Double-Strand Break-Induced Mitotic Intrachromosomal Recombination in the Fission Yeast *Schizosaccharomyces pombe*

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ABSTRACT

The *Saccharomyces cerevisiae HO* gene and *MAT a* cutting site were used to introduce site-specific double-strand breaks (DSBs) within intrachromosomal recombination substrates in *Schizosaccharomyces pombe*. The recombination substrates consisted of nontandem direct repeats of *ade6* heteroalleles. DSB induction stimulated the frequency of recombinants 2000-fold. The spectrum of DSB-induced recombinants depended on whether the DSB was introduced within one of the *ade6* repeats or in intervening unique DNA. When the DSB was introduced within unique DNA, over 99.8% of the recombinants lacked the intervening DNA but retained one copy of *ade6* that was wild type or either one of the heteroalleles. When the DSB was located in duplicated DNA, 77% of the recombinants were similar to the deletion types described above, but the single *ade6* copy was either wild type or exclusively that of the uncut repeat. The remaining 23% of the induced recombinants were gene convertants with two copies of *ade6* and the intervening sequences; the *ade6* heteroallele in which the DSB was induced was the recipient of genetic information. Half-sectored colonies were isolated, analyzed and interpreted as evidence of heteroduplex DNA formation. The results are discussed in terms of current models for recombination.

REPETITIVE DNA sequences, characteristic of higher eukaryotes, are subject to general homologous recombination. Although mitotic recombination between specific repetitive DNA sequences fulfills a clear biological role (ENGLER and STORB 1988; KALLENBACH and ROUGEON 1992), recombination between repetitive sequences is also a potentially major form of mutagenesis (LEHRMAN *et al.* 1985; GILMAN 1987; MYEROWITZ and HOGIKYAN 1987; MOREL *et al.* 1989; VNENCAK-JONES and PHILLIPS 1990; MARSHALL 1991; WÜRGLER 1992).

Artificially created DNA duplications have been used to understand the mechanism of homologous intramolecular recombination between repeated sequences. Typically, the duplication consists of either two different alleles of the same gene or two overlapping segments of a gene separated by unique DNA, commonly with a marker gene also present within the intervening sequence. Recombination between the duplicated DNA in such substrates produced two classes of recombinants: conversion-type recombinants that still have two copies of the repeat element and have presumably arisen by nonreciprocal transfer of information from one element to the other without loss of the intervening sequences and deletion-type recombinants that have a single copy of the repeat element with accompanying loss of the intervening sequences.

Such systems in *Saccharomyces cerevisiae* have allowed an analysis of mitotic recombination between artificially created duplications both in the chromosome (JACKSON and FINK 1981; NICKOLOFF *et al.* 1986, 1989; RAY *et al.* 1988; RUDIN and HABER 1988; SCHIESTL and PRAKASH 1988, 1990; THOMAS and ROTHSTEIN 1989a,b; YUAN and KEIL 1990; SUGAWARA and HABER 1992; JINKS-ROBERTSON *et al.* 1993) and using plasmids (RUDIN *et al.* 1989; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992). The spontaneous recombinant frequency and the proportion of conversion-type *vs.* deletion-type recombinants depended on the substrates used. With some substrates conversion-type events predominated over deletion-type events (JACKSON and FINK 1981; RAY *et al.* 1988); while with others, deletion-type events predominated over conversion-type events (NICKOLOFF *et al.* 1986, 1989; JINKS-ROBERTSON *et al.* 1993).

Several experimental systems suggest that DNA double-strand breaks (DSBs) either initiate, or provide efficient substrates for, mitotic recombination in *S. cerevisiae*, and that recombination between homologous sequences is the predominant process for the repair of DSBs (HAYNES and KUNZ 1981; ORR-WEAVER *et al.* 1981; STRATHERN *et al.* 1982; KOSTRIKEN *et al.* 1983; ORR-WEAVER and SZOSTAK 1983; KOSTRIKEN and HEFFRON 1984). The DSB/gap repair model was proposed to explain the recombinogenic effects of DSBs (RESNICK 1976; SZOSTAK *et al.* 1983). This model could account for the formation of conversion- and deletion-type recombinants from the duplication substrates. Subsequently, studies of extrachromosomal recombination in mammalian cells suggested that a DSB flanked by homologous sequences may also be efficiently repaired

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by an alternative recombination pathway, based on single-strand annealing (SSA) (LIN *et al.* 1984, 1990a,b). The SSA pathway gives rise to deletion-type recombinants from duplication substrates.

In *S. cerevisiae,* the analysis of DSB-induced mitotic recombination between repeats has been made possible by expressing HO endonuclease and incorporating its target site, the *S. cerevisiae MATa y/z* junction (KOSTRIKEN *et al.* 1983), into the duplication substrates. The HO recognition site has been placed either within duplicated DNA in one of the repeat elements (NICKOLOFF *et al.* 1986, 1989; RAY *et al.* 1988; RUDIN *et al.* 1989; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992), or within unique DNA in the intervening sequence between the repeats (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989; SUGAWARA and HABER 1992).

In studies of DSB-induced mitotic intrachromosomal recombination in which the DSB was in duplicated DNA, both conversion- and deletion-type events were stimulated (NICKOLOFF *et al.* 1986, 1989; RAY *et al.* 1988; RUDIN *et al.* 1989). The spectrum of DSB-induced recombination events remained the same as spontaneous events, indicating that the pathways involved in spontaneous and DSB-induced mitotic recombination might be the same. For the induced conversion-type recombinants, the cleaved repeat sequence acted exclusively as the recipient of genetic information, and these recombinants could be accounted for by the DSB/gap repair pathway. Spontaneous and induced deletion-type recombinants could result by any of the following means: gene conversion associated with crossing over, an unequal sister chromatid exchange at G2, or by SSA. If gene conversion was accompanied by crossing over, the segment of DNA internal to the two halves of the repeat would be excised as a circle. The experimental evidence, however, suggested that deletion-type recombinants arose primarily via the SSA pathway (NICKOLOFF *et al.* 1986, 1989; RAY *et al.* 1988; RUDIN *et al.* 1989; THOMAS and ROTHSTEIN 1989a).

Similarly, in studies of DSB-induced direct-repeat recombination on plasmids in which the *MATa* site was within duplicated DNA, both conversion- and deletion-type recombinants were recovered (RUDIN *et al.* 1989; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992). The conversion-type recombinants could again be explained by the DSB/gap repair model, but the large fraction of deletion-type recombinants (~80%) and the absence of the reciprocal circular product accompanying their formation (which would be expected from a DSB/gap repair intermediate resolved with an exchange) could best be explained by the SSA model. A physical analysis of the kinetics of both DSB-induced conversion- and deletion-type product formation provided evidence that DSB/gap repair and SSA are two independent competing pathways of DSB-induced recombination with a common intermediate (RUDIN *et al.* 1989; FISHMAN-LOBELL *et al.* 1992).

The SSA mechanism accounts for the predominance (>99%) of deletion-type mitotic recombinants obtained from intrachromosomal and extrachromosomal recombination substrates in which the DSB was induced within unique DNA between the repeats (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989; SUGAWARA and HABER 1992).

Additional evidence for at least two distinct pathways of homologous recombination between nontandem intramolecular repeats in *S. cerevisiae* comes from an examination of its genetic control. Spontaneous and DSB-induced recombination rates were affected by mutations in the *RAD1, RAD10* and *RAD52* genes, and analysis of double mutants suggested that *RAD52* and *RAD1/RAD10* act in different but overlapping mitotic recombination pathways (JACKSON and FINK 1981; ROTHSTEIN *et al.* 1987; KLEIN 1988; RUDIN and HABER 1988; SCHIESTL and PRAKASH 1988, 1990; NICKOLOFF *et al.* 1989; THOMAS and ROTHSTEIN 1989b; ZEHFUS *et al.* 1990; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992; SUGAWARA and HABER 1992). The RAD52 protein appears to be primarily involved in the formation of conversion-type recombinants via the DSB/gap repair pathway, although it also plays a role in the formation of deletion-type recombinants (JACKSON and FINK 1981; RUDIN and HABER 1988; NICKOLOFF *et al.* 1989; WHITE and HABER 1990; OZENBERGER and ROEDER 1991; FISHMAN-LOBELL *et al.* 1992; SUGAWARA and HABER 1992). The *RAD1/RAD10* genes are involved in the formation of deletion-type recombinants via the SSA pathway (SCHIESTL and PRAKASH 1988, 1990; THOMAS and ROTHSTEIN 1989b; FISHMAN-LOBELL and HABER 1992; BARDWELL *et al.* 1994).

