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Gene Conversion Adjacent to Regions of Double-Strand Break Repair

TERRY L. ORR-WEAVER,†* ALAIN NICOLAS,‡ AND JACK W. SZOSTAK

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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The repair of double-strand breaks and gaps can be studied in vegetative yeast cells by transforming the DNA with restriction enzyme-cut plasmids. Postulated models for this repair process require the formation of heteroduplex DNA on either side of the region of break or gap repair. We describe the use of restriction site mutations in the *his3* gene to detect conversion events flanking but outside of a region of a double-strand break repair. The frequency with which a mutation was converted declined with increasing distance between the mutation and the edge of the gap repair region. The data are consistent with heteroduplex DNA tracts of at least several hundred base pairs adjacent to regions of double-strand break repair.

According to the double-strand break repair model for meiotic recombination (23), recombination events are initiated by double-strand breaks, and gene conversion events can occur either by the repair of a double-strand gap or by mismatch repair in associated heteroduplex DNA, as proposed in earlier models (6, 10). The double-strand break repair model evolved from the demonstration that double-strand breaks and gaps on plasmids introduced into *Saccharomyces cerevisiae* cells by transformation could be repaired by using chromosomal information as a template (13). An example of gene conversion by double-strand gap repair is the observation that a chromosomal mutation can be transferred to a homologous plasmid sequence when the plasmid contains a gap covering the site of the mutation (14).

In previous experiments we have shown that the process of double-strand gap repair in the yeast transformation system is similar to meiotic recombination in two important respects. Both double-strand gap repair and meiotic gene conversion are associated with a high frequency of reciprocal crossing over, and both processes require the *RAD52* gene product (13, 15). Other genetic properties of meiotic recombination can also be explained by the double-strand gap repair model (23). Recently, Kolodkin et al. (9) and Klar and Miglio (8) have shown that meiotic recombination can be initiated by experimentally induced double-strand breaks.

We sought to test experimentally, in the plasmid transformation system, the possibility that gene conversion events can be mediated by heteroduplex DNA adjacent to regions of double-strand break repair. There is genetic evidence for the occurrence of gene conversion events that are mediated by heteroduplex DNA during mitotic and meiotic recombination, if not for its role as an obligatory intermediate in gene conversion (for reviews, see references 12, 20, and 25). The existence of heteroduplex DNA in yeasts and other fungi is inferred from the occurrence of postmeiotic segregation. Postmeiotic segregation is quite rare in yeast cells for most mutations (4); however, mutations in the *PMS1* gene result in an increased frequency of postmeiotic segregation and a reduced frequency of gene conversion (6:2 segregation), and a mitotic mutator phenotype (26). Bishop and co-workers (2, 3) have shown that mitotic mismatch correction is defective in *pms1* strains by examining mismatch correction of heteroduplex DNA introduced into mitotic yeast cells by transformation. These results suggest that at least some meiotic gene conversion events in *S. cerevisiae* proceed through a heteroduplex intermediate.

Our previous observation that mutant chromosomal *his3* alleles could be replaced by wild-type information from a plasmid during gap repair suggested the existence of heteroduplex DNA (11). However, in these experiments, we could not measure either the extent or frequency of heteroduplex formation. Following transformation of yeast cells with linear DNA fragments, Rothstein (18) observed colonies that were sectored for markers on the fragment. These sectored colonies, described as postdivision segregations, are most simply explained as the consequence of replication of a heteroduplex intermediate formed adjacent to a double-stranded end (18). Heteroduplex DNA is also associated with mitotic recombination between direct repeats on chromosomal DNA (17). In this report we describe the use of restriction site mutations in the *his3* gene to select for and analyze gene conversion events adjacent to a region of double-strand break repair in the yeast *S. cerevisiae*. The mutations were arranged on the plasmid and the chromosome so that conversion events flanking a region of gap repair could be distinguished from events caused by gap repair itself. These experiments indicated that mutations adjacent to, but outside of, a region of double-strand gap repair can undergo gene conversion, presumably through a heteroduplex intermediate.

MATERIALS AND METHODS

Strains. *S. cerevisiae* J19 (α *can1 his3*$^-$ *Kpn*I$^-$ *leu2-3,112*) is a substitution transformant of strain T415 (α, *can1 leu2-3,112*), in which the *his3*$^-$ *Kpn*I$^-$ mutation from plasmid pSZ513 was transplaced into the *HIS3* gene (19). Strain AN1 (α *can1 his3*$^-$ *Hin*dIII$^-$ *leu2-3,112*) is a similar derivative of TA1191 (α *can1 leu2-3,112*) in which the *his3*$^-$ *Hin*dIII$^-$ mutation from plasmid pSZ537 was transplaced into the *HIS3* gene. These constructions were verified by Southern blot restriction mapping of genomic DNA.

