

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

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### 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; KOSTRIKEN *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,

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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 *LACZ* gene on a centromere-containing plasmid such that no functional  $\beta$ -galactosidase was produced. This construction also includes another promoterless copy of *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<sup>+</sup> 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 physical analysis has recently been carried out on *MAT* switching (CONNOLLY, WHITE and HABER 1988; D. RAVEH and J. N. STRATHERN, personal communication). Like *MAT* switching, *HO*-induced conversion of *LACZ* sequences was found to be slow, requiring as much as 60 min from the time of *HO* endonuclease cleavage to completion of DSB repair. Possible explanations for the slowness of this event have been discussed in CONNOLLY, WHITE and HABER 1988; RUDIN and HABER 1988. In addition, we find evidence for two temporally distinct events, indicating that repair and recombination initiated by a DSB may not always proceed with the concerted participation of both cut ends.

## MATERIALS AND METHODS

**Plasmid construction:** Plasmid pRK113 (a gift from R. KOSTRIKEN) consists of a 117-bp *HincII*-*BglII* fragment surrounding the *MATaHO* cut-site inserted into *Bam*HI-*HincII* cut pUC9 (KOSTRIKEN and HEFFRON 1984). The *HincII* junction was changed to a *Bam*HI site using linkers, and the fragment released using *XhoII* which will cut at a *Bam*HI site as well as a *Bam*HI/*BglII* junction. The *Eco*RI *ARS* fragment was removed from plasmid pLG199 Leu (a gift from L. GUARENTE) (GUARENTE, YOCUM and GIFFORD 1982) in which the *LACZ* gene is under control of the *CyC1 UAS*, and the *Tth* IIII site changed to a *Hind*III site using linkers; The construction also contains a 300-bp segment of the *LEU2* gene promoter region ('*leu2'*) between *CYC1 UAS* and the *LACZ* sequence. The *XhoII* *MATa* cut-site fragment was inserted into the *Bcl*I site of *LACZ* and both orientations of the cut-site relative to *LACZ* were obtained. In both cases it was subsequently discovered that two copies of the cut-site,

in direct orientation, had been inserted (*LACZ::CS<sub>2</sub>*). In the plasmid used for the construction of plasmids pNR17 and pNR18, the *BglII*-*HincII* *MATa* cut-sites were oriented with the *MAT Z1* region (originally *HincII*) closest to the 5' side of *LACZ* (orientation I). In the predecessor to plasmids pNR50 and pNR51, the cut-sites were oriented with the *Ya* regions (originally *BglII*) closest to the 5' side of *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 *LACZ* ( $\Delta$ P-*LACZ*) was obtained by removing a H2A-H2B histone promoter from a *LACZ* fusion (plasmid pCAL $\Delta$ 16, a gift from M. A. OSLEY) (OSLEY and HEREFORD 1982). This plasmid also contains a 1.4-kb *XhoI* *CEN4* fragment embedded in the 3' untranslated region of a 4.1-kb *LEU2* gene, as well as an *ARS* sequence derived from the histone genes.

*Hind*III fragments from either of the two different cut-site orientation plasmids, containing *URA3*, *CYC1 UAS*, and *LACZ::CS<sub>2</sub>*, were inserted into a *Hind*III site at the 5' end of the promoterless *LACZ* copy. Bacterial transformants were selected for Ura<sup>+</sup> and Leu<sup>+</sup> and screened for the relative orientation of the two *LACZ* genes. Plasmids pNR17 (cut-site orientation I, Figure 1A) and pNR50 (cut-site orientation I, Figure 1C) contain a duplication of *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 *LACZ*.

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

pSE271::*GAL10-HO* was a gift from F. HEFFRON laboratory (NICKOLOFF, CHEN and HEFFRON 1986), which will be referred to as p*GAL10-HO*, also contains *CEN4* and *TRP1 ARS1*.

pSp65-*LACZ* (a gift from the M. WORMINGTON laboratory) was constructed by inserting the *LACZ* gene into the multiple cloning site of pSP65. Either the entire plasmid, or a 2.5-kb *PvuII* gel-isolated fragment of *LACZ* was nick-translated with [ $\alpha$ -<sup>32</sup>P]dCTP for use as a hybridization probe.

**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 *MATa* allele of R126 (*ho HMLa MATa hmr-3 $\Delta$  leu2-3,112 ura3-52 trp1 thr4 GAL*) with *mat $\Delta$ ::LEU2* from plasmid pJH124. The *hmr-3 $\Delta$*  deletion of *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 p*GAL10-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 *ura3-52* in strain tNR85.

**Media:** Cells were grown in YEP medium (1% w/v yeast extract, 2% w/v Bacto-peptone) supplemented with dextrose (2% w/v, YEPE) 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.

**Scoring of *HO*:** When necessary, *HO* was scored by taking advantage of the strains U60 and U90 as described in WEIFFENBACH and HABER (1981).

**Scoring for  $\beta$ -galactosidase activity in yeast:** We have

TABLE 1

## Yeast strains

DBY745	<i>ho HML<math>\alpha</math> leu2-3,112 MAT<math>\alpha</math> HMR<math>\alpha</math> MAL2 ura3-52 ade1-110</i>
R167	<i>ho HML<math>\alpha</math> leu2 mat<math>\Delta</math>::LEU2 hmr<math>\Delta</math>3 mal2 ura3-52 thr4 trp1</i>
tNR85	R167[pGAL10-HO]
tNR86	tNR85[pNR17] (inverted <i>LACZ</i> duplication, cut-site orientation I)
tNR87	tNR85[pNR18] (direct <i>LACZ</i> duplication cut-site orientation I)
tNR118	tNR85[pNR50] (inverted <i>LACZ</i> duplication, cut-site orientation II)
tNR119	tNR85[pNR51] (direct <i>LACZ</i> duplication, cut-site orientation II)
tNR101	tNR85[pNR27] (inverted <i>LACZ</i> duplication, integrated at <i>ura3-52</i> )
tNR102	tNR85[pNR28] (direct <i>LACZ</i> duplication, integrated at <i>ura3-52</i> )
U60	<i>ho HML<math>\alpha</math> mata1 HMR<math>\alpha</math> ade2 leu1 ura3 cmt</i>
U90	<i>ho HML<math>\alpha</math> mata1 HMR<math>\alpha</math> ade2 leu1 ura3</i>