Mitotic recombination between repeats has been studied in mammalian cells utilizing extrachromosomal (FINN *et al.* 1989; WAHLS and MOORE 1990; LIN *et al.* 1990a,b) and intrachromosomal (LIN and STERNBERG 1984; STRINGER *et al.* 1985; HELLGREN *et al.* 1990; BOLLAG and LISKAY 1992) substrates. There are similarities and some important differences between recombination in the two organisms.

With DSB-induced extrachromosomal recombination substrates in mammalian cells, one of the most striking differences is that nonhomologous recombination (end-joining) events to repair the DSB predominate over homologous recombination events (reviewed by ROTH and WILSON 1988). Nevertheless, appropriately placed DNA DSBs stimulate extrachromosomal homologous recombination in mammalian cells and linearized DNA molecules undergo homologous events similar to those in yeast, albeit at a reduced frequency (for review see BOLLAG *et al.* 1989). As with yeast, in mammalian cells SSA represents a major pathway by which extrachromosomal elements recombine (LIN *et al.* 1984, 1990a,b).

The majority of spontaneous intrachromosomal recombination events between direct repeats in mamma-

lian cells are simple gene conversions unaccompanied by crossing over rather than deletion-type events, and the DSB/gap repair mechanism seems to be more prevalant than SSA (LISKAY and STACHELEK 1983; LISKAY *et al.* 1984; SUBRAMANI and RUBNITZ 1985; BOLLAG and LISKAY 1988). As discussed earlier, this is similar to results observed for some substrates in *S. cerevisiae* but contrary to that seen for others. Unlike the situation in *S. cerevisiae*, BOLLAG and LISKAY (1991) concluded that the majority of events occur by unequal sister chromatid exchange, with perhaps 15% occuring by intrachromosomal recombination.

Studies on DSB-induced intrachromosomal recombination using defined DSBs in mammalian chromosomes have been more difficult. Indirect evidence suggests that DSBs stimulate mitotic intrachromosomal recombination in mammalian cells (WANG *et al.* 1988; WALDMAN and WALDMAN 1991; HELLGREN 1992; LUDWIG and STRINGER 1994). However, in the only study that has examined recombination in a substrate with a defined *in vivo* DSB, in marked contrast to the results with *S. cerevisiae*, all induced events examined were due to end-joining (GODWIN *et al.* 1994).

In the fission yeast *Schizosaccharomyces pombe*, strains containing a nontandem heteroallelic *ade6* duplication had previously been used to study spontaneous mitotic intrachromosomal recombination by recovery of Ade$^+$ recombinants (SCHUCHERT and KOHLI 1988). As described above, the *ade6* heteroalleles could recombine with each other to generate a wild-type *ade6*$^+$ gene by four different recombination processes (Figure 1). However, since only selected Ade$^+$ mitotic recombinants were recovered and analyzed, conclusions about the recombination pathways operating were limited.

DSBs at the mating-type locus can initiate mitotic (BEACH 1983; EGEL *et al.* 1984) and meiotic (KLAR and MIGLIO 1986) recombination in *S. pombe*. These studies illustrated some of the general features of the DSB/gap repair model. No studies have examined DSB-induced mitotic intrachromosomal recombination at loci other than the mating-type loci in *S. pombe*. Given the differences between *S. cerevisiae* and mammalian cells, it is unclear whether the pathways of DSB-induced intrachromosomal recombination in *S. cerevisiae* are conserved in other eukaryotic organisms. In evolutionary terms, *S. pombe* is as distant from *S. cerevisiae* as it is from mammalian cells (SIPICZKI 1989), and several features of *S. pombe* have more in common with higher eukaryotes than do those of *S. cerevisiae*. We have therefore examined DSB-induced mitotic recombination in *S. pombe*. This understanding should make *S. pombe* an attractive and complementary system to help elucidate the mechanisms and genetic control of intrachromosomal mitotic recombination in eukaryotes.

MATERIALS AND METHODS

***S. pombe* strains:** Strains of *S. pombe* (Table 1) with one of six *ade6* duplications were used. The duplication in strains PS1 and PS3 (SCHUCHERT and KOHLI 1988) consisted of nontandem direct repeats of *ade6* heteroalleles separated by pUC sequence and a functional *ura4*$^+$ gene (the endogenous *ura4* gene is deleted). The repeated *ade6* genes are separated by 4.4 kb and the length of homology is 1.9 kb. Strains SL1, SL2, SL3 and SL4 contain similar substrates (Figure 2) but include a fragment of the *S. cerevisiae MAT a* locus containing the *y/z* junction target site for *S. cerevisiae* HO endonuclease (KOSTRIKEN *et al.* 1983). The duplications in these strains were constructed using the integrating plasmids pDUP12-MATa-A and pDUP12-MATa-B (see below), in a manner similar to that described for the construction of duplications PS1 and PS3 (SCHUCHERT and KOHLI 1988). Their structures were confirmed by Southern blot hybridization analysis, probing with the *ade6* gene and the *MAT a* site (Figure 3). In all duplications the left-hand heteroallele (with respect to the *ura4*$^+$ gene) was *ade6-L469*. The mutation *L469*, a C to T transition, abolishes an *Xho*I site present in the wild-type sequence and is close to the end of the open reading frame (SZANKASI *et al.* 1988). Both *M26* and *M375* are G to T transversions and are located only three nucleotides apart (SZANKASI *et al.* 1988). The *M26* mutation causes a meiotic recombination hot-spot but has no effect on spontaneous mitotic intrachromosomal recombination (SCHUCHERT and KOHLI 1988). For clarity, henceforth all strains will be referred to by the duplication substrate they contain rather than by the strain designation.

Plasmids: Plasmid pDUP12 contained *ura4*$^+$ and *ade6-L469* cloned, respectively, into the *Hin*dIII and *Sma*I polylinker sites of pUC8 (provided by P. SZANKASI, Fred Hutchinson Cancer Research Center, Seattle, WA). A 117-bp *Bgl*II-*Hin*dII fragment of *S. cerevisiae MAT a* containing the target site of endonuclease HO (the *y/z* junction) (KOSTRIKEN *et al.* 1983) was cloned into *Bam*HI-*Hin*dII-digested pUC8 and cut out with *Sma*I-*Hin*dIII as a 137-bp fragment; *Cla*I polylinkers were added and cloned into the *Cla*I site of pBlueScript II to produce plasmid pCla7 (provided by M. HOEKSTRA, ICOS Corp., Bothell, WA). A 175-bp *Pst*I-*Sal*I fragment of pCla7 containing *MAT a* sequence was cloned into the *Pst*I-*Sal*I sites of the polylinker of pDUP12 between the *ura4*$^+$ and *ade6-L469* sequences to give plasmid pDUP12-MATa-A (Figure 4a). Similarly, a 179-bp *Sma*I-*Hin*dII fragment was cloned into the *Eco*NI site of *ade6-L469* to give plasmid pDUP12-MATa-B (Figure 4b).

The pUC8 sequence of plasmid pREP81X (provided by S. FORSBURG, Salk Institute, San Diego, CA) (FORSBURG 1993) was removed by digestion with *Pvu*II and replaced by ligation of the rest of the plasmid to pACYC184 linearized with *Sca*I to give plasmid pACYCREP81X (Figure 4c). A 1.8-kb fragment containing the HO endonuclease coding sequence was cloned into the *Sma*I site of pREP81X in front of the modified *nmt1* promoter to give plasmid pREP81X-HO (not shown). The pUC8 sequence of pREP81X-HO was replaced with pACYC184 sequence as described above to give plasmid pACYCREP81X-HO (Figure 4d). Plasmid pACYCREP81X-HO therefore contains the *S. cerevisiae HO* gene expressed from an inducible modified *nmt1* promoter. Expression from the *nmt1* promotor is repressed in the presence of thiamine and induced in the absence of thiamine (FORSBURG 1993). Plasmid pACYCREP81X is the same as pACYCREP81X-HO but lacks the *HO* gene.

