Genetic and Physical Analysis of Double-Strand Break Repair and Recombination in *Saccharomyces cerevisiae*

Norah Rudin,¹ Elaine Sugarman and James E. Haber

Rosenstiel Basic Medical Sciences Research Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02254

Manuscript received August 6, 1988
Accepted for publication March 20, 1989

**ABSTRACT**

We have investigated HO endonuclease-induced double-strand break (DSB) recombination and repair in a *LACZ* duplication plasmid in yeast. A 117-bp *MATa* fragment, embedded in one copy of *LACZ*, served as a site for initiation of a DSB when HO endonuclease was expressed. The DSB could be repaired using wild-type sequences located on a second, promoterless, copy of *LACZ* on the same plasmid. In contrast to normal mating-type switching, crossing-over associated with gene conversion occurred at least 50% of the time. The proportion of conversion events accompanied by exchange was greater when the two copies of *LACZ* were in direct orientation (80%), than when inverted (50%). In addition, the fraction of plasmids lost was significantly greater in the inverted orientation. The kinetics of appearance of intermediates and final products were also monitored. The repair of the DSB is slow, requiring at least an hour from the detection of the HO-cut fragments to completion of repair. Surprisingly, the appearance of the two reciprocal products of crossing over did not occur with the same kinetics. For example, when the two *LACZ* sequences were in the direct orientation, the HO-induced formation of a large circular deletion product was not accompanied by the appearance of a small circular reciprocal product. We suggest that these differences may reflect two kinetically separable processes, one involving only one cut end and the other resulting from the concerted participation of both ends of the DSB.

Two types of double-strand break (DSB) repair events have been characterized in *Saccharomyces cerevisiae*. In the repair of broken chromosomes, a single end may recombine with homologous sequences on an intact chromosome arm (Resnick 1975; Mccusker and Haber 1981; Haber and Thorburn 1984; Dunn et al. 1984). Alternatively, both ends of a broken DNA molecule may ultimately interact with an homologous segment, as seen both in the repair of cut or gapped plasmid molecules (Orr-Weaver, Szostak and Rothstein 1981; Orr-Weaver and Szostak, 1983) and when both ends of a broken chromosome recombine with an intact homologue (Mccusker and Haber, 1981). A specialized and highly efficient example of such “two-ended” DSB repair is homothallic mating-type (MAT) switching, in which the site-specific HO endonuclease creates a DSB at MAT (Strathern et al. 1982; Kostrikien et al. 1983) and initiates the replacement of MAT sequences by gene conversion from unexpressed mating-type sequences located at two donor loci. In contrast to the homologous repair of gapped plasmids, in which conversion is accompanied by exchange about 50% of the time, MAT conversions generally occur with virtually no associated crossing over (reviewed by Nasmyth 1982; Haber 1983; Klar, Strathern and Hicks, 1984). Suppression of crossing over is maintained when cloned fragments of MAT and HMR are present on the same plasmid (H. Friesen, B. Andrews, P. Sadowski, X. Wu and J. E. Haber, unpublished results) as well as when HMR is used as an interchromosomal donor (Klar and Strathern 1984). Exceptions to the constraint on reciprocal crossing over occur when switching takes place either mitotically or meiotically in a diploid deleted for the normal donors, HML and HMR, so that one MAT allele is converted using a MAT gene on the homologous chromosome as its donor (Klar and Strathern 1984; Kolodkin, Klar and Stahl 1986). Additionally, the mating-type switch is unique among mitotic gene conversions in that it occurs very efficiently, as often as once per generation.

In order to address the issues of efficiency and lack of exchange associated with mating-type switching, it would be useful to analyze HO endonuclease-induced recombination of other DNA sequences lacking any special properties of MAT and its donors. Previous studies have shown that when either a 24-bp or 117-bp segment, containing the HO endonuclease recognition site from MATa, was inserted into one member of a pair of directly repeated sequences in a chromosome, expression of HO endonuclease stimulated homologous gene conversion between direct repeats.

1 Present address: Cell and Molecular Biology Division, Lawrence Berkeley Laboratory 984-47A, University of California, Berkeley, California 94720.

Genetics 122: 519–534 (July, 1989)
both with and without associated exchange (Nickoloff, Chen and Heffron 1986; Ray et al. 1988). Analogous constructs with homologous sequences in inverted orientation have not previously been studied.

In the present study, the 117-bp fragment containing the HO endonuclease cut-site was inserted into a \textit{LACZ} gene on a centromere-containing plasmid such that no functional \(\beta\)-galactosidase was produced. This construction also includes another promoterless copy of \textit{LACZ}, containing wild-type coding sequences, from which repair of an \(HO\)-induced cut could take place. Using this system, gene conversions to produce \(Lac^+\) plasmids and associated crossing-over are readily scored. In addition, the efficiency of the process could be monitored by scoring the proportion of events in which the cut plasmid was not repaired, and subsequently lost. A similar analysis was carried out when these same sequences were integrated into the chromosome. There was a substantial difference in both the efficiency and spectrum of events obtained from centromere-containing plasmids, or when these sequences were present on the chromosome.

It was possible to study these recombination events in more detail by synchronously inducing \(HO\) endonuclease in a population of cells and extracting DNA at intervals for Southern blot analysis. A similar physiological analysis has recently been carried out on \textit{LACZ}, containing wild-type coding sequences, from which repair of the \(5\)' side of \(LACZ\) was monitored by scoring the proportion of events in which the cut plasmid was not repaired, and subsequently lost. A similar analysis was carried out when these same sequences were integrated into the chromosome. There was a substantial difference in both the efficiency and spectrum of events obtained from centromere-containing plasmids, or when these sequences were present on the chromosome.

\textbf{Materials and Methods}

\textbf{Plasmid construction:} Plasmid pRK113 (a gift from R. Kostriken) consists of a 117-bp \textit{HindII-BglII} fragment surrounding the \textit{MATa} cut-site inserted into \textit{BamHI-HindII} cut pUC9 (Kostriken and Heffron 1984). The \textit{HindII} junction was changed to a \textit{BamHI} site using linkers, and the fragment released using \textit{XhoI} which will cut at a \textit{BamHI} site as well as a \textit{BamHI/BglII} junction. The EcoRI \textit{ARS} fragment was removed from plasmid pLG199 Leu (a gift from L. Guarente) (Guarente, Yocum and Gifford 1982) in which the \textit{LACZ} gene is under control of the \textit{Cyc1 UAS}, and the \textit{Tet1111} site changed to a \textit{HindIII} site using linkers. The construction also contains a 300-bp segment of the \textit{LEU2} gene promoter region (\textit{lev2}) between \textit{Cyc1 UAS} and the \textit{LACZ} sequence. The \textit{XhoI} \textit{MATa} cut-site fragment was inserted into the \textit{BclI} site of \textit{LACZ} and both orientations of the cut-site relative to \textit{LACZ} were obtained. In both cases it was subsequently discovered that two copies of the cut-site, in direct orientation, had been inserted (\textit{LACZ::CS2}). In the plasmid used for the construction of plasmids pNR17 and pNR18, the \textit{BglII-HindII} \textit{MATa} cut-sites were oriented with the \textit{MATa} region (originally \textit{HindII}) closest to the 5' side of \textit{LACZ} (orientation I). In the predecessor to plasmids pNR50 and pNR51, the cut-sites were oriented with the 3' regions (originally \textit{BglII}) closest to the 3' side of \textit{LACZ} (orientation II). In the case of plasmids pNR17 and pNR18, the structure was confirmed by DNA sequence analysis (C. White, personal communication).