developed a new method for scoring  $\beta$ -galactosidase activity in yeast. An aliquot of 0.2 ml of 10 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -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 3MM 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  $5 \times 10^5$  cells/ml. The cultures were grown at 30° with vigorous aeration to a final cell density of  $1-3 \times 10^7$  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 ( $5-10 \times 10^7$  cells) were collected onto a 0.45- $\mu$ m pore nitrocellulose filter by filtration, and washed with 1-2 ml 10 mM Tris-HCl, 1 mM EDTA, pH 7.9 (TE). Cells were washed from the filter with 0.5 ml of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2% NaDodSO<sub>4</sub> 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  $2 \times 15$  sec and then cooled on ice. The aqueous phase was subsequently extracted with an equal volume of TE-saturated phenol/chloroform and the DNA precipitated with an equal volume of isopropanol. The precipitate was pelleted, dissolved in TE, treated with pancreatic RNase (20  $\mu$ g/ml, 30 min, 37°C) and Proteinase K (50  $\mu$ g/ml, 30 min, 37°C). The RNase and Proteinase K were removed by phenol/chloroform extraction, and the DNA recovered by precipitation with an equal volume of isopropanol in the presence of 0.3 M NaOAc. The DNA was pelleted, resuspended in TE and ethanol precipitated. The purified DNA was washed in 70% ethanol, 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 radioactively-labeled [ $\alpha$ -<sup>32</sup>P]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 *Bgl*II-*Hinc*II fragment of *MAT $\alpha$*  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 *MAT $\alpha$*  *HO* endonuclease cut-sites at the *Bcl*I site (*LACZ*::*CS*<sub>2</sub>, MATERIALS AND METHODS). Two plasmids were constructed that additionally contained a second, promoterless, copy of *LACZ* ( $\Delta$ P-*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*::*CS*<sub>2</sub> 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<sup>+</sup> 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.

tNR86 and tNR87 were first analyzed genetically after galactose induction of *HO* endonuclease. Prior

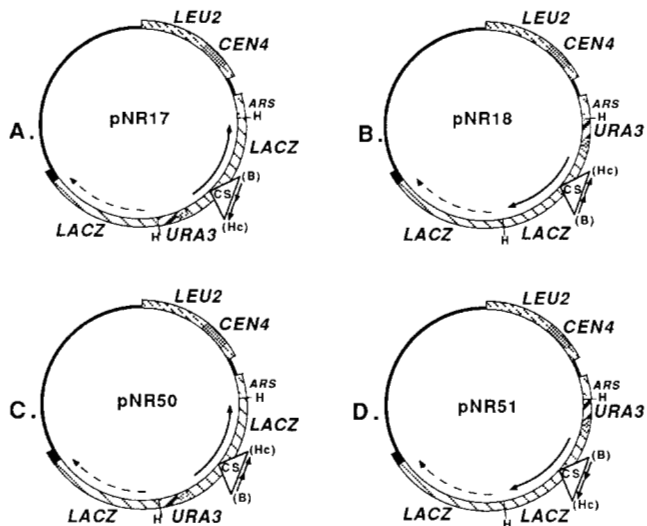


FIGURE 1.—Structure of the *LACZ* duplication plasmids. Plasmid pNR17 contains the inverted *LACZ* duplication and the *HO*-endonuclease cut site in orientation I (see MATERIALS AND METHODS). Plasmid pNR18 contains the direct *LACZ* duplication, and the cut-site in orientation I. Plasmids pNR50 and pNR51 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 *CYC1 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, *Bgl*III; Hc, *Hinc*II; H, *Hind*III. ( ) indicates that the site was destroyed in cloning. CS indicates the pair of 117-bp *MATa* cut-sites.

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*<sup>+</sup> 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. Cells microscopically observed to be in G<sub>1</sub> phase (unbudded) were separated from the population, grown into colonies and then replica-plated to plates lacking either uracil or tryptophan, or containing X-gal. Cells containing a *LACZ* plasmid were Ura<sup>+</sup>, and those containing the *GAL10-HO* plasmid, Trp<sup>+</sup>. Gene conversions resulting in wild type  $\beta$ -galactosidase activity were recognized on X-gal plates as blue colonies; cells which had lost the *LACZ* plasmid, either due to plasmid instability, or to an unrepaired cut, were identified as Ura<sup>-</sup>. In a separate experiment, it was found that both the parental constructions and *HO*-induced *Lac*<sup>+</sup> derivatives of the *LACZ* plasmids are lost at only about 1% per generation (data not shown), hence *HO*-specific loss is easily identifiable above this low background. The results in Table 2 are corrected for loss of p*GAL::HO*, which ranged from 18–22% by the end of the 8-hr time course. tNR86 (inverted orientation) showed an increase in loss of the *LACZ* duplication plasmid through 8 hr, at which point 22% of the cells

TABLE 2  
Time course<sup>a</sup> of *LACZ::CS*<sub>2</sub> plasmids

Time (hr)	Strain	Trp <sup>+</sup>	Trp <sup>+</sup> Ura <sup>+</sup>	Trp <sup>+</sup> Ura <sup>+</sup> Lac <sup>+</sup>
0	tNR86 <sup>b</sup>	575/613 (94%)	516/608 (85%)	0/516 (<1%)
	tNR87 <sup>c</sup>	353/356 (99%)	298/329 (90%)	0/298 (<1%)
1	tNR86	43/46 (93%)	32/46 (70%)	4/32 (13%)
	tNR87	62/67 (92%)	54/67 (81%)	21/54 (39%)
3	tNR86	52/57 (91%)	23/57 (40%)	7/23 (30%)
	tNR87	30/35 (86%)	27/35 (77%)	19/27 (70%)
8	tNR86	42/51 (82%)	11/51 (22%)	11/11 (100%)
	tNR87	29/37 (78%)	20/37 (54%)	18/20 (90%)

<sup>a</sup> Galactose induction was performed as described in MATERIALS AND METHODS. For all points except 0 hr, individual unbudded (G<sub>1</sub>) 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 G<sub>1</sub>.