Media and genetic methods: Media and general genetic methods for *S. pombe* have been described by GUTZ *et al.* (1974). Synthetic minimal media (EMM) was supplemented with 150 mg/l of appropriate amino acids. Yeast extract (YE) media supplemented with 200 mg/l guanine was used to select for Ade$^+$ recombinants (CUMMINS and MITCHISON 1967). Low adenine (10 mg/l) and thiamine (40 μM) were used where necessary. Ura$^-$ colonies were selected on EMM containing 50 mg/l uracil and 1 mg/ml 5-fluoroorotic acid (FOA).

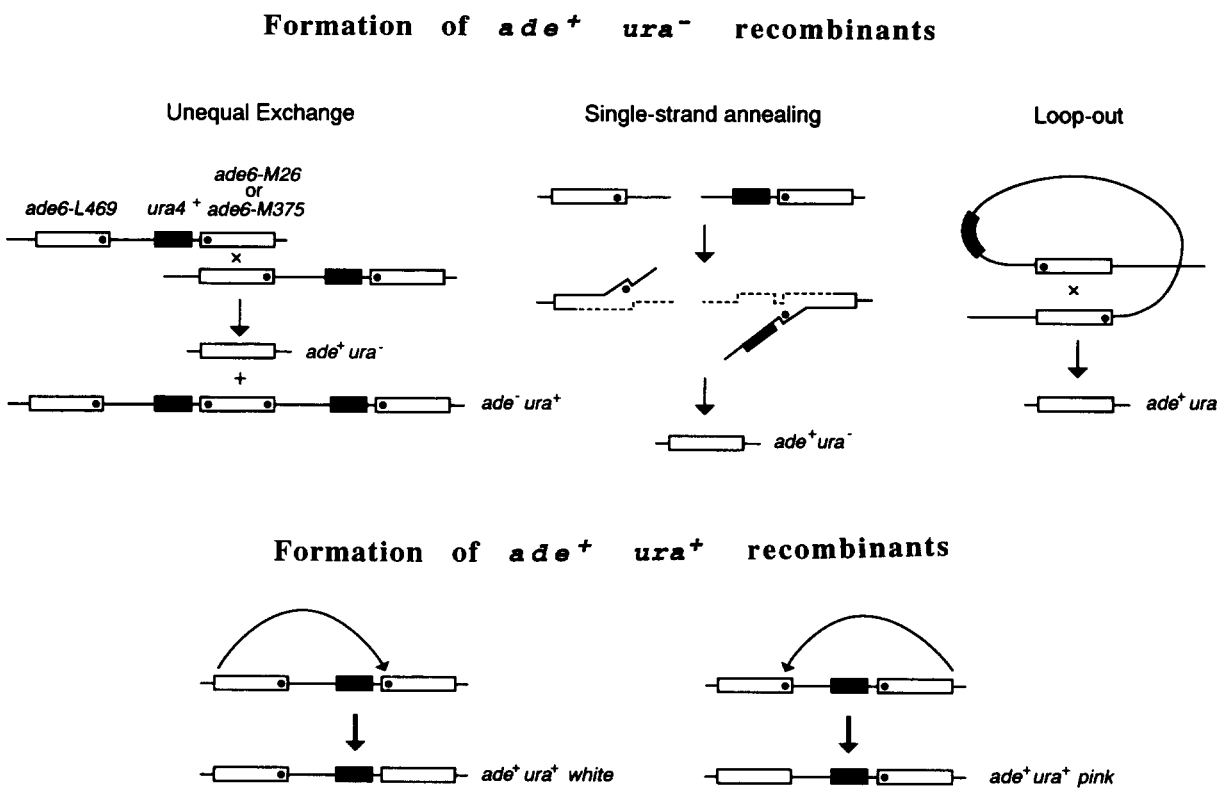

FIGURE 1.—Recombination events at the *ade6* duplication that can generate Ade$^+$ recombinants. Deletion-type recombinants (Ade$^+$ Ura$^-$) can arise via three mechanisms: unequal sister chromatid exchange, single-strand annealing and intrachromatid loop-out. Conversion-type Ade$^+$ Ura$^+$ recombinants arise by transfer of wild-type information from one heteroallele to the other.

Molecular biological methods: *S. pombe* transformations were performed by the method of ITO *et al.* (1983). *S. pombe* genomic DNA was prepared according to HOFFMAN and WINSTON (1987). Southern blot hybridization analysis was performed using Hybond-N nylon filters (Amersham, Arlington Heights, IL) and nonradioactive digoxigenin-labelled probes following manufacturer's protocols (Genius System, Boehringer Mannheim, Indianapolis, IN).

Determining spontaneous recombinant frequencies by fluctuation tests: To determine spontaneous frequencies, strains containing pACYCREP81X and growing on minimal media containing excess adenine (to prevent selection for Ade$^+$ recombinants) and lacking leucine (to maintain the plasmid) were used. Ten independent colonies were assayed per strain. Cells from each colony were plated at a density of $\sim 10^5$ cells per plate on media selective for Ade$^+$ recombinants. The cell titer was determined by appropriate dilution and plating onto complete media. After 4 days growth, the number of recombinants and cell titer were determined. The Ade$^+$ recombinants on the selective plates were replicated onto media lacking uracil to determine the proportion of convertant-type (Ade$^+$ Ura$^+$) to deletion-type (Ade$^+$ Ura$^-$) recombinants. The proportion of pink [*ade6-L469* heteroallele converted to wild-type (SCHUCHERT and KOHLI 1988)] to white [*ade6-M26/M375* converted to wild type (SCHUCHERT and KOHLI 1988)] convertants was also determined. In addition, the frequency of spontaneously arising Ade$^+$ Ura$^-$ and Ade$^-$ Ura$^-$ deletion-type recombinants was determined separately by plating onto FOA plates that select for Ura$^-$ cells.

Determining DSB-induced recombinant frequencies by fluctuation tests: To determine DSB-induced recombinant frequencies, strains containing the *MATa* site and pACYCREP81X-HO were used. Cells were harvested from colonies growing on minimal media containing excess adenine, lacking leucine and lacking thiamine (HO expression induced). For the DSB-induced recombination assays, the recombinant frequencies were high enough so that cells were plated ($\sim$200 cells/plate) onto media nonselective for adenine requirement to allow scoring of all resulting colonies.

Two sets of DSB-induced recombination assays were performed, assaying cells on two types of media: cells were plated onto either YE media containing thiamine to repress further expression of HO or onto EMM media lacking thiamine to allow continued expression of HO. Results from using the two different plates were consistent. The media in these assay plates contained low adenine to distinguish Ade$^+$ and Ade$^-$ strains. (*ade6*$^+$ strains are white on low adenine plates; *ade6*$^-$ strains grow on low adenine plates but are red.) The EMM plates lacking thiamine allowed the identification of colonies with a DSB-induced red/white sectoring phenotype indicative of retention of the *MATa* site and *ade6* heteroalleles (see Figure 5).

