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Transcription and Double-Strand Breaks Induce Similar Mitotic Recombination Events in *Saccharomyces cerevisiae*

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Manuscript received April 9, 2002
Accepted for publication July 1, 2002

ABSTRACT

We have made a comparative analysis of double-strand-break (DSB)-induced recombination and spontaneous recombination under low- and high-transcription conditions in yeast. We constructed two different recombination substrates, one for the analysis of intermolecular gene conversions and the other for intramolecular gene conversions and inversions. Such substrates were based on the same *leu2-HOr* allele fused to the *tet* promoter and containing a 21-bp HO site. Gene conversions and inversions were differently affected by *rad1*, *rad51*, *rad52*, and *rad59* single and double mutations, consistent with the actual view that such events occur by different recombination mechanisms. However, the effect of each mutation on each type of recombination event was the same, whether associated with transcription or induced by the HO-mediated DSB. Both the highly transcribed DNA and the HO-cut sequence acted as recipients of the gene conversion events. These results are consistent with the hypothesis that transcription promotes initiation of recombination along the DNA sequence being transcribed. The similarity between transcription-associated and DSB-induced recombination suggests that transcription promotes DNA breaks.

DNA is a reactive molecule that can be damaged by radicals, chemicals, or radiation. Such forms of damage can result directly or indirectly in DNA breaks (Pâques and Haber 1999). A major mechanism of DNA break repair in vegetatively growing cells is homologous recombination. However, DNA metabolic processes such as replication and transcription can strongly influence the incidence of homologous recombination in mitotic cells (Pâques and Haber 1999; Aguilera *et al.* 2000). Thus, replication fork blockage may be an important source of spontaneous mitotic recombination (Cox 2001; Michel *et al.* 2001).

Particularly intriguing is the observation that high-transcription levels of a DNA sequence can strongly stimulate its frequency of recombination (Aguilera 2001). The first evidence of transcription-associated recombination (TAR) was shown in *Escherichia coli* (Ikeda and Matsumoto 1979), in which recombination of phage λ was stimulated by Rpo-mediated transcription. Afterward, TAR was shown in yeast cells by the identification of *HOT1*, which is a *cis*-acting recombination hotspot present in the rDNA tandem repeats (Voelkel-Meiman *et al.* 1987). Hyper-recombination caused by this sequence was dependent on RNA polymerase I (RNAPI) transcription (Stewart and Roeder 1989; Huang and Keil 1995). Subsequently, RNA polymerase II (RNAPII)-driven transcription was also shown to stimulate recombination in *Saccharomyces cerevisiae* (Thomas and Rothstein 1989; Nevo-Caspi and Kupiec 1994; Bratty *et al.* 1996; Saxe *et al.* 2000) and *Schizosaccharomyces pombe* (Grimm *et al.* 1991). There is a group of proteins in yeast (Hpr1, Tho2, Mft1, Thp2, and Thp1) from which the null mutations lead to transcription defects linked to an increase in direct-repeat recombination (Chavez and Aguilera 1997; Prado *et al.* 1997; Piruat and Aguilera 1998; Chavez *et al.* 2000; Gallardo and Aguilera 2001). In mammalian cells, RNAPII-driven transcription is linked to two important developmentally regulated recombination processes, V(D)J recombination (Blackwell *et al.* 1986; Lauster *et al.* 1993; Oltz *et al.* 1993) and immunoglobulin class switching (Daniels and Lieber 1995).

Homologous recombination is catalyzed by a number of Rad proteins whose biochemical activities are being identified (Pâques and Haber 1999; Sung *et al.* 2000). *RAD51*, *RAD59*, and *RAD52* are among those most representative ones due to their particular relevance in different types of recombination events.

Rad51 is the eukaryotic homolog of RecA (Aboussekhra *et al.* 1992; Basile *et al.* 1992; Shinohara *et al.* 1992; Ogawa *et al.* 1993). Rad51 promotes homologous pairing and strand-exchange reactions (Sung 1994). Together with Rad54, Rad55, and Rad57, the Rad51 protein is required for gene conversions and crossovers in which a strand-exchange reaction is a landmark step. However, Rad51 is not required either for deletions or for a large proportion of inversion events occurring between DNA repeats (McDonald and Rothstein 1994; Aguilera 1995; Rattray and Symington 1995; Bai and Symington 1996; Jablonovich *et al.* 1999;

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Kang and Symington 2000; Malagon and Aguilera 2001). These observations are consistent with the actual idea that deletions and inversions may occur in the absence of Rad51 by mechanisms not requiring strand exchange such as break-induced replication (BIR) and single-strand annealing (SSA; Bartsch *et al.* 2000; Kang and Symington 2000; Malagon and Aguilera 2001; Rattray *et al.* 2001). In these events, Rad59 seems to play a major role. This hypothesis has raised the possibility that Rad59 is more relevant in reactions occurring in the absence of Rad51-mediated strand exchange. In addition, it explains why the major effect on recombination of *rad59* is observed in a *rad51* background (Bai and Symington 1996; Aguilera 2001; Davis and Symington 2001; Malagon and Aguilera 2001). Rad59 seems to be involved in the removal of nonhomologous sequences from the ends of single-strand DNA (ssDNA) and in the reannealing of complementary DNA sequences (Petukhova *et al.* 1999; Sugawara *et al.* 2000; Davis and Symington 2001).

Rad52 is a strand-annealing protein that forms ring structures at the ends of ssDNA (Mortensen *et al.* 1996; Shinohara *et al.* 1998; Parsons *et al.* 2000). *In vitro*, Rad52 stimulates the Rad51-promoted strand-exchange reaction presumably by overcoming the inhibitory effects of the single-strand binding protein RPA (Sung 1997; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998). Rad52 seems to be essential at an early step of double-strand-break (DSB) recombinational repair prior to or during strand invasion (Sung *et al.* 2000). This explains why Rad52 is required for all types of homologous recombination, including deletions between direct repeats, inversions, gene conversions, and crossovers between homologous chromosomes (Pâques and Haber 1999).

An additional relevant gene for the study of recombination is *RAD1*, whose product constitutes, together with Rad10, the nucleotide (nt) excision-repair endonuclease activity that cleaves ssDNA "flap" structures (Tomkinson *et al.* 1993). Rad1 is necessary in recombination for removal of nonhomologous 3′ tails, a step essential for the mechanism of SSA responsible for deletions between repeats (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995).

Our main interest is to understand how transcription stimulates recombination. Consequently, we have determined the effect of transcription on two different types of homologous recombination events: Rad51-dependent gene conversions and Rad51-independent inversions. We have undertaken for the first time a comparative genetic and molecular analysis of TAR and double-strand-break (DSB)-induced recombination using the same recombination substrates. We have determined the effect of *rad1*, *rad51*, *rad52*, and *rad59* mutants on each type of recombination event. Our results suggest that transcription of a DNA sequence increases the formation of DNA breaks or DNA lesions that are processed into DNA breaks, which could be repaired by double-strand-break repair (DSBR), synthesis-dependent strand annealing (SDSA), BIR, or SSA, depending on the structure and location of the donor sequence.

