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Genetics

Recombinational substrates designed to study recombination between unique and repetitive sequences *in vivo*

(sister-chromatid recombination/reciprocal recombination/gene conversion/*Saccharomyces cerevisiae*)

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ABSTRACT **Three recombination events, reciprocal recombination, sister-chromatid recombination, and gene conversion, were studied using substrates designed *in vitro*. Each type of recombination event can be monitored at any chromosomal location. We have shown that sister-chromatid recombination is induced mitotically by DNA damaging agents, such as methyl methanesulfonate and γ-rays, but is decreased mitotically in strains defective in *rad52*. Reciprocal recombination by which circular plasmids integrate into the genome is unaffected by *rad52* defective alleles and occurs by a different recombination pathway. Mechanisms are suggested by which gene conversion between sister chromatids can generate chromosome rearrangements.**

Homologous recombination between eukaryotic repeated DNA (nonallelic) sequences generates gene conversion events, reciprocal translocations, tandem duplications, and deletions. Nonalleles include members of gene families, such as immunoglobulin and histone genes, that share homology but not chromosomal location. Recombination between tandem or dispersed nonalleles is termed ectopic (1) or nonallelic recombination. Genetic diseases may result from the consequences of such ectopic (nonallelic) recombination, and some are characterized by an elevated level of somatic recombination. Familial hypercholesterolemia is correlated with a deletion event that occurred by homologous recombination between *Alu* sequences in the low density lipoprotein receptor (2). Bloom syndrome is characterized by an elevated frequency of mitotic sister-chromatid (SC) recombination (3). However, little is understood concerning the different mechanisms of mitotic and meiotic ectopic recombination between repeated nonallelic sequences on SCs, nonhomologous chromosomes, or homologous chromosomes.

Saccharomyces cerevisiae is a useful organism in which to study how recombination is initiated and how recombination is resolved between repeated sequences on SCs, or on the same or different chromosomes. Recombination between two heteroalleles (homologous fragments) of a known structural gene, such as *HIS3* (4) and *HIS4* (5), can provide models for ectopic recombination. These homologous fragments can be placed at different locations on the same chromosome or on nonhomologous chromosomes by DNA transformation.

The type of recombinant that arises from this selection depends on the nature of the recombination substrates and their genomic position (4, 6). When these recombination substrates contain point mutations or internal deletions, the recombinants that arise include gene conversion events (not associated with rearrangements) and sometimes rearrangements. For example, when *his3* alleles are on the same chromosome in inverted orientation with respect to each other, primarily gene conversion events arise (6). When *his3* alleles are on different chromosomes, both gene conversion events and translocation events arise (4). However, specific rare rearrangements may not be detected when screened from a background of such events.

To investigate whether repair mutants, mutagens, and carcinogens affect specific types of interactions between repeated sequences, a set of recombination substrates (gene fragments) were designed that can be integrated into any *S. cerevisiae* chromosomal locus. These recombination substrates were then used to select for prototrophic revertants that are due to specific recombination events, excluding others. Specific recombination events studied include interchromosomal reciprocal exchange, gene conversion, and SC recombination.

MATERIALS AND METHODS

Constructing Yeast Strains and Plasmids. The yeast strains we have used in this study are listed in Table 1. The plasmids YIp5 and YRp17 have been described (7). YIp5 *his3*-Δ3′, *his3*-Δ5′ was constructed by separately subcloning the 800-base-pair *Bam*HI–*Bgl* II *his3* fragment and the 1.4-kilobase (kb) *Bam*HI *his3*-Δ5′ (*his3*-Δ2619) (8) into YIp5. YIp5 *his3*-Δ3′ was constructed by deleting a *Bam*HI–*Bgl* II fragment from YIp5Sc3103, a plasmid originally constructed by Kevin Struhl (28). YIp5 *his3*-Δ(5′,3′) was constructed by subcloning a *Bam*HI–*Kpn* I fragment of *his3*-Δ2639 (8) into YIp5. Additional plasmids were made by subcloning the appropriate *Eco*RI restriction fragment into these plasmids. Restriction digests, DNA ligations, and bacterial transformations were done according to published procedures (9).

Yeast strains were constructed by transformation of the appropriate plasmids. All yeast strains used to monitor sister-chromatid recombination or reciprocal exchange contained *his3*-Δ200. Plasmids were targeted to homologous sites in the genome by restriction endonuclease digestion at the appropriate site in the plasmids.