After 4 days growth the number of red (Ade$^-$) and white (Ade$^+$) colonies and, for the EMM plates lacking thiamine, the number of red/white sectoring colonies, were scored. As before, the colonies were replicated onto media lacking uracil and onto media lacking uracil and adenine. The different phenotypic classes of colonies obtained in the spontaneous assays (Ade$^+$ Ura$^-$, Ade$^-$ Ura$^-$, Ade$^+$ Ura$^+$-pink, Ade$^+$ Ura$^+$-white) could thus be distinguished. In addition, three types of DSB-induced Ade$^-$ Ura$^+$ colonies could be distinguished phenotypically: Ade$^-$ Ura$^+$ colonies that retained the DSB-induced red/white sectoring phenotype indicative of retention of the *MATa* site and *ade6* heteroalleles (Figure 5), Ade$^-$ Ura$^+$ colonies that had lost the sectoring phenotype but could still give rise to Ade$^+$ recombinants at spontaneous frequencies, and Ade$^-$ Ura$^+$ colonies that had lost the sectoring phenotype and could not give rise to Ade$^+$ recombinants. From

TABLE 1

***S. pombe* strains used in this study**

Strain	Genotype
FO1	*h⁺ leu1-32 ura4-D18 ade6-M26*
FO2	*h⁻ leu1-32 ura4-D18 ade6-M26*
FO3	*h⁺ leu1-32 ura4-D18 ade6-M375*
FO4	*h⁻ leu1-32 ura4-D18 ade6-M375*
GP1593	*h⁻ ura4-D18 ade6-L469*
GP1594	*h⁺ leu1-32 ura4-D18 ade6-L469*
PS1	*h⁻ leu1-32 ura4-D18 ade6-M26 int::pUC8/ ura4⁺/ade6-L469*
PS3	*h⁻ leu1-32 ura4-D18 ade6-M375 int::pUC8/ ura4⁺/ade6-L469*
SL1	*h⁺ leu1-32 ura4-D18 ade6-M26 int::pUC8/ ura4⁺/MATa/ade6-L469*
SL2	*h⁻ leu1-32 ura4-D18 ade6-M375 int::pUC8/ ura4⁺/MATa/ade6-L469*
SL3	*h⁺ leu1-32 ura4-D18 ade6-M26 int::pUC8/ ura4⁺/ade6-MATa-L469*
SL4	*h⁺ leu1-32 ura4-D18 ade6-M375 int::pUC8/ ura4⁺/ade6-MATa-L469*
PS10	PS1 containing pACYCREP81X
PS30	PS3 containing pACYCREP81X
SL10	SL1 containing pACYCREP81X
SL20	SL2 containing pACYCREP81X
SL30	SL3 containing pACYCREP81X
SL40	SL4 containing pACYCREP81X
PS11	PS1 containing pACYCREP81X-HO
PS31	PS3 containing pACYCREP81X-HO
SL11	SL1 containing pACYCREP81X-HO
SL21	SL2 containing pACYCREP81X-HO
SL31	SL3 containing pACYCREP81X-HO
SL41	SL4 containing pACYCREP81X-HO

Strains PS1 and PS3 were provided by J. KOHLI (University of Bern, Switzerland). Strains GP1593 and GP1594 were provided by G. SMITH (Fred Hutchinson Cancer Research Center, Seattle, WA).

the YE assay plates containing thiamine these three types of Ade⁻ Ura⁺ colonies were distinguished by further testing. Subculturing onto low adenine media lacking thiamine was used to determine whether they retained the red/white sectoring phenotype. The nonsectoring (*i.e.*, do not exhibit high frequency sectoring as in Figure 5) Ade⁻ Ura⁺ colonies were further tested on media lacking adenine to determine whether they could give rise to spontaneous Ade⁺ recombinants. From the EMM assay plates lacking thiamine these three classes of Ade⁻ Ura⁺ colonies could be distinguished immediately by visual scoring and replica plating.

For SL1 and SL2, a separate assay was performed by plating ($\sim 10^5$ cells/plate) onto media lacking adenine and uracil to determine the frequency of DSB-induced conversion-type recombinants, since these did not occur at high frequencies for these strains.

Physical and genetic analyses to establish the genotypes of the different phenotypic classes of colonies from DSB-induced recombination assays: Genomic DNA was isolated from the different phenotypic classes of colonies arising after DSB induction. For each strain containing substrates SL1–SL4, 10 of each class of colony (of independent origin) were analyzed. The DNA was digested with *Xho*I and analyzed by Southern blot hybridization, probing with *ade6* sequences and with *MATa* sequences.

All Ade⁺ Ura⁻, Ade⁺ Ura⁺-white and Ade⁺ Ura⁺-pink colonies probed with *ade6* and *MATa* gave hybridization patterns (Figure 6, A and F) consistent with the genotypes reported by SCHUCHERT and KOHLI (1988).

The single copy of *ade6* in the Ade⁻ Ura⁻ deletion-type colonies could be one of three genotypes: *ade6-L469*, *ade6-M26/M375*, or *ade6-L469-M26/M375* (both point mutations present). The presence or absence of the *L469* point mutation (which abolishes an *Xho*I cutting site) could be readily determined physically. The *ade6-L469* and *ade6-L469-M26/M375* colonies could not be distinguished physically since they would give the same *Xho*I-generated hybridization pattern, but they could be distinguished genetically by sexual crosses to *ade6-L469*, and *ade6-M26* or *ade6-M375* strains, as appropriate, assaying for the frequency of Ade⁺ recombinants.

The Ade⁻ Ura⁺ colonies that failed to show the DSB-induced red/white sectoring phenotype, but could give rise to Ade⁺ recombinants spontaneously, were shown by Southern hybridization to be convertants in which the *MATa* site had been lost without coconverting the *L469* mutation 148 bp downstream (Figure 6, E and F). The Ade⁻ Ura⁺ colonies that failed to show the DSB-induced red/white sectoring phenotype and could not give rise to Ade⁺ recombinants spontaneously were also convertants in which the genetic information in the *ade6-L469* heteroallele was replaced by information from the right-hand *ade6-M26/M375* heteroallele, such that the left-hand heteroallele was also converted to *ade6-M26/M375* (Figure 6, E and F).

The phenotype of the Ade⁻ Ura⁺ colonies that showed the DSB-induced red/white sectoring phenotype was indicative of *ade6* heteroallelic repeats with retention of the *MATa* site. Southern hybridization was used to determine whether they contained the original duplication substrate or possibly a triplication generated by unequal sister chromatid exchange (Figure 1).

Demonstration that HO expression results in the introduction of a DSB within the *MATa* site: Cells with substrate SL2 and containing pACYCREP81X-HO were grown for 12 hr in liquid EMM containing excess adenine, lacking leucine and in the presence of thiamine to repress HO expression. Cells were collected by centrifugation, washed three times in water to remove thiamine, resuspended in fresh medium lacking thiamine (to induce HO expression), and grown for 48 hr. At 8-hr time intervals cells were collected and resuspended in fresh media, and genomic DNA was prepared from aliquots of the dividing cells digested with *Xho*I. The appearance of a DSB within the *MATa* site was monitored by Southern hybridization analysis using the *MATa* probe.

Isolation of DSB-induced red/white half-sectored colonies: Strains with substrates SL1–4 and containing pACYCREP81X-HO were grown and induced for HO expression as above but for 72 hr to saturation without thiamine. The cells were diluted and plated onto EMM media lacking leucine and containing low adenine. After 4 days growth, the two half-sectors of the sectored colonies were subcultured, and their phenotypes were determined by plating onto appropriate selective plates.