<sup>b</sup> Strain tNR86 contains plasmid pNR17 (inverted repeat plasmid).

<sup>c</sup> Strain tNR87 contains plasmid pNR18 (direct repeat plasmid).

retained both plasmids; the proportion of cells that were *Lac*<sup>+</sup> 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*<sup>+</sup>.

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 G<sub>1</sub> 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 micromanipulated cells, was due to the small sample size in the G<sub>1</sub> experiment. All Ura<sup>+</sup>, Trp<sup>+</sup> colonies were *Lac*<sup>+</sup>, indicating that a precise repair event had taken place to restore functional  $\beta$ -galactosidase activity.

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

**TABLE 3**  
**Fate of *LACZ::CS<sub>2</sub>* plasmids following an 8-hr galactose induction<sup>a</sup>**

A. Retention of plasmids		
	Ura <sup>+</sup> Trp <sup>+</sup>	Ura <sup>+</sup> Trp <sup>+</sup> Lac <sup>+</sup>
tNR86 <sup>b</sup>	160/368 (43%)	160/160 (100%)
tNR118 <sup>c</sup>	705/1616 (43%)	705/705 (100%)
tNR87 <sup>d</sup>	758/1279 (59%)	758/758 (100%)
tNR119 <sup>e</sup>	758/1380 (55%)	758/758 (100%)
B. Association of exchange with conversion <sup>f</sup>		
	Structure of plasmid	
pNR17 (inverted)	Parental	Inversion
	17/33 (52%)	16/33 (48%)
pNR18 (direct)	Parental	Deletion
	10/59 (17%)	49/59 (83%)

<sup>a</sup> 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<sup>+</sup> Trp<sup>-</sup> Lac<sup>-</sup>, since some had lost the *TRP1 ARS1 GAL10-HO* plasmid subsequent to the repair event.

<sup>b</sup> Strain tNR86 contains plasmid pNR17 (inverted repeat plasmid, cut-site orientation I).

<sup>c</sup> Strain tNR118 contains plasmid pNR50 (inverted repeat plasmid, cut-site orientation II).

<sup>d</sup> Strain tNR87 contains plasmid pNR18 (direct repeat plasmid, cut-site orientation I).

<sup>e</sup> Strain tNR119 contains plasmid pNR51 (direct repeat plasmid, cut-site orientation II).

<sup>f</sup> DNA from subclones of independent Lac<sup>+</sup> 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/MATα* 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 G<sub>1</sub> 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<sup>+</sup> (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 [ $\alpha$ -<sup>32</sup>P]-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<sup>+</sup> derivatives from galactose-induced G<sub>1</sub> 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  $\chi^2 = 12$ ,  $P < 0.05$ ) (Table 3B). DNA samples from 10 Lac<sup>+</sup> 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 *BclI* site was restored in all of these Lac<sup>+</sup> 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 *Yα* 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 inverting 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<sup>+</sup>, 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<sup>+</sup>. 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-