One-step gene disruption (10) of *RAD52* with *TRP1* to generate *rad52*-8 was done by transformation with a *Bam*HI fragment in which *TRP1* (but not *ARS1*) had been inserted (11).

Determining the Presence and Linkage of *his3* Alleles. To detect the *his3* fragments, DNA from His^+ revertants was digested with *Eco*RI and *Bam*HI endonucleases and probed with a 1.4-kb fragment that contains homology to all three *his3* alleles. To determine the linkage of the *his3* fragments, DNA from the His^+ revertants was digested with *Eco*RI and *Sal* I endonucleases (sites that flank *his3*-Δ3′ and *his3*-Δ5′, respectively). A 4.3-kb fragment was obtained instead of the 2.6-kb fragment found in the His^- parent. This larger fragment is expected if $HIS3^+$ is flanked by the *his3* fragments.

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Abbreviations: SC, sister chromatid; ARS, autonomously replicating sequence.

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Table 1. Relevant yeast and plasmid strains

Strain (synonym)	Relevant genotype
Yeast strains	
YNN217	a *his3*-Δ200, *ura3*-52, *lys2*-801, *ade2*-101
YNN285	α *his3*-Δ200, *his3*-Δ5′ at *GAL1*, *ura3*-52, *trp1*-289, *ade2*-101
YNN282 (CMY135)	α *his3*-Δ200, *trp1*-Δ1, *lys2*-801, *ade2*-101, *ura3*-52
YNN305 (CMY133)	a *his3*-Δ200, *trp1*-Δ1, *lys2*-801, *ade2*-101, *ura3*-52, *rad52*-8 (*TRP1*)
YNN299	pSC4131 transformed into YNN217
YNN302	pSC*ARO1* integrated into YNN282
YNN301	pSC4131 integrated into YNN282
YNN36	α *his3*-Δ1, *trp1*$^-$, *gal2*$^-$, *ura3*-52
YNN300	YNN299 *rad52*-8(*TRP1*)
YNN287 (parent of translocation II, IV)	PNN275 integrated into YNN285
YNN296	α *his3*-Δ200, *ura3*-52, *trp1*-289, *ade2*-101, *his3*-Δ5′ at *Gal1*, *rad52*-8(*TRP1*)
YNN304	YNN299 X YNN217
Plasmid strains	
PNN286 (pSC)	Amp^r, *URA3*, *his3*-Δ5′, *his3*-Δ3′
PNN287 (pSC4131)	Amp^r, *URA3*, *his3*-Δ3′, *his3*-Δ5′, Sc4131
PNN288 (pSC*ARO1*)	Amp^r, *URA*3, *his3*-Δ3′, *his3*-Δ5′, Sc*ARO1*
PNN275 (YIp5 Sc4131a, *his3*-Δ3′)	Amp^r, *URA*3, *his3*-Δ3′ oriented to *CEN4*
PNN293 (pGC)	Amp^r, *URA3*, *his3*-Δ(5′,3′)
PNN295 (pRE)	Amp^r, *URA3*, *TRP1*, *his3*-Δ3′

To monitor stability of the His$^+$ phenotype, colony-purified His$^+$ revertants were grown in YPD (rich medium, 29) for 10 generations and plated on YPD plates. Colonies were then scored for their histidine requirement.

Determining Rates and Frequencies of Recombination. Rates (events per cell division) of SC recombination were determined using the method of the median, as described by Lea and Coulson (12). Rates for specific constructions were averaged for independent transformants. Frequencies (events per number of cells) of plasmid integration were determined by culturing independent transformants and then plating 0.1 ml onto histidine-free medium.

An Assay for the Induction of SC Recombination. A qualitative assay was used to determine whether particular chemicals induce SC recombination. Cells were grown logarithmically in rich (YPD) media. Approximately 10^7 cells are plated on minimal medium lacking histidine. Aliquots of a particular chemical are then placed in the center of the plate and allowed to absorb into the medium. The plates are then incubated at 30°C.

Quantitative Assay for γ-Ray Induction. Cells were grown logarithmically in YPD to an OD_{650} of 2 and plated at a dilution of 10^{-5} on YPD plates. Plates were subsequently irradiated using a ^{137}Cs source for indicated doses.