A promoterless copy of \textit{LACZ} (\(\Delta LACZ\)) was obtained by removing a H2A-H2B histone promoter from a \textit{LACZ} fusion (plasmid pGAL16, a gift from M. A. Osley) (Osley and Hereford 1982). This plasmid also contains a 1.4-kb \textit{XhoI} \textit{CEN4} fragment embedded in the 3' untranslated region of a 4.1-kb \textit{LEU2} gene, as well as an \textit{ARS} sequence derived from the histone genes.

\textit{HindIII} fragments from either of the two different cut-site orientation plasmids, containing \textit{LACZ}, \textit{Cyc1 UAS}, and \textit{LACZ::CS2}, were inserted into a \textit{HindIII} site at the 5' end of the promoterless \textit{LACZ} copy. Bacterial transformants were selected for \textit{Ura} and \textit{Leu} and screened for the relative orientation of the two \textit{LACZ} genes. Plasmids pNR17 (cut-site orientation I, Figure 1A) and pNR50 (cut-site orientation II, Figure 1C) contain a duplication of \textit{LACZ} in the inverted orientation. Plasmids pNR18 (cut-site orientation II, Figure 1B) and pNR51 (cut-site orientation II, Figure 1D) contain a direct duplication of \textit{LACZ}.

Plasmids pNR27 and pNR28 were obtained by partially digesting plasmid pNR17 and plasmid pNR18 with \textit{XhoI}, religating, and screening for loss of the 1.4-kb \textit{CEN4} fragment.

\(pSE271::GAL10-HO\) was a gift from F. Heffron laboratory (Nickoloff, Chen and Heffron 1986), which will be referred to as \(pGAL10-HO\), also contains \textit{CEN4} and \textit{TRP1 ARS1}.

\(Psp65-LACZ\) (a gift from the M. Wormington laboratory) was constructed by inserting the \textit{LACZ} gene into the multiple cloning site of \(Psp65\). Either the entire plasmid, or a 2.5-kb \textit{Psp65} gel-isolated fragment of \textit{LACZ} was nick-translated with [\(\alpha-\text{32P}\)]CTP for use as a hybridization probe.

\textbf{Strains:} Strains used in this study are listed in Table 1. Strain DBY745 was originally obtained from D. Botstein. Strain R167 was constructed in this laboratory by replacing (Rothstein 1983) the normal \textit{MATa} allele of R126 (\textit{ho HMLa MATa hmr-3A leu2-3,112 ura3-52 trpl thr4 GAL}) with \textit{matA::LEU2} from plasmid pJH124. The \textit{hmr-3A} deletion of \textit{HMRa} was originally obtained from A. J. S. Klar (Klar, Hicks and Strathern 1982). Strain tNR85 was obtained by transformation of strain R167 with plasmid pGAL10-HO. Strains tNR85, tNR87, tNR118 and tNR119 were constructed by transforming strain tNR85 with respectively: plasmids pNR17, pNR18, pNR50 and pNR51. Strains tNR101 and tNR102 were the result of integrating plasmids pNR27 and pNR28, respectively, at \textit{ura3-52} in strain tNR85.

\textbf{Media:} Cells were grown in \textit{YP} medium (1% w/v yeast extract, 2% w/v Bacto-peptone) supplemented with dextrose (2% w/v, YE PD) or lactic acid (3.7% pH 5.5, YEPL). When required, galactose (2% w/v) was added as a 20% solution to YEPL medium. Synthetic complete media lacking uracil, leucine or tryptophan and minimal medium plus threonine, were made according to Sherman, Fink and Hicks 1986. Bacto-Agar was added (2%) for solid media.

\textbf{Scoring of \textit{HO}}: When necessary, \textit{HO} was scored by taking advantage of the strains U60 and U90 as described in Weiffenbach and Haber (1981).

\textbf{Scoring for \(\beta\)-galactosidase activity in yeast:} We have
developed a new method for scoring β-galactosidase activity in yeast. An aliquot of 0.2 ml of 10 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) is spread on synthetic complete plates containing 8.3% (v/v) glycerol and 2.7% (v/v) ethanol as a carbon source, and buffered to pH 9 with KOH. After replica plating yeast colonies to these plates, they are inverted, and a 7-cm Whatman 3M circle is placed in the lid and saturated with ethyl ether. After replacing the bottom of the plate, they are stored upside-down in a hood overnight. Blue color, due to the metabolism of X-gal, is apparent overnight, and increases over several days.

**Yeast transformation:** Yeast cells were transformed using lithium acetate by the method of ITO *et al.* (1983).

**HO induction:** A stationary phase culture grown in medium selective for the plasmid(s) was diluted 1:100 into YEPl 1/100 (v/v) to yield a final concentration of approximately 5 x 10^6 cells/ml. The cultures were grown at 30° with vigorous aeration to a final cell density of 1-3 x 10^7 cells/ml. Galactose was added as a 20% (w/v) solution and the incubation continued. Cells were removed at various points for subsequent analysis.

**DNA extraction:** DNA from stationary phase cells was extracted using the potassium acetate method as described in SHERMAN, FINK and HICKS (1986), with minor modifications. DNA from time course material was extracted in the following manner. Aliquots of 25 ml of culture (5-10 x 10^7 cells) were collected onto an 0.45-μm pore nitrocellulose filter with 0.5 ml of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2% NaDodSO4 into a 1.5-ml microcentrifuge tube. Glass beads (0.45-0.5-mm diameter, acid washed and baked) were added to the level of the meniscus, 0.5 ml TE-saturated phenol was added and the tube vortex mixed for 15 sec and then cooled on ice. The aqueous phase was subsequently extracted with an equal volume of isopropanol. The precipitate was pelleted, dissolved in 100% ethanol, air dried and dissolved in TE and stored at -20°C.

**Southern blot analysis:** Restriction endonuclease digested DNA was electrophoresed on 0.5%-0.8% agarose gels, at 40-60 V for appropriate lengths of time, transferred to nitrocellulose membrane (Millipore), hybridized to radiactively-labeled [α-32P]dCTP probe, washed and autoradiographed using standard procedures (SOUTHERN 1975; MANIATIS, FRITCH and SAMBROOK 1982). Nick-translated probes were prepared using standard procedures.

**RESULTS**

**HO-induced gene conversion of LACZ sequences:**

The 117-bp BglII-HincII fragment of MATα containing the HO cut-site has been shown to be cut efficiently in *vivo* by HO endonuclease (RUDIN and HABER 1988). A plasmid-borne LACZ gene that is expressed in yeast (GUARENTE, YOCUM and GIFFORD 1982) was disrupted by the insertion of a pair of 117-bp *MATα* HO endonuclease cut-sites at the BglI site (LACZ::CS2, MATERIALS AND METHODS). Two plasmids were constructed that additionally contained a second, promoterless, copy of LACZ (ΔP-LACZ) (Figure 1). Plasmid pNR17 contains the LACZ duplication in an inverted orientation, separated by a 1.2-kb URA3 gene; in plasmid pNR18 the two LACZ genes are oriented directly head to tail. Both of these plasmids contain URA3 and LEU2, as well as *CEN4* and an ARS sequence, and are maintained at 0-2 copies per cell. A yeast strain containing either plasmid scores as white on X-gal medium, since no functional copy of LACZ is present. In the presence of HO endonuclease, LACZ::CS2 undergoes a double strand cut and possible repair by gene conversion from the intact promoterless gene. The entire 234-bp insertion is removed and replaced by wild-type sequences, producing a Lac+ gene harbored by cells that form blue colonies on X-gal plates.

