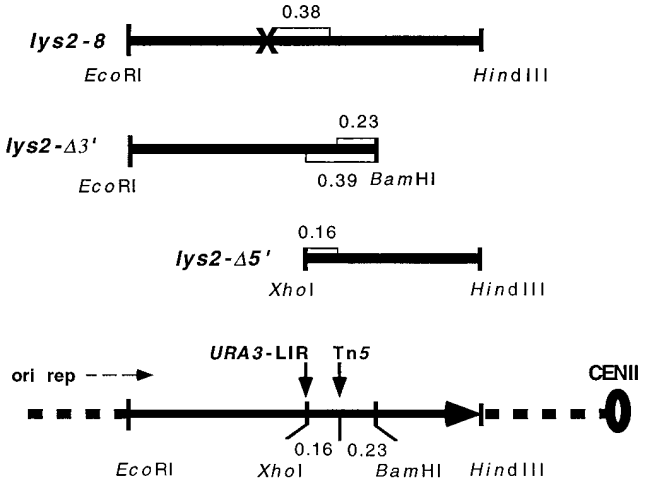

Figure 4.—The *lys2* heteroalleles used to study the effect of IRs on ectopic recombination. *lys2-8* is a 4795-bp *Eco*RI-*Hin*dIII fragment of *LYS2* containing a point mutation 384 bp upstream from the Tn*5*-based insertion site; *lys2*-Δ*5′* is a 1691-bp *Xho*I-*Hin*dIII fragment of *LYS2*; *lys2*-Δ*3′* is a 3486-bp *Eco*RI-*Bam*HI fragment of *LYS2*. The position of the restriction sites and their location relative to the sites of Tn*5* based inserts (above the line) or *URA3* based inserts (below the line) in *LYS2* are shown in order to indicate where exchanges are expected. The direction of *LYS2* transcription and the direction of replication fork movement (ori rep) through the *LYS2* (Freudenreich *et al.* 1997; Tran *et al.* 1995) are indicated in the bottom of the figure.

Δ*5′* alleles are 3′- and 5′-truncated copies of the *LYS2* gene, corresponding to a 3486-bp *Eco*RI-*Bam*HI fragment and a 1691-bp *Xho*I-*Hin*dIII fragment, respectively. These mutant alleles were cloned into pFL26 and pFL34 plasmids taking into account the orientation of *LEU2*, *URA3* and *LYS2* in the chromosome relative to the centromere, so that crossing-over would not create dicentrics incapable of propagation. Only this orientation enables detection of interchromosomal crossing-over. Uncut plasmids with *lys2* mutant alleles were introduced into the strains containing different insertions in the *LYS2* gene. Transformants with *lys2* mutant alleles integrated into the *LYS2*, *LEU2* or *URA3* locus were selected and analyzed using genetic and Southern analysis. Yeast strains carrying *lys2* heteroalleles are listed in Table 1.

Genetic and molecular procedures: Genetic and molecular procedures were described previously (Gordenin *et al.* 1991–1993). Rates of recombination and deletion (precise excision) and their 95% confidence intervals were determined in fluctuation tests as previously described using 12–30 independent cultures (Tran *et al.* 1995). Two rates were considered to be significantly different if the 95% confidence intervals for those rates did not overlap. Since some of the strains carried the temperature-sensitive mutation, *pol3-t*, all tests were performed at 25°. Temperature sensitive strains were maintained at 20°.

In order to determine the association of intrachromosomal and interchromosomal conversion with exchange, about 100 independent Lys$^+$ recombinants were isolated and genetically characterized. For studies of intrachromosomal recombination, the isolated Lys$^+$ recombinants were replica plated on complete and selective media lacking uracil or leucine, depending on which gene, *URA3* or *LEU2*, was placed between the *lys2* repeats.

Intrachromosomal conversion not associated with crossing-over leads only to changes inside *lys2* repeats leaving the sequence between repeats intact (Leu$^+$ or Ura$^+$ phenotype of the Lys$^+$ revertants). Crossing-over or single-strand annealing (Haber 1995) between direct *lys2* repeats in the same chromosome results in deletion of one of the *lys2* sequences together with plasmid DNA, which can be identified by the loss of the central *LEU2* or *URA3* marker (Leu$^-$ or Ura$^-$ phenotype of the Lys$^+$ revertants; Figures 5A and 6C). In the case of interchromosomal recombination, Lys$^+$ recombinants were characterized according to their ability to papillate on media containing 5-FOA, as described in Harris *et al.* (1993). Lys$^+$ recombinants which arose by conversion not associated with crossing-over yielded many papillae due to frequent intrachromosomal recombination between the *ura3-x* and *URA3* genes flanking one of the *lys2* sequences located on chromosome *V* (see Figure 5). In contrast, Lys$^+$ recombinants carrying reciprocal chromosome *II:V* translocations rarely papillate on 5-FOA media because the *URA3* genes placed on different chromosomes recombine very rarely. A limited number of