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used in this study are listed in Table 1. All strains used for the analyses of recombination of inverted-repeat systems were isogenic with W303. Those used with the plasmid-chromosome recombination construct were derivatives of W303 and its congenic strain AYW3-1Bu$^-$. Deletions of *RAD1*, *RAD51*, *RAD52*, and *RAD59* genes were accomplished with the PCR-based method using the kanMX4 (Wach *et al.* 1994) or the hphMX4 (Goldstein and McCusker 1999) cassettes and the 60-mer oligonucleotides described previously for each *rad* mutation (Malagon and Aguilera 2001). Correct deletions were confirmed by methyl methanesulfonate or UV sensitivity, PCR, and Southern blot analysis as previously described (Malagon and Aguilera 2001). Isogenic double mutants were constructed by genetic crosses.

Plasmids constructed for this study are as follows. Plasmid pCM189-L2 is pCM189 (Gari *et al.* 1997) in which the 1.34-kb *Bam*HI-*Ssp*I *LEU2* fragment of p414G*LEU2* (Piruat and Aguilera 1998) has been inserted at *Bam*HI-*Hpa*I in the polylinker under the *tet* promoter (P_{tet}). Plasmid p414GL2HOr is p414G*LEU2* with a 25-bp insertion at the *Eco*RI site of *LEU2* (*leu2-HOr* allele). Such a 25-bp fragment contains the 21-bp HO site (Nickoloff *et al.* 1986) made by hybridization of oligonucleotides AATTTCAGCTTTCCGCAACAGTATA and AATT TATACTGTTGCGGAAAGCTGA. Plasmid pCM189-L2HOr is pCM189 in which the 1.34-kb *Bam*HI-*Ssp*I *leu2-HOr* fragment of p414GL2HOr has been inserted at *Bam*HI-*Hpa*I in the polylinker. Plasmid pRS316-L2Δ is pRS316 (Sikorski and Hieter 1989) in which the 1.4-kb blunt-ended *Cla*I-*Sal*I *LEU2* fragment has been inserted at the *Sma*I site in the appropriate orientation. pRS316-INV is pRS316-L2Δ in which the 2.36-kb *Xho*I-*Hin*dIII P_{tet}*::leu2-HOr* fragment of pCM189-L2HOr has been inserted at *Xho*I-*Hin*dIII of the polylinker. pRS316-TINV is pRS316-INV in which the 1.8-kb blunt-ended *Eco*RI-*Xho*I fragment of pCM189 containing the *tTA* transactivator has been inserted at the blunt-ended *Xba*I site in the same orientation as the P_{tet}*::leu2-HOr* fragment.

Genetic and molecular analysis of recombination: Recombination frequencies are the median values of fluctuation tests performed with six independent yeast colonies each, as previously described (Prado and Aguilera 1995). For every genotype, the fluctuation test was repeated three times with three different yeast transformants. The final frequency shown for each genotype corresponds to the median value of the three median frequencies obtained from the tests. For low- or no-transcription conditions, frequencies were obtained from yeast colonies grown on medium containing 2% glucose medium and 5 μg/ml doxycycline (dox). TAR frequencies were obtained from yeast colonies grown on SC-2% glucose without dox. For the analysis of HO-mediated DSB recombination, mid-log phase yeast cells carrying the *HO* gene under the control of *GAL1* were obtained from SC-3% glycerol/2% lactate + dox liquid cultures and split into two halves. One-half was maintained in liquid SC-3% glycerol/2% lactate + dox (no HO expression) and the other was cultured in SC-2% galactose + dox for 6 hr for transient expression of HO, before performing fluctuation tests. Recombinants were selected on SC-leu-ura containing 2% glucose. HO-induced recombination values were obtained by subtracting the frequency of nonhomologous end-joining (NHEJ) from the total frequency of Leu$^+$ events.

Gene conversions and inversions in the inverted-repeat construct were determined by Southern and PCR analyses. The

TABLE 1

Strains

Name	Genotype
W303-1B	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
AYW3-1Bu	*MAT*α *ade2 can1-100 his3 leu2-k trp1 ura3*
M279	*MAT*α*-inc ade2-1 can1-100 his3-11,15 leu2,3-112 trp1-1 ura3-1 ade3::GAL-HO*
OI-15	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade3::GAL-HO*
WS	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO*
WSR1	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad1Δ::kanMX4*
WSR51	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad51Δ::kanMX4*
WSR52	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad52Δ::kanMX4*
WSR59	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad59Δ::kanMX4*
WSR1-1C	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad1Δ::kanMX4*
WSR51-8A	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 rad51Δ::kanMX4*
MKOS-3B	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad51Δ::kanMX4*
WSR52-5C	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 rad52Δ::kanMX4*
WSR159-3B	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad1Δ::kanMX4 rad59Δ::kanMX4*
WSR151-10D	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad1Δ::kanMX4 rad51Δ::kanMX4*
WSR5951-1D	*MAT***a-inc** *ade2-1 can1-100 his3-11,15 leu2Δ::SFA1 trp1-1 ura3-1 ade3::GAL-HO rad59Δ::kanMX4 rad51Δ::kanMX4*
AWR1-2B	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k rad1Δ::kanMX4*
AWR1-2BH	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k rad1Δ::hphMX4*
AWR51-2B	*MAT*α *ade2 can1-100 his3 trp1 ura3 leu2-k rad51Δ::kanMX4*
AWR52-3B	*MAT*α *ade2 can1-100 his3 trp1 ura3 leu2-k rad52Δ::kanMX4*
AWR59-5C	*MAT*α *ade2 can1-100 his3 trp1 ura3 leu2-k rad59Δ::kanMX4*
AWR159-13C	*MAT*α *ade2 can1-100 his3 trp1 ura3 leu2-k rad1Δ::kanMX4 rad59Δ::kanMX4*
AWR151-2BH	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k rad1Δ::hphMX4 rad51Δ::kanMX4*
AWR5159-1A	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO rad51Δ::hphMX4 rad59Δ::kanMX4*
MAWR-4C	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO*
MAWR1-3C	*MAT*α*-inc ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO rad1Δ::kanMX4*
MAWR59-3C	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO rad59Δ::kanMX4*
MAWR51-3D	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO rad51Δ::kanMX4*
MAWR52-8D	*MAT***a-inc** *ade2 can1-100 his3 trp1 ura3 leu2-k ade3::GAL-HO rad52Δ::kanMX4*

All strains were constructed for this study except W303-1B (Thomas and Rothstein 1989), AYW3-1Bu$^-$ (Santos-Rosa and Aguilera 1995), and M279 and OI-15 (M. Kupiec).

first was performed on total DNA of independent Leu$^+$ recombinants digested with *Ssp*I and probed with the ^{32}P-labeled 1.2-kb *Cla*I-*Ssp*I internal *LEU2* fragment. The latter was determined with reactions using a mix of the three oligonucleotides CCGGCAGATCAATTCCTCGATC (a), TTAGAGCGGATGT GGGGGAG (b), and GAAGGTTTTGGGACGCTCGAAG (c).