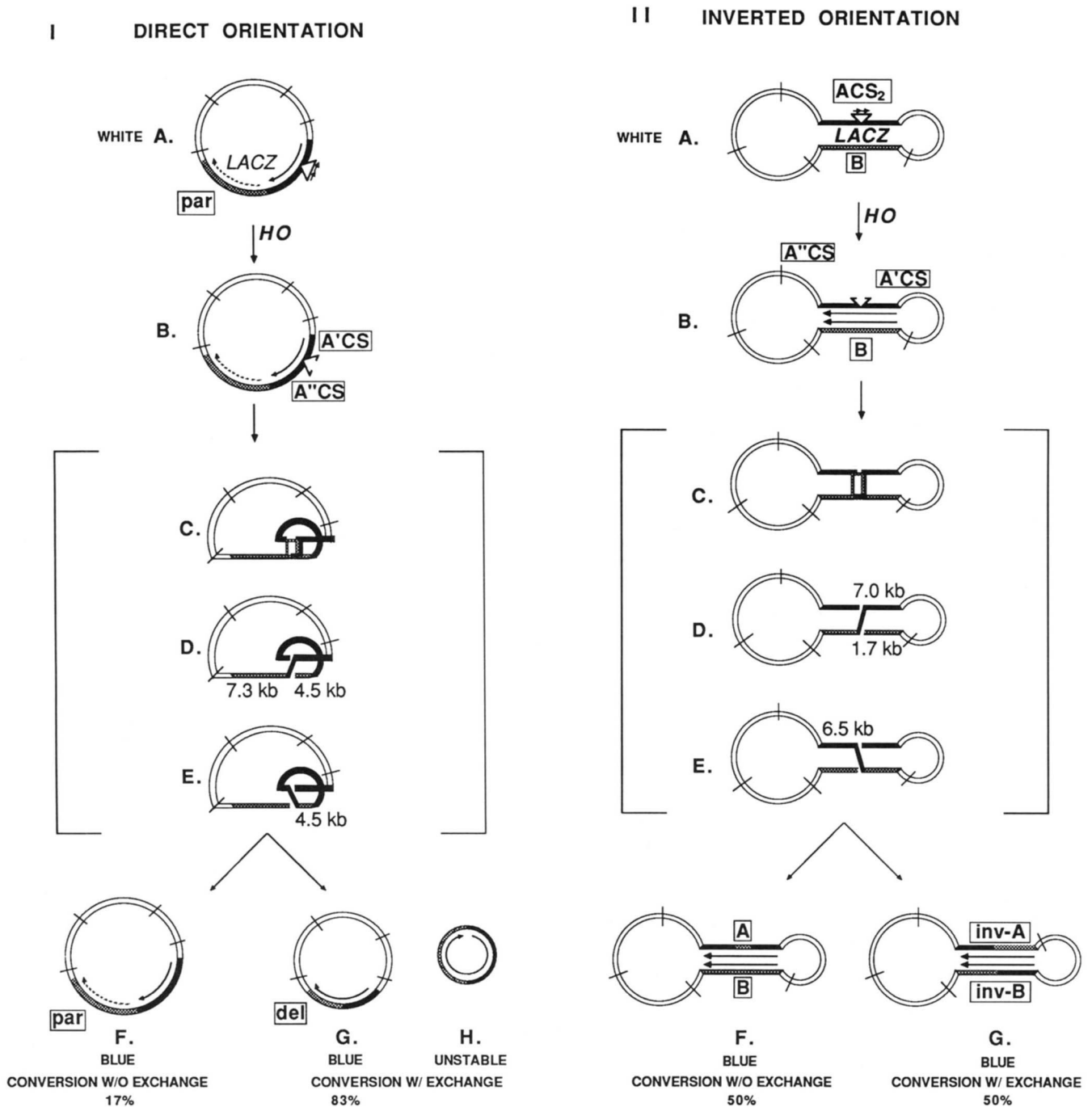


FIGURE 2.—Products of *HO*-induction of the *LACZ* duplication plasmids. tNR86 (containing pNR17, Figure 1A) and tNR87 (containing pNR18, Figure 1B) were induced with galactose as described in MATERIALS AND METHODS. After cutting by *HO* endonuclease, each plasmid is either lost (pNR17, 57%; pNR18, 41%), or undergoes gene conversion to Lac<sup>+</sup> 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::CS<sub>2</sub>* 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::CS<sub>2</sub>* 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. **IIA**, 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::CS<sub>2</sub>*. **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 *Pst*I 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-sites in orientation I. When the DNA was cut with *Pst*I (Figure 2, I) and probed with a nick-translated 2.5-kb *Pvu*II 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 slow, 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 *Pst*I 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

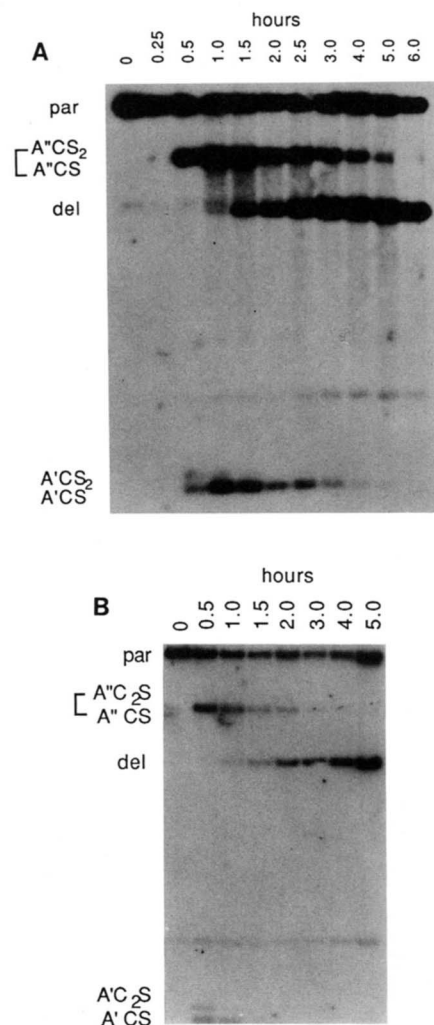


FIGURE 3.—Physical monitoring of DSB repair in a direct *LACZ* duplication. **A**, Strain tNR87 (cut-site orientation I) was galactose-induced as described in MATERIALS AND METHODS, and DNA extracted from 11 samples taken 6 hr. The DNA was cut with *Pst*I, run on a 0.5% agarose gel at 40 V for 24 hr, transferred to nitrocellulose membrane, and probed with gel isolated, nick translated *LACZ* fragment. The *Pst*I digest produces only one parental band (**par**), containing both copies of *LACZ* (11.8 kb). Both fragments resulting from the *HO* endonuclease cut were seen at 30 minutes (8.8 kb, **A"CS** and 3.0 kb, **A'CS**), although the higher molecular weight band was not resolved from the parental molecule. The small amount of non-exchange conversion product, i.e. the parental plasmid minus the cut-sites, is too large to be resolved from the starting material on these gels. The deletion band (7.3 kb, **del**) becomes visible at about 1.5 hr. **B**, The same experiment was performed with strain tNR118 (cut-site orientation II). The light band towards the bottom of the gel results from a small amount of pBR322 contamination of the gel-isolated *LACZ* probe, which hybridizes to sequences in the *GAL::HO* plasmid. The increase in intensity across the time course is proportional to the amount of total DNA.

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<sup>+</sup> 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

(Figure 2, IG). 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 *Pst*I (Figure 2, II). The Southern blot of the reaction time course is shown in Figure 4A. Lane 1 (0 min) shows the parental *Pst*I fragments: the upper band contains the copy of *LACZ* interrupted by the cut-sites (designated **ACS<sub>2</sub>**, 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<sup>+</sup> without exchange, and is 234 bp smaller than **ACS<sub>2</sub>** due to conversional loss of the cut-site sequences. Band **B** remains unchanged and cannot be distinguished from that of the parental plasmid. The *Pst*I 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''CS<sub>2</sub>**, 4.7 kb; **A''CS**, 4.5 kb; **A'CS<sub>2</sub>**, 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 *Pst*I 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  $\Delta$ P-*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