Most plasmids were maintained in *Escherichia coli* JA300 (*thr-1 leuB6 thi-1 thyA trpC1117 hsr*$_k$ *hsm*$_k$ Str^r), which was obtained from J. Carbon (24). Strain BA1 (a *hisB* derivative

* Corresponding author.
† Present address: Whitehead Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142.
‡ Present address: Laboratoire de Génétique, Université Paris-Sud, Centre d'Orsay, 91405F, Orsay, France.

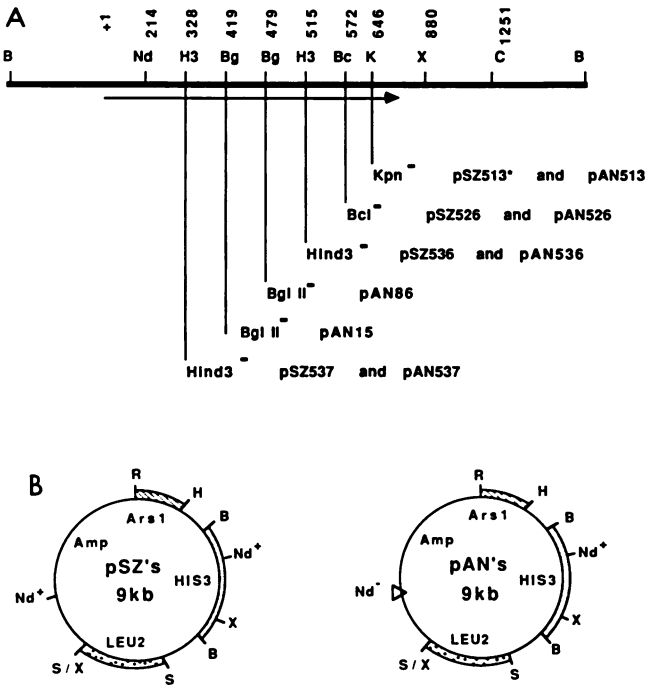

FIG. 1. (A) Restriction endonuclease cleavage map of the 1.7-kb *Bam*HI DNA fragment containing the entire *HIS3* gene. The nucleotide coordinates are relative to the site of *HIS3* transcriptional initiation, which was defined as position +1 (22). Below the restriction map are indicated the various *his3* mutations and the corresponding plasmids that were used. (B) Restriction maps of plasmids. The pSZ and pAN series of plasmids contained the 1.7-kb *Bam*HI *HIS3* fragment in either the wild-type form (pSZ528 and pAN528) or carried one of the mutations indicated above. In all of them, a 2.2-kb *LEU2* fragment was inserted at the *Sal*I site of pBR322. All plasmids except pSZ513 had the 0.7-kb *ARS1* (autonomously replicative sequence) between the *Eco*RI and *Hin*dIII sites of pBR322. The *Nde*I site of pBR322 was destroyed in the pAN series of plasmids. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; H or H3, *Hin*dIII; K, *Kpn*I; Nd, *Nde*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I. Symbols: Stippled, *HIS3*; dots, *LEU2*; stripes, *ARS1*; solid line, pBR322.

of JA300) was constructed by Andrew Murray and was used in the construction of some of the *his3* mutations. Plasmid pSZ526 was constructed in the *dam*⁻ strain RK1007 (*dam-3 dcm-6 thr-1 thi-1 leuB6 lacY1 galK2 galT22 ara-14 tonA31 tsx-18 supE44*).

Plasmid and mutation construction. The restriction endonuclease cleavage map of the *HIS3* gene is diagrammed in Fig. 1. Plasmids pSZ513 and pAN513 carried a deletion of the unique *Kpn*I site in the *HIS3* gene. This mutation was produced initially in pSZ62, a pBR322 derivative containing a 1.7-kilobase-pair (kb) *Bam*HI fragment that carries the yeast *HIS3* gene (13). pSZ62 DNA was cleaved with *Kpn*I, the overhanging ends were degraded with T4 DNA polymerase in the presence of 100 μM deoxynucleoside triphosphates, and the blunt ends were ligated together with T4 ligase. A 2.1-kb *Sal*I-*Xho*I fragment containing the yeast *LEU2* gene was inserted into the unique *Sal*I site to generate pSZ513. The *Bam*HI fragment carrying the mutated *his3-Kpn*I gene was inserted into the *Bam*HI site of the *ARS1 LEU2* plasmid pAN93 to generate pAN513. pAN93 was a derivative of pSZ93 (14) in which the unique *Nde*I site was destroyed. DNA of pSZ93 was cleaved with *Nde*I, and the 5′ overhanging ends were filled in with T4 DNA polymerase. The DNA was blunt-end ligated and then used to transform *E. coli* JA300.