The directionality of gene conversion in the plasmid-chromosome recombination construct was determined genetically. First, independent Leu$^+$ gene convertants that lost their plasmid were selected on SC + FOA. Afterward the Ura$^-$ segregants were scored for their ability to grow on SC-leu, where only gene convertants of the chromosomal *leu2-k* allele were able to form colonies.

Miscellaneous: Growth conditions and genetic analyses were performed according to previously published methods (Prado and Aguilera 1995). Southern and Northern analyses were performed as described (Chavez and Aguilera 1997). RNA levels were quantified with a Fuji FLA3000 analyzer and were normalized with respect to the 28S rRNA values.

RESULTS

A new P_{tet}*::leu2-HOr* allele, containing a 21-bp HO site, for the analyses of transcription-associated and DSB-induced recombination: Our goal in this study was to determine the genetic requirements of TAR in comparison with spontaneous recombination occurring under no- or low-transcription conditions and with DSB-induced recombination. To construct recombination substrates that were valid for the analysis of transcription-associated and DSB-induced recombination we first placed the *LEU2* open reading frame under a modified P_{tet} promoter, which is negatively controlled by the presence of dox (Gari *et al.* 1997). Using a P_{tet}*::LEU2* fusion (plasmid pCM189-L2) we first showed that dox both impeded growth in SC-leu and reduced the *LEU2* mRNA up to 11% of the levels observed without dox (Figure 1, A and B).

We next constructed a *leu2-HOr* allele containing the 21-bp HO site at the *LEU2 Eco*RI site, instead of the full 117-bp HO site used in most previously reported recombination assays (Pâques and Haber 1999). We used a *leu2-HOr* substrate under the control of P_{tet} to be able to use the P_{GAL1}*::HO* fusion to study TAR and DSB-induced recombination with the same substrates. Transcript levels of P_{tet}*::leu2-HOr* were the same as in P_{tet}*::LEU2* as determined by Northern analyses (Figure 1; data not shown). We analyzed by Southern hybridization the kinetics of HO cleavage in this allele, under conditions of high (−dox) or low transcription (+dox) of the *leu2-HOr* allele. As expected, HO did not cleave

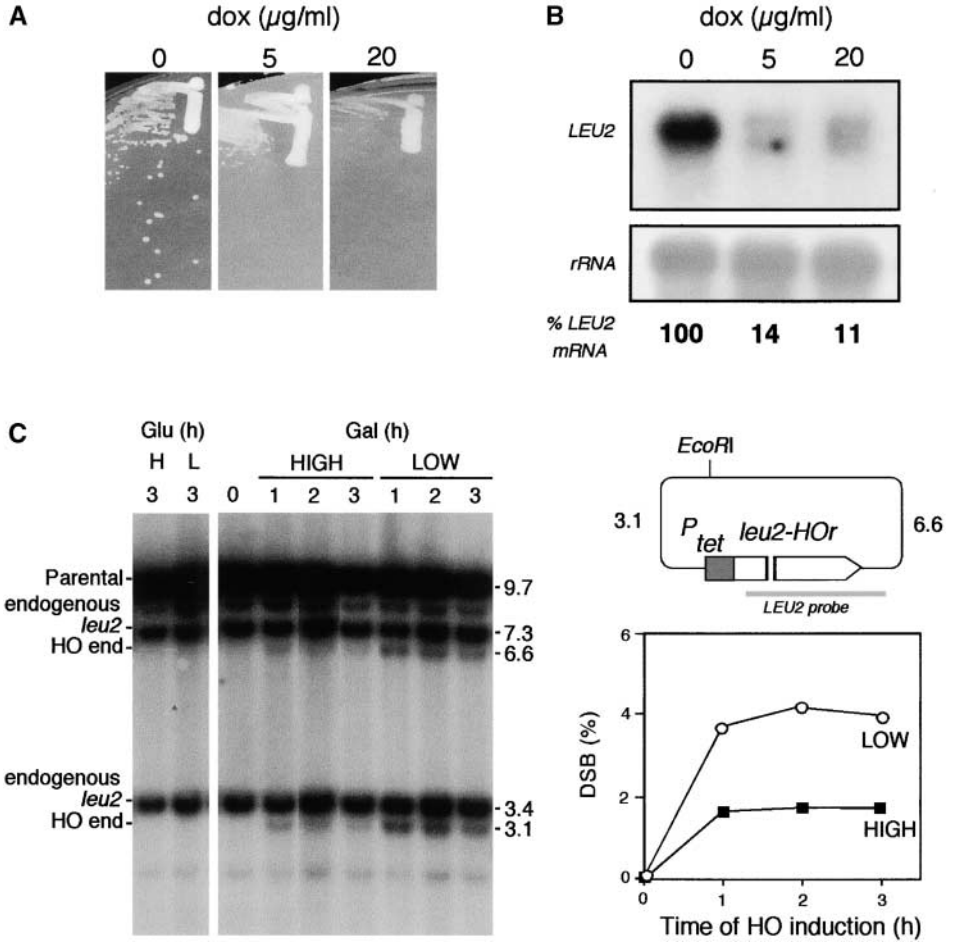


FIGURE 1.—Expression of P_{tet}::*LEU2* and kinetics of HO cleavage at the P_{tet}::*leu2-HOr* fusion. (A) Molecular and genetic analysis of expression of a P_{tet}::*LEU2* fusion. Colony formation of the wild-type strain (WS) containing the plasmid pCM189-L2 (P_{tet}::*LEU2*) after 3 days on SC-ura-leu supplemented with 5 or 20 μg/ml dox. (B) Northern analysis of the WS wild-type transformant grown in SC-ura supplemented with 5 or 20 μg/ml dox. The percentages of *LEU2* mRNA are referred to the zero time values and were obtained after normalizing values with respect to the 28S rRNA. Identical results were obtained with the *leu2-HOr* allele (not shown). (C) Southern analysis of HO cleavage in the *leu2-HOr* allele under low (L; 5 μg/ml dox) and high (H; −dox) transcription conditions in the OI-15 wild-type strain. SC-3% glycerol-2% lactate-ura with or without dox mid-log phase cultures were split into two halves. One-half was supplemented with 2% glucose and the other with 2% galactose. DNA samples were taken at hourly intervals and digested with *Eco*RI for Southern analysis. A scheme of plasmid pCM189-L2HOr containing the *leu2-HOr* allele and the quantification data of the 6.6-kb band resulting from HO cleavage and *Eco*RI restriction is shown. A ^{32}P-labeled 1.2-kb *Cla*I-*Ssp*I internal *LEU2* fragment was used as a probe (gray line). The 7.3- and 3.4-kb bands correspond to the chromosomal *leu2-3,112* marker. Sizes are indicated in kilobases.

the 21-bp HO site with full efficiency (Figure 1C). The levels of DSBs reached a maximum value of ~4% of total DNA after 2 hr of shifting cells to galactose. Interestingly, this efficiency of HO cleavage was obtained with low transcription. Under high-transcription conditions the overall efficiency of HO cleavage was ~2.5-fold lower (Figure 1C). This result suggests that the accessibility or affinity of the HO endonuclease to the 21-bp HO site was significantly reduced by transcription. Relative frequencies of HO-induced recombination were the same under both high- and low-transcription conditions. For this reason only the results with low transcription will be shown.