RESULTS

Experimental system: Cells with duplication substrates SL1–SL4, which contain *MATa*, and harboring plasmid pACYCREP81X-HO were grown as single colonies in the absence or presence of thiamine on minimal media lacking leucine (to maintain the plasmid) and containing low adenine to distinguish Ade⁺ (white) and Ade⁻ (red) colonies. In the presence of thiamine, HO

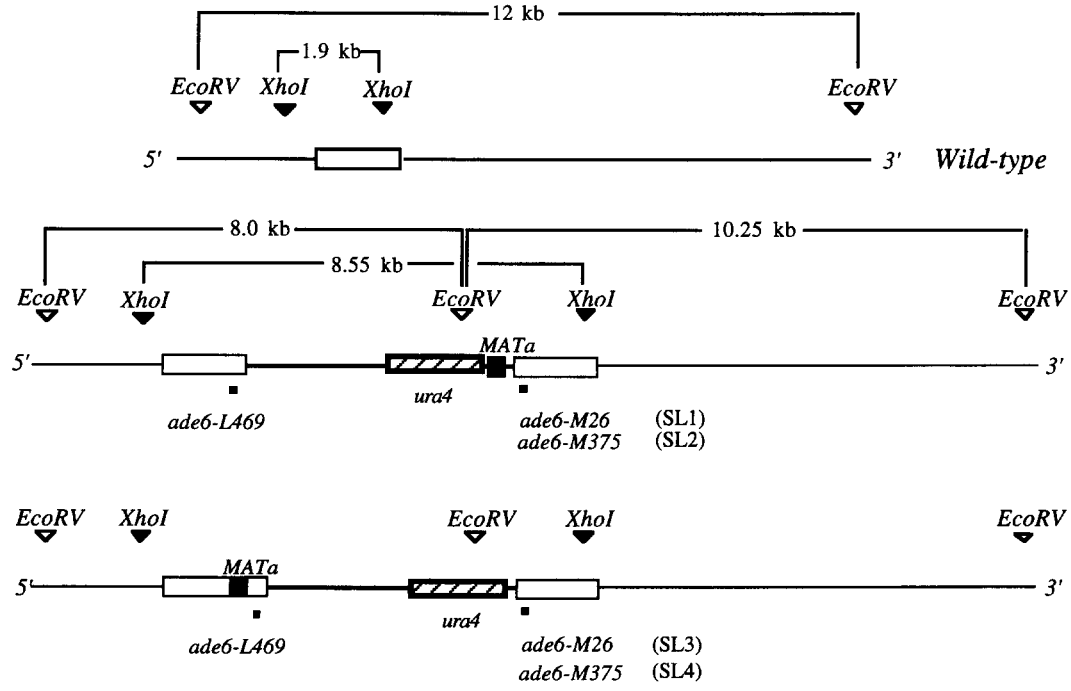

FIGURE 2.—Schematic drawing of the *ade6* duplication recombination substrates containing the *MATa* cutting site in strains SL1, SL2, SL3 and SL4. □, *ade6* coding regions. In strains PS1, SL1 and SL3 the right-hand cassette has the *ade6-M26* allele, whereas in PS3, SL2 and SL4 the right-hand cassette has the *ade6-M375* allele. ▨, 1.8-kb *Hin*dIII fragment with the *ura4*$^+$ marker gene; ■, *MATa* cutting site. The thick lines represent pUC8 vector and the thin lines the chromosomal DNA. In SL1 and SL2 the HO-induced DSB is made 86 bp upstream from the right-hand *ade6* heteroallele, 404 and 401 bp from the *M26* and *M375* mutations, respectively. In SL3 and SL4 the HO-induced DSB is made 148 bp upstream from the *L469* mutation, 1358 and 1361 bp downstream from the *M26*$^+$ and *M375*$^+$ bases in the left-hand *ade6-L469* heteroallele, respectively.

expression from the *nmt1* promotor is repressed, spontaneous Ade$^+$ recombinants arose at a low frequency (10^{-4}) and colonies appeared uniformly red. In the absence of thiamine, HO expression is switched on, a DSB is induced in the *MATa* target site that stimulates recombination in the *ade6* duplication, and resulting colonies were red/white sectored (Figure 5). PS1 and PS3, which lack the *MATa* target site, still appeared uniformly red after HO expression. Similarly, all the strains transformed with pACYCREP81X (lacking *HO*) appeared uniformly red in the presence or absence of thiamine. Thus, strains with recombination substrates SL1–SL4 containing the *HO* plasmid can be used to study DSB-induced recombination, while those transformed with pACYCREP81X can be used to study spontaneous recombination.

Cells in which a DSB was induced grew slower than those in which a DSB was not generated. Cells growing on plates or in liquid culture in DSB-inducing and non-inducing conditions were treated with methylene blue, which darkly stains dead cells, and observed microscopically. There was no appreciable difference between the number of dead cells in cultures grown in the two conditions, indicating that DSB-induction did not lead to cell death. The slower growth could be due to a delay in cell division to allow repair of the DSB.

Spontaneous and DSB-induced recombinant frequencies, and the phenotypic and genotypic classes of the various colonies were determined as described in MATERIALS AND METHODS. Combined results for all assays are shown in Table 2. The relatively large standard deviations associated with the frequencies are not unexpected for these assays, given the fact that measurements are subject to fluctuation due to different clonal populations within different colonies.

Several observations indicated that most recombination events were occuring in G1 of the cell cycle, before DNA replication, rather than in G2. DSB-induced Ade$^+$ recombinant frequencies determined by selecting for only Ade$^+$ recombinants on medium lacking adenine (data not shown) were no higher than those shown in Table 2 obtained by assaying unselected colonies. Also, there were few (<1%) mixed (red/white) colonies on the nonselective assay plates, and triplications arising from unequal sister chromatid interactions in G2 were not observed (see later).

Spontaneous recombinant frequencies: Spontaneous recombinant frequencies (Table 2) were similar to those reported by SCHUCHERT and KOHLI (1988). The majority of spontaneous Ade$^+$ recombinants (75%, average of all six strains) were Ade$^+$ Ura$^-$ deletion types. Deletion-type Ade$^-$ Ura$^-$ recombinants, which could not give rise to Ade$^+$ recombinants and appear to have lost the duplication by a recombination event that does not give an *ade6*$^+$ gene, also arose spontaneously but at a threefold lower frequency. Spontaneous conversion-type recombi-

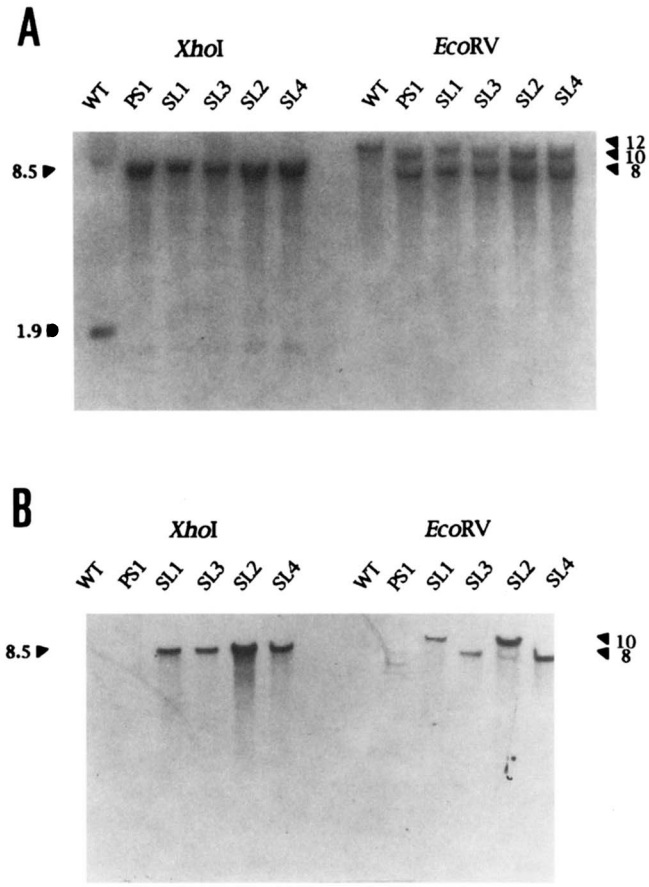

FIGURE 3.—Comparison of wild-type *ade6* duplication (PS1) and *ade6* duplication containing *MATa* (SL1–4) strains by restriction enzyme digestion and Southern blot hybridization. The restriction enzymes used were *Xho*I and *Eco*RV. (A) The hybridization probe was a *Aha*III fragment of the *ade6* gene (shown in Figure 4). Fragments of 1.9 (*Xho*I) and 12 kb (*Eco*RV) were observed with the wild-type strain. As expected from the scheme in Figure 2, fragments of 8.3–8.5 kb were observed with *Xho*I, and fragments of ~10 and 8 kb with *Eco*RV for strains containing the *ade6* duplication. (B) The hybridization probe was a fragment of the *MATa* locus. As expected *MATa* was absent from wild-type and PS1 strains. With *Xho*I digests, 8.5-kb fragments were observed with strains SL1–4. With *Eco*RV digests, as expected from the scheme in Figure 2, *MATa* hybridized to fragments of ~10 kb with SL1 and SL2, and to fragments of 8 kb with SL3 and SL4.

nants in which the right-hand *ade6-M26/M375* heteroallele was converted to wild type (average 88% of conversion-types) were more common than those in which the left-hand *ade6-L469* heteroallele had been converted to wild type (average 12% of conversion types). It is likely that conversion-type recombinants in which the mutations (*L469* or *M26/M375*), rather than the wild-type information, are transferred from one heteroallele to the other can also arise spontaneously. Such convertants (*e.g.*, *ade6-M26-L469/ura4*$^+$*/ade6-M26*) would be Ade$^-$ Ura$^+$ but unable to give rise to Ade$^+$ recombinants. However, selection for these types of recombinants is not possible. If they arose, their frequency was low since of the red (Ade$^-$) colonies growing on the nonselective viability plates used for these assays, all of those tested (>1000 per strain) were Ade$^-$ Ura$^+$ and could give rise to Ade$^+$ recombinants, suggesting that they contained the unaltered substrates.