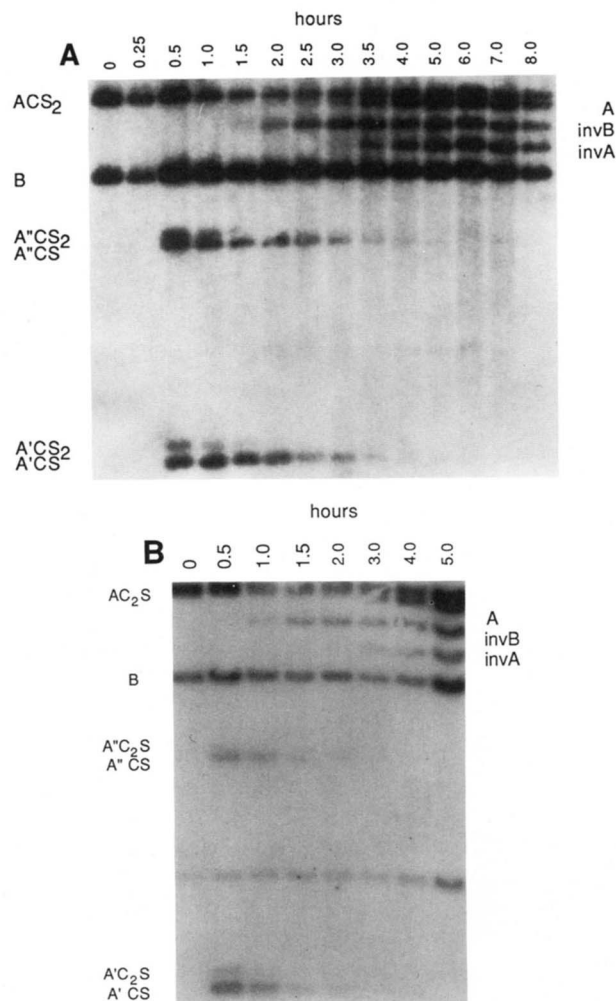


FIGURE 4.—Physical monitoring of DSB repair in an inverted *LACZ* duplication. **A**, Strain tNR86 (cut-site orientation I) was galactose-induced as described in MATERIALS AND METHODS, and the DNA extracted from 14 samples taken up to 8 hr. The DNA was cut with *Pst*I, electrophoresed on a 0.5% agarose gel at 40 V for 24 hr, transferred to nitrocellulose membrane and probed with gel-isolated, nick-translated *Pvu*II *LACZ* fragment. Lane 1 (0 min) shows the parental fragments: the upper band contains the copy of *LACZ* interrupted by the cut-sites (**ACS<sub>2</sub>**, 7.5 kb), and the bottom band the promoterless copy (5.9 kb). All of the predicted products are visible in equimolar quantities by 5 hr. Bands **A** (7.3 kb) and **B** (5.9 kb) represent the conversion without exchange; band **A** is 234 bp smaller than **ACS<sub>2</sub>** due to loss of the cut-site sequences, while band **B** remains unchanged. The *Pst*I fragments produced by the inverted products are marked as **inv-A** (7.0 kb) and **inv-B** (6.5 kb). The first evidence of cutting was seen at 30 min (bands **A'CS<sub>2</sub>** (3.2 kb), **A'CS** (3.0 kb); **A''CS<sub>2</sub>** (4.7 kb), **A''CS** (4.5 kb)). The doublet observed for each of the cut bands is presumably the result of non-coincident cutting of the two *HO* cut-sites. At first appearance of the cut a majority of the molecules appear to have already been cut at both sites, and by 1 hour, almost all of the cut material was in the lower of the two bands of each doublet. **B**, The same experiment was performed with strain tNR119 (cut-site orientation II).

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.



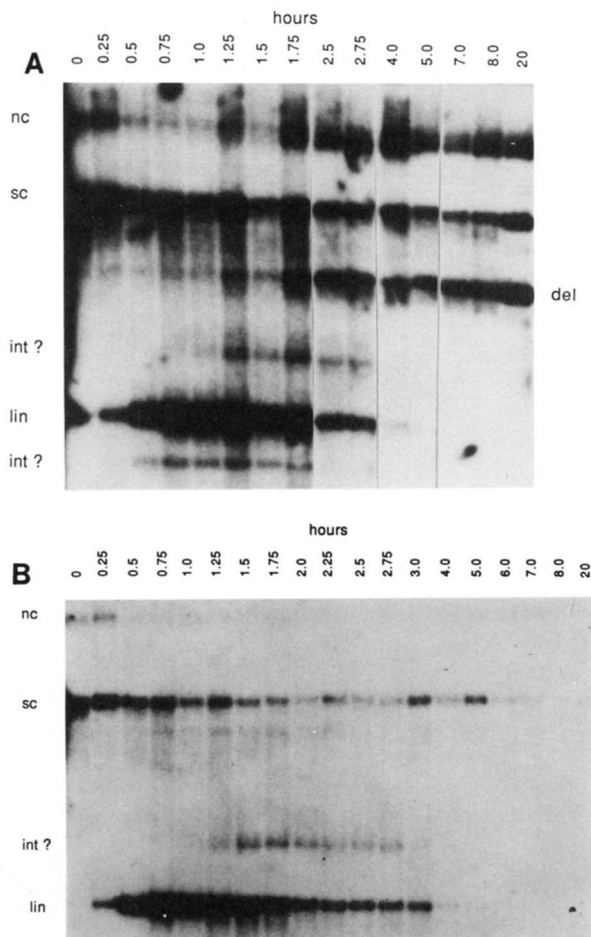


FIGURE 5.—Southern analysis of undigested DNA from time courses of direct and inverted *LACZ* duplication plasmids. **A**, DNA from a 20-hr time course of tNR87, that was uncut by any restriction enzyme, was run on a 0.5% agarose gel at 40 V for 48 hr, transferred to nitrocellulose membrane, and probed with nick-translated pSP65-*LACZ*. The probe is not homologous to any genomic yeast sequences. In this instance, the unreacted plasmid, pN18, runs as a supercoiled circle (sc), and the product of an *HO* endonuclease cut (lin), as a linear molecule. The linear *HO*-cut molecule is detectable as early as 15 min (lane 2), and under these electrophoresis conditions, migrates ahead of the 21.5-kb supercoiled plasmid, and its nicked circular counterpart (nc). Two additional bands (int), might correspond to intermediates of the double strand break repair process. The light band present in the same location as the deletion in Figure 5A, but present at the zero time point as well, appears to be a small amount of the nicked circular form of the *GAL10-HO* plasmid. **B**, Uncut DNA from a 20 hr time course of strain tNR86 shows a similar pattern, except that the inverted orientation plasmid does not produce any novel product bands in undigested DNA, since the inversion is detectable only by a restriction digest. Only the 21.5-kb putative intermediate was detectable in the inverted repeat.