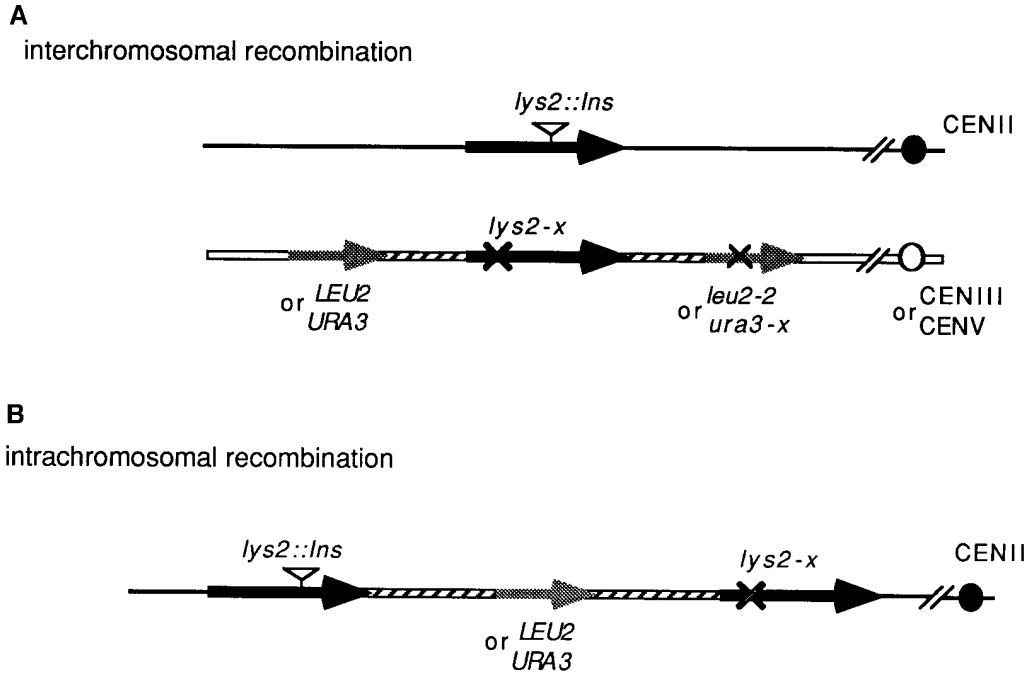

Figure 5.—Ectopic location of *lys2* repeats. (A) Interchromosomal recombination. Normal location of *LYS2* is on chromosome *II*. Various inserts were created at this position. To create a *lys2* duplication, plasmids carrying *lys2* mutant alleles were integrated into the *URA3* locus of chromosome *V* or into the *LEU2* locus of chromosome *III*. The second *lys2* allele is flanked by *URA3* or *LEU2* locus of chromosome *III*. The second *lys2* allele is flanked by *URA3* or *LEU2* sequences (wild-type and mutant alleles). (B) Intrachromosomal recombination. Integration of plasmids into the *LYS2* locus of chromosome *II* creates intrachromosomal *lys2* repeats separated by either the *URA3* or *LEU2* genes. The *lys2-x* corresponds to a point mutation *lys2-8* (analogous to point mutation shown in the figure), the *lys2-D3′* truncation, or the *lys2-D3′* truncation (see materials and methods and Figure 4). (It should be noted that, of the two possible ways of *lys2-8* integration, centromere distal and centromere proximal, we used the centromere distal *lys2-8* shown in the figure.) The *lys2::Ins* represents the *lys2* allele containing an IR or a control insert (indicated as a triangle). pFL26 (5558 bp) and pFL (3799 bp) sequences are shown as a diagonally striped line.

recombinants were checked by Southern blot analysis to prove the genetic changes corresponded to actual physical changes.

RESULTS

Experimental systems: A series of isogenic haploid strains was developed to study deletions of IRs and recombination stimulated by IRs (Figure 6). The strains used to study deletions (Figure 6A) were Lys$^-$ and carried a *lys2* allele with either the Tn*5* IR-insert or various sizes of inserts that were derived from Tn*5* (Figure 2) and retained the Tn*5* external ends. Each insert was flanked by short (9 bp) direct repeats of the *LYS2* sequence present at the ends of Tn*5* (Gordenin *et al.* 1992). Deletions removing the insert and one copy of the repeat can lead to Lys$^+$ reversion. Since deletions of IRs separated by a long spacer were infrequent in wild type strains, these deletions were studied in strains carrying the *pol3-t* mutation which increases the deletion rate as much as 1000-fold (Gordenin *et al.* 1992, 1993).

The Lys$^-$ haploid strains used to study recombination (Figure 6, B and C) contained two *lys2* alleles in ectopic positions. One *lys2* allele at the normal chromosome *II* position carried either an IR or a non-IR insert as a control. In addition to the Tn*5*-based IRs (Figure 2) we developed a series of IRs based on the yeast *URA3* gene (Figure 3). This allowed us to address the generality of recombinagenic effects of IRs as well as to construct the long palindrome *URA3*-PAL (Figure 3 and materials and methods). The second *lys2* allele (Figure 4) carried a homologous sequence overlapping the site containing the insert in the first allele. In one group of strains, the second *lys2* allele was placed as a direct repeat in the same chromosome and the *lys2* repeats were separated by vector sequence carrying either a *URA3* or a *LEU2* marker (Figures 5B and 6C). In the other group of strains, the second *lys2* allele was placed in another chromosome, either chromosome *V* near the *URA3* locus, or chromosome *III* near the *LEU2* locus (Figures 5A and 6B). The rate of appearance of Lys+ was 10–1000-fold more with the constructs allowing recombination (Figure 6, B and C) than with the constructs that could revert only by deletions (Figure 6A). (Two exceptions are described in the footnotes for Table 7.)