Both pNR17 and pNR18 were transformed into tNR85, a heterothallic (*ho*) yeast strain deleted for *MAT* and *HMR*. This strain also contains a galactose-inducible *HO* gene (JENSEN and HERSKOWITZ 1984) on a *TRP1 ARS1 CEN4* plasmid (pGAL10-HO, NICKOLOFF, CHEN and HEFFRON 1986). The yeast transformant strain containing pNR17 (inverted orientation) will be referred to as tNR86, and the strain containing pNR18 (direct orientation) as tNR87.

**TABLE 1**

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY745</td>
<td>ho HMLα leu2-3,112 MATα HMRα MAL2 ura3-32 ade1-110</td>
</tr>
<tr>
<td>R167</td>
<td>ho HMLα leu2 maηΔ LEU2 hmrΔS mal2 ura3-32 thr4 trp1</td>
</tr>
<tr>
<td>tNR85</td>
<td>R167[pGAL10-HO]</td>
</tr>
<tr>
<td>tNR86</td>
<td>tNR85[pNR17] (inverted LACZ duplication, cut-site orientation I)</td>
</tr>
<tr>
<td>tNR87</td>
<td>tNR85[pNR18] (direct LACZ duplication cut-site orientation I)</td>
</tr>
<tr>
<td>tNR118</td>
<td>tNR85[pNR50] (inverted LACZ duplication, cut-site orientation II)</td>
</tr>
<tr>
<td>tNR119</td>
<td>tNR85[pNR51] (direct LACZ duplication, cut-site orientation II)</td>
</tr>
<tr>
<td>tNR101</td>
<td>tNR85[pNR27] (inverted LACZ duplication, integrated at ura3-32)</td>
</tr>
<tr>
<td>tNR102</td>
<td>tNR85[pNR28] (direct LACZ duplication, integrated at ura3-32)</td>
</tr>
<tr>
<td>U60</td>
<td>ho HMLα maη1 HMRα ade2 leu1 ura3 cmt</td>
</tr>
<tr>
<td>U90</td>
<td>ho HMLα maη1 HMRα ade2 leu1 ura3</td>
</tr>
</tbody>
</table>
to induction, an average of 85% of cells retained both the LACZ and GAL10-HO plasmids, and the LACZ plasmid itself was retained in over 90% of the cells; fewer than 1% of the cells had undergone recombination to produce Lac+ colonies. Samples of the culture taken at 1, 3 and 8 hr after galactose addition were diluted into YEPD (to repress further expression of the HO gene) and then spread on YEPD plates. The numbers for the zero time point were taken from a direct plating in which cells were not selected to be in GI.

Consistent results were also obtained using strains where a genomic copy of HO was expressed under normal physiological control. In these experiments, retained both plasmids; the proportion of cells that were Lac+ also increased through this period to 100% of those containing both plasmids. tNR87 (direct orientation) showed a less dramatic loss of the LACZ plasmid, such that 54% of the cells retained both plasmids at 8 hours; again, virtually all the cells that contained both plasmids at 8 hr were Lac++.

In order to assay a much larger number of events, a similar experiment was performed using mass cultures in which five independent cultures were assayed at 8 hr after galactose induction (Table 3A). Of the total colonies, an average of 43% of the cells containing the inverted repeat retained both the LACZ and GAL10-HO plasmids, and 59% in the case of the direct repeat; these values differ significantly from each other (z = 5.3, P < 0.05; SOKAL and ROHLF 1969). The value of 54% retention of pNR18 among selected G1 cells agrees very well with the 59% plasmid retention seen in mass cultures; we suspect that the value of 22% retention obtained for pNR17 among micro-manipulated cells, was due to the small sample size in the G1 experiment. All Ura+, Trp+ colonies were Lac+, indicating that a precise repair event had taken place to restore functional β-galactosidase activity.

Consistent results were also obtained using strains where a genomic copy of HO was expressed under normal physiological control. In these experiments,

![Image](Figure 1 — Structure of the LACZ duplication plasmids. Plasmid pNR17 contains the inverted LACZ duplication and the HOendonuclease cut site in orientation I (see MATERIALS AND METHODS). Plasmid pNR18 contains the direct LACZ duplication, and the cut-site in orientation II. Plasmids pNR50 and pNR51 both contain the cut-site in orientation II; pNR50 contains the inverted LACZ duplication and pNR51, the inverted orientation. The stippled region of the plasmids represents the GCI UAS 'leu2' promoter region constructed for LACZ and the large curved arrows indicate the direction of each LACZ gene. Each parental plasmid is about 21.5 kb. Restriction sites are indicated as follows: B, BglII; Hc, HincII; H, HindIII. ( ) indicates that the site was destroyed in cloning. CS indicates the pair of 117-bp MATa cut-sites.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Strain</th>
<th>Trp+</th>
<th>Trp+ Ura+</th>
<th>Trp+ Ura+ Lac+</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>tNR86</td>
<td>575/613</td>
<td>(94%)</td>
<td>516/608</td>
</tr>
<tr>
<td></td>
<td>tNR87</td>
<td>353/356</td>
<td>(99%)</td>
<td>298/329</td>
</tr>
<tr>
<td>1</td>
<td>tNR86</td>
<td>43/46</td>
<td>(93%)</td>
<td>32/46</td>
</tr>
<tr>
<td></td>
<td>tNR87</td>
<td>62/67</td>
<td>(92%)</td>
<td>54/67</td>
</tr>
<tr>
<td>3</td>
<td>tNR86</td>
<td>52/57</td>
<td>(91%)</td>
<td>23/57</td>
</tr>
<tr>
<td></td>
<td>tNR87</td>
<td>30/35</td>
<td>(86%)</td>
<td>27/35</td>
</tr>
<tr>
<td>8</td>
<td>tNR86</td>
<td>42/51</td>
<td>(82%)</td>
<td>11/51</td>
</tr>
<tr>
<td></td>
<td>tNR87</td>
<td>29/37</td>
<td>(78%)</td>
<td>20/37</td>
</tr>
</tbody>
</table>

* Galactose induction was performed as described in MATERIALS AND METHODS. For all points except 0 hr, individual unbudded (Gi) cells were micromanipulated away from other cells and allowed to grow into colonies on YEPD plates. The numbers for the zero time point were taken from a direct plating in which cells were not selected to be in Gi.

* Strain tNR86 contains plasmid pNR17 (inverted repeat plasmid).

* Strain tNR87 contains plasmid pNR18 (direct repeat plasmid).
TABLE 3
Fate of LACZ:CS2 plasmids following an 8-hr galactose induction*

<table>
<thead>
<tr>
<th>Structure of plasmid</th>
<th>Parental</th>
<th>Inversion</th>
<th>Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNR17 (inverted)</td>
<td>17/33 (52%)</td>
<td>16/35 (48%)</td>
<td></td>
</tr>
<tr>
<td>pNR18 (direct)</td>
<td>10/59 (17%)</td>
<td>49/59 (83%)</td>
<td></td>
</tr>
</tbody>
</table>

* Galactose induction was performed as described in MATERIALS AND METHODS. Each data point represents the summation of results from five independent cultures. A small proportion of total colonies (typically 0–3%) were Ura+ Trp- Lac-, since some had lost the TRP1 ARS1 GAL10-HO plasmid subsequent to the repair event. 4 Strain tNR86 contains plasmid pNR17 (inverted repeat plasmid, cut-site orientation I). 5 Strain tNR118 contains plasmid pNR50 (inverted repeat plasmid, cut-site orientation II). 6 Strain tNR87 contains plasmid pNR18 (direct repeat plasmid, cut-site orientation I). 7 Strain tNR119 contains plasmid pNR51 (direct repeat plasmid, cut-site orientation II). 8 DNA from subclones of independent Lac+ colonies, produced by galactose induction of HO (described in MATERIALS AND METHODS) for 1, 3 or 8 hr, was examined by Southern analysis (see text) to determine plasmid structure.