RESULTS

Vectors were constructed to study three types of nonallelic (ectopic) recombination events. These vectors contain recombination substrates that can be introduced into any chromosome locus. Substrates for reciprocal recombination (Fig. 1*A*) and SC recombination (Fig. 1*B*) contain fragment(s) of the *his3* gene that overlap by 300 base pairs of homology. One fragment *his3*-Δ5′ (*his3*-Δ2619) lacks the 5′ end of the amino acid coding sequence (amino terminus), and the other fragment *his3*-Δ3′ lacks the 3′ end of the amino acid coding sequence (carboxyl terminus) (8). Substrates for gene conversion (Fig. 1*C*) consists of two fragments of *his3*; one fragment, *his3*-Δ(5′,3′), lacks both the 5′ and 3′ ends of the gene, and the other fragment, *his3*-Δ1, lacks an internal portion of the gene (13). The regions of *his3* homology for all recombination substrates are shown in Fig. 2.

Reciprocal Recombination Selection. Construction of a substrate to monitor reciprocal recombination specifically depends upon the appropriate orientation and position of *his3*-Δ5′ and *his3*-Δ3′ with respect to each other. His$^+$ revertants due to gene conversion not associated with chromosome rearrangements can occur if these *his3* fragments are directly flanked by repeated sequences or if sufficient homology exists at the wild-type *HIS3* locus on chromosome XV. Gene conversion of the "wild-type" locus is blocked by complete elimination of all sequences essential for *HIS3* promotion and expression (*his3*-Δ200, see Fig. 2) as described (14). If the *his3* fragments are on two nonhomologous vectors or chromosomes then reversion of His$^+$ can only occur by reciprocal recombination between truncated *his3* fragments as shown in Fig. 3.

Reciprocal recombination between nonhomologous chromosomes can be directly selected by the following strain constructions. *His3*-Δ5′ was integrated in chromosome II near *GAL(7,10,1)*, and *his3*-Δ3′ was present either on a replicating plasmid or integrated in chromosome IV near *TRP1*. In both cases there is no homologous DNA that flanks these fragments. His$^+$ revertants due to reciprocal recombination can be easily verified by detecting the presence of both *HIS3*$^+$ and *his3*-Δ(5′,3′).

Plasmid integration was directly selected in transformants of YNN285 that contain an autonomously replicating plasmid, YRp17 *his3*-Δ3′. His$^+$ revertants were selected from these transformants, and integration of this plasmid by way of *his3* homology occurred at a frequency of 10^{-6}. His$^+$ revertants contain an integrated plasmid as shown by the stability of the Ura$^+$ and His$^+$ phenotypes and by the presence of *his3*-Δ(5′,3′). An isogenic *rad52*-8 strain of YNN285 was made by one-step gene disruption. Spontaneous His$^+$ revertants due to plasmid integration occurred at an indistinguishable frequency in *rad52*-8 and *RAD52*$^+$.

Recombination between nonhomologues can be directly selected and results in a reciprocal translocation of chromo-

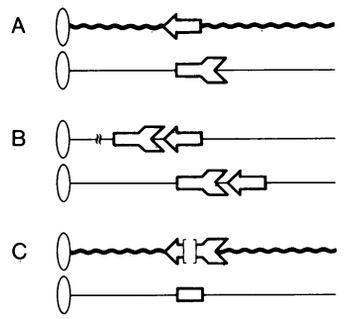

FIG. 1. Wild-type *HIS3* is depicted as an arrow with feathers and an arrowhead. An arrow without feathers represents *his3*-Δ3′. The direction of the arrow is indicative of the polarity of the amino acid coding sequence. A fragment lacking both arrowhead and feathers represents *his3*-Δ(5′,3′). Three different arrangements of the *his3* fragments are shown. (*A*) *his3* fragments are on two completely nonhomologous replicons. His$^+$ revertants are due to reciprocal recombination. (*B*) *his3* fragments are in tandem with respect to each other on the same chromosome. His$^+$ revertants are due to SC recombination. (*C*) *his3* fragments are *his3*-Δ1 and *his3*-Δ(5′,3′). Recombination between these *his3* fragments generate His$^+$ revertants due to gene conversion.