**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* endonucle-

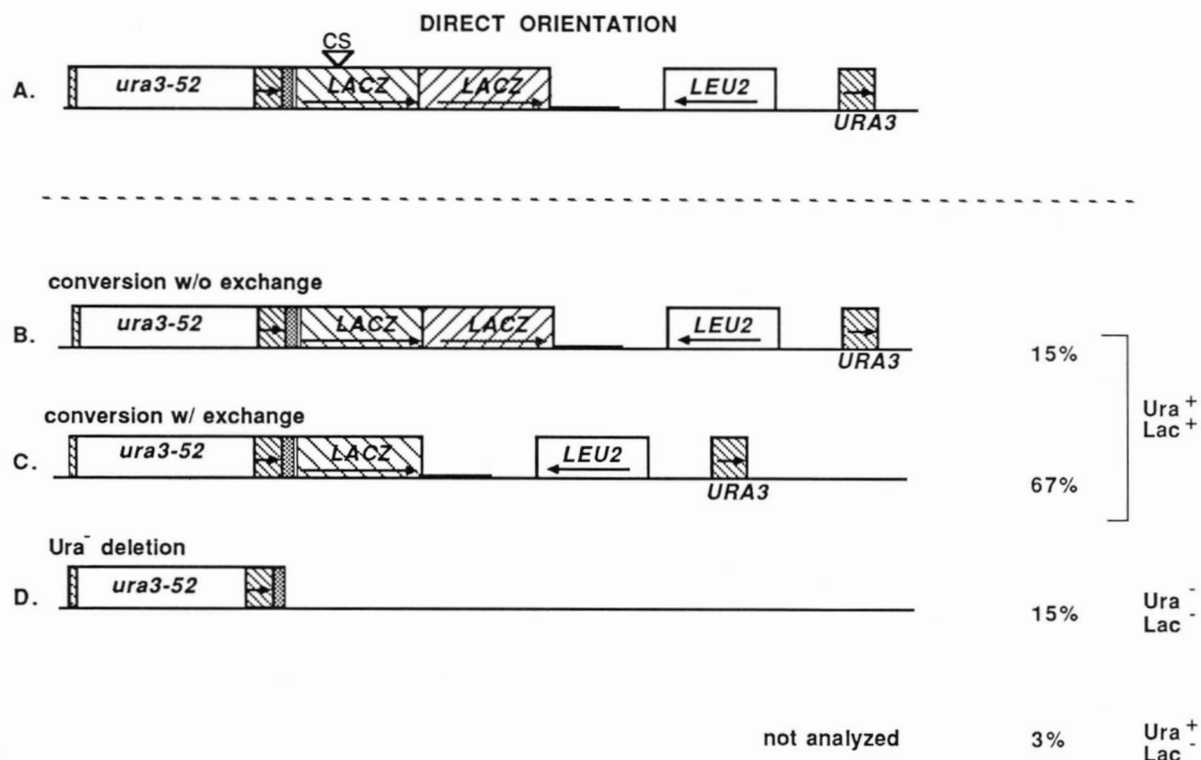
ase cleavage migrates as a linear molecule, regardless of the orientation of the *LACZ* duplication. In this experiment, the entire pPS65-*LACZ* plasmid was labeled with [ $\alpha$ - $^{32}$ P]dCTP for use as a hybridization probe. pPS65-*LACZ* lacks homology to any yeast genomic sequences, but in addition to *LACZ* will hybridize to pBR322-derived sequences in p*GAL10-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 *Pst*I digest (Figure 3A). Additional bands (int) might correspond to intermediates of the DSB repair process, such as  $\chi$  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.

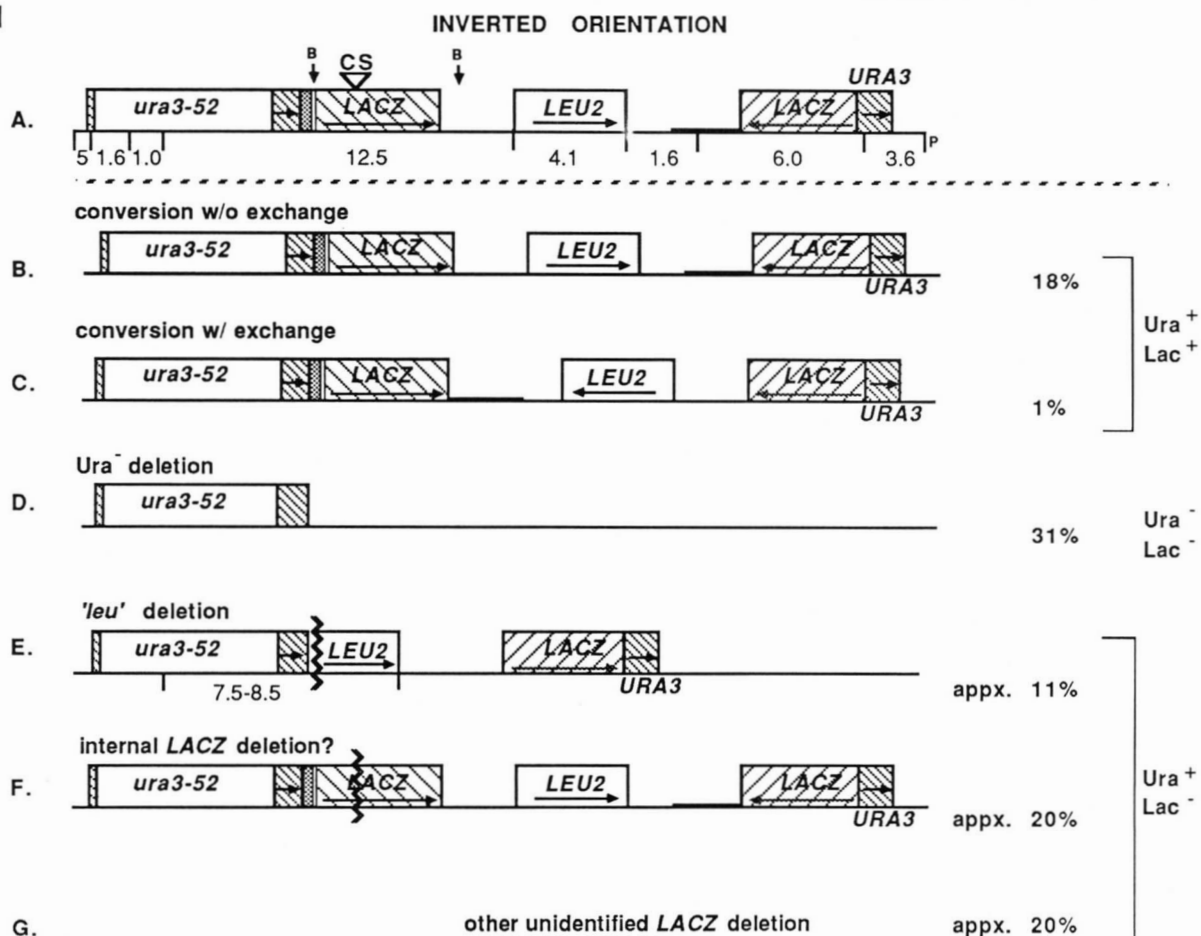
As expected for the inverted orientation plasmid (strain tNR86) the products of *HO*-induced recombination are the same size in undigested DNA as the original plasmid. There appears to be a loss of total material by the end of reaction, which agrees with the genetic result that 50% of the plasmid is 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 *Xho*I 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, IA) and tNR101 (Figure 6, IIA). An unrepaired *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 *MATa* 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