Plasmids pSZ526 and pAN526 contained a *his3* gene in which the unique *Bcl*I site was changed to a *Cla*I site. The *Bcl*I mutation was made on pSZ62 DNA that was grown in the *dam*⁻ strain RK1007. The plasmid was cleaved at the *Bcl*I site, and the 5′ overhanging ends were filled in with T4 DNA polymerase. The DNA was blunt-end ligated and used to transform RK1007. The transformants were screened for plasmids carrying the extra *Cla*I site. The *Bam*HI fragment carrying the mutated *his3* gene was inserted into the *Bam*HI site of pSZ93 to generate pSZ526. pAN526 was generated by insertion of the 2.5-kb *Stu*I-*Sph*I fragment containing the *his3-Bcl*I mutation of pSZ526 into pAN93 digested by the same restriction enzymes.

Insertion mutations were also generated at each of the two *Hin*dIII sites in the *HIS3* gene. Plasmid pSZ528, a plasmid containing *HIS3*, *LEU2*, and *ARS1*, was partially digested with *Hin*dIII. The DNA was treated with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates to fill in the 5′ overhanging ends. After blunt-end ligation, the DNA was used to transform *E. coli* BA1 (*hisB*). Since the yeast *HIS3* gene complements the *hisB* mutation, we screened for *his*⁻ transformants. In pSZ536, the *Hin*dIII site closest to the *Kpn*I site was mutated, and in pSZ537 the site farther from the *Kpn*I site was mutated. The 2.5-kb *Stu*I-*Sph*I fragment containing the *his3 Hin*dIII mutations in pSZ536 and pSZ537 was inserted into the *Stu*I-*Sph*I digested pAN93 plasmid to generate pAN536 and pAN537, respectively.

Insertion mutations were also generated at each of the two *Bgl*II sites in the *HIS3* gene. The *ARS1 HIS3 LEU2* pAN528 plasmid was generated by insertion of the 1.8-kb *Bam*HI- and *HIS3*-containing fragment of pSZ528 into pAN93. Plasmid pAN528 was partially digested with *Bgl*II, the 5′ overhanging ends were filled in, and the DNA was blunt-end ligated. In plasmid pAN86, the *Bgl*II closest to the *Kpn*I site was mutated, and in pAN15 the site farther from the *Kpn*I site was mutated.

Yeast transformation. CsCl plasmid DNA preparations, restriction digestion of plasmid DNA, and protoplast yeast transformations were carried out as described previously (14). LiAc yeast transformations were performed as described previously (7). *Xho*I experiment 1 was done with spheroplasts. All others were done with LiAc. Carrier DNA was not used in any of these yeast transformations. To minimize the variability in transformation efficiency in each set of experiments, all the individual reactions involving the various plasmids were performed in parallel. Equal amounts of DNA (about 10 μg for each datum point) were mixed with equal numbers of the same competent cells. Southern blot analysis of yeast transformants was carried out as described previously (14).

RESULTS

Experimental design. We designed a system that enabled us to select for conversion events in DNA flanking a region of double-strand break or gap repair (Fig. 2A and B). Recombination between the plasmid and the chromosome was initiated by a double-strand break in the plasmid that was made by restriction enzyme digestion at a unique site. The chromosomal copy of the gene was genetically and physically marked with a restriction site mutation. The plasmid copy of the *his3* gene was also genetically and physically marked by a restriction site mutation at a point

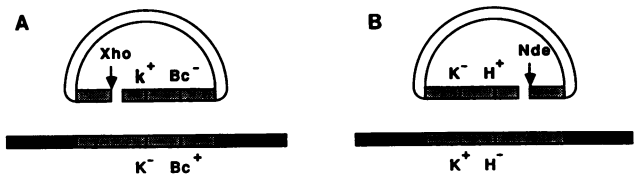

FIG. 2. (A) Experimental design. The recipient strain contained a mutated *Kpn*I site in the *his3* gene. This strain was transformed with a plasmid containing a 4-base-pair insertion in the *Bcl*I site in the *his3* gene that generated a new *Cla*I site. The plasmid was linearized at the *Xho*I site so that the chromosomal *Kpn*I mutation lies between the double-strand break and the plasmid *Bcl*I mutation. A *His*$^+$ transformant was produced only if the wild-type *Kpn*I site on the plasmid was maintained, i.e., only if the gap produced by degradation from the *Xho*I ends did not extend beyond the *Kpn*I site. This figure corresponds to the configuration of experiments with *Xho*I 1, 2, and 3 (Table 1) involving transformation with plasmids pSZ526 and pAN526. (B) Same experimental design as for panel A, but the plasmid was linearized on the other side of the *HIS3* gene. The recipient strain contained a mutated *Hin*dIII site. The various *his3*$^-$ plasmids were linearized at the unique *Nde*I site of the *HIS3* fragment. The plasmid contained the *Kpn*I site mutation in the transformation with pAN513 in experiments *Nde*I 1 and 2 (Table 1). Abbreviations: Bc, *Bcl*I; H, *Hin*dIII; K, *Kpn*I; Nde, *Nde*I; Xho, *Xho*I. Symbols: Solid line, flanking chromosomal DNA; open line, plasmid DNA; stippled line, *HIS3* gene; arrow, cut site.

farther from the site of plasmid cutting than the chromosomal mutation.