A plasmid-chromosome construct to study transcription-associated and DSB-induced gene conversions: We first developed a recombination substrate that permitted us to analyze spontaneous gene conversion events occurring under low- and high-transcription conditions as well as by induction with HO. The recombination construct was based on the *leu2-k* allele (fill-out of the *Kpn*I site) at the *LEU2* chromosomal locus and on the P_{tet}::*leu2-HOr* allele in the monocopy plasmid pCM189. In this construct, recombinants were scored as Leu$^+$ colonies. They could arise by gene conversion of either the chromosomal *leu2-k* allele or the plasmidic *leu2-HOr* allele (Figure 2A). Leu$^+$ reciprocal exchange events, leading to the integration of the plasmid into the chromosome, were never recovered. This was expected as a consequence of the instability of the resulting dicentric chromosomes (SUROSKY and TYE 1985). Therefore, this recombination construct was designed specifically to study gene conversion events.

Transcription-associated gene conversions show the same patterns of *RAD51* and *RAD59* dependency as do DSB-induced gene conversions: Figure 2B shows that high-transcription levels stimulate gene conversion between the *leu2-HOr* and *leu2-k* alleles 8 times the frequency obtained with low transcription. Increases in gene conversions were observed in *rad1*, *rad51*, *rad59*, and *rad52* cells as well as in double-mutant combinations. Recombination in both cases was independent of *RAD1* and strongly dependent on *RAD52*. Recombination was significantly reduced in *rad51* cells (35 and 45 times with respect to the wild type under low and high levels of transcription, respectively), but weakly affected in *rad59* cells (3.8 and 2.1 times). None of the double-

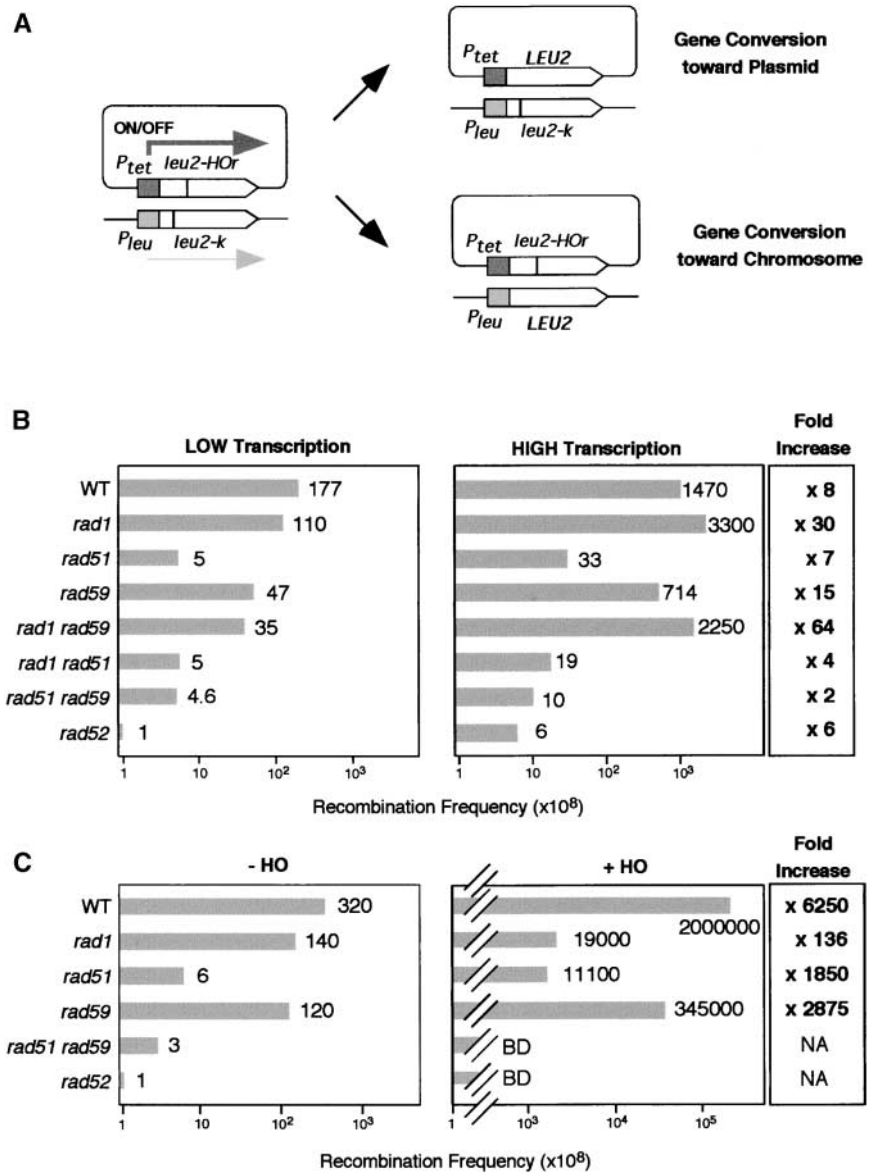


FIGURE 2.—Plasmid-chromosome recombination constructs used to study transcription-associated and HO-induced gene conversion. (A) Recombination between pCM189-L2HOr, carrying the *leu2-HOr* allele under P_{tet}, and chromosome III, carrying the *leu2-k* allele under its own promoter, P_{LEU2}. Leu^+ recombinants can arise by gene conversion of either allele, *leu2-HOr* or *leu2-k*. (B) Recombination frequencies at low (5 μg/ml dox) and high levels of transcription (−dox). (C) Low-transcription spontaneous (−HO) and DSB-induced (+HO) Leu^+ recombination frequencies after 6 hr of HO activation in 2% galactose under low-transcription conditions. Each value represents the median of three different fluctuation tests, each performed with three different transformants. Strains used were AYW3-1Bu$^-$ (wild-type), AWR1-2B (*rad1*), AWR1-2BH (*rad1*), AWR51-2B (*rad51*), AWR52-3B (*rad52*), AWR59-5C (*rad59*), AWR159-13C (*rad1 rad59*), AWR151-2BH (*rad1 rad51*), AWR5159-1A (*rad51 rad59*), MAWR-4C (wild-type), MAWR1-3C (*rad1*), MAWR59-3C (*rad59*), MAWR51-3D (*rad51*), and MAWR52-8D (*rad52*). HO-induced homologous recombination values were obtained by subtracting the frequency of illegitimate Leu^+ recombinants (2.2×10^{-4}) from the total frequency of Leu^+ events (see text). Those cases (*rad52* and *rad51 rad59*) with resulting frequencies below 1×10^{-4} are indicated as below detection (BD). In such cases, the fold-increase value was not applicable (NA).

mutant combinations done with *rad1*, *rad51*, and *rad59* showed synergistic effects, *rad51* being epistatic over *rad1* and *rad59*, and *rad59* being epistatic over *rad1* at both low- and high-transcription conditions. However, some quantitative differences existed between low and high transcription. Thus, the highest stimulation of gene conversions by transcription was obtained in *rad1 rad59* cells (64 times) and the lowest in *rad51 rad59* cells (2 times). The observation that recombination in *rad51 rad59* cells was 3 times lower than that in *rad51* cells confirms that Rad59 is only slightly required for transcription-associated gene conversion events.