To estimate the effect of IRs on recombination, the relative and absolute increases in recombination rate were determined when the allele in chromosome *II* contained or lacked an IR. As a non-IR control for Tn*5*-

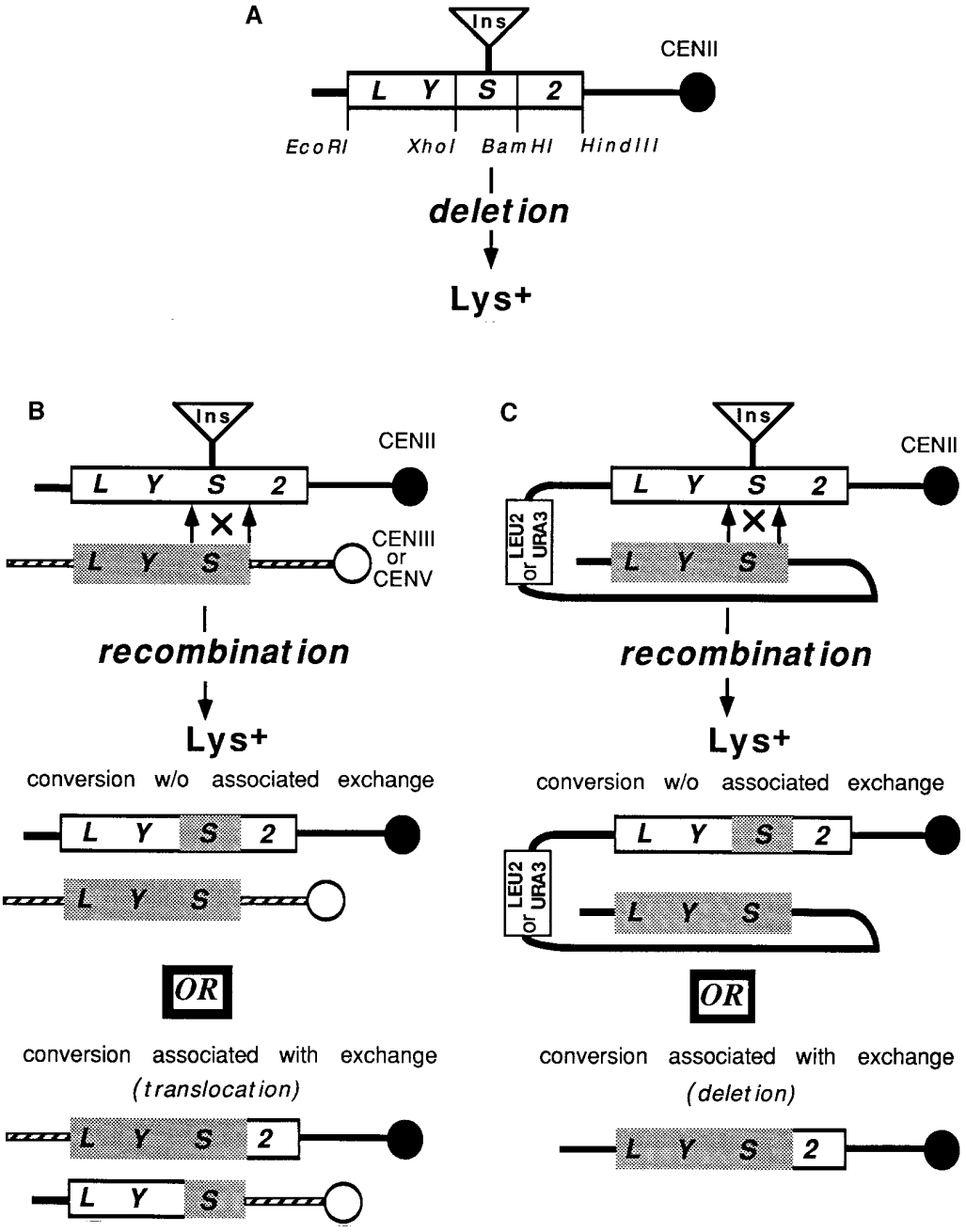

Figure 6.—Selective system to study the effect of IRs on deletion and recombination. One of the Tn*5*-based (Figure 2) or *URA3*-based (Figure 3) inserts was placed into the *LYS2* gene. (A) Selection for deletion. Deletion of the insert and of one flanking 9 bp direct repeat (precise excision) restores the *LYS2* reading frame on chromosome *II*. (B) Selection for interchromosomal recombination. The second mutant *lys2* allele was inserted into chromosome *III* (*LEU2* locus) or chromosome *V* (*URA3* locus; see materials and methods and Figures 4 and 5). Lys^+ recombinants could occur by gene conversion of the insert with or without crossing-over, or infrequently by reciprocal recombination in the region of the *LYS2* gene (at the "S" in the figure). The exchange between nonhomologous chromosomes leads to translocations which, for the exchanges involving chromosomes *II* and *V*, could be identified genetically (Harris *et al.* 1993), as described in materials and methods. (C) Selection for intrachromosomal recombination. Recombination between *lys2* repeats in the same chromosome occurs by gene conversion either without or with crossing-over (identified by the loss of the central *LEU2* or *URA3* marker as described in materials and methods).

based IRs, we used the 61-bp InsE insertion derived from the Tn*5* allele (Figure 2) and located at the same site as Tn*5-13* (Gordenin *et al.* 1992). As a non-IR control for the *URA3*-based IRs with the *ADE2* spacer, we used two different orientations of direct repeats of *URA3* separated by the *ADE2* gene and two different orientations of unique *URA3* and *ADE2* sequences (DIR and SOLO in Figure 3). A single *URA3* sequence served as a non-IR control for the *URA3*-based perfect palindrome *URA3*-PAL.

Some of the Lys^+ recombinants were due to conversion associated with crossing-over (Figure 6, B and C) or due to rare events of reciprocal recombination in the region denoted by an "S" in Figure 6. Exchanges between nonhomologous chromosomes leading to translocations, or between repeats in the same chromosome leading to deletions, were identified both genetically and by Southern analysis, as described in materials and methods. We established earlier the synergistic interaction between the recombinagenic effects of IRs and a defect in DNA-Polδ, *pol3-t* (Gordenin *et al.* 1993; Tran *et al.* 1997). [The *pol3-t* mutation was later identified as a missense mutation Gly_{641} (GAC) to Ala_{641} (AAC) (Tran *et al.* 1997) near the conserved region VI putative nucleotide binding and active site domains of the enzyme (Wang 1996).] Therefore studies of recombination were performed in isogenic *POL* and *pol3-t* strains.