I



II



**TABLE 4**  
**Overnight induction of *HO* in *LACZ::CS<sub>2</sub>* integrants<sup>a</sup>**

A.	Trp <sup>+</sup>	Trp <sup>+</sup> Ura <sup>+</sup>	Trp <sup>+</sup> Ura <sup>-</sup>	Trp <sup>+</sup> Ura <sup>+</sup> Lac <sup>+</sup>	Trp <sup>+</sup> Ura <sup>+</sup> Lac <sup>-</sup>
tNR101 <sup>b</sup>	769	534 (69%)	235 (31%)	145 (19%)	389 (50%)
tNR102 <sup>c</sup>	827	705 (85%)	122 (15%)	680 (82%)	25 (3%)
B. Analysis of Trp <sup>+</sup> Ura <sup>+</sup> Lac <sup>+</sup> colonies <sup>d</sup>				tNR101	tNR102
Conversion without exchange				19/20	4/20
Conversion with exchange				1/20	16/20
C. Analysis of Trp <sup>+</sup> Ura <sup>+</sup> Lac <sup>-</sup> colonies <sup>e</sup>					
Deletion/repair event involving <i>LEU2</i> and <i>CYC1 UAS::LEU2::LACZ</i>				4/18	
Deletion/repair event within <i>LACZ</i>				7/18	
Other unidentified <i>LACZ</i> deletion				7/18	

<sup>a</sup> Galactose induction was performed as described in MATERIALS AND METHODS. The results represent the summation of results from five independent cultures for each strain.

<sup>b</sup> Strain tNR101: *ura3-52-LACZ::CS-LEU2-pBR322-ΔP-LACZ-URA3*.

<sup>c</sup> Strain tNR102: *ura3-52-LACZ::CS-ΔP-LACZ-pBR322-LEU2-URA3*.

<sup>d</sup> Twenty of the Ura<sup>+</sup> Lac<sup>+</sup> colonies were analyzed by SOUTHERN blots.

<sup>e</sup> Eighteen of the Ura<sup>+</sup> Lac<sup>-</sup> colonies were analyzed by SOUTHERN blots.

restored on the chromosome, resulting in a Ura<sup>-</sup> phenotype; a single *URA3* gene (Ura<sup>+</sup>) was recovered only rarely. This was defined as a deletion/repair event. By extrapolation, a high degree of *HO*-specific lethality was not expected in the chromosomal duplications, and in fact, the viability of colonies resulting from single *HO*-induced cells was not visibly different than those from uninduced cells. As in the *LACZ* plasmids, exchange in the chromosomal direct repeat would result in a deletion to yield one copy of *LACZ*, while a crossover in the inverted duplication would produce an inversion of 15–19 kb of DNA. Failure to repair using the *LACZ* sequences was expected to yield

Ura<sup>-</sup> cells resulting from the deletion/repair event.

Five independent cultures of tNR102 (direct orientation) were galactose-induced overnight, and plated for single colonies on YEPD; colonies were scored for Ura, Trp and β-galactosidase (Table 4). A majority (705/827, 85%) of the colonies resulting from galactose induction of tNR102 (direct repeat) were Lac<sup>+</sup>, Ura<sup>+</sup>. Out of 20 independent Lac<sup>+</sup>, Ura<sup>+</sup> subclones examined by SOUTHERN blotting (data not shown, Table 4B), 4 (20%) gave the parental pattern (Figure 6, IB), while 16 (80%) showed a deletion (Figure 6, IC), in close agreement with the plasmid results. In a separate experiment, 7 out of 9 Lac<sup>+</sup> Ura<sup>+</sup> colonies (77%) contained a deletion plasmid, and only 2 (23%) the parental form. The combined total from these two experiments gives 79% conversion with apparent exchange and 21% conversion without exchange for the direct repeat integration. In terms of total direct repeat events, these data represent, respectively, 67% and 15%. The remaining 147/827 (18%) were Lac<sup>-</sup> colonies; 122 (15%) were also Ura<sup>-</sup>, and were assumed to have resulted from recombination between the flanking *URA3* and *ura3-52* genes (Figure 6, ID), as described above. Several Lac<sup>-</sup> Ura<sup>-</sup> colonies from a separate induction (data not shown) were found to contain the predicted structure. The remaining 25 (3%) colonies remained Lac<sup>-</sup> and Ura<sup>+</sup> and were not further analyzed; they might have been cases in which a cut had never taken place, or in which the deletion/repair process yielded a *URA3* gene.