Gene conversions were strongly induced by HO in the plasmid-chromosome assay. When yeast colonies were grown in media containing 2% galactose, all cells became recombinants in wild-type and *rad* mutant cells as a consequence of recurrent HO cleavage (our unpublished data). Nevertheless, *rad51* and *rad52* mutants took longer to grow under these conditions, due to their low efficiency of DSB recombinational repair (data not shown). For this reason, we did all experiments with a transient expression of HO for 6 hr in liquid media. Under these conditions, we could obtain a reliable recombination frequency, clearly above spontaneous levels. Figure 2C shows that HO-mediated DSBs induced recombination strongly in all strains tested. However, the pattern of dependency on *rad51*, *rad59*, and *rad52* was similar to that of the spontaneous recombination under low and high levels of transcription. A strong dependency on *RAD51* and *RAD52* and a weak dependency on *RAD59* was observed (Figure 2C). Thus the frequency of HO-induced recombination was 180-, $>10^6$-, and 5.8-fold below wild-type levels in *rad51*, *rad52*, and *rad59* cells, respectively. These results contrast with the lower dependency on *RAD* genes observed for spontaneous (non-HO-induced) recombination in which *rad51*,

rad52, and *rad59* reduced recombination 53-, 320-, and 2.7-fold below wild-type levels (Figure 2C). The only exception was *rad1* in which the frequency of HO-induced gene conversions was 106 times below wild-type levels but the frequency of spontaneous events was reduced only 2.3-fold. This result might be caused by the requirement for Rad1 in recombination events that initiate at heterologous regions (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995; Pâques and Haber 1997; Colaiacovo *et al.* 1999). Most spontaneous events could initiate at the homologous *leu2* sequences, outside the HO site, which would not require Rad1/Rad10 to be processed.

Transcription-associated gene conversions initiate at the highly transcribed DNA sequence: Genetic analysis of the directionality of gene conversions in the plasmid-chromosome assay revealed that under high-transcription conditions, most spontaneous recombination events occurred by gene conversion of the strongly transcribed *leu2-HOr* allele in wild-type and *rad* strains (Table 2). This is clearly observed in wild-type cells, in which all spontaneous recombination events converted the *leu2-HOr* allele. As expected, all HO-induced gene conversion events, among a total of 62 Leu^+ events analyzed, converted the *leu2-HOr* allele into *LEU2* in wild-type and all *rad* mutant strains. As all HO-induced Leu^+ events were initiated by a DSB at the *leu2-HOr* allele, it was expected that they occur by copying the wild-type information from the *leu2-k* allele. Our results, therefore, indicate that transcription facilitates initiation of the recombination event at the strongly transcribed sequence, *leu2-HOr*, which acts as recipient of information in the gene conversion event. Interestingly, a large majority of spontaneous Leu^+ events occurring with low transcription also underwent gene conversion of the *leu2-HOr* allele in wild-type cells, although to a minor degree (Table 2). This implies that most spontaneous events were also initiated at the *leu2-HOr* allele. It is likely that the low levels of transcription at the *leu2-HOr* were enough to facilitate initiation of recombination at this allele.

At low transcription, although *leu2-HOr* is the allele preferentially converted in most strains tested, in most of the *rad* mutant combinations tested the proportion of conversion of *leu2-k* (4-bp insertion) is lower than that of *leu2-HOr* (25-bp insertion) relative to high transcription (Table 2). The case of the *rad1 rad51* cells is worth mentioning, in which spontaneous gene conversion of the chromosomal *leu2-k* allele was clearly favored as compared to other *rad* mutants, including *rad1* and *rad51* single mutants. This may reflect a higher difficulty in conversion of long heterologies in *rad* mutants.

TAR between inverted repeats occurs similarly to DSB-induced recombination in a *RAD51*-independent and *RAD59*-dependent manner: Recombination was next analyzed between inverted repeats. The rationale was that inversions can also occur by a mechanism different from gene conversions and reciprocal exchange (Bartsch *et al.* 2000; Malagon and Aguilera 2001; Rattray *et al.* 2001). Therefore, we constructed an inverted-repeat assay (TINV) on the basis of the P_{tet}*::leu2-HOr* allele and a *leu2*Δ*5′* allele. This *leu2*Δ*5′* copy was not transcribed, as determined by Northern analysis (data not shown). With this assay, Leu^+ recombinants could occur in principle either by gene conversion of the *leu2-HOr* copy, whether or not associated with an inversion of the region located between the repeats, or by a crossover upstream of the HO site of *leu2-HOr* (Figure 3A).

TABLE 2

Spontaneous mitotic gene conversion proportions in the plasmid-chromosome system

Genotype[a]	Low transcription: % *leu2-HOr* convertants[b,c]	High transcription: % *leu2-HOr* convertants[b,c]
Wild type	87 (150)	100 (271)
rad1	64* (195)	99 (282)
rad51	58* (62)	86* (64)
rad52	97* (89)	97 (71)
rad59	97* (145)	89* (141)
rad1 rad59	63* (84)	98 (208)
rad1 rad51	36* (151)	75* (194)
rad51 rad59	91 (46)	100 (50)

[a] Strains are the same used in Figure 2.
[b] Percentage of events that converted the *leu2-HO* allele to wild-type *LEU2* and total Leu^+ independent recombinants tested are in parentheses.
[c] An asterisk indicates that the difference from the wild-type value is statiscally significant according to a χ^2 test ($P < 0.05$).

High-transcription conditions stimulated recombination in both wild-type and *rad* mutants, although at low level (2- to 11-fold). We believe that this is the case because, in contrast to gene conversions in the plasmid-chromosome system, the basal recombination levels in this system were already high (above 10^{-4}). This introduces a background noise above which TAR is less clearly detected.