HO/ho MATa-inc/MATa diploids containing the LACZ plasmids pNR17 or pNR18 were sporulated and dissected to obtain HO MATa-inc segregants containing the plasmids, in which HO endonuclease is expressed only in the G1 stage of the cell cycle in haploid mother cells, ho MATa-inc segregants served as controls. Under these conditions, it was again found that plasmids in the inverted orientation were lost more frequently than those with direct repeats, and that all of the plasmids that were retained were Lac+ (RUDIN 1987).

Exchange is associated with gene conversions initiated by HO endonuclease cleavage in the LACZ-duplication plasmids: In the case of the inverted repeat plasmid, reciprocal recombination yields an inversion of the nonhomologous sequences between the LACZ repeats (Figure 2, IIG). Exchange in the direct repeat produces a deletion plasmid containing only one copy of LACZ (Figure 2, IG); its reciprocal product in this case contains no plasmid maintenance sequences (Figure 2, IH), so is quickly lost. Association of exchange with gene conversion was assessed by Southern blots of PstI digests probed with [α-32P]-dCTP-labeled pSP65-LACZ (data not shown). An inversion in pNR17 produces LACZ specific PstI fragments of 6.5 kb and 7.0 kb, as compared to the 7.5-kb and 5.9-kb fragments indicative of the parental configuration. A deletion in pNR18 is detectable as a 7.3-kb PstI band instead of an 11.8-kb parental band. When DNA samples from independent subclones of Lac+ derivatives from galactose-induced G1 cells were analyzed (data not shown), 16/33 (49%) of the plasmids initially containing an inverted repeat had undergone an inversion indicative of reciprocal exchange. In contrast 49/59 (83%) of the direct repeat plasmids were deletions (contingency χ² = 12, P < 0.05) (Table 3B). DNA samples from 10 Lac+ colonies resulting from physiological induction of HO, containing each plasmid orientation, were also checked (data not shown), and the results found to be in agreement with those produced by a galactose-induced copy of HO. The BelI site was restored in all of these Lac+ plasmids (data not shown), indicating that a precise repair event had occurred in each case.

Inversion of the cut-site relative to LACZ has little effect on the nature of recombination events: Plasmids pNR17 and pNR18 consistently showed different loss and recombination frequencies after HO induction. As the two plasmids have different relative orientations of the two LACZ genes, it was possible that the orientation of the inserted 117-bp HO recognition sites, relative to LACZ sequences, might affect the outcome of the recombination event. In MAT switching, HO endonuclease cleaves in the homologous Z1 region, at the edge of the nonhomologous Ya or Ya information that is replaced, suggesting that strand exchange is initiated asymmetrically, by the end containing Z1 sequences (STRATHERN et al. 1982; HABER 1983). Thus it was important to test the effect of inserting the 117-bp MATa cut site, which contains the Y/Z junction, relative to LACZ sequences. Plasmids pNR50 (with LACZ segments in inverted orientation) and pNR51 (direct orientation) contain the 117-bp cut-sites in the opposite orientation compared to pNR17 and pNR18, respectively. Transformants carrying the new LACZ plasmids were galactose-induced for 8 hr, plated on YEPD plates, and scored for plasmid retention (Table 3A). The results represent the summation of 12 independent experiments for each strain. Of the total colonies that were also Trp+, the conversion products of the inverted LACZ plasmid (pNR50) were retained in 705/1616 cases (43%), and in the direct orientation (pNR51), in 758/1380 cases (55%), almost identical to the results obtained for cut-site orientation I. As previously, all colonies that retained both the pGAL::HO and LACZ plasmids were Lac+. Thus the difference in the proportion of successful repair events between the two orientations was maintained, as well as the absolute recombination frequencies. Physical analysis, presented below, further confirmed that the difference in proportion of crossing over between the inverted (pNR50) and di-
Figure 2.—Products of HO-induction of the LACZ duplication plasmids. tNR86 (containing pNR17, Figure 1A) and tNR87 (containing pNR18, Figure 1B) were induced with galactose as described in MATERIALS AND METHODS. After cutting by HO endonuclease, each plasmid is either lost (pNR17, 57%; pNR18, 41%), or undergoes gene conversion to Lac* without crossing over (IF and IIF), or with associated exchange (IG and IIG). Hypothetical intermediates are described within the brackets. IC, A two-ended event in a direct repeat results in a viable plasmid with the possibility of 50% exchange. In this case reciprocal exchange results in the formation of a 17-kb, stable, deletion plasmid, and a 4.5-kb unstable circle with no plasmid maintenance sequences. The structure represents invasion of one strand, and displacement of a D-loop. ID, A one-ended event initiated by invasion of the left half of the promoterless LACZ by the right half of LACZ::CS2 results in a viable deletion plasmid, and a 4.5-kb linear fragment. Such a fragment has not been detected. IE, A one-ended event initiated by invasion of the right half of the promoterless LACZ by the left half of LACZ::CS2 results in a 4.5-kb unstable circle, bearing no plasmid maintenance sequences, and a 17-kb linear fragment. No evidence is available to support the formation of this structure. II A, A two-ended event within an inverted repeat results in a viable plasmid with the possibility of 50% exchange. In this case reciprocal exchange results in an inversion of plasmid sequences between the two LACZ genes. The structure shown represents invasion of one strand, and displacement of a D-loop. IIB, A one-ended event within an inverted repeat results in an inviable plasmid. DNA analysis suggests formation of a transient structure involving invasion of the left half of the promoterless LACZ with the right half of LACZ::CS2. IIC, A one-ended event in an inverted repeat results in an inviable plasmid. No evidence is available for the formation of this structure. In all cases, the intersecting lines indicate PstI sites.
rect (pNR51) LACZ duplications containing the cut-site in orientation II, was the same as previously observed for the plasmids with the cut-site in orientation I (pNR17 and pNR18).

**Physical monitoring of HO-induced LACZ recombination in the direct repeat plasmid:** The kinetics of HO-induced DSB repair events were investigated. HO endonuclease activity was induced synchronously in a population of cells by the addition of galactose and samples for DNA analysis were taken at various time points. Events were monitored over a 5-8-hr time period, since the genetic results showed that most of the cells were committed to recombination during this time.

Events resulting from HO-induction of plasmids in which the LACZ duplication is present in direct orientation will be considered first. A conversion-associated exchange event in this configuration results in the deletion of one copy of LACZ (Figure 2, IG). Figure 3A shows a Southern blot of DNA samples taken during a time course of strain tNR87, which contains the MATa cut-sizes in orientation I. When the DNA was cut with PstI (Figure 2, I) and probed with a nick-translated 2.5-kb PvuII fragment of LACZ, the parental plasmid (lane 1, 0 min) produces one 11.8-kb band containing both copies of LACZ (designated par). The expected fragments resulting from HO endonuclease cleavage (8.8 kb, designated A'CS; 3.0 kb, designated A'CS) are present at 30 min. A band corresponding to the deletion product (7.3 kb, designated del) becomes visible 60 min after HO cutting was seen. Thus this recombination reaction is quite similar, to what was observed for GAL10-HO induced switching of the MAT locus (CONNOLLY, WHITE and HABER 1988). It was not possible in this digest to determine the time of appearance of the conversion event not accompanied by crossing-over in tNR87, as the resulting PstI fragment is only 234 bp smaller than the 11.8-kb parental molecule, and not resolved on these gels.