This arrangement of plasmid and chromosomal mutations relative to the plasmid-cut site could not yield a His$^+$ transformant by simple gap repair. We have previously shown (11) that when a linear or gapped plasmid is introduced into yeast by transformation, the ends can be degraded and the gap can be enlarged. If the gap is enlarged to the extent that it covers the chromosomal mutation, only *his*$^-$ transformants will be recovered.

His$^+$ transformants could arise by two possible mechanisms. First, plasmid information could be transferred to the chromosome in a substitution type of event, removing the chromosomal mutation and leading to a His$^+$ chromosomal gene. Second, chromosomal information could be transferred to the plasmid, removing the plasmid mutation and leading to a His$^+$ plasmid. We describe the characterization of such His$^+$ recombinants and show that they arise by both pathways.

Gene conversion flanking a double-strand break. The plasmid pSZ526 used in the first set of transformation experiments contains a *Bcl*I$^-$ mutation in the *his3* gene, the *LEU2*$^+$ gene, and *ARS1* (Fig. 1). Plasmid DNA was linearized by digestion with the restriction enzyme *Xho*I, used to transform the *his3*$^-$ *Kpn*I$^-$ recipient strain J19, and His$^+$ transformants were obtained. The fraction of recombination events that produced a wild-type *HIS3* gene was measured by plating the transformation mixture either for Leu$^+$ transformants or for His$^+$ transformants. By using the linearized plasmid, His$^+$ transformants were detected at 0.5% of the frequency of Leu$^+$ transformants. In contrast, in this experiment we were unable to obtain His$^+$ transformants with uncut, circular pSZ526 DNA, even when we used amounts of DNA that would yield 10^5 Leu$^+$ transformants. Since the position of the mutations and the double-strand break precluded our obtaining His$^+$ transformants by simple gap repair, gene conversion can occur adjacent to a region of double-strand break (or gap) repair. In this experiment we failed to recover His$^+$ transformants using uncut plasmid DNA, confirming that these conversion events are associated with double-strand break repair. His$^+$ transformants were not obtained when a plasmid with the same mutation as the chromosomal mutation was used.

Transformants that were simultaneously selected to be His$^+$ and Leu$^+$ were characterized genetically by analyzing the mitotic stability of the *HIS3* and *LEU2* markers. The markers that are present on extrachromosomal, replicating plasmids are mitotically unstable in yeast cells (21). The stability of the transformants was determined by streaking them to single colonies on nonselective medium and then replica-plating them on selective medium. Conversion of the plasmid mutation to the wild type without an associated crossover (i.e., without plasmid integration) would produce a transformant that was unstable for both the His$^+$ and the Leu$^+$ markers. Conversion of either the plasmid or the chromosomal mutations accompanied by crossing over (plasmid integration) would yield a transformant that was stable for both His$^+$ and Leu$^+$. Conversion of the chromosomal mutation without plasmid integration would yield a stable His$^+$, unstable Leu$^+$ phenotype, while conversion of the plasmid mutation accompanied by the integration of a second unrelated plasmid molecule would yield an unstable His$^+$, stable Leu$^+$ transformant. All four classes of His$^+$ transformants were obtained (Table 1), indicating that conversion of both the plasmid and the chromosomal mutations occurred.

Both classes of unstable His$^+$ transformants were caused by the conversion of the plasmid mutation, while conversion of the chromosomal mutation without plasmid integration produced the stable His$^+$, unstable Leu$^+$ class. These transformants represent the simplest and most easily interpreted events. However, the most abundant class of transformants was the stable His$^+$, stable Leu$^+$ class, which arose by integration of the plasmid.

When transformants were selected for Leu$^+$ only, both stable and unstable transformants were obtained. His$^+$ colonies were present in each class. Three classes of stable Leu$^+$ transformants were obtained: stable His$^+$ (17 of 180), *his*$^-$ but able to papillate to His$^+$ (113 of 180), and stable *his*$^-$ (50 of 180). His$^+$ papillae arose from secondary recombination events between two integrated copies of the *his3* gene that carried different mutations. Therefore, a papillation-positive phenotype corresponds to plasmid integration without gene conversion of either mutation. A papillation-negative phenotype indicates that the homologous copies of *his3* shared at least one common mutation. This could result from gene conversion of either mutation to the homozygous state, for example, by the repair of an enlarged double-strand gap.