The pattern of dependency of the *RAD* genes of both TAR and recombination under low-transcription conditions was similar. Figure 3B shows that spontaneous inverted-repeat recombination was strongly affected in *rad52* cells. In contrast to the gene conversion events of the plasmid-chromosome assay, inverted-repeat recombination was not affected by the *rad51* mutation and was slightly reduced by *rad59* (4- to 8-fold below wild-type levels). Indeed, as inversions in *rad59* cells represent half the proportion of total inversions as compared to wild-type cells (Figure 4), the decreases caused by *rad59* in the frequency of total Leu^+ inversions are between 6.8- and 12.4-fold (Figures 3 and 4). The *rad59* and *rad51* mutations show additive effects on the reduction of the frequency of inverted-repeat recombination, consistent with previous observations (Bai and Syming-

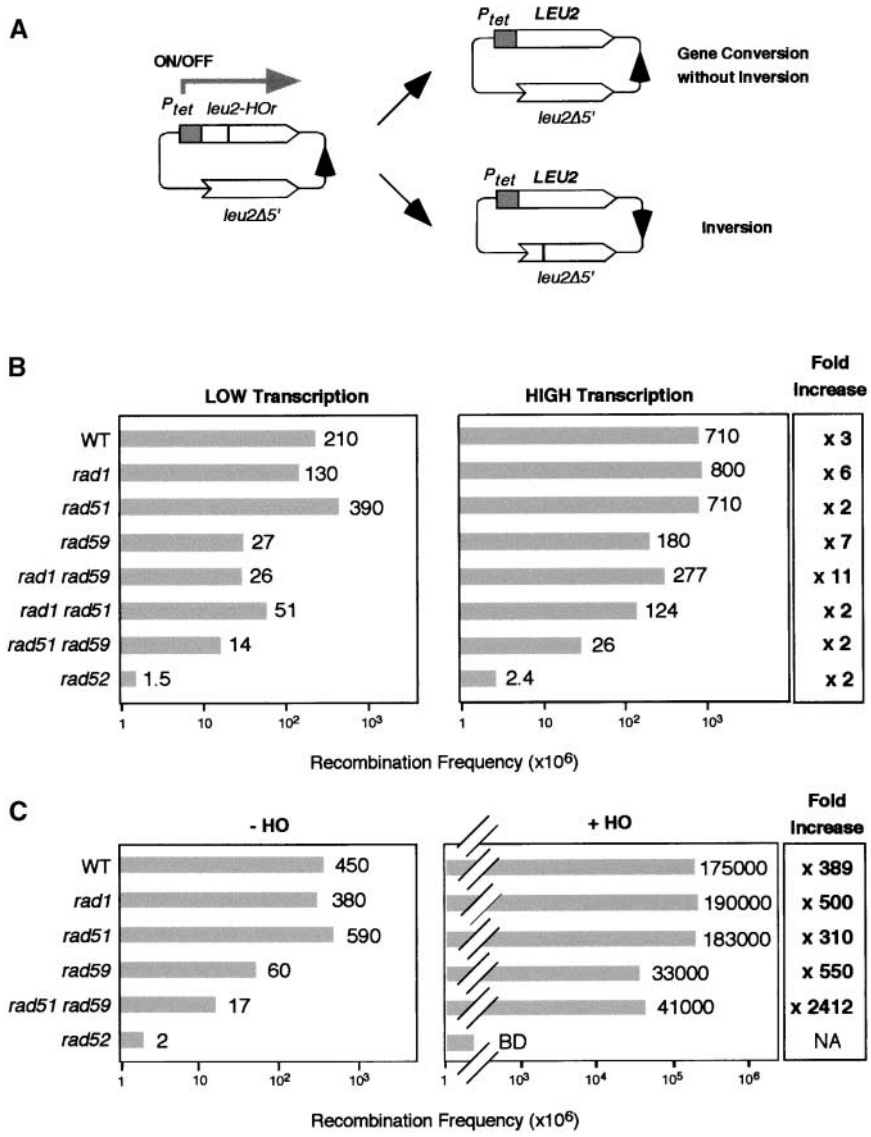


FIGURE 3.—TINV inverted-repeat construct used to study TAR and HO-induced recombination. (A) Plasmid substrate pRS316-TINV carrying two inverted *leu2* sequences. One copy is the *leu2-HOr* allele under P_{tet} and the other is a 5′-end truncated allele (*leu2Δ5′*). Transcription of *leu2* is driven only from P_{tet}. Leu$^+$ recombinants can arise by gene conversion of *leu2-HOr* without an associated inversion or by crossover occurring upstream of the HO site, whether or not associated with gene conversion. (B and C) As in Figure 2. Strains used were WS (wild type), WSR52 (*rad52*), WSR59 (*rad59*), WSR1-1C (*rad1*), WSR51-8A (*rad51*), MKOS-3B (*rad51*), WSR52-5C (*rad52*), WSR159-3B (*rad1 rad59*), WSR151-10D (*rad1 rad51*), and WSR5951-1D (*rad51 rad59*). Other details as in Figure 2.

TON 1996; MALAGON and AGUILERA 2001). As reported with other assays, Rad1 becomes important in spontaneous inverted-repeat recombination when Rad51 is absent (AGUILERA 1995; RATTRAY and SYMINGTON 1995).

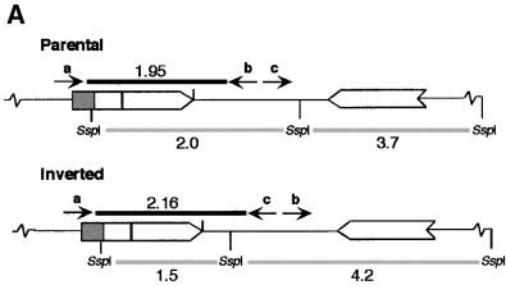
This result, different from those of the plasmid-chromosome system, indicates that gene conversion may not be the major spontaneous recombination event between inverted repeats under both low- and high-transcription conditions. Instead, a Rad51-independent and Rad1- and Rad59-dependent mechanism might be the primary pathway leading to Leu$^+$ events between inverted repeats, presumably BIR and SSA, as suggested previously (BARTSCH *et al.* 2000; MALAGON and AGUILERA 2001; RATTRAY *et al.* 2001).

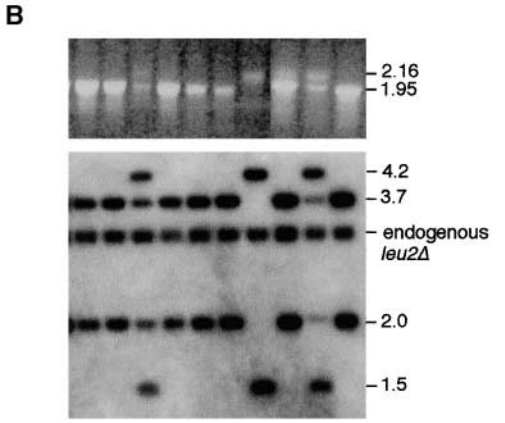
When HO was induced for 6 hr in 2% galactose, recombination was strongly stimulated. Again, DSB-induced recombinants were almost abolished in *rad52* cells, unaffected in *rad1* and *rad51* cells, and reduced in *rad59* cells (5.3-fold below wild-type levels; Figure 3C), showing, therefore, the same pattern of genetic requirements as spontaneous recombination.