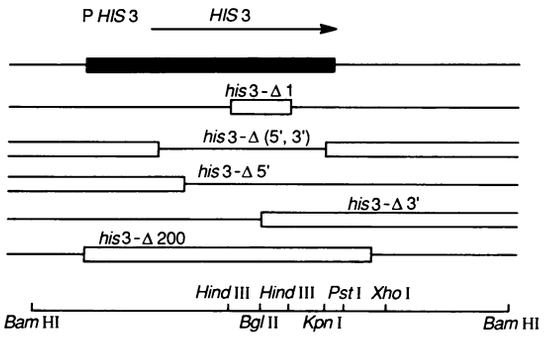

FIG. 2. Restriction maps of *HIS3* and deletions *his3*-Δ3′, *his3*-Δ5′, *his3*-Δ200, and *his3*-Δ(5′,3′). The map includes the 1.7-kb *Bam*HI fragment of *HIS3*. Open boxes represent sequences missing or fused to *HIS3* DNA.

some arms (Fig. 3). A plasmid containing *his3*-Δ3′ was integrated on chromosome IV by virtue of homology to Sc4131, an *Eco*RI restriction fragment mapping just on the centromere-proximal side of *TRP1* (7). In this strain, both *his3* fragments are oriented so that their coding sequences are read toward the centromere. His^+ revertants arise at a rate of 10^{-8}. Of 15 independent events, 14 were determined by orthogonal field-alternating gel electrophoresis to be revertants where a reciprocal translocation occurred.

SC Recombination Selection. To monitor nonallelic SC recombination, vectors were constructed whose prototype is shown in Fig. 4. The most essential feature is that the nontruncated ends of the *his3* fragments are juxtaposed. This special arrangement of the *his3* fragments will be termed the SC assay. Vectors containing the SC assay can be integrated into the yeast genome. Upon selection for His^+ revertants two types of recombination events can be hypothesized. One type, intrachromosomal recombination, excises the wild-type *HIS3* and leaves a fragment of *his3*, *his3*-Δ(5′,3′), in the genome. Since *HIS3* has no autonomously replicating sequence (*ARS*), this extrachromosomal element cannot be maintained through successive cell divisions unless it is reintegrated into the genome. Such events are too rare to be detected in our assay system. However, if the locus has been replicated, the *his3* fragments can interact through SCs to recreate wild-type *HIS3*. These events result in *HIS3* flanked by *his3*-Δ5′ on one side and *his3*-Δ3′ on the other (Fig. 4), and the original position and orientation of these fragments are maintained. Since the His^- SC segregates and is not selected for, the reciprocity of the recombination event cannot be assumed. Mechanisms for these events are presented in the *Discussion*.

As an example of this system, pSC4131, a plasmid containing the SC assay, was integrated adjacent to the *TRP1* locus. His^+ revertants were selected and occurred at a rate of 4×10^{-6}. To verify that these His^+ revertants are due to

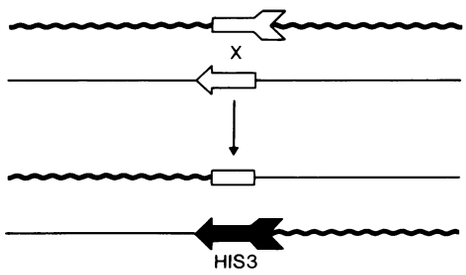

FIG. 3. Reciprocal exchange between two fragments of *his3*, *his3*-Δ3′, and *his3*-Δ5′ on nonhomologous chromosomes generates *HIS3*$^+$ and *his3*-Δ(5′,3′).

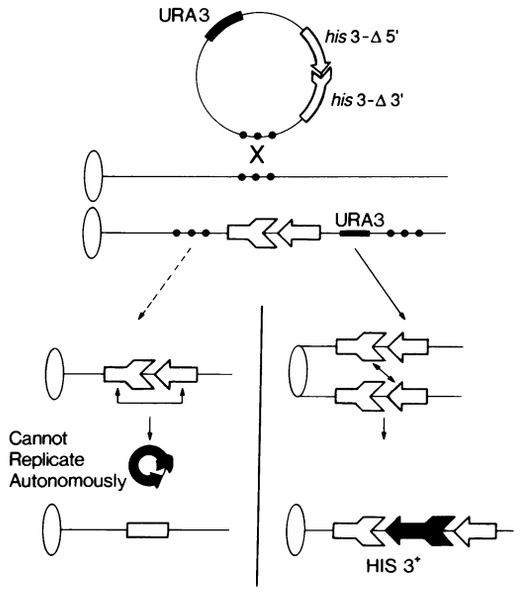

FIG. 4. Unequal SC recombination between two fragments of *his3*. The prototype vector used in these studies is shown (*Top*). Dotted area represents yeast DNA sequences by which this vector can integrate at the desired locus in the genome. (*Middle*) Recombination events. (*Bottom*) The left-hand side represents consequences of intrachromosomal recombination between the *his3* fragments and the formation of the *HIS3* circle. Right-hand side depicts the outcome of SC recombination between the *his3* fragments.