Physical characterization of conversion events. Since the mutations that we used altered the restriction sites, it was possible to confirm and extend the genetic analysis of the transformants by physical analysis of their DNAs. The class of unstable His$^+$, unstable Leu$^+$ transformants was shown by Southern blot analysis (Fig. 3) to result from conversion of the plasmid mutation to the wild type. By using a probe containing both pBR322 and *HIS3* sequences, we were able to determine the structure of both the plasmid and chromosomal copies of the *HIS3* genes. We determined the structure of the plasmid and chromosomal *HIS3* genes for 15 unstable His$^+$, unstable Leu$^+$ transformants. The Southern blot data for four of these are shown in Fig. 3. In all of the transformants analyzed, the plasmid contained the wild-type *HIS3 Kpn*I site, while the chromosomal gene retained the mutation. Both the plasmid and the chromosomal gene contained the wild-type *Bcl*I site. Thus, this class of trans-

TABLE 1. Effect of distance on gene conversion

Restriction enzyme, expt no., and plasmid[a]	Plasmid mutation	Distance (bp) between chromosomal and plasmid mutation	No. (%) of transformants[b]			
			HIS^{+s} *LEU*$^{+s}$	*HIS*$^{+u}$ *LEU*$^{+u}$	*HIS*$^{+s}$ *LEU*$^{+u}$	*HIS*$^{+u}$ *LEU*$^{+s}$
*Xho*I, expt 1						
pSZ526	*Bcl*I$^-$	74	135	33 (19)	3	2
pSZ536	*Hin*dIII$^-$	131	186	16 (8)	6	3
pSZ537	*Hin*dIII$^-$	318	236	13 (5)	13	0
*Xho*I, expt 2						
pAN526	*Bcl*I$^-$	74	9	4 (31)	0	0
pAN536	*Hin*dIII$^-$	131	10	8 (38)	2	1
pAN86	*Bgl*II$^-$	167	129	32 (19)	4	2
pAN15	*Bgl*II$^-$	227	96	18 (15)	3	2
pAN537	*Hin*dIII$^-$	318	87	7 (7)	1	0
*Xho*I, expt 3						
pAN526	*Bcl*I$^-$	74	129	80 (36)	8	7
pAN536	*Hin*dIII$^-$	131	122	79 (39)	3	1
pAN86	*Bgl*II$^-$	167	180	39 (17)	3	1
pAN15	*Bgl*II$^-$	227	158	54 (24)	8	2
pAN537	*Hin*dIII$^-$	318	171	35 (16)	9	1
*Nde*I, expt 1						
pAN15	*Bgl*II$^-$	91	128	56 (30)	1	0
pAN86	*Bgl*II$^-$	151	71	22 (23)	3	0
pAN536	*Hin*dIII$^-$	187	166	38 (20)	2	2
pAN526	*Bcl*I$^-$	244	18	2 (10)	0	1
pAN513	*Kpn*I$^-$	318	160	25 (13)	4	1
*Nde*I, expt 2						
pAN15	*Bgl*II$^-$	91	36	26 (41)	0	2
pAN86	*Bgl*II$^-$	151	64	22 (25)	2	1
pAN536	*Hin*dIII$^-$	187	59	29 (32)	2	0
pAN526	*Bcl*I$^-$	244	120	42 (25)	3	0
pAN513	*Kpn*I$^-$	318	115	36 (23)	5	2

[a] *Xho*I and *Nde*I indicate the restriction sites used to linearize the plasmid. The number designates the individual experiment. The first experiment with *Xho*I was done by spheroplast formation. All others were done by LiAc transformation.
[b] s, Stable transformant; u, unstable transformant.

formant most likely results from a single recombination event: the conversion of the plasmid mutation without an associated crossover. We did not observe the transfer of the plasmid mutation to the chromosome in any of these transformants.

The types of events proposed to explain each of the other classes of transformants were also confirmed by Southern blot restriction mapping. In the two stable His$^+$, unstable Leu$^+$ transformants studied, the plasmid retained its original mutational structure, while the chromosomal *HIS3* gene had wild-type information at both the *Kpn*I and *Bcl*I sites.

Seven of eight stable His$^+$, stable Leu$^+$ transformants analyzed contained multiple, tandemly integrated copies of the plasmid (data not shown). All contained at least one wild-type *HIS3* gene. The simplest way that a wild-type *HIS3* gene could have arisen was through conversion of the chromosomal allele. The remaining *his3* mutant genes were of the original chromosomal genotype, the plasmid genotype, or the double mutant and could have arisen through a variety of mechanisms.

Southern blot restriction mapping of eight of the stable Leu$^+$, papillation-negative transformants confirmed that they were unable to produce His$^+$ recombinants because all of the *his3* genes contained at least one common mutation. Six of the eight transformants contained a single integrated copy of the plasmid; two others had multiple copies. In all of the transformants except one, all of the copies of the *his3* gene were in the original mutant chromosomal configuration, which is consistent with the transfer of both chromosomal alleles to the plasmid by repair of an enlarged gap. The exception had a single integrated copy of the plasmid, with one *his3* gene with the original chromosomal allele and the other having both the chromosomal *Kpn*I mutation and the plasmid *Bcl*I mutation. The two papillation-positive transformants analyzed contained copies of the *his3* gene with different mutations. One of these had multiple copies of the integrated plasmid.