Finally, PCR analysis of independent Leu$^+$ recombination events showed that 30% were associated with an inversion in wild-type cells under low- and high-transcription conditions (Figure 4). There are no significant differences ($P < 0.05$) in the proportion of inversions in wild-type and *rad* cells. Indeed, the percentage of inversions was also similar for DSB-induced recombination in wild-type, *rad51*, and *rad59* cells (our unpublished data). These results confirm that inversions may occur efficiently in the absence of Rad51, via a Rad1- and Rad59-dependent recombination mechanism with both low and high transcription. It is worth noting that inversions are frequently in association with plasmids carrying noninverted repeats (our unpublished data).

Illegitimate end-joining causes high levels of Leu$^+$ events at the *leu2-HOr* allele: In wild-type and *rad* strains HO induced Leu$^+$ recombinants two to three orders

of magnitude above spontaneous levels. Unexpectedly, HO-induced Leu$^+$ events were very high in *rad52* strains (1.7 and 3.3 $\times$ 10^{-4} for the plasmid-chromosome and inverted-repeat recombination assays, respectively). Here we show that such a high frequency of recombination was the result of NHEJ. We know that *leu2*Δ strains with plasmid pCM189-L2HOr, carrying only a *leu2-HOr* copy, should never lead to Leu$^+$ events by homologous recombination because they lack wild-type *LEU2* sequences acting as donors of information. However, using these assays, HO-induced Leu$^+$ events were obtained at frequencies ranging from 1.5 to 3.0 $\times$ 10^{-4} in wild-type, *rad51 rad59*, and *rad52* strains. These frequencies were similar to those of HO-induced Leu$^+$ events obtained with the plasmid-chromosome system in *rad51 rad59* (1.96 $\times$ 10^{-4}) and *rad52* cells (1.7 $\times$ 10^{-4}) or with the TINV inverted-repeat constructs in *rad52* cells (3.3 $\times$ 10^{-4}). This is explained by the capability of our 25-bp insertion mutation containing the 21-bp HO site to be converted by illegitimate NHEJ into 27-bp insertions that reestablish the *LEU2* wild-type frame (Moore and Haber 1996). Therefore, our results indicate that HO can induce detectable gene conversion events in all *rad* mutants analyzed except *rad51 rad59* and *rad52* and inverted-repeat recombination in all *rad* mutants except *rad52*. For this reason, HO-induced recombination values were obtained by subtracting the frequency of NHEJ from the total frequency of Leu$^+$ events (Figures 2 and 3).





C

Genotype	% INVERSIONS LOW Tr.	HIGH Tr.
WT	31 (112)	30 (114)
rad1	17 (48)	17 (48)
rad51	35 (48)	40 (48)
rad52	12* (49)	8* (107)
rad59	18 (66)	17 (69)
rad1 rad59	N.D.	N.D.
rad1 rad51	23 (48)	25 (48)
rad51 rad59	21 (47)	37 (48)

Figure 4.—Molecular analysis of inversions in the TINV inverted-repeat system. (A) Scheme of the inverted-repeat substrate, indicating the primers (a, b, and c), PCR products (black line), and *Ssp*I fragments (gray line) expected from PCR and Southern analyses. (B) PCR analysis using a combination of primers a, b, and c and Southern analysis after digestion with *Ssp*I of total DNA from Leu$^+$ independent recombinants. Sizes are indicated in kilobases. (C) Percentage of inversions in each strain under low and high levels of transcription. Strains are those used in Figure 3. The total number of Leu$^+$ independent recombinants tested are shown in parentheses. An asterisk indicates that the difference from the wild-type value is statistically significant according to a χ^2 test ($P < 0.05$).

DISCUSSION

For the comparative analysis of TAR and DSB-induced recombination we developed new inter- and intramolecular recombination substrates on the basis of a *leu2-HOr* allele fused to the regulated P_{tet} promoter and containing a 21-bp HO site. These substrates allowed the study of different types of recombination events, including gene conversions and inversions, which may occur by distinct mechanisms as deduced from their different dependencies on Rad51 and Rad59. We have shown that highly transcribed sequences act as recipients of gene conversions. Importantly, TAR shows similar dependencies such as HO-induced recombination on the DSB-repair genes *RAD1*, *RAD51*, *RAD52*, and *RAD59*. These results suggest that high transcription facilitates the formation of DNA breaks along the DNA sequence being transcribed. We argue that transcription is able to increase all types of homologous recombination events, whether occurring by DSBR, SDSA, or BIR/SSA mechanisms.

Intra- and intermolecular recombination events show different Rad51 and Rad59 requirements: All homologous recombination events analyzed in this study were Rad52 dependent. However, we observed different Rad51 and Rad59 genetic requirements between intramolecular gene conversions and intermolecular gene conversion and inversions, regardless of whether they initiated spontaneously (under low- and high-transcription conditions) or by an HO-induced DSB. As expected, intermolecular gene conversions were dependent on the Rad51 strand-exchange protein. This is consistent

with a number of published observations (Sugawara *et al.* 1995; Bratty *et al.* 1996; Elias-Arnanz *et al.* 1996; Bartsch *et al.* 2000), confirming that the major mitotic mechanism leading to intermolecular gene conversions is either DSBR (Szostak *et al.* 1983) or SDSA (Hastings 1988; McGill *et al.* 1989), which requires Rad51-mediated strand exchange (Pâques and Haber 1999). However, it is worth noting that the levels of plasmid-chromosome gene conversions in *rad51* mutants were still fivefold above *rad52* levels. There is evidence that the most likely mechanism for recombination in the absence of Rad51-mediated strand exchange is BIR (Malkova *et al.* 1996; Signon *et al.* 2001). It is possible, therefore, that in *rad51* cells plasmid-chromosome gene conversions occurred via BIR initiated at either 3′-end of the DSB. This would result in a linear plasmid containing *leu2* repeats that would yield a converted Leu^+ circular plasmid by SSA.

In contrast to intermolecular events, gene conversion and inversion between inverted repeats occur with wild-type efficiency in *rad51* cells. This result confirms that inverted-repeat recombination leading to Leu^+ events is efficient in the absence of a Rad51-mediated strand-exchange reaction. It is likely that in the absence of Rad51 many Leu^+ inverted-repeat recombination events occur via BIR followed by SSA as previously proposed (Bartsch *et al.* 2000; Malagon and Aguilera 2001). Indeed, the detection of duplicated inverted-repeat fragments among the recombination products of *mre11*, *rad50*, and *sae2/com1* cells is consistent with BIR as a mechanism responsible for inversions (Rattray *et al.* 2001).