The spontaneous recombinant frequencies for PS1, PS3 and SL1–4 were not significantly different. Therefore, inclusion of the *MATa* site within the substrates had no detectable effect on recombinant frequencies. Additionally, the spontaneous and HO-induced recombinant frequencies for PS1 and PS3 were not significantly different. This shows that expression of HO in the absence of the *MATa* target site has no effect on recombinant frequencies.

Effect of induction of a DSB within unique DNA, between the *ade6* heteroalleles: The total frequency of Ade$^+$ recombinants was stimulated ~2000-fold relative to the frequency of spontaneous recombinants. Almost all Ade$^+$ recombinants (>99.8%) were deletion-type Ade$^+$ Ura$^-$ recombinants. The frequencies of Ade$^-$ Ura$^-$ deletion-type recombinants were stimulated >5000-fold compared to spontaneous frequencies. Appropriate test crosses were used to confirm the presence and absence of particular *ade6* alleles remaining in the Ade$^-$ Ura$^-$ recombinants. The Southern hybridization analysis (Figure 6, B and F) and the genetic analysis (see MATERIALS AND METHODS; data not shown) showed that the Ade$^-$ Ura$^-$ colonies had lost the *MATa* site and contained a single copy of *ade6* in which either the *L469* mutation (10-kb *Xho*I fragment) or the *M26/M375* mutation (1.9-kb *Xho*I fragment) was retained. For SL1, of 20 Ade$^-$ Ura$^-$ colonies tested, 14 were *ade6-M26* and six were *ade6-L469*. For SL2, 13 were *ade6-M375* and seven *ade6-L469*. Genetic test crosses showed that in no cases were both point mutations present within the recombinant *ade6* gene (data not shown). Thus a DSB in unique DNA stimulated recombination between adjacent duplicated DNA, giving rise primarily to deletion-type recombinants.

For SL2, in which the right-hand heteroallele is *M375*, there was no significant effect of DSB induction on the frequency of Ade$^+$ Ura$^+$ conversion-type recombinants compared to spontaneous frequencies. However, for SL1, in which the right-hand heteroallele is *M26*, there was a 20-fold increase in the frequency of conversion-type recombinants in which the *ade6-M26* heteroallele had been converted to wild type, although no significant effect was observed on the frequency of those in which *ade6-L469* had been converted to wild type. All (20/20) of the conversion-type recombinants analyzed by Southern hybridization had retained the *MATa* site (Figure 6F).

A significant difference was also observed between the total frequency of induced recombinants obtained with SL1 (*ade6-M26*) and SL2 (*ade6-M375*). Although the frequencies of Ade$^-$ Ura$^-$ recombinants were virtually the same, the frequency of Ade$^+$ Ura$^-$ colonies was ~1.5-fold higher for SL2 than for SL1. Conversely, the

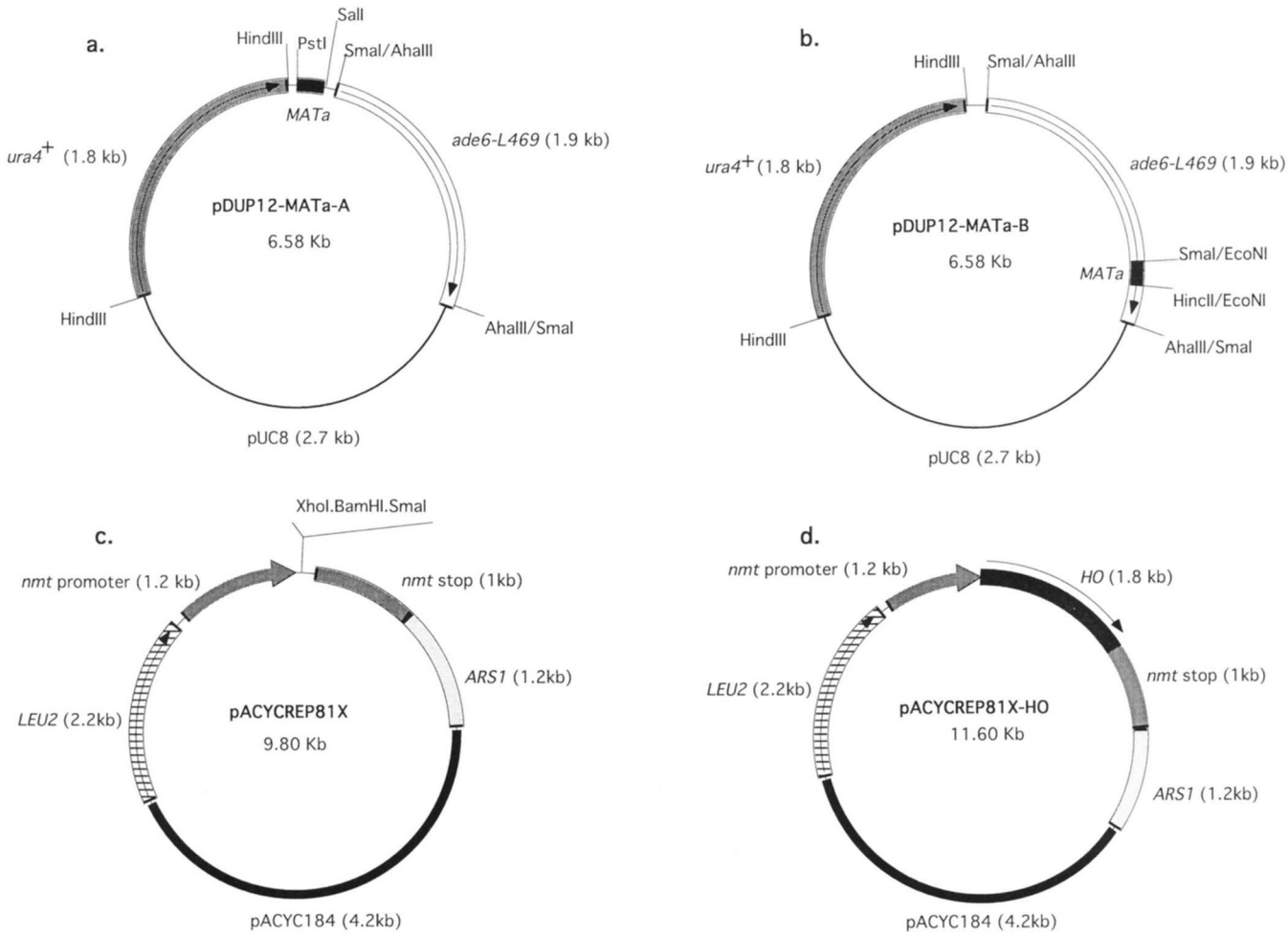

FIGURE 4.—Plasmids. (a) A 175-bp *Pst*I-*Sal*I fragment containing part of *S. cerevisiae MATa,* including the target site of HO endonuclease (the *y*/*z* junction) (KOSTRIKEN *et al.* 1983), was cloned into the *Pst*I-*Sal*I sites of the polylinker of pDUP12 between the *ura4*$^+$ and *ade6-L469* sequences to give plasmid pDUP12-MATa-A. (b) Similarly, a 179-bp *Sma*I-*Hin*dII fragment was cloned into the *Eco*NI site of *ade6-L469* in pDUP12 to give plasmid pDUP12-MATa-B. (c) pACYCREP81X was constructed by replacing the pUC119 sequences in pREP81X with pACYC184 sequences. (d) pACYCREP81X-HO contains the *S. cerevisiae HO* gene under the control of a modified *nmt1* promotor.

frequency of colonies with an unaltered parental phenotype (Ade$^-$ Ura$^+$, DSB-induced red/white sectoring) was almost 40-fold greater for SL1 than for SL2; virtually all of the colonies from SL2 were recombinants whereas ~20% of the colonies from SL1 had the parental phenotype.