Distance effect on conversion frequency. While the results described above establish that gene conversion of a marker can occur in DNA flanking a region of double-strand break repair, it is difficult, based on this result alone, to exclude the possibility that the His$^+$ recombinants were derived from multiple recombination events involving several plasmid molecules instead of repair of heteroduplex DNA initiated at one edge of the double-strand break (double-strand break repair model). We studied the effect of increasing distance between the double-strand break and the plasmid mutation to distinguish between these alternatives. Recombination frequencies increase the increasing distance between two markers. Thus, a multiple exchange model would predict that the number of recombinants should increase with distance. However, that double-strand break repair model predicts that the frequency of conversion of a marker decreases with increasing distance between the double-

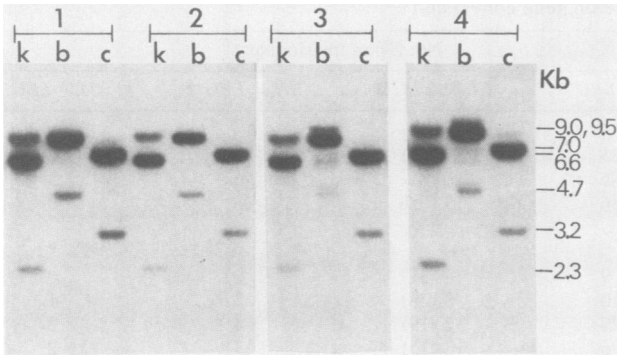

FIG. 3. Structure of unstable His^+, unstable Leu^+ transformants. Southern blots are shown for four unstable transformants of strain J19 with *Xho*I-cut pSZ526. The DNA from each transformant was digested with *Kpn*I, *Bcl*I, and *Cla*I; and Southern blots were probed with the pBR322 *HIS3* plasmid pSZ63 (14). If the wild-type *Kpn*I site in the *HIS3* gene was present on the plasmid, two fragments of 6.6 and 2.3 kb would be present on the Southern blot. If the mutation that destroys the *Kpn*I site was present in the chromosomal gene, a *Kpn*I digest would yield a 9.5-kb fragment, rather than the two wild-type fragments of 3.5 and 6.0 kb. In the four transformants shown, the *Kpn*I digest gave a chromosomal fusion fragment of 9.5 kb and two plasmid fragments of 6.6 and 2.3 kb. Thus, the plasmid had the wild-type *Kpn*I site and the chromosome had the mutated site. The presence of the single copy of the *Bcl*I site on the plasmid, which was expected from a $HIS3^+$ gene, would yield a single 9.0-kb fragment after *Bcl*I digestion. A wild-type *Bcl*I site produced a 4.7-kb chromosomal fragment; this was replaced by a 6.4-kb fragment if the *Bcl*I site was missing. In all four transformants, a 4.7-kb *Bcl*I band was present in the chromosome. In addition, the plasmid had the *Bcl*I site because a linear plasmid fragment was generated by *Bcl*I digestion. Since the *Bcl*I mutation generated a *Cla*I site, the status of the *Bcl*I mutation was confirmed by a *Cla*I digest. The chromosomal *Cla*I fragment was 3.2 kb, or 2.4 kb if the extra site was present. A 3.2-kb chromosomal fragment was produced and therefore the mutant *Cla*I site was absent. Abbreviations: b, *Bcl*I digest; c, *Cla*I digest; k, *Kpn*I digest. Fragment sizes are labeled on the side.

strand break and the marker. The effect of distance on conversion of the marker on the plasmid was studied by analyzing the unstable His^+, Leu^+ transformants, because these arose by conversion of the plasmid marker.

To test the effect of distance on conversion frequency, we constructed four other *his3* mutations by filling in the overhanging ends of the two *Hin*dIII and the two *Bgl*II sites located in the coding region of the *HIS3* gene (Fig. 1). All of these mutations were 4-base-pair insertions, thus minimizing the possibility of marker effects. The plasmids containing these mutations also contained the *LEU2* gene and *ARS1* (Fig. 2). The results presented in Table 1 correspond to three independent experiments in which we transformed the $his3^-$ $Kpn I^-$ yeast strain J19 with the various $his3^-$ plasmids either as uncut circular DNA or after linearization with *Xho*I, selected for His^+ Leu^+ transformants, and determined the mitotic stability of the transformants for the *HIS3* and *LEU2* markers.

In experiment 1 with *Xho*I linearization, J19 cells were transformed with plasmids pSZ526, pSZ536, and pSZ537. We obtained His^+ Leu^+ transformants only with linearized DNA. To compare the frequency of conversion of the marker on the plasmid between the three different plasmids, we compared the percentage of the class of unstable His^+, unstable Leu^+ transformants of the total His^+ Leu^+ transformants. The class of unstable His^+, unstable Leu^+ transformants, which reflects conversion of the plasmid mutation, decreased significantly from 19% of His^+ transformants for pSZ526 to 8 and 5% of pSZ536 and pSZ537, respectively. Thus, plasmid mutations farther from the position of the double-strand break are converted at a lower frequency.