SC recombination, the stability of the His^+ phenotype was determined, the presence of *his3*-Δ3′ and *his3*-Δ5′ was shown, and their physical linkage to *HIS3* was demonstrated. Thirteen His^+ revertants from six independent clones show the presence of *his3*-Δ3′, *his3*-Δ5′, and *HIS3*$^+$ by Southern blot analysis (15). Indepenent His^+ revertants show stability for the His^+ phenotype.

This system was used to study the effect of four different parameters on SC recombination; namely, ploidy, distance from the centromere, distance from *ARS1*, and *rad52*$^-$. Rates

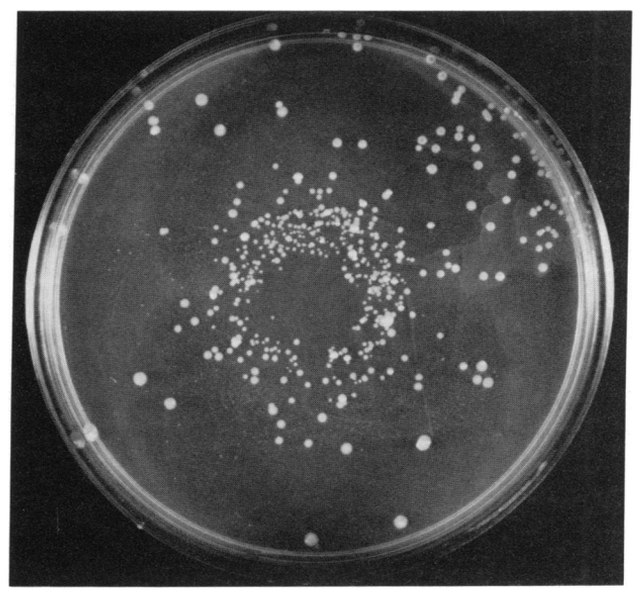

FIG. 5. A simple qualitative assay for the induction of SC recombination. A small aliquot of methyl methanesulfonate (1 μl) was placed in the center of a plate containing a lawn of His^- yeast. A halo of His^+ revertants is seen surrounding the center of the plate.

of SC recombination have been determined in diploid strains ($\mathbf{a}/\alpha$) where one homologue contains the SC assay and the other homologue does not. In these strains, the rate of SC recombination does not significantly vary from the haploid parent. Rates of SC recombination were determined in a haploid strain (YNN282) at two loci on chromosome IV (Table 2). One locus, *TRP1*, is <1 cM (centimorgan) away from *CEN4*, and the other locus *ARO1* is unlinked to *CEN4* and >30 cM away. The rate of SC recombination at *ARO1* is 2×10^{-6} and at *TRP1* is 4×10^{-6}. Mitotic recombination (crossing-over) between homologues also does not decrease dramatically as one approaches *CEN7* (16).

The effect of proximity to *ARS1* on SC recombination was determined because *ARS1* may be an origin of replication, and SCs would first be generated near this locus. pSC4131 was integrated near the *ARS1* locus in a strain deleted for *ARS1* on chromosome IV and in an isogenic strain that did not contain this deletion. In both strains the SC assay was the same distance and orientation with respect to *CEN4*. The *ARS1* deletion encompasses the entire 1.4-kb *Eco*RI *TRP1–ARS1* fragment (19). The rate of SC recombination appears to be higher for the strain with the *ARS1* deletion.

To determine the effect of *rad52*$^-$ on SC recombination, an isogenic strain was made by one-step gene replacement into YNN299 that inserted *TRP1* into *RAD52* generating the *rad52*-8 allele. SC recombination decreases from 4×10^{-6} in *RAD52*$^+$ to 2×10^{-7} in *rad52*-8. His$^+$ revertants from this strain show the same stability and genotype as Rad$^+$ strains.