As expected from the observation that Rad51 and Rad59 control different genetic recombination pathways (Bai and Symington 1996), Rad51-independent spontaneous and DSB-induced inverted-repeat gene conversions and inversions are more dependent on Rad59 than are Rad51-dependent plasmid-chromosome gene conversions (Figures 2 and 3). These results are consistent with the idea that Rad59 plays an important role in the formation of inversions (Sugawara *et al.* 2000; Davis and Symington 2001; Malagon and Aguilera 2001). Rad59 has strand-annealing activity (Petukhova *et al.* 1999; Davis and Symington 2001) and might be important, together with Rad52, for strand invasion in the absence of Rad51 (Bartsch *et al.* 2000; Aguilera 2001; Davis and Symington 2001). Therefore, Rad59 may be required in inverted-repeat recombination for the initial BIR event and for the putative SSA event following BIR.

It is likely that in wild-type cells BIR is responsible for only a low proportion of events (Malkova *et al.* 1996; Signon *et al.* 2001). However, if both inter- and intramolecular events can theoretically occur in *rad51* cells by BIR/SSA, the question arising is why this mechanism is much more efficient in intramolecular events. We believe that invasion of a 3′-end into a "closed" DNA structure clearly requires Rad51, as has been suggested previously (Sugawara *et al.* 1995). However, in intrachromosomal repeats, where invasion occurs at a sequence adjacent to the site of initiation, recombination can efficiently take place in the absence of Rad51. It is likely that the DSB will change the supercoiling (Duguet 1997) and chromatin structure of the DNA (Downs *et al.* 2000; Paull *et al.* 2000), leading to an "open" DNA structure at the adjacent repeat sequence and facilitating, in consequence, Rad59-Rad52-dependent strand invasion in the absence of Rad51.

Finally, it is worth noting that Rad1 had little effect on both spontaneous and DSB-induced interchromosomal gene conversion and inverted-repeat recombination, with one exception: HO-induced plasmid-chromosome gene conversions are Rad1 dependent. This was unexpected, because cleavage of the 21-bp HO site leads to tails containing 16- and 9-nt heterologous 3′-ended strands (Nickoloff *et al.* 1986). Nevertheless, Rad1/Rad10 has been shown not to be required for the removal of heterologous tails <30 bp in intramolecular events (Prado and Aguilera 1995; Pâques and Haber 1997). Considering the hypothesis mentioned above, it is possible that the 16- and 9-bp heterologous tails significantly diminish the invasion efficiency of the 3′ end into a closed DNA sequence (intermolecular event) but not into a less restrictive open DNA sequence (intramolecular events). Removal of the heterologous tails by Rad1 would therefore facilitate strand invasion in intermolecular events and the formation of plectonemic joint molecules.

Transcription-associated and DSB-induced recombination events are similar and show identical gene requirements: We have used two different constructs containing the same *leu2* alleles to show that transcription induces any type of recombination event. Our results, together with previously reported data on deletions (Thomas and Rothstein 1989) and ectopic recombination (Bratty *et al.* 1996; Nevo-Caspi and Kupiec 1996; Saxe *et al.* 2000), indicate that transcription does not specifically stimulate a particular type of recombination mechanism, whether DSBR, SDSA, BIR, or SSA. Gene conversions occurring between different DNA molecules are strongly stimulated by transcription (Figure 2), consistent with the observations that ectopic gene conversions between heterologous chromosomes are stimulated by transcription (Bratty *et al.* 1996; Nevo-Caspi and Kupiec 1996; Saxe *et al.* 2000). As reported for ectopic recombination (Saxe *et al.* 2000), we observed that the highly transcribed DNA sequence is preferentially converted; that is, it acts as a recipient of information in our plasmid-chromosome assay (Table 2). According to the DSBR (Szostak *et al.* 1983) and SDSA models (Hastings 1988; McGill *et al.* 1989), the results are consistent with the idea that transcription stimulates the initiation of recombination.

Transcription stimulates recombination in all *rad* mu-

tants tested, confirming that transcription induces all types of recombination events, whether or not Rad51 and Rad59 dependent. Consistent with our results, a parallel study showed that transcription of the recipient molecule increases ectopic recombination between heterologous chromosomes in both *rad51* and *rad59* mutants (J. A. Freedman and S. Jinks-Robertson, unpublished results). Importantly, using the same recombination systems we show for the first time that spontaneous recombination occurring under both low and high transcription has the same *RAD51*, *RAD52*, and *RAD59* gene requirements as HO-induced recombination (Figures 2 and 3). This is so, despite the different pattern of *RAD* dependency observed for each type of recombination event studied. The similar gene requirements of TAR and HO-induced recombination suggest that the initiation events stimulated by transcription are DNA breaks and/or lesions that subsequently lead to DNA breaks.

The main emerging question is how transcription through one DNA sequence can contribute to the initiation of recombination, that is, to the formation of DNA breaks. We envision two possible scenarios (Aguilera 2002). First, transcription can encounter the replication machinery and lead to a replication fork blockage. Such a blockage could cause a fork reversal leading to Holliday junctions with one arm formed with the newly synthesized leading and lagging strands and with a recombinogenic double-strand tail (Seigneur *et al.* 1998; Michel *et al.* 2001). Interestingly, experiments using *E. coli* strains with high levels of the (p)ppGp signal molecules that modulate RNAP activity or with a mutated RNAP suggest that the RNAP might contribute to the formation of recombinogenic Holliday junctions by promoting fork reversal (McGlynn and Lloyd 2000).

In an alternative scenario, the opening of the chromatin structure of the DNA strands, facilitated by the transient accumulation of supercoiled negative DNA behind the advancing RNAPII, could increase the accessibility of DNA-damaging agents, such as free radicals (Aguilera 2002). This could lead to recombinogenic DNA breaks, whether directly or after the subsequent action of the replication fork. The rationale for this hypothesis is that ssDNA is more reactive than double-strand DNA (Frederico *et al.* 1990). This would be consistent with the observations that the spontaneous mutagenesis of a gene increases with transcription, as shown in *E. coli* (Beletskii and Bhagwat 1996) and *S. cerevisiae* (Datta and Jinks-Robertson 1995). Indeed, the mutation rate is higher with a mutant T7 polymerase having a slower elongation rate (Beletskii *et al.* 2000).

In summary, our results provide evidence that transcription elongation may contribute to the formation of DNA breaks or lesions that are subsequently converted into DSBs. Such breaks would be repaired by DSBR, SDSA, BIR, or SSA, depending on the structure and location of the donor sequence, and therefore can potentially lead to all types of homologous recombination events.

We thank F. Prado for reading the manuscript, M. Kupiec for providing strains MK279 and OI-15, S. Jinks-Robertson for communicating unpublished data, and D. Haun for style supervision. Research was funded by grants from the Spanish Ministry of Science and Technology (BMC200-0439) and the Human Frontier Science Program (RG1999/0075). S.G.-B. was a recipient of a predoctoral training grant from the Spanish Ministry of Education and Culture.

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Communicating editor: L. S. Symington