The doublet observed in conjunction with the HO-cut band is presumably the result of noncoincident cutting of the two HO cut-sites. At first appearance of the cut fragments, a majority of the molecules appear to have already been cut at both sites, and by 1 hr, almost all of the cut material was in the lower band of the doublet. As will be discussed in more detail, the inclusion of two HO cut-sites appears not to influence significantly the outcome of the remainder of the recombination event.

A time course was carried out with strain tNR119, which differs from strain tNR87 only in the orientation of the HO cut-sites inserted into LACZ (orientation II). As shown by the Southern blot in Figure 3B, the reaction appears identical to that for strain tNR87 (Figure 3A). Surprisingly, in neither of these blots was there evidence for the expected reciprocal product of gene conversion associated with a crossing over. As shown in Figure 2, I, formation of a Lac+ recombinant with an accompanying exchange should yield both the deletion plasmid (Figure 2, IG) and a 4.5-kb closed circular molecule containing only LACZ sequences.
Neither a supercoiled nor closed circular nor linear version of this product is evident in these time courses. Although this circular product lacks an ARS sequence that would allow it to be replicated in yeast, we would have expected to see it immediately after recombination. This result raises the possibility that the deletion product is formed by a nonreciprocal process.

Physical monitoring of HO-induced LACZ recombination in the inverted repeat plasmid: A similar kinetic analysis was carried out with strain tNR86, which carries the inverted LACZ duplication plasmid, pNR17. Conversion-associated exchange in this situation results in an inversion of plasmid sequences (Figure 2, IIG), detectable by subsequent cleavage with PstI (Figure 2, II). The Southern blot of the reaction time course is shown in Figure 4A. Lane 1 (0 min) shows the parental PstI fragments: the upper band contains the copy of LACZ interrupted by the cut-sites (designated ACS2, 7.5 kb), and the bottom band the promoterless copy (designated B, 5.9 kb). All of the predicted products of HO-induced recombination are visible in equimolar quantities by 5 hr. Band A represents conversion to Lac+ without exchange, and is 234 bp smaller than ACS2 due to conversional loss of the cut-site sequences. Band B remains unchanged and cannot be distinguished from that of the parental plasmid. The PstI fragments produced by reciprocal exchange accompanying gene conversion are marked as inv-A (6.5 kb) and inv-B (7.0 kb). The first evidence of cutting is seen at 30 min (bands A’CS2, 4.7 kb; A’CS, 4.5 kb; A’CS2, 3.2 kb; A’CS, 3.0 kb). Almost three hours elapse between the first evidence of the cut and the time when all of the products are finally visible. It is striking that one of the two expected crossover products, inv-B, is visible at least an hour before any other endpoint products. In a PstI digest, this structure represents the novel fragment generated by the joining of the 5’ half of the HO-cut LACZ to the 3’ half of ΔP-LACZ (Figure 2, IID). The time of appearance of inv-B is approximately the time at which the deletion is first observed in the direct repeat plasmid (Figure 3). The reciprocal crossover product, inv-A, appears with the same kinetics as the nonexchange product, band A, approximately 60 min after inv-B.

It is possible that the apparent asymmetry of the reaction depends on the orientation of the cut-site in relation to LACZ. If this were true, one might expect a reversal in the order of appearance of the two reciprocal crossover fragments if the cut-sites were inverted; inv-A would become visible prior to inv-B. In order to test this possibility, a kinetic analysis was performed on strain tNR118 (Figure 4B), which contains plasmid pNR50 in which the cut sites are in the reverse orientation (orientation II) from pNR17 in strain tNR86. Exactly the same pattern is observed as with strain tNR86; the half crossover band, inv-B, was visible long before band inv-A, indicating that the asymmetry of this recombination reaction is independent of the orientation of the cut-site.
Analysis of undigested DNA shows evidence of possible intermediates: DNA from the time courses was also examined by Southern analysis in the absence of subsequent restriction enzyme digestion in order to detect any possible intermediates that might be obscured by linearizing circular molecules. Under these conditions the unreacted plasmid runs as a supercoiled circle, and the product of an HO endonuclease cleavage migrates as a linear molecule, regardless of the orientation of the LACZ duplication. In this experiment, the entire pPS65-LACZ plasmid was labeled with [α-32P]dCTP for use as a hybridization probe. pPS65-LACZ lacks homology to any yeast genomic sequences, but in addition to LACZ will hybridize to pBR322-derived sequences in pGAL10-HO.

During HO-induction of strain tNR87 (Figure 5A), the linear cleavage product is detectable as early as 15 min. The deletion resulting from recombination between the two direct repeats of LACZ is initially detectable at 1.5 hr, consistent with the PstI digest (Figure 3A). Additional bands (int) might correspond to intermediates of the DSB repair process, such as χ structures, or other hybrid molecules. One such band, migrating above the 21-kb linear form, appears between 1 and 2.75 hr. In addition there is a band at approximately 17 kb, which is present from 30 min through about 3 hr. This band is approximately 4.5 kb smaller (the size of one copy of LACZ) than the parental plasmid and may represent a noncovalently closed intermediate in the formation of the deletion.

As expected for the inverted orientation plasmid (strain tNR86) the products of HO-induced recombination are the same size in undigested DNA as the original plasmid. There appears to be a loss of total material by the end of reaction, which agrees with the genetic result that 50% of the plasmid is lost during exposure to HO-endonuclease. Similarly to strain tNR87, a band which migrates above the 21-kb linear form appears at 1 hr, but is completely undetectable by 2.75 hr (Figure 5B). The smaller putative intermediate band seen in the direct repeat plasmid does not appear to be formed during HO-induction of the inverted repeat plasmid.

**Analysis of chromosomal integration of the LACZ duplications:** In order to compare our results more directly with other chromosomal events that have been studied, both plasmids were integrated on chromosome V at ura3-52. The XhoI 1.4-kb CEN4 fragment was deleted from plasmids pNR17 and pNR18, and the resulting plasmids, pNR27 (inverted orientation) and pNR28 (direct orientation), integrated into strain tNR85 to yield respectively, tNR102 (Figure 6, IIA) and tNR101 (Figure 6, IIA). An un repaired HO-induced chromosomal break might likely result in cell death (Klar, Strathern and Abraham 1984). However, previous results (Rudin and Haber 1988) showed that a similar HO-induced intrachromosomal repair event between flanking URA3 sequences was extremely efficient. When the 117-bp MATα cut site was embedded in non-yeast sequences, and located between the ura3-52 and URA3 genes, HO-induction almost always resulted in recombination between homologous URA3 regions, and loss of the internal sequences. Most of the time, a single ura3-52 locus was
DIRECT ORIENTATION

A. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LACZ</td>
<td>LEU2</td>
<td>URA3</td>
</tr>
</tbody>
</table>
```

conversion w/o exchange

B. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LACZ</td>
<td>LEU2</td>
<td>URA3</td>
</tr>
</tbody>
</table>
```

conversion w/ exchange

C. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LEU2</td>
<td>URA3</td>
<td></td>
</tr>
</tbody>
</table>
```

Ura deletion

D. 