IRs can stimulate inter- and intrachromosomal recom-

TABLE 2

Recombination between *lys2* repeats stimulated by the Tn*5* LIR

		Interchromosomal recombination[a]				Intrachromosomal recombination[b]			
		lys2-Tn5		*lys2-InsE*		*lys2-Tn5*		*lys2-InsE*	
POL3 genotype	Second *lys2* allele	Rate[c] ($\times 10^7$)	Percent exchange[d]	Rate[c] ($\times 10^7$)	Percent exchange[d]	Rate[c] ($\times 10^7$)	Percent exchange[e]	Rate[c] ($\times 10^7$)	Percent exchange[e]
POL3	*lys2-Δ5′*	9.4	5.1 (6/117)	2.3	4.8 (4/83)	650	72 (83/116)	50	51 (61/120)
	lys2-Δ3′	22	5.0 (6/119)	3.6	12 (13/111)	750	66 (79/120)	220	66 (78/119)
	lys2-8	23	7.3 (7/96)	11	13 (13/98)	1,700	54 (64/119)	490	34 (32/95)
pol3-t	*lys2-Δ5′*	92	7.8 (9/116)	8.6	6.4 (9/139)	8,800	58 (67/116)	630	57 (67/118)
	lys2-Δ3′	104	7.6 (10/120)	12	12 (13/99)	16,000	59 (64/109)	2,500	85 (93/109)
	lys2-8	343	7.6 (9/118)	60	13 (15/118)	15,300	46 (54/117)	3,400	44 (51/117)

[a] In all cases the second *lys2* allele was located in chromosome *V* (see Figure 5). Not shown are the data where *lys2-Δ3′* and *lys2-8* alleles were also inserted into chromosome *III*. The presented recombination rates did not differ significantly from those when pFL26 spacer was used instead of pFL34.

[b] The *lys2* direct repeats were separated from each other by the pFL34 sequence carrying a *URA3* marker (see Figure 5). The presented recombination rates did not differ significantly from those where the pFL34 spacer was used instead of pFL26 (data not shown).

[c] Median rates and 95% confidence intervals (not shown) were calculated as stated in materials and methods. In all cases, the recombination rates when LIR was present were significantly higher than when the control insert (InsE) was present.

[d] The percent and ratio (in parentheses) of revertants associated with interchromosomal exchange.

[e] The percent and ratio (in parentheses) of revertants associated with intrachromosomal exchange.

bination: We determined the effect of two types of IRs, Tn*5* and *URA3-ADE2*, on both inter- and intrachromosomal recombination (Tables 2 and 3). There was as much as a 50-fold stimulation in recombination by IRs, that for interchromosomal recombination was independent of the chromosomal location of ectopic sequence, and for intrachromosomal recombination was independent of the marker between intrachromosomal repeats. The absolute increase in recombination rate caused by IRs was much greater in *pol3-t* than in *POL* strains carrying wild-type DNA Pol genes. The synergistic interaction between IRs and the *pol3-t* mutation was previously shown for allelic recombination (Gordenin *et al.* 1993) and for intrachromosomal recombination between homologous and homeologous *ade2* repeats (Tran *et al.* 1997).

Recombination events that are associated with exchange (crossing-over or single-strand annealing) will lead to rearrangements (deletions and translocations). The overall incidence of exchange associated with IR-stimulated recombination was the same as for recombination where IRs were absent (22–85% for intrachromosomal recombination and 5–13% for interchromosomal recombination). For the intrachromosomal recombination, the variation of the exchange fraction was statistically significant. This could be due to the differences in particular constructs and/or the presence of the *pol3-t* mutation. Based on the presented results we cannot draw a conclusion about the origin of such variation. Nevertheless, the higher level of associated exchange for all cases of intrachromosomal recombination compared to interchromosomal recombination is consistent with the results of Jinks-Robertson *et al.* (1993) using other recombination systems.

Effects of an internal replication origin on IR-stimulated recombination and deletion: According to the replication model described in Figure 1, the likelihood of deletion and recombination is increased when an IR is encountered during nascent strand elongation (see discussion). We, therefore, investigated the consequences of placing a bi-directional replication origin between IRs. The *TRP1* fragment containing an ARS1 sequence was inserted between the 1515-bp IRs of Tn*5* (Figure 2). The ARS1 functions as a strong replication origin when placed at various chromosomal locations (Ferguson and Fangman 1992; Brewer and Fangman 1993). There was an approximately five-fold decrease in the deletion rate in the *pol3-t* strain caused by ARS1 (*TRP1* ARS1$^+$; Table 4) as compared to the ARS1$^-$ control in which the same *TRP1* fragment had a 20-bp deletion that inactivates ARS1 (*TRP1* ARS1$^-$; see materials and methods). The *TRP1* ARS1$^+$ spacer also caused a statistically significant decrease in the rate of intrachromosomal recombination in both *POL3* and *pol3-t* strains. There was no effect of *ARS1* on recombination when either *TRP1* ARS1$^+$ or *TRP1* ARS1$^-$ was placed between the short (185 bp) versions of the Tn*5* IRs. These repeats lost their ability to induce recombination (see next section). The lack of an ARS1$^+$ effect when placed between non-recombinagenic IRs supports the conclusion that the ARS1$^+$ suppression of recombination is specific to recombination events induced by IRs.