Using the strains described above where the SC assay is integrated at *TRP1* and *ARO1*, the ability of radiation, carcinogens, and mutagens to stimulate SC recombination was investigated. Whereas methyl methanesulfonate has a dramatic effect in increasing SC recombination, the mutagen ethyl methanesulfonate had no such effect as visualized in a qualitative plate assay (Fig. 5). The effect is seen in both haploid and diploid. γ-Irradiation of the strain YNN299 was seen to stimulate recombination at 35 krads (60% lethality; 1 rad = 0.01 Gy). The mean frequency of recombination increased for 9×10^{-6} to 9.3×10^{-5} at 42 krads. This level of irradiation is capable of generating between 40 and 100 double-strand breaks in the genome (17).

Other chemicals that increase SC recombination in higher eukaryotes have not been seen to have a dramatic effect in yeast. Intercalating agents, such as ethidium bromide or acridine orange, do not qualitatively enhance SC recombination.

Gene Conversion Selection. To insert the *his3* fragment in any particular orientation to measure gene conversion, a plasmid was constructed that contains a *his3* allele lacking the 5′ end important in *HIS3* promotion and the 3′ end of the structural gene. When such an allele is on an autonomous replicating vector and introduced into a strain containing *his3*-Δ1, His$^+$ revertants arise at a frequency of 10^{-6}. These His$^+$ revertants are stable under nonselective conditions, indicating that the genomic allele has been repaired. However, the occurrence of double-reciprocal recombination during the G_2 phase of the cell cycle cannot be disproved using the assay system described above. When a similar allele is integrated into the genome, interchromosomal gene conversion of *his3*-Δ1 to *HIS3*$^+$ occurs at a frequency of 10^{-6} (13). A gene conversion assay similar to the one described here has been used for the *TK* (thymidine kinase) gene (18).

Table 2. Rates and frequencies of SC recombination and reciprocal exchange

Recombination	Locus	Rate		
		Spont.	+ MMS	*rad52*-8(*TRP1*)
SC				
Diploid	*ARS1*$^-$	2×10^{-6}	+	NT
Haploid	*ARS1*$^-$	4×10^{-6}	+	2×10^{-7}
	ARS1$^+$	1×10^{-6}	+	NT
	ARO1	2×10^{-6}	+	NT
Reciprocal				
Integration	*GAL1*	1×10^{-6}*	−	1×10^{-6}*
Translocation	II, IV	1×10^{-8}	−	NT

NT, Not tested; Spont., spontaneous; +, induced in Rad$^+$; −, not induced in Rad$^+$; MMS, methyl methanesulfonate.
*This is a frequency, not a rate.

DISCUSSION

A series of vector systems were constructed to study ectopic (nonallelic) recombination *in vivo*. A pair of fragments, one lacking an internal fragment (*his3*-Δ1) and the other lacking both the 5′ and the 3′ ends of *HIS3*, can be used to select directly for gene conversion events. Two tandem fragments of the *his3* gene (*his3*-Δ3′ and *his3*-Δ5′) can be used to select directly for SC recombination. To select His$^+$ revertants due only to reciprocal exchange, the two fragments (*his3*-Δ3′ and *his3*-Δ5′) must be on two nonhomologous chromosomes or plasmids.

By selecting for His$^+$ revertants generated by recombination between two tandem *his3* fragments in the SC assay, rearrangements are generated that recreate the *HIS3* gene flanked by *his3*-Δ5′ and *his3*-Δ3′. His$^+$ revertants can only arise by recombination of replicated genomic *his3* fragments. Theoretically, the entire chromosome does not need to be replicated for this recombination event to occur, and this event may occur either in the S or G_2 phase of the cell cycle. Plasmids containing *ARS* or *CEN* sequences cannot be guaranteed to be in single copy and may possibly recombine prior to replication (19). To accurately assay for SC recombination, these *his3* fragments must be present in the genome. The variance in the rate of SC recombination in wild-type strains could be due to the following three parameters: distance from the centromere or chromosomal location, distance from an *ARS* sequence or origin of replication, and interactions among repeated sequences flanking the *his3* fragments. Proximity to the *CEN4* does not decrease the rate of SC recombination.