```
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

not analyzed

3% Ura

Lac

INVERTED ORIENTATION

A. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LACZ</td>
<td>LEU2</td>
<td>URA3</td>
</tr>
</tbody>
</table>
```

conversion w/o exchange

B. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LEU2</td>
<td>LACZ</td>
<td></td>
</tr>
</tbody>
</table>
```

conversion w/ exchange

C. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LEU2</td>
<td>LACZ</td>
<td></td>
</tr>
</tbody>
</table>
```

Ura deletion

D. 

```
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

'leu' deletion

E. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LEU2</td>
<td>LACZ</td>
<td></td>
<td>URA3</td>
</tr>
</tbody>
</table>
```

Internal LACZ deletion?

F. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ura3-52</td>
<td>LACZ</td>
<td>LEU2</td>
<td>LACZ</td>
<td></td>
</tr>
</tbody>
</table>
```

other unidentified LACZ deletion

G. 

```
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```
The putative junction has not yet been confirmed by sequence analysis.

About 15 kb of DNA at their closest points.

The result of conversion without exchange. The cut-site in direct orientation, was converted to wild-type sequences, accompanied by a deletion to one copy of LACZ.

The deletion to one copy of LACZ, which results in deletion of the internal sequences (RUDIN and HABER 1988).

A cut by HO-endonuclease stimulates a cross-over between the direct repeats, ura3-52 and URA3, which results in deletion of the internal sequences (RUDIN and HABER 1988).

The cut-site, but did not fall into any of the above classes. These events were not pursued.
(95%) found to retain a parental configuration (Figure 6, IIB), while only 1 (5%) was the product of a reciprocal exchange (Figure 6, IIC), resulting in an inversion. Of the total inverted repeat events, these represent, respectively, 18% and 1%. This result dramatically contrasts with that obtained from the comparable CEN-containing plasmid, pNR17, where 50% reciprocal recombination was associated with the conversion event, but is consistent with the intrachromosomal constraint on crossing over associated with spontaneous gene conversion (Klein and Peters 1981; Jackson and Fink 1981). Of the colonies, 235/769 (31%) were Lac-, Ura-, again presumably resulting from a deletion/recombination event involving URA3 flanking sequences (data not shown; Figure 6, IID, Table 4A).

The remaining 50% (389/769) of the colonies from the above experiment (Table 4B), as well as 52 out of 56 examples from a separate induction of tNR101 remained Lac+ Ura+. Upon reinduction, no further conversion to Lac+ in 52/52 colonies was observed, indicating that reduced cutting by HO was not the reason for the apparent reduction in events restoring a functional Lac+ gene, and suggested that the cut was often repaired to an aberrant Lac+ structure. Preliminary Southern blot analysis (data not shown) has revealed at least four putative recombination events that have produced a Lac+Ura+ phenotype, all apparently the result of homologous exchange between different sets of other, smaller direct repeats in the vicinity of the HO cut. One event appears to involve deletion of LACZ::CS1 by recombination between LEU2 and the 300 bp of 5′ LEU2 sequences present in the CYC1 UAS::LEU2 promoter construction (Figure 6, IIE). In this case, a novel band with the predicted size (approximately 8 kb) and homology (LEU2 and URA3, but not LACZ, data not shown) replaced a LACZ-specific PstI band on a Southern blot. Another similarly sized but distinct band, of unexplained origin, was also sometimes observed when the LACZ-specific PstI band was missing. A third class of events also removed LACZ::CS1, but no corresponding novel bands have been detected. The fourth class of events were those in which LACZ::CS1 remained resistant to cutting with BclI, indicating that this site was not restored, yet no hybridization to a MA7a cut-site specific riboprobe was evident (data not shown). One possible explanation for this class is that the DSB repair took the form of a very small deletion involving small direct repeats close to the BclI site into which the cut-site had been inserted (Figure 6, IIF). A computer search has revealed that a perfect 8-bp repeat (AATGAATC) occurs at bases 1264–1277 and 1376–1389 of the LACZ sequence (Kalinina et al. 1983); the cut-site was inserted at the BclI site at base 1358.

**DISCUSSION**

**HO endonuclease stimulates DSB repair in a LACZ duplication system which results in gene conversion associated with exchange:** It is clear from the results presented, that a 117-bp fragment containing the HO endonuclease cut-site can stimulate DSB repair within a LACZ duplication in vivo. The efficiency and spectrum of HO-induced repair events in LACZ duplication plasmids are dependent on the relative orientations of the LACZ genes, but do not seem to be affected by the orientation of the cut-sites within LACZ. Additionally, we note that each of the constructions examined contains two 117-bp cut-sites in direct orientation, which accounts for the presence of doublets corresponding to the HO-cut fragments on the gels if noncoincident cutting is taking place. When probed with LACZ, most of the material was present in the bottom band of each doublet, indicating that cutting is in fact quite efficient, and that the presence of two cut-sites does not affect the outcome of the rest of the recombination event. The fact that the reactions are apparently independent of the cut-site orientation reinforces this conclusion. In addition, preliminary experiments using plasmids containing only a single 117-bp cut-site (J. Fishman and J. E. Haber, unpublished data) give results essentially identical to those presented here.

On a CEN plasmid, the HO-cut LACZ sequence is repaired using the unexpressed copy of LACZ approximately half the time, and is no longer constrained with regard to reciprocal recombination. In contrast, a similar intraplasmidic MAT system retains both the high repair efficiency and the constraint on crossing over of a normal mating-type switch (H. Friesen, B. Andrews, P. Sadowski, X. Wu and J. E. Haber, unpublished data). These data are consistent with an hypothesis that suggests that cis-acting sequences adjacent to MAT and its donors play a role in their efficient pairing and act to prevent exchange.

The deletion resulting from exchange in the direct repeat was observed about 80% of the time, both on plasmids and on the chromosome. This result does not seem different from the results of Nickoloff, Chen and Heffron (1986), who found that HO-induced recombination between direct repeats of the URA3 gene yielded deletions 52–67% of the time. However, these results contrast sharply with those of Ray et al. (1988), who used an apparently similar chromosomal construct involving repeats of the ADE4 gene. They found that only 10% of the gene conversions of ade4::HO cut site to ADE4 were accompanied by crossing over. The variation among these results may indicate that the proportion of crossing over associated with different gene-conversion events is strongly influenced by adjacent sequences. Indeed Ahn et al. (1988) have noted strong effects of adjacent
sequences on the proportion of spontaneous HIS3 gene conversions associated with crossing over.

The level of associated exchange in general spontaneous mitotic gene conversion ranges from 10 to 55% (reviewed in Esposito and Wagstaff 1981; Orr-Weaver and Szostak 1985) the lower range representing intrachromosomal events (Klein and Petes 1981; Jackson and Fink 1981). There are no known examples of either induced or spontaneous conversion associated exchange rate as high as 80% in a wild-type genetic background. These differences may reflect on the mechanism of spontaneous recombination events versus those induced by an HO-initiated double strand break.

A possible distinction between recombination events involving one or two ends of a DSB: Several key observations have been reported here. (1) HO-induced loss of plasmids containing inverted repeats of LACZ is significantly more frequent than of plasmids with direct repeats. (2) The proportion of gene conversions accompanied by crossing-over is much higher for the direct orientation than in the inverted orientation both in plasmids and on the chromosome. (3) When the LACZ genes are in inverted orientation, one strand exchange (inv-B) occurs decidedly earlier than the other (inv-A).