For SL1 and SL2, all Ade$^-$ Ura$^+$ colonies exhibited the DSB-induced red/white sectoring phenotype on media lacking thiamine and containing low adenine, indicating the presence of *ade6* heteroalleles with no loss of *MATa.* This indicates that there is nothing to inherently prevent cutting at the *MATa* site in cells which failed to recombine initially upon HO induction. For SL1, all 10 of the Ade$^-$ Ura$^+$ sectoring colonies analyzed by probing with *ade6* and *MATa* gave the hybridization pattern expected for an intact *ade6* duplication (Figure 6, D and F). None gave the pattern expected for a triplication of *ade6,* which would be one of the expected products of unequal exchange between sister chromatids in G2 (Figure 1). The same result was obtained for 7/10 of the rare Ade$^-$ Ura$^+$ colonies from SL2, but three colonies gave patterns indicative of multiple copies of the duplication or part of the duplication (data not shown). None gave the pattern indicative of a simple triplication, and so they appear to have arisen by more complex events.

Effect of induction of a DSB within duplicated DNA in the left-hand *ade6-L469* heteroallele: The total frequency of Ade$^+$ recombinants and of deletion-type Ade$^+$ Ura$^-$ and Ade$^-$ Ura$^-$ recombinants was again stimulated ~2000-fold. Southern hybridization analysis (Figure 6, C and F) and genetic analysis (data not shown) of the Ade$^-$ Ura$^-$ colonies showed that the 10 examined per strain had lost the *MATa* site and contained a single copy of *ade6* in which only the *M26* or *M375* mutation sites were present.

There was also an ~1750-fold increase in the frequency of Ade$^+$ Ura$^+$ convertants. The vast majority of the induced Ade$^+$ convertants were wild type for the left-hand (cut) *ade6* repeat. In contrast, among sponta-

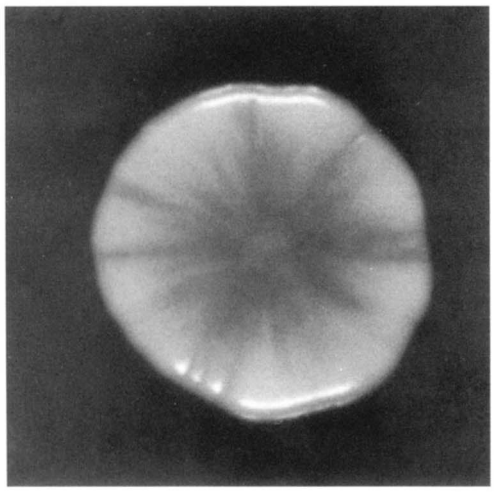

FIGURE 5.—Effect of HO induction on intrachromosomal recombination in an *ade6* duplication strain containing *MATa*. Cells with substrate SL1 containing pACYCREP81X-HO were plated on low adenine minimal media lacking thiamine and grown for 4 days. On low adenine plates *ade6*$^-$ cells are red, whereas *ade6*$^+$ cells are white. When HO expression is switched on (no thiamine), DSB-induced recombinants arise at a high frequency and the colonies appear with red/white sectors.

neous convertants the right-hand *ade6-M26* or *-M375* heteroallele was more frequently converted to wild type. Thus, for DSB-induced recombination, the *ade6* gene containing the *MATa* site (*i.e.,* the DSB) is the recipient of genetic information during conversion. This result is in agreement with the DSB/gap repair model for recombination (SZOSTAK *et al.* 1983). The site of the DSB in *MATa* is 148 bp from the *L469* point mutation. The *L469* mutation must be coconverted to wild type to give rise to these Ade$^+$ Ura$^+$ convertants. As described in MATERIALS AND METHODS, there were two other classes of DSB-induced conversion-type recombinants from SL3 and SL4 that differed in the extent of the conversion tract in the left-hand *ade6* heteroallele: convertants in which only *MATa* was converted to wild-type without coconversion of the *L469* point mutation and convertants in which the information in the left-hand heteroallele had been extensively replaced by information from the right-hand *ade6-M26/M375* so that the duplicated *ade6* heteroalleles contained the same information and therefore could not recombine to give Ade$^+$ recombinants. Convertants in which the right-hand *ade6-M26/M375* heteroallele was converted to wild type occurred at low frequencies, retained the *MATa* site (Figure 6F), and probably arose spontaneously.

For SL3 and SL4, all 20 of the Ade$^-$ Ura$^+$ sectoring colonies analyzed by probing with *ade6* and *MATa* gave the hybridization pattern expected for an intact *ade6* duplication (Figure 6, D and F).

A disparity between *ade6-M26*-containing (SL3) and *ade6-M375*-containing (SL4) substrates was again observed. The substrate with the *M26* allele gave more Ade$^-$ Ura$^+$ conversion-type recombinants that had just lost *MATa* (10 and 5% of colonies for SL3 and SL4, respectively) and a lower frequency of colonies with the unrecombined substrate (4% of colonies for SL3, 12% of colonies for SL4).

Demonstration that HO expression results in the introduction of a DSB within the *MATa* site: We tested whether expression of HO produces a DSB within the *MATa* site in *S. pombe*. The SL2 substrate was chosen because its recombinant products were primarily deletion types that had lost the *MATa* site, so that the appearance of a DSB could be easily diagnosed by Southern-hybridization analysis. DSB-induction was clearly observed 24 hr after switching to growth in media lacking thiamine (Figure 7), consistent with the time frame reported for the induction of the *nmt1* promoter (FORSBURG 1993). The production of DSBs coincided with the appearance of DSB-induced deletion-type recombinants (data not shown).

DSB-induced red/white half sectored colonies: DSB-induced red/white half-sectored colonies were isolated from strains containing substrates SL1–4 as described in MATERIALS AND METHODS. These half-sectored colonies likely arose from genetic recombination intermediates containing unrepaired heteroduplex DNA (hDNA). The half-sectored colonies were analyzed for phenotype. The results obtained were as follows: for SL1 (15 out of 15) and SL2 (18 out of 18), all red/white half-sectored colonies were Ade$^-$ Ura$^-$/Ade$^+$ Ura$^-$. This was also the case for most half-sectored colonies from SL3 (13 out of 15) and SL4 (14 out of 16). For SL3 one colony was Ade$^+$ Ura$^+$-pink/Ade$^-$ Ura$^+$, sectoring. One colony had the phenotype Ade$^+$ Ura$^+$-pink/Ade$^-$ Ura$^+$, nonsectoring. For SL4 one colony was Ade$^+$ Ura$^+$-pink/Ade$^-$ Ura$^-$, and one Ade$^+$ Ura$^+$-pink/Ade$^-$ Ura$^+$, nonsectoring.

These results are consistent with each sectored colony representing a single recombination event in which hDNA formed but was not repaired before DNA replication, but at least two alternatives are possible. One alternative interpretation invokes two G2-associated recombination events, one on each sister chromatid. However, the similar frequencies of selected and unselected Ade$^+$ recombinants and the absence of triplications suggest that most recombination occurs in G1. Second, the sectored colonies could represent the chance immediate adjacent seeding of two different recombinant cells. However, the shape of the half-sectored colonies, the low density of cells plated, and the low frequency of recombinants observed in liquid cultures at this time point suggest that this is less likely.