When tNR101 (inverted repeat) was scored after a galactose induction that was allowed to go to completion (overnight), only 145/769 (19%) colonies were Lac<sup>+</sup>, Ura<sup>+</sup>. DNA samples from 20 independently derived Lac<sup>+</sup> Ura<sup>+</sup> subclones were analyzed by SOUTHERN hybridization (data not shown, Table 4B) and 19

FIGURE 6.—Structure of the direct *LACZ* duplication (tNR102) and the inverted *LACZ* duplication (tNR101) integrated at *ura3-52* and its *HO*-induced products. The percentage of each class was in some cases extrapolated from examination of a representative sample (see text). **IA**, pNR28, which was constructed from pNR18 by removing the *CEN4* region, was integrated at *ura3-52* on chromosome V (tNR102). The integration results in a direct head to tail *LACZ* duplication. *LACZ::CS<sub>2</sub>* is located closest to *ura3-52*. The *URA3* genes are in direct orientation. **IB**, The result of conversion without exchange. The cut-site in *LACZ::CS<sub>2</sub>* is converted to wild-type sequences, and two copies of *LACZ* are retained. **IC**, The result of conversion with exchange. The cut-site in *LACZ::CS<sub>2</sub>* is converted to wild-type sequences, accompanied by a deletion to one copy of *LACZ*. **ID**, 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). **HA**, pNR27, which was constructed from pNR17 by removing the *CEN4* region, was integrated at *ura3-52* on chromosome V (tNR101). The integration results in an inverted *LACZ* duplication, separated by about 15 kb of DNA at their closest points. *LACZ::CS<sub>2</sub>* is located closest to *ura3-52*. The *URA3* genes are in direct orientation. *PstI* sites, and the distances between them in kb, are indicated by the vertical lines below IIA, and the two internal *BamHI* sites are shown by arrows above. **IIB**, The result of conversion without exchange. The cut-site in *LACZ::CS<sub>2</sub>* is converted to wild-type sequences, and the sequences between the two copies of *LACZ* remain in their parental orientation. **IIC**, The result of conversion with exchange. The cut-site in *LACZ::CS<sub>2</sub>* is converted to wild-type sequences, accompanied by an inversion of the sequences between the *LACZ* duplication. **IID**, A cut by *HO*-endonuclease stimulates a crossover between the direct repeats, *ura3-52* and *URA3* which results in deletion of the internal sequences (RUDIN and HABER 1988). **IIE**, A cut by *HO*-endonuclease stimulates a deletion consistent with a crossover between *leu2* sequences located in the promoter construction for *LACZ::CS<sub>2</sub>*, and the *LEU2* gene situated between the *LACZ* repeats. The *LEU2* sequences are in direct orientation. The putative junction has not yet been confirmed by sequence analysis. **IIF**, A cut by *HO*-endonuclease stimulates a deletion consistent with a recombination between two 8-bp direct repeats surrounding the *BclI* site in *LACZ*, into which the cut-site has been inserted in *LACZ*. The putative junction has not yet been confirmed by sequence analysis. **IIG**, About 20% of the events resulted in deletion of the *LACZ* sequences originally containing the *MATa* 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 PETES 1981; JACKSON and FINK 1981). Of the colonies, 235/769 (31%) were Lac<sup>-</sup>, Ura<sup>-</sup>, 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<sup>-</sup> Ura<sup>+</sup>. Upon reinduction, no further conversion to Lac<sup>+</sup> 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<sup>+</sup> gene, and suggested that the cut was often repaired to an aberrant Lac<sup>-</sup> structure. Preliminary SOUTHERN blot analysis (data not shown) has revealed at least four putative recombination events that have produced a Lac<sup>-</sup>Ura<sup>+</sup> 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::CS<sub>2</sub>* 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 *Pst*I band on a Southern blot. Another similarly sized but distinct band, of unexplained origin, was also sometimes observed when the *LACZ*-specific *Pst*I band was missing. A third class of events also removed *LACZ::CS<sub>2</sub>*, but no corresponding novel bands have been detected. The fourth class of events were those in which *LACZ::CS<sub>2</sub>* remained resistant to cutting with *Bcl*I, indicating that this site was not restored, yet no hybridization to a *MATa* 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 *Bcl*I 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 (KALNINS *et al.* 1983); the cut-site was inserted at the *Bcl*I 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, ID and IE). 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 *Hind*III fragment of  $\lambda$  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 IIE). However, in the direct repeat plasmid, either single-ended interaction would produce a closed circular product (Figure 2, ID and IIE), 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, ID) yields a plasmid containing *ARS* and *CEN* sequences that can replicate in yeast; the opposite event (Figure 2, IE) will be lost. Thus in 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 3/4 of the Lac<sup>+</sup> 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<sup>+</sup> products of gene conversional repair of the *HO* endonuclease cut in *LACZ::CS<sub>2</sub>*, as evidenced by the relative abundance of the **A**, **inv-A** and **inv-B** *Pst*I 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<sup>+</sup> 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<sup>+</sup> 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*<sub>2</sub>, 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 *CYC1 UAS* enhancer/promoter

region adjacent to *LACZ::CS<sub>2</sub>* 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 *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 *LACZ* duplications were present on the chromosome, the viability of colonies resulting from single *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 *LACZ* chromosomal duplication system.

When *HO*-induced recombination was examined in inverted *LACZ* repeats, located 10 kb apart on the chromosome, a minority of events results in Lac<sup>+</sup> 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 *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 (*URA3*, *LEU2*) take place by a more favorable mechanism than those events which would lead to a *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 *rad52* mutation as well as spontaneous mitotic intergenic recombination (ESPOSITO *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 *rad52* diploids was frequently (35%) accompanied by the

formation of 2N-1 diploids in which the homolog carrying the prototrophic recombinant *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 Rad<sup>+</sup> diploids (CAMPBELL and FOGEL 1977). The much higher proportion of such apparent one-ended events that we describe here for *HO*-induced Lac<sup>+</sup> recombinants in Rad<sup>+</sup> cells may reflect our use of centromere-containing plasmids to examine intrachromosomal recombination rather than interchromosomal events involving normal chromosomes. We suggest that the *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 *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.

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