Dependence of IR-stimulated recombination and de-

TABLE 3

Recombination between *lys2* repeats stimulated by the *URA3*-IR

		Recombination		
		Interchromosomal[b]	Intrachromosomal[c]	
Strain	*lys2* allele[a]	Rate[d] ($\times 10^7$)	Rate[d] ($\times 10^7$)	Percent associated with exchange[e]
POL3	*lys2-URA3-LIR*	4.7	500	40 (48/120)
	lys2-URA3-DIR-A[f]	0.6	7	48 (77/160)
	lys2-URA3-DIR-B[f]	0.6	6	ND[g]
	lys2-URA3-Solo-A	0.5	10	41 (49/119)
	lys2-URA3-Solo-B	0.7	15	ND[g]
pol3-t	*lys2-URA3-LIR*	74	2500	22 (26/118)
	lys2-URA3-DIR-A	5.1	50	50 (61/120)
	lys2-URA3-DIR-B	3.1	100	ND[g]
	lys2-URA3-Solo-A	4.1	54	50 (214/475)
	lys2-URA3-Solo-B	3.2	85	ND[g]

[a] Only one *lys2* repeat is specified; the second *lys2* allele was always *lys2-Δ3′*.
[b] The second *lys2* allele was located in chromosome *III* (see Figure 5).
[c] The *lys2* direct repeats were separated from each other by the pFL26 sequence (see Figure 5).
[d] Median rates and 95% confidence intervals (not shown) were calculated as stated in materials and methods. In all cases recombination rates in the presence of the LIR were significantly higher than rates with the control insert.
[e] Number of reversions that are associated with exchange divided by the number of reversions examined.
[f] In the A-orientation, *URA3* transcription is in the opposite direction to that of *LYS2*; in the B-orientation, *URA3* transcription is the same as *LYS2* (see Figure 3).
[g] Not determined.

letions on the size of IRs and the distance between IRs: Based on the model in Figure 1, increasing the length of IRs or decreasing the spacer between IRs should provide more opportunity to form a secondary structure, and thus increase the rate of IR stimulated deletion or recombination. Therefore, several Tn*5* derivatives were constructed in which the size of the IR or the distance between IRs was varied (Table 1; Figure 2). There was a gradual increase of both deletion and intrachromosomal recombination rates either with increase in IR size or with decrease in the distance between IRs (Tables 5 and 6). Interactions between IRs may still be possible for the smallest (185 bp) IRs and for the largest spacer (8457 bp) based on the incidence of deletions in these constructs. However, since recombination rates when these inserts were present did not exceed the rates for the non-IR insert InsE (compare with the data in Table 2), most of these recombination events were not initiated by IR interaction.

Based on results described in the previous section, closely spaced IRs should be very recombinagenic. We, therefore, investigated the effect of Tn*5*- and *URA3*-based closely spaced IRs on inter- and intrachromosomal recombination (Table 7). Reducing the distance between IRs to only 58 bp results in relatively short IRs (323 bp) becoming recombinagenic (compare InsQ to

TABLE 4

Influence of an ARS sequence on intrachromosomal recombination and deletion stimulated by the Tn*5* LIR

Insertion in *LYS2*		Intrachromosomal recombination with *lys2-del 3′*[a] (rate $\times 10^7$)		Deletion[b] (rate $\times 10^7$)
Tn5 inverted repeats (bp)	Spacer between IRs	*POL3*	*pol3-t*	*pol3-t*
1515	*TRP1* ARS1$^-$	610 (550–1090)	23,000 (21,600–27,500)	4.4 (3.5–5.4)
1515	*TRP1* ARS1$^+$	130 (120–160)	12,800 (11,700–15,800)	0.9 (0.7–1.2)
185	*TRP1* ARS1$^-$	30 (20–100)	790 (440–1,000)	ND[c]
185	*TRP1* ARS1$^+$	30 (20–30)	750 (520–830)	ND[c]

95% confidence intervals are given in parentheses.
[a] The *lys2* repeats were separated from each other by the pFL26 sequence (see Figure 5).
[b] The deletion rate was measured in strains containing only one copy of the *lys2* with the insert (see Figure 6).
[c] Not determined.

TABLE 5

Effects of the size of IRs on recombination and deletion

Size of IRs[c] (bp)	Intrachromosomal recombination with *lys2Δ3′*[a] (rate × 10^7)		Deletion[b] (rate × 10^7)
	POL3	*pol3-t*	*pol3-t*
1515	800 (540–1,540)	8,530 (6,540–12,830)	1.9 (1.6–2.2)
1115	710 (550–1,290)	9,060 (6,820–11,500)	1.7 (1.3–2.5)
705	360 (310–470)	5,330 (3,780–6,210)	1.5 (1.2–1.9)
485	200 (150–390)	2,350 (1,940–2,800)	1.0 (0.76–1.1)
323	200 (100–300)	1,800 (1,470–2,300)	0.84 (0.70–1.1)
185	100 (70–140)	850 (480–1,340)	0.43 (0.38–0.54)

95% confidence intervals are given in parentheses.
[a] The *lys2* direct repeats were separated from each other by the pFL26 sequence (see Figure 5)
[b] Deletion rate was measured in strains containing only one copy of the *lys2* with the insert (see Figure 6).
[c] All IRs were derived from Tn5 and are separated by the *URA3* spacer (1.2 kb) (see Figure 2).

InsL and InsE). The strongest recombinagenic effect of the closely spaced 323-bp IRs (InsQ) was observed in the *pol3-t* background. Reduction of the spacer from 1.1 kb (InsL) to 58 bp (InsQ) caused a 15-fold increase in the interchromosomal, and a 24-fold increase in the intrachromosomal, recombination in the *pol3-t* background. This correlated with an approximately 50-fold higher deletion rate of InsQ compared with InsL in the *pol3-t* strain (data not shown). Even short (69 bp) closely spaced IRs of the quasipalindrome InsH (compare with InsE) stimulated intrachromosomal recombination (6-fold in the *pol3-t* background). Among the intrachromosomal recombinants induced by quasipalindrome InsH, 20% were associated with exchange resulting in deletions of the *LEU2* marker between the *lys2* repeats. No conclusion could be drawn about the effect of InsH on interchromosomal recombination, since reversion rates via deletions were very high (see footnotes *b*, *c* for Table 7).