The decrease in the percentage of unstable His^+, unstable Leu^+ transformants could, in theory, be a consequence of an increase in the number of stable His^+, stable Leu^+ transformants. Since these were the majority of transformants, such an increase would have been reflected by an overall increase in the percentage of His^+ Leu^+ transformants relative to the total number of Leu^+ transformants. However, the number of His^+ Leu^+ transformants did not increase with increasing distance between the double-strand break and the plasmid mutation. In this experiment a 10-fold dilution of the transformation mix was plated, selecting for Leu^+ transformants, and an undiluted portion of the same volume was plated to select for His^+ Leu^+ transformants. When corrected for the dilution, the percentages of His^+ Leu^+ transformants relative to the total number of Leu^+ transformants for pSZ526, pSZ536, and pSZ537 were 0.53% [219 His^+/(4,156 × 10 Leu^+)], 0.29% [81 His^+/(2,770 × 10 Leu^+)], and 0.37% [128 His^+/(3,464 × 10 Leu^+)], respectively. While a chi-square contingency test showed that these values were not identical, the decrease in conversion frequency for the plasmid mutation was not merely a reflection of an increase in the number of stable His^+ Leu^+ transformants. This class also showed a slight decrease in frequency.

In experiments 2 and 3 with *Xho*I, we examined more plasmid mutations in a similar protocol. J19 yeast cells were transformed with the pAN series of plasmids that were either uncut or cut with *Xho*I. His^+ Leu^+ transformants were selected. As described above, transformants were obtained almost exclusively with linearized plasmids (His^+ Leu^+ transformants from uncut plasmids were obtained at less than 1% of the frequency as they were from cut plasmids). As in experiment 1 with *Xho*I, we observed a distance effect on the fraction of unstable Leu^+, unstable His^+ transformants (Table 1). In all experiments, the mutations closest to the double-strand break were converted at a significantly higher frequency than the more distant mutations ($P < 0.05$ by the G test), although the pattern of decrease was quite variable from experiment to experiment. These results indicate that gene conversion can occur in a region adjacent to a region of double-strand break repair and that the frequency of these conversion events decreases with distance from the site of the break. This last observation weakens a model proposing multiple events. It is possible that the decrease in conversion frequencies is caused by marker effects, although this is unlikely since several markers were tested.

To eliminate the possibility of marker effects, we performed an additional set of experiments in which we introduced the initiating double-strand break on the other side of the same set of *his* plasmid mutations used above. In our experimental design the double-strand break repair model predicted a reversed polarity, whereas marker effects would show no difference in polarity. To do this experiment, we used the 5′-most *Hin*dIII mutation in place of the *Kpn*I mutation as the chromosomal allele defining the edge of the possible gap repair region (Fig. 2B). The AN1 yeast strain was then transformed with the pAN series of plasmids that were either uncut or linearized at the unique *Nde*I site located 114-base-pair 5′ of the *Hin*dIII mutation. We performed two independent experiments with *Nde*I in which we selected Leu^+ His^+ transformants and determined their mitotic stabilities (Table 1). Such transformants were ob-

tained almost exclusively with linearized plasmids. The percentage of His^+ Leu^+ transformants relative to that of Leu^+ transformants was determined by plating a 20-fold dilution of the transformation mix on plates lacking leucine and plating the same volume of the undiluted mix on plates lacking both leucine and histidine. Correcting for the 20-fold dilution on the Leu^+ plate, the percentage of His^+ Leu^+ transformants relative to Leu^+ were 0.80% [32 His^+/(198 × 20 Leu^+)] for pAN15, 0.37% [28 His^+/(381 × 20 Leu^+)] for pAN86, 0.61% [35 His^+/(285 × 20 Leu^+)] for pAN536, 0.85% [82 His^+/(483 × 20 Leu^+)] for pAN526, and 0.79% [82 His^+/(521 × 20 Leu^+)] for pAN513. A chi-square contingency test showed that only pAN86 gave a different number of His^+ Leu^+ transformants than pAN15 ($P < 0.01$). This allowed us to compare (with the exception of pAN86, which decreased) the frequency of conversion of the plasmid marker by comparing the percentage of unstable His^+, unstable Leu^+ transformants for each plasmid.

As in the previous experiments, the mutations closest to the site of the double-strand break tended to show conversion at a higher frequency than did the mutations that were farther away. Thus, the polarity was reversed. Mutations that converted at high frequencies in one case were converted at lower frequencies in the other. Conversion frequencies were a consequence of distance from the double-strand break and were not a property of the marker itself.