A number of models may be considered in order to explain these data. For instance, differences in the proportion of crossover products for the different orientations of LACZ might be attributed to a bias in the resolution of recombination structures. This explanation, however, fails to account in any simple way for the differences in plasmid recovery, or for the appearance of one of the products of a reciprocal exchange before the other.

Another possible explanation for the difference in the apparent exchange frequencies between the two different orientations of the LACZ genes involves the topology of the pairing interaction. In the direct repeat (pNR18), the homologous sequences are arranged with no space in between them, such that pairing involving both ends of the break (Figure 2, IC) might be less favorable than an invasion utilizing only one end (Figure 2, 1D and 1E). In the inverted orientation (pNR17), pairing of regions adjacent to the DSB with their intact homologous sequences can occur by a simple “hairpin” structure, such that conversion might have an equal chance of occurring with or without exchange. The pairing of both ends of the DSB with their homologous sequences in the direct repeat orientation appears to require a more complex folding of the DNA (Figure 2, IC), which may be hindered by the relatively short distance (4.5 kb) separating the cut end from its homologous site. In an experiment in which a 2-kb HindIII fragment of λ DNA was inserted between the direct repeats as a spacer (Rudin 1987), it was not possible to demonstrate a change in the proportion of conversions that occurred without crossing over. Topology may also play a role in determining whether either end of a DSB interacts with intact, homologous sequences, thus determining the proportion of plasmids that are repaired or lost.

We suggest that a model based on differential initiation of recombination best explains all of our data. An HO endonuclease cut produces two potentially recombinogenic ends. One or both of these ends may theoretically participate in recombination. It is possible that when only one end becomes involved in recombination, the event proceeds along a pathway that excludes converting an intermediate into one produced by the coordinate involvement of both ends. In either LACZ duplication plasmid, the coordinate participation of both ends of the break would be expected to produce a viable gene conversion product, accompanied by an associated exchange of flanking markers 50% of the time (Figure 2, IC and IIC). In the case of the inverted orientation plasmid, either of the events involving a single end would produce a linear product, resulting in loss of the plasmid (Figure 2, IID and IIIE). However, in the direct repeat plasmid, either single-ended interaction would produce a closed circular product (Figure 2, 1D and 1IE), but only the structure produced by joining the upstream half of the HO-cut LACZ to the downstream half of the promoterless copy (Figure 2, 1D) yields a plasmid containing ARS and CEN sequences that can replicate in yeast: the opposite event (Figure 2, 1E) will be lost. Thus the case of the direct orientation, there are two ways to produce deletions, either by crossing over associated with two-ended events or from one of the two single-ended pathways. Assuming these pathways occur with equivalent frequencies, one would expect that about ¼ of the Lac+ plasmids would be deletions; in fact 80% are. The suggested scheme also accounts for the higher degree of plasmid loss from the inverted orientations, as neither of the single-ended events will be retained. Plasmid loss will occur in either case if neither cut end interacts with its homologous sequence.

Events seen during physical monitoring of the inverted repeat plasmid support the hypothesis that single-ended repair events producing linear products are lost. At the end of the experiment, there appear to be essentially equivalent amounts of the expected Lac+ products of gene conversional repair of the HO endonuclease cut in LACZ::GS2, as evidenced by the relative abundance of the A, inv-A and inv-B PstI fragments (Figure 4). The amount of the other expected product (B) is not resolved from the band derived from plasmids that were not cleaved by HO endonuclease. These results are consistent with the
genetics, in which all of the stable products were Lac\(^*\) and 50% had undergone an associated exchange. **inv-A** and **inv-B** each represent one half of a reciprocal cross-over structure (Figure 2, IID and IIE). However, **inv-B** appears much earlier in the time course than **inv-A**.

The fact that **inv-B** is not in excess over **inv-A** at the end of the time course can be interpreted in two ways. The early appearance of **inv-B** might be viewed as an intermediate in a sequential reaction that eventually involves the second end of the DSB. In the simplest representation of this reaction, however, no homology remains between the two unjoined ends (Figure 2, IID); direct ligation remains the only way to rejoin the circle. It should also be noted that **inv-A** and **A**, which are alternative restriction fragments carrying the Lac\(^*\) product, both appear later and at the same time. This argues that common intermediates, leading to either parental or recombined molecules, are resolved with the same kinetics.

Thus, we suggest that the early appearance of **inv-B** may represent the formation of linear molecules resulting from invasion of only one of the two cut ends (Figure 2, IID) but that they are not intermediates on the pathway to the final inversion product. These products of a "one-ended" event yield **inv-B**, but would be degraded by the end of the experiment, and would also be lost genetically. The results with LACZ sequences in direct orientation are also in accord with this model. The appearance of the deletion product follows the same, more rapid kinetics of the appearance of the equivalent single-ended event in the case of the inverted orientation. Moreover, its appearance is not accompanied by the formation of the expected reciprocal product, a 4.5-kb circle of LACZ sequences. Thus, from a comparison of the genetic and physical data for plasmids with LACZ segments in either inverted or direct orientation, we propose that single-ended recombination structures are not necessarily intermediates, but may sometimes represent a separate pathway of recombination.

**Detection of intermediates in recombination:** Concurrent with the formation of the stable genetic products, other transient species of recombination might formally be predicted. In the inverted repeat plasmid, both products of reciprocal recombination are recovered, both molecularly and genetically, in equal amounts. In the direct repeat, the reciprocal product of the deletion is a 4.5-kb closed circular molecule containing \(\Delta P-LACZ\) (Figure 2, IH) which does not contain an ARS sequence and would be expected to be lost (diluted out) from a colony of growing cells. Nevertheless, one would expect this product to be present in stoichiometric amounts immediately after the recombination events were completed. No such band was detected in SOUTHERN blots, even at longer exposures (data not shown). This might suggest that a majority of deletions are formed in a non-reciprocal fashion from one-ended events. This product would also be expected from a non-reciprocal event in which the downstream half of the HO-cut LACZ recombined with the intact \(\Delta P-LACZ\) segment (Figure 2, IE). This possibility will be discussed below.

In both the direct and inverted orientations, transient linear molecules might be produced by the non-reciprocal event resulting from the invasion of one of the two cut ends. In the direct repeat, invasion of the intact \(\Delta P-LACZ\) sequence by the downstream end adjacent to the 5' half of the HO-cut LACZ (Figure 2, ID), could produce a transient 4.5-kb linear PstI fragment. No such band was visible in Figure 3, even with longer exposures. Similarly, in the inverted repeat, if a completed exchange event occurs by joining the upstream half of the promoterless LACZ, to the downstream half of LACZ::CS\(_2\), a novel PstI fragment of 1.7 kb might be predicted (Figure 2, IID); again a fragment of this length was not detectable even on a higher percentage gel designed specifically to detect fragments in this size range (data not shown). In other words, it was not possible to detect any of the linear products that might formally be predicted from the invasion and completion of recombination of a single end of the DSB. This might simply be due to extreme heterogeneity in the restriction fragments with HO-cut ends due to variation in the crossover site; fragments including the two ends which had already been rejoined would not be affected. This is testable by the construction of substrates containing heterologies designed to block branch migration. In addition, the undetectable fragments might be rapidly degraded.