SC recombination is also induced by many of the recombinogens that induce mitotic gene conversion between homologues. Agents that induce this recombination, such as γ-irradiation and methyl methanesulfonate, are also agents that induce double-strand breaks. The ability of methyl methanesulfonate and the inability of ethyl methanesulfonate to induce SC recombination may be due to the cytotoxicity of methyl methanesulfonate (20), although the molecular basis of the induction is not understood. These data are consistent with the observation that G_2 haploids are more resistant to γ-irradiation than G_1 haploids; presumably, double-strand breaks can be repaired in G_2 (21). Conversion of two *cdc* alleles that arrest the cell cycle in G_2 at the restricted temperature also suggests that mitotic gene conversion can occur in G_2 (22).

A defect in *RAD52*, *rad52*-8, also decreases SC recombination between *his3* fragments. Another allele, *rad52*-1, blocks mitotic gene conversion between nontandem *his4* heteroalleles but does not abolish unequal SC exchange (5). We suggest that SC recombination between *his3* fragments occurs primarily by gene conversion (*RAD52*-dependent mechanism) and at a lower frequency by reciprocal exchange of SCs (*RAD52*-independent mechanism). A double-strand break model has been suggested for repairing double-strand gaps on linear, transformed plasmids and for mediating mating-type interconversion (23). This may also be evoked for repairing double-strand breaks between SCs that could lead to SC recombination. A model for this type of recom-

bination is shown in Fig. 6. After the SC replicates, a break is initiated adjacent to one of the *his3* fragments on either one of the SCs. The repair of this break is initiated by misalignment or unequal pairing of one *his3* fragment adjacent to the break with its complementing fragment on the SC. At the other end of the break, homologous sequences are free to pair anywhere along the SC since SCs are completely homologous. The gap created is then repaired as mentioned in detail by Szostak *et al.* (23). The SC that is used as a template for this repair segregates away from the His^+ SC through repeated cell divisions. This model cannot be confirmed unless the His^- SC is isolated and shown to have the SC assay. At present the ratio of His^+ SC exchange events due to reciprocal exchange and to gene conversion is unknown.

Models for SC recombination have relied on assays that detect recombination events due to reciprocal exchange. These assays include unequal SC exchange at the rRNA gene (rDNA) locus (24) and SC exchange in human lymphocytes as detected by bromodeoxyuridine incorporation (3). SC recombination between *his3* fragments differs from unequal SC exchange at the rDNA locus in yeast in that the latter is unaffected by *rad52*-1 and is not induced by γ-irradiation or methyl methanesulfonate (25). However, nonreciprocal recombination (gene conversion) between SCs at the rDNA locus has been suggested (26) and is possibly stimulated by *HOT1*, a sequence that stimulates intrachromosomal mitotic gene conversion and maps to the rDNA region (27). Since the *his3* SC assay does not *a priori* exclude gene conversion events, it is a useful tool to monitor all events due to SC recombination.

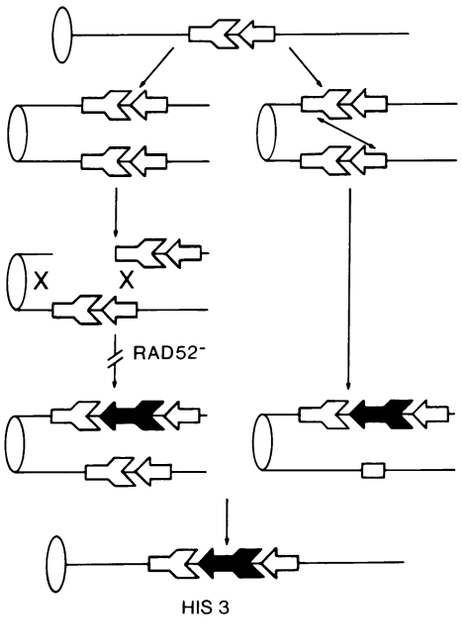

FIG. 6. Two alternative ways of generating *HIS3*$^+$ by SC recombination. (*Left*) Gene conversion by way of gap repair. (*Right*) Unequal SC exchange.

The *his3* recombination substrates that have been described provide models for nonallelic recombination, and their modes of recombination can be compared with that observed between dispersed repeated elements or genes. The induction and genetics of specific recombination events, including SC recombination, gene conversion, and reciprocal exchange can be directly studied. Similar substrates can be constructed for different genes from yeast or for genes from different organisms.

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