DISCUSSION

We detected gene conversion events in DNA adjacent to but outside of a region of double-strand break repair in *S. cerevisiae*. The recombination events we studied were between homologous regions containing the *his3* gene on a plasmid and a chromosome. The events were initiated by a double-strand break in the plasmid, which was introduced by restriction enzyme cleavage. Such breaks are enlarged to gaps by DNA degradation during transformation, and the gaps are repaired by recombination with the intact chromosomal sequence (11). In order to detect gene conversion events occurring in DNA flanking the region of gap repair, we marked both the chromosomal and plasmid genes with mutations made by altering restriction sites (Fig. 1). *HIS3*$^+$ recombinants could be generated only by conversion events adjacent to the region of gap repair. If the gap extended beyond the site of the chromosomal mutation, both the plasmid and the chromosome would carry that mutation after repair.

The observation that *HIS3*$^+$ recombinants were readily obtained from linearized plasmid DNA, but only rarely from uncut circular DNA, strongly supports the idea that the recombinants occur as a result of the process of gap repair. The recombinants were analyzed both genetically and by restriction enzyme mapping to determine their structures and fell into several classes corresponding to conversion of the chromosomal or plasmid mutations, with or without associated crossing over and plasmid integration. To test the hypothesis that these conversion events arose from recombination intermediates (presumably, heteroduplex DNA) that were formed adjacent to the region of gap repair and not from multiple recombination events, we tested the effect of varying the distance between the chromosomal and plasmid mutations. We expected that plasmid mutations farther from the edge of the double-strand gap would be less likely to fall within the presumptive heteroduplex DNA adjacent to the gap, and would therefore be less likely to be converted. We observed a decrease in conversion of the plasmid mutation as a function of distance, which was reversed when the break was made on the other side of the set of mutations.

We measured the frequency of conversion of the marker on the plasmid by comparing the percentage of the unstable His^+, unstable Leu^+ transformants out of the total His^+ Leu^+ transformants. Therefore, the possibility exists that the observed decrease in the percentage of unstable His^+, unstable Leu^+ transformants with increasing distance between the double-strand break and the plasmid mutation actually reflects an increase in the stable class of transformants. Since this class was the majority, such an increase would increase the total frequency of His^+ Leu^+ transformants. We did not observe such an increase. Thus, the decrease in the frequency of unstable His^+, unstable Leu^+ transformants reflected a decrease in the conversion of the plasmid marker. However, we saw some variation in that the percentage of His^+ transformants obtained with different plasmids was not always identical. Consequently, it is possible that there are other parameters affecting the various classes of transformants that are measurable in this experimental system. For example, the frequency of crossing over may vary with conversion tract length (1), altering the ratio of unstable to stable His^+ transformants.

The simplest explanation for the class of unstable His^+ transformants is that heteroduplex DNA extended from one edge of the double-strand gap through both the chromosomal and the plasmid alleles. Independent mismatch repair of the two alleles would result in a *HIS3*$^+$ gene. Since we had no markers on the other side of the double-strand gap, we could not determine whether, as proposed in the double-strand break repair model, there was heteroduplex DNA on both sides of the gap.

The heteroduplex DNA that formed adjacent to a region of gap repair appeared to be largely asymmetric in our experiments. Asymmetric heteroduplex DNA could be formed by the initial pairing of an overhanging plasmid end with chromosomal DNA and could be extended by branch migration, perhaps driven by replication as in the Meselson-Radding model (10, 13, 23). Symmetric heteroduplex DNA could be generated by branch migration of the two Holliday junctions postulated to flank the region of gap repair (23). We examined the nature of the heteroduplex DNA by determining the structure of the chromosomal *his3* gene in cells in which the plasmid was converted to *HIS3*$^+$. Because the chromosomal gene had to have been the source of the wild-type information that was transferred to the plasmid, if the heteroduplex DNA was symmetric, the plasmid mutation should have been transferred to the chromosome in 50% of the events (assuming random mismatch repair). No such events occurred among the 15 transformants studied. Thus, extensive symmetric heteroduplex DNA does not appear to form flanking a region of double-strand gap repair in the plasmid transformation system. While this is in contrast to chromosomal mitotic gene conversion in which symmetric heteroduplex DNA has been reported to be frequently present (5, 16), the results of Ahn and Livingston (1) are also consistent with a preponderance of asymmetric relative to symmetric heteroduplex DNA in mitotic plasmid recombination.

We have proposed that meiotic recombination in *S. cerevisiae* is initiated by double-strand breaks and that at least some meiotic gene conversion in *S. cerevisiae* results from double-strand gap repair. One prediction of this model was that the repair of double-strand breaks and gaps should generate sufficient heteroduplex DNA to explain the levels of postmeiotic segregation observed in *S. cerevisiae*. The experiments discussed here suggest that regions of asymmet-

ric heteroduplex DNA extending over several hundred base pairs are formed flanking regions of double-strand gap repair in mitotic cells. If double-strand gap repair is involved in meiotic recombination, it would therefore not be surprising if such events were also associated with heteroduplex DNA.

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