Alternatively, these recombination events may take a completely different pathway, for instance resolution by replication, in which the aforementioned intermediates are never formed. Bands which might potentially correspond to higher order recombinational intermediates were evident both in time courses in which the DNA had not subsequently been cleaved with restriction enzymes (Figure 5), and in PstI-digested DNA which had been run on a higher percentage gel (data not shown).

**Both ends of the HO-induced cut are not equivalent:** There is no evidence, in either plasmid orientation, either physically or genetically, for a non-reciprocal event that involves only the cut end adjacent to the downstream half of LACZ (Figures 2, IE and IIE). As this apparent asymmetry in one-ended events is not attributable to the orientation of the HO cut-sites, the difference in reactivity of the two ends might either be due to the arrangement of the LACZ genes on the circular plasmid, or a structural feature of the LACZ gene that favors invasion of the cut end adjacent to the 5' half. Since the CYCI UAS enhancer/promoter
region adjacent to \textit{LACZ::CS} \textsubscript{a} gene permits transcription in both lactate and galactose (Guarente, Yocum and Gifford 1982), it is possible that continued transcription from the 5' end keeps this half of the gene in an "open" state after \textit{HO} endonuclease cleavage, facilitating strand invasion. This hypothesis is testable with the appropriate plasmid constructions.

**Implications of the proposed model for chromosomal events:** When the \textit{LACZ} duplications were present on the chromosome, the viability of colonies resulting from single \textit{HO}-induced cells was not observably different than those from uninduced cells. Thus, as expected from other chromosomal deletion/recombination events that have been examined (Rudin and Haber 1988), repair by flanking region recombination is also fairly efficient in the \textit{LACZ} chromosomal duplication system.

When \textit{HO}-induced recombination was examined in inverted \textit{LACZ} repeats, located 10 kb apart on the chromosome, a minority of events results in \textit{Lac} \textsuperscript{+} gene conversions, and of those that did, 95% occurred without reciprocal exchange. This is a very different result than was seen for the analogous plasmid, pNR17, and might reflect the increase in distance between the repeats, the particular sequences present, or their arrangement relative to each other. In light of the fact that on a plasmid, these same sequences show a high level of exchange, this lack of chromosomal crossing-over is probably not a property of the \textit{HO} cut-site itself, but specific to this construction. We suspect that deletion events involving at least two different sets of direct repeats in the vicinity (\textit{URA3, LEU2}) take place by a more favorable mechanism than those events which would lead to a \textit{LACZ} conversion between inverted repeats, especially associated with a crossover. This is suggested by the fact that nearly all of the aberrant events appear to have involved recombination between direct repeats, thus could proceed via a mechanism requiring only one end of homology, as discussed previously.

An interaction involving only one end of a DSB can produce a viable product in the context of a direct repeat, but is an abortive event in the case of an inverted repeat. In a chromosomal situation, the abortive event would lead to chromosome loss. This may have implications regarding results obtained for both intra- (Klein and Petes 1981; Jackson and Fink 1981) and interchromosomal (Haber and Hearn 1985) repeats in the presence of the \textit{rad52} mutation as well as spontaneous mitotic intergenic recombination (Esposito \textit{et al.} 1984). In the first two cases, the total number of events was drastically reduced, but a residual pool of gene-conversions associated with a crossover remained. Haber and Hearn (1985) specifically showed that intragenic recombination in \textit{rad52} diploids was frequently (35%) accompanied by the formation of 2N-1 diploids in which the homolog carrying the prototrophic recombined \textit{HIS4} gene was associated with a crossing over and the other participating homolog was lost. These events can be explained by single-ended recombination events involving one homolog containing a DSB and an intact chromosome, analogous to the events depicted in Figure 2, IID. Similar chromosome losses accompanying gene conversions have also been observed at lower (10%) frequencies in \textit{Rad} \textsuperscript{+} diploids (Campbell and Fogel 1977). The much higher proportion of such apparent one-ended events that we describe here for \textit{HO}-induced Lac \textsuperscript{+} recombinants in \textit{Rad} \textsuperscript{+} cells may reflect our use of centromere-containing plasmids to examine intrachromosomal recombination rather than interchromosomal events involving normal chromosomes. We suggest that the \textit{RAD52} gene product, which is thought to be essential for DSB repair (Resnick and Martin 1976) might play a role in holding the two ends of a DSB together, while in its absence, at least some proportion of single-ended events could proceed normally. It is not known what proportion of spontaneous recombination is initiated by DSBs.

In the inverted \textit{LACZ} integration described above, where interactions may take place over a total of 28 kb, it is also possible that higher order chromosome structure may come into play in determining thermodynamically favorable pairing structures.

We are grateful to Fred Heffron and Rich Kostriken for providing the plasmid containing the 117-bp \textit{HO} cut-site, and the \textit{TRP1-GAL10-HO} plasmid. We thank Michael Esposito, Rodney Rothstein, Michael Rossbash, Michael Lichten and Paul Metts for helpful discussion and suggestions concerning this manuscript, and Charles White for sequencing the duplicated cut-site. This work was supported by National Institutes of Health grant GM20056 and by National Science Foundation grant DGB8409086. N.R. was a predoctoral trainee of Training Grant GM07122. E.S. was the recipient of an undergraduate summer research award from the Genetics Society of America.

**LITERATURE CITED**


CYCI hybrid yeast promoter identifies the GAL4 regulatory
region as an upstream site. Proc. Natl. Acad. Sci. USA 79:
7410–7414.

HABER, J. E., 1983. Meiotic recombination in Saccharolyces cerasii
that accompany MATa × MATα matings. Genetics 99:
388–403.


NICKOLOFF, J. A., E. Y. CHEN and F. HEFFRON, 1986. A 24-base-
pair DNA sequence from the MAT locus stimulates intergenic
recombination in yeast. Proc. Natl. Acad. Sci. USA 83:
7831–7835.

OBR–WEAVER, T. L., and J. W. SZOSTAK, 1983. Yeast recombina-
tion: the association between double-strand gap repair and

OBR–WEAVER, T. L., and J. W. SZOSTAK, 1985. Fungal Recombina-

Yeast transformation: a model system for the study of recombi-

OSLEY, M. A., and L. HEREFORD, 1982. Identification of a se-
quence responsible for periodic synthesis of yeast histone 2A

RAY, A. I., SIDDALL, A. L. KOLODKIN and F. W. STAHL, 1988. Intrachro-
nosomal gene conversion induced by a DNA double-
strand break in Saccharomyces cerevisiae. J. Mol. Biol. 201:
247–260.

RICK, M. A., 1975. The repair of double strand breaks in
DNA: a model involving recombination. J. Theor. Biol. 59:
97–106.

RICK, M. A., and P. MARTIN, 1976. The repair of double-
strand breaks in the nuclear DNA of Saccharomyces cerasii

Enzymol. 101: 202–211

RUDIN, N., 1987. Co-operating regions involved in mating-type inter-
conversion in the yeast Saccharomyces cerevisiae. Ph.D. thesis,
Brandeis University.

chromosomal breaks in Saccharomyces cerevisiae by recombi-
nation between flanking homologous sequences. Mol. Cell. Biol. 8:
3918–3928.

Genetics, Laboratory Manual. Cold Spring Harbor Laboratory,
Cold Spring Harbor, N.Y.

San Francisco.

DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:
503–517.

STRATHORN, J. N., A. J. S. KLER, J. B. HICKS, J. A. ABRAHAM, J. M.
IVY, K. A. NASMYTH and C. MCGILL, 1982. Homothallic
switching of yeast mating type cassettes is initiated by a double-

WEIFFENBACH, B., and J. E. HABER, 1981. Homothallic mating
type switching generates lethal chromosome breaks in rad52

Communicating editor: N. KLECKNER.