Since the recombinagenic effect of IRs was greatly increased when the distance between IRs was decreased, we constructed a long perfect palindrome *URA3*-PAL (Figure 3). The final step in construction of *URA3*-PAL involved transformation of the ligated DNA directly into yeast (see materials and methods), since long perfect palindromes cannot be propagated in bacteria (for review, see Leach 1994). In order to confirm that the perfect palindrome structure did not undergo rearrangement in yeast, we checked by Southern analysis the size of the insert and the retention of the *Bgl*II site at the junction between the inverted repeats and the *Nde*I sites located close (144 bp) to the internal ends of repeats (data not shown). In the strains used to measure deletion (Figure 6A), reversions of the *lys2::URA3-PAL* allele were infrequent ($<10^{-8}$). This was probably due to the IRs being flanked by only small (4 bp) direct repeats. We observed the frequency of at least 10^{-6} of Ura$^-$ 5-FOA-resistant mutants. This is several orders of magnitude higher than the expected frequency of two independent mutations inactivating both *URA3* genes in the *URA3*-PAL. If these events represent imprecise excision (deletions) of the *URA3*-PAL, the rate of such deletions is 100-fold more than the rate of imprecise deletions of Tn*5* in a *POL* strain, where 1.5-kb IRs were separated by a 2.7-kb spacer (Gordenin *et al.* 1993). The rate of deletions for *URA3*-PAL could be even higher, since many deletions may alter only the center

TABLE 6

Effects of the distance between IRs on recombination and deletion

Distance between IRs[c] (kb)	Intrachromosomal recombination with *lys2-Δ3′*[a] (rate × 10^7)		Deletion[b] (rate × 10^7)
	POL3	*pol3-t*	*pol3-t*
1.2	380 (250–620)	9,470 (7,850–12,080)	1.9 (1.6–2.2)
3.3	240 (170–420)	4,870 (4,040–7,670)	0.9 (0.7–1.3)
4.8	140 (80–290)	2,860 (2,350–3,260)	0.46 (0.4–0.7)
8.5	80 (60–180)	1,940 (1,740–2,450)	0.13 (0.07–0.22)

95% confidence intervals are given in parentheses.
[a] The *lys2* direct repeats were separated from each other by the pFL26 sequence (see Figure 5).
[b] Deletion rate was measured in the strains containing only one copy of the *lys2* with the insert (see Figure 6).
[c] All spacers are flanked by the Tn*5*-derived IRs (1515 bp) (see Figure 2).

of the palindrome, leaving one or both *URA3* genes functional. Deletions of *URA3*-PAL were not investigated further in this study. *URA3*-PAL was extremely recombinagenic, both for interchromosomal and intrachromosomal recombination (Table 7). The recombination rates were 17,000-fold higher for interchromosomal recombination, and 2400-fold higher for intrachromosomal recombination, than when the insert contained a single *URA3*. The recombinagenic effect of *URA3*-PAL was 50–340 times stronger than the recombinagenic effect of *URA3*-LIR separated by the 2.5-kb *ADE2* spacer (compare with data in Table 3). The long palindrome was efficient in stimulating rearrangements, since 22% of the intrachromosomal recombinants (43 out of 198 Lys^+ recombinants) had lost the DNA between the *lys2* direct repeats.

DISCUSSION

DNA sequence motifs that are at-risk for genetic change in wild-type or mutation prone cells have been identified in various organisms. Among them are the following commonly occurring at-risk motifs: microsatellites, minisatellites, triplet repeats, short separated repeats, mirror repeats, and inverted repeats. IRs are an important class of at-risk motifs, prone to deletions, that have been extensively studied in bacteria. Relatively little is known about IR-stimulated deletions in eukaryotes and there have been only a few studies on recombination stimulated by IRs. We developed several genetic systems to understand the role of DNA structure and genetic factors in IR-stimulated deletion and recombination in yeast and to assess the potential for the instability caused by this at-risk motif.

Our results suggest common events in IR-induced deletion and various kinds of recombination, as well as a high potential for IRs to stimulate these events. We shall discuss our data in the framework of the replication model for IR deletions and IR-stimulated recombination.

Mechanism of IR-stimulated recombination and deletion in yeast: In the replication model for IR-stimulated deletions and recombination (Figure 1), ssDNA regions are developed within the IRs that lead to the formation of duplexes and stem structures. We have suggested that single-strand regions can occur during replication (especially lagging strand) and they may be more extensive in mutants such as *pol3-t* (Gordenin *et al.* 1992). [By way of example, we show the IRs that form a stem structure in Figure 7A (bottom part) as completely single-stranded, although the ssDNA could be interrupted by double-strand DNA as long as complementary ssDNA regions are sufficient to support stable association of IRs.] We suggest that the likelihood of long complementary ssDNAs occurring within both repeats would be greater if replication across the IRs were accomplished by a single replication complex (Figure 7A) than by two

TABLE 7

The effect of various IRs on recombination rates

		Recombination rate ($\times 10^7$) in presence of Tn5-based insert (size of IR/size of spacer, in bp)					*URA3*-based inserts (size of IR/size of spacer, in bp)	
Recombination	Strain	Tn5 (1535/2750)	InsL (323/1112)	InsQ (323/58)	InsH (69/9)	InsE (0/61)	*URA3*-PAL (1020/0)	*URA3* (0/1255)
Interchromosomal[a]	*POL3*	9.2	3.7	7.3	6.5[b]	2.5	6,706	0.4
	pol3-t	185	27	406	442[c]	46	ND[d]	ND[d]
Intrachromosomal[e]	*POL3*	420	90	260	200	80	37,980	16
	pol3-t	12,850	1,980	48,440	12,280	1,950	ND[d]	ND[d]

Median rates and 95% confidence intervals (not shown) were calculated as stated in materials and methods. In all cases except InsL, recombination rates in the presence of the IR were significantly higher than rates with the control insert (InsE or *URA3*).

[a] The second *lys2* allele (*lys2-Δ3′*) was placed in chromosome *III* (see Figure 5).

[b] The rate of reversion is not significantly different from the rate of deletions in the isogenic *POL* strain carrying only one copy of the *lys2::InsH* (see Figure 6 and *Experimental system*).

[c] The rate of reversion is only four-fold more than the rate of deletions in the isogenic *pol3-t* strain carrying only one copy of the *lys2::InsH* (see Figure 6 and *Experimental system*).

[d] Not determined.

[e] The second *lys2* allele was always *lys2-Δ3′*. *lys2* repeats were separated by the pFL26 sequence (see Figure 6).

independent replication complexes moving in opposite directions (Figure 7B). Even if single-strand regions form in the lagging strand templates of both replication forks (not shown on Figure 7) they would not lead to intrastrand stem-like structures. In support of this, we have demonstrated that the presence of a strong replication origin between repeats suppresses IR deletions and IR-stimulated intrachromosomal recombination (Table 4). The suppressing effect is clearly due to the presence of the functional ARS1, since suppression of intrachromosomal recombination was observed only for recombination events stimulated by IRs. However, while there is suppression of both deletions and recombination by a functional ARS1, it is incomplete. This could be due to the ARS1 not always serving as an origin. The efficiency of the ARS1 between IRs as a replication origin can vary in different chromosomal contexts (*lys2* duplication *vs.* single *lys2*) and between genotypes (*POL3 vs. pol3-t*). In this case both IRs would be replicated from the origin located upstream to the *LYS2* gene (Figures 4 and 7A), as determined by Freudenreich *et al.* (1997) and by Tran *et al.* (1995). Another possibility is that not all IR-stimulated rearrangements are sensitive to the replication origin between the IRs. A related observation concerning position of the replication origin has been made in *E. coli*. When the spacer between IRs of bacterial transposon Tn5 was replaced by a large fragment of pUC19 containing a unidirectional replication origin, the rate of IR deletions decreased more than 100-fold (Kil' *et al.* 1994).

The observation that stimulation of deletion, as well as recombination, increased with size of IR (Tables 5–7), excludes models where only a small region of an IR is genetically active. The increase in the rate of IR deletions with an increase in IR size provides further evidence for the IR motif, not just sequence, being able to stimulate deletions via interaction between repeats.

We also found that increasing the distance between IRs led to decreased rates of both deletion and recombination. In the framework of the replication model (Figure 1), this suggests that concerted formation of ssDNA in IRs becomes less likely with increased distance between the repeats.

IR-stimulation of rearrangements via homologous recombination is a general phenomenon: IRs appeared capable of stimulating both intrachromosomal recombination leading to deletions and interchromosomal recombination leading to translocations. The lower incidence of associated exchange for the interchromosomal as compared to the intrachromosomal recombination is consistent with the results of Jinks-Robertson *et al.* (1993) obtained in different genetic systems and backgrounds. The actual association with exchange for interchromosomal recombination may be higher, since half of the crossovers occurring in G2 would produce unbalanced translocations resulting in lethality and would thus not be recoverable in haploids (discussed in Jinks-Robertson *et al.* 1993).

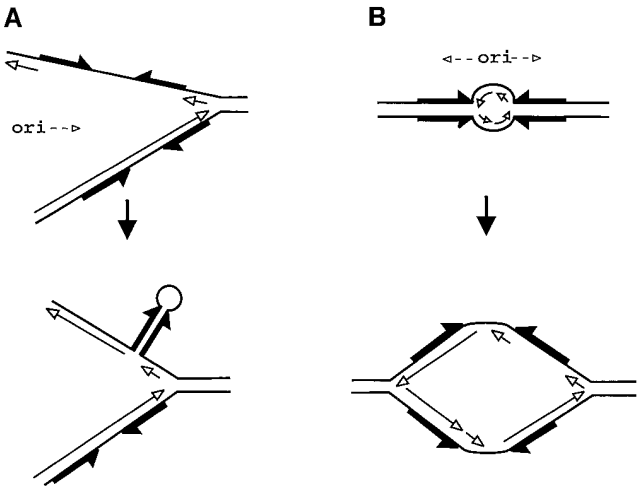

Figure 7.—The effect of position of replication origin on opportunity for IRs to form secondary structure. Inverted repeats are shown as thick black arrows. Open triangular arrowheads show 3′ nascent strands. The direction of movement of the replication fork (dashed arrows) is indicated. (A) Initiation of replication from a distant origin (ori). (B) Initiation of replication from an origin located between IRs.

Based on our previous (Gordenin *et al.* 1993; Tran *et al.* 1997) and current results we conclude that regardless of the sequence (Tn*5* terminal repeats and *URA3* repeats) the IR is a recombinagenic at-risk motif. IR-stimulated recombination was found for nearly all sizes of IRs studied. Shorter IRs appeared to be highly recombinagenic only when separated by a relatively short spacer and when present in the *pol3-t* mutants.

IRs have a high potential for stimulating both deletions and recombination: Since palindromes and quasipalindromes have the highest capability to form a secondary structure, these IRs are expected to be the most efficient in causing deletions and (or) recombination. This agrees with their being the most deletion-prone IRs in several yeast systems (Henderson and Petes 1993; Ruskin and Fink 1993). Deletions of long palindromes in mice also occur frequently (Collick *et al.* 1996; Akgun *et al.* 1997). It is noteworthy, that long (up to 15 kb) palindromes are maintained in mice although they are very unstable, while replicons with >150–200 bp palindromes in bacteria cannot be propagated (reviewed in Erlich 1989; Leach 1994). The long (1020 bp) perfect palindrome *URA3*-PAL in yeast also appeared capable of propagation [overall rate of deletions measured by loss of *URA3* function was 10^{-6}–10^{-5} (data not shown)]. It remains to be determined which factors are responsible for maintaining longer palindromes in eukaryotes.

While IRs can stimulate recombination and deletion, correlation between the two events is not complete. The quasipalindrome InsH was deleted about 1000-fold more frequently than Tn*5* in *POL* and about 50 times

more in the *pol3-t* background (Gordenin *et al.* 1993; Tran *et al.* 1995), while there was more stimulation of interchromosomal recombination by Tn*5* as compared to InsH in the *POL* strains (Table 7). However, the InsH was more recombinagenic in the *pol3-t* background. These results appear, at first, to be inconsistent with our replication model since IR-stimulated genetic changes would be expected to increase with the likelihood of forming a secondary structure. We suggest that additional factors responsible for completing the genetic events are likely to influence the outcomes. For example, there is also the possibility of recombination between sister chromatids which would be undetected in the present systems (discussed by Sargent *et al.* 1997, and by Zou and Rothstein 1997). The recombination pathway could depend on the type of IR. Similarly, the choice of deletion *vs.* recombination (Figure 1, A and B) could also depend on the IR structure. The systems described in this study should be useful in identifying genetic factors, such as *pol3-t*, that can influence the impact of the IR at-risk motif.

The fact that weakly recombinagenic IRs, *i.e.*, InsQ and InsH, are strongly recombinagenic in *pol3-t* mutants, demonstrates that there are genetic backgrounds where relatively stable IRs (and possibly other at-risk motifs) can become unstable. There are motifs in the human genome that are analogous to InsQ and InsH IRs. The 323-bp IRs of InsQ (323 bp) are close in size to Alu repeats. These Alu repeats are abundant in the human genome and can be associated with rearrangements that lead to disease (Calabretta *et al.* 1982; Lehrman *et al.* 1985, 1986, 1987; Weiner *et al.* 1986; Jalanko *et al.* 1995; Macina *et al.* 1995). Based on Alu-PCR (Nelson *et al.* 1989) and indirect physical methods (Deininger and Schmid 1976; Deininger 1989), many Alu-repeats form closely spaced IRs although they are nonidentical. Sequencing of the human genome revealed examples of closely spaced Alu-IRs (Macina *et al.* 1995; Pauly *et al.* 1995; Unfried *et al.* 1995; Kiyosawa and Chance 1996; Koda *et al.* 1997) including some that are nearly palindromic (Hanke *et al.* 1995). Genetic factors that enhance recombination stimulated by InsQ in yeast could be homologous to human genetic factors that might increase rearrangements associated with Alu-IRs. These factors would be important in genomic stability and disease.

It is important to note that Alu-repeats, as well as other repeats in the human genome, are highly diverged. Thus, sequence divergence and genetic factors that might affect interactions between diverged sequences could affect the potential for IR-induced genome instability. In addition to diverged IRs formed by long repeats, some of the small repeats, microsatellites and minisatellites are analogous to diverged long IRs. The unstable triplet repeats CTG (CAG) or CGG (CCG) (Warren 1996; Wells 1996; Mandel 1997) and minisatellites rich in dA-T dinucleotide (Fearon *et al.* 1990; Yu *et al.* 1997) are capable of expansion and deletion, and are associated with chromosomal fragile sites and disease. These sequences can form imperfect hairpins analogous to the hairpins that can be adopted by a ssDNA of diverged inverted repeats (Mitas 1997). While IRs induce recombination between diverged repeats (Tran *et al.* 1997), the influence of IR divergence within repeats on IR-induced instability has not been examined. Since the long unstable palindrome *URA3*-PAL causes up to 17,000-fold induction of recombination, this motif provides opportunities for investigating the consequences of DNA divergence.

The hyperrecombination effect of the *URA3*-PAL motif demonstrates the high potential of IR-processing mechanisms in stimulating rearrangements. This structure enhanced recombination up to 17,000-fold, creating the strongest recombinational hotspot identified. The level of recombination induced by *URA3*-PAL may indicate a highly reactive intermediate such as a double-strand break (DSB). DSBs are efficient at causing homologous recombination in mitotically dividing yeast cells (Haber 1995; Resnick *et al.* 1995) and they are initiators of meiotic recombination (Lichten and Goldman 1995). Based on indirect data it was proposed (Bierne and Michel 1994; Kuzminov 1995) that stalled elongation could result in the collapse of replication forks and DSB in *E. coli*. Later it was shown that arrested replication leads to DSBs in *E. coli* (Michel *et al.* 1997). A DSB in the vicinity of the 140-bp perfect palindrome was recently demonstrated in yeast during meiosis, although a role for replication remains to be established (Nag and Kurst 1997). If a DSB arises at the secondary structure of an IR during replication, this lesion could irreversibly direct subsequent events toward a recombination pathway. If a DSB is not the cause of IR-stimulated recombination, another highly recombinagenic intermediate should exist to account for high recombination rates.

The replication model of IR-stimulated recombination (Figure 1) may be applicable to other kinds of DNA sequences capable of forming secondary structures interfering with replication, for example sequences capable of forming triplex DNA and Z-DNA. These motifs have been shown to be recombinagenic. We propose that there are mutations, such as the *pol3-t* defect in DNA-Polδ described in this study, that can act synergistically with these motifs. Further studies in model systems such as yeast will lead to the identification of additional at-risk motifs and factors that can act synergistically to cause genome instability.

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