DISCUSSION

***S. cerevisiae* HO endonuclease and *MATa* can be used to introduce site-specific DSBs within *S. pombe* DNA:**

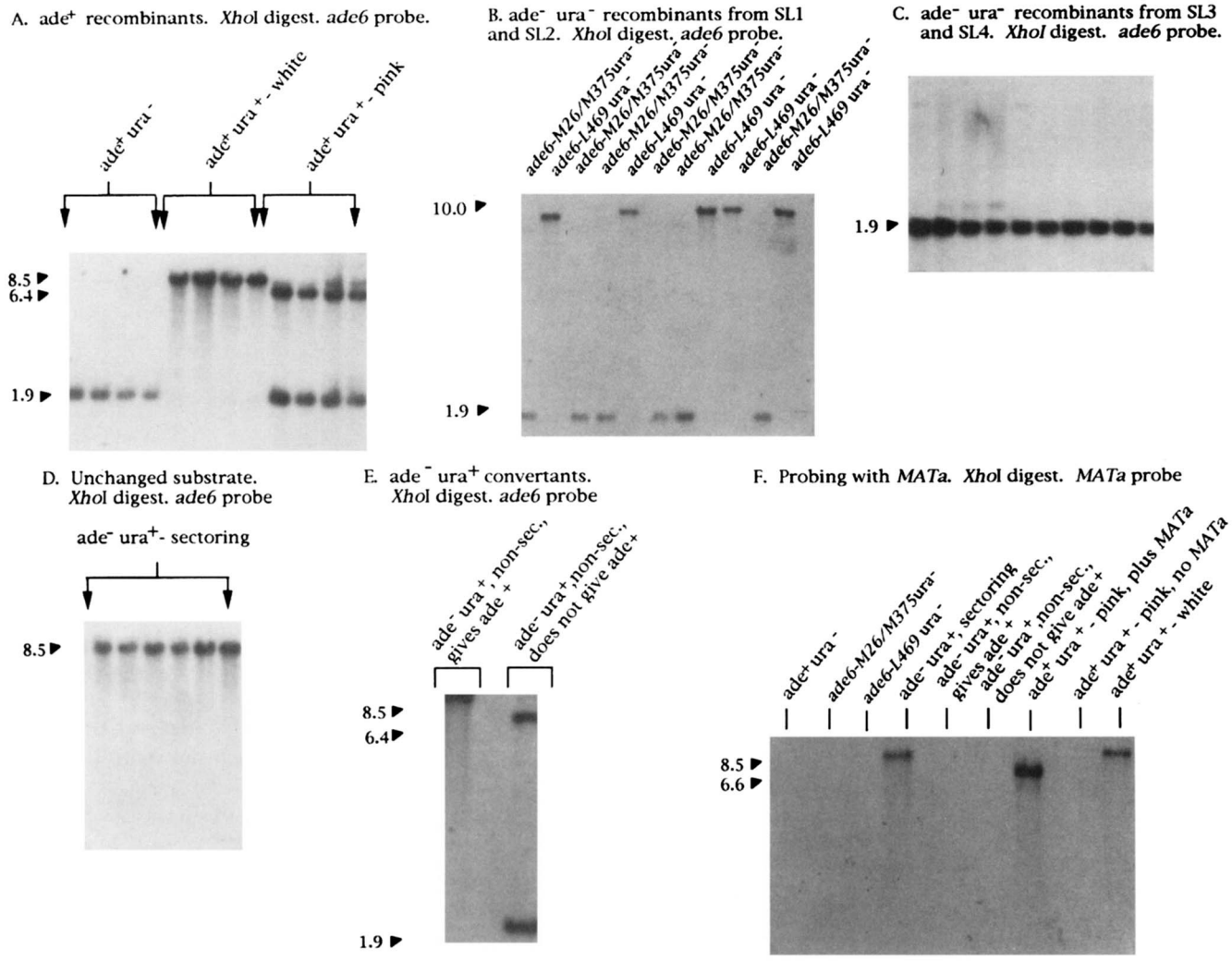

FIGURE 6.—Southern blot hybridization analysis of DSB-induced recombinants. Genomic DNA was digested with *Xho*I and probed with the *ade6* gene (A–E) or *MATa* (F). With the *ade6* probe, the Ade$^+$ Ura$^-$ recombinants produced a 1.9-kb band expected for a single copy of wild-type *ade6* (A) with the *MATa* site deleted (F). With the *ade6* probe, Ade$^+$ Ura$^+$-white recombinants produced a single band of ~8.5 kb indicative of two copies of *ade6* with conversion of the right-hand allele to wild-type. All Ade$^+$ Ura$^+$-white recombinants still contained *MATa* (F). With the *ade6* probe, Ade$^+$ Ura$^+$-pink recombinants produced two bands of ~6.4 and 1.9 kb indicative of two copies of *ade6* with conversion of the left-hand *L469* mutation to wild type restoring the *Xho*I site. The Ade$^+$ Ura$^+$-pink recombinants from SL1 and SL2 still contained *MATa,* whereas those from SL3 and SL4 lacked *MATa* (F). The Ade$^-$ Ura$^-$ recombinants from SL1 and SL2 produced one of two possible patterns when probing with *ade6* (B). In some cases a 1.9-kb fragment was produced that indicated a single *ade6* gene in which the *L469* mutation has been repaired to wild type restoring the *Xho*I site. The *M26* or *M375* mutation is still present since the strain is still Ade$^-$ (confirmed genetically by crossing to *M26* or *M375*). Alternatively, a 10-kb fragment was produced indicative of a single copy of *ade6* in which the *L469* mutation was retained eliminating the *Xho*I site at this location. This pattern would also arise from a *ade6-M26/M375-L469* double mutant, but the *M26/M375* locations were shown to be wild type genetically. The Ade$^-$ Ura$^-$ recombinants from SL3 and SL4 all contained a single copy of *ade6* in which the *M26/M375* mutation was retained and the *L469* mutation had been repaired to wild type restoring the *Xho*I site (C). The pattern from Ade$^-$ Ura$^+$ sectoring strains (D) indicates no change in the recombination substrate with retention of *MATa* (F). The Ade$^-$ Ura$^+$ nonsectoring colonies (*i.e.,* no high-frequency sectoring as in Figure 5) gave the patterns (E and F) expected for an intact duplication in which either just the *MATa* site had been converted to wild type, or the whole of the left-hand heteroallele had been converted to the right-hand *ade6* heteroallele.

We have shown that the *S. cerevisiae* HO endonuclease, expressed from an *S. pombe* promotor, and its *MATa* target site can successfully be used to introduce site-specific DSBs within DNA in *S. pombe* (Figure 7). The *S. pombe* genome does not contain sequences that cross-hybridize to the *MATa* target site (Figures 3B and 6F), and expression of HO endonuclease in the absence of the *MATa* site had no effect on recombination in the *ade6* duplication substrate (Table 2). The *HO* gene and the *MATa* site should prove useful in the study of site-specific DSB-induced recombination in a wide variety of recombination substrates in *S. pombe,* as they have in *S. cerevisiae.* In this study we used these tools to introduce the DSB within intrachromosomal recombination substrates. DSB induction increased the recombinant frequency ~2400-fold (average of all strains), so that

TABLE 2

Spontaneous and DSB-induced intrachromosomal mitotic recombinant frequencies

Duplication	Location of *MATa*	HO	Deletion type (Ura$^-$)		Conversion type (Ura$^+$)				
			Ade$^+$	Ade$^-$	*M26/M375* → *ade6*$^+$	*L469* → *ade6*$^+$	*ade6-MATa-L469* → *ade6-L469*	*ade6-MATa-L469* → *ade6-M26/M375*	Unchanged substrate
PS1	Absent	−	1.34 ± 0.92	0.62 ± 0.36	0.25 ± 0.15	0.03 ± 0.03	NA	NA	NA
(*M26*)		+	0.81 ± 1.11	ND	0.10 ± 0.07	0.01 ± 0.01	NA	NA	NA
PS3	Absent	−	2.46 ± 0.95	0.61 ± 0.27	0.67 ± 0.58	0.19 ± 0.50	NA	NA	NA
(*M375*)		+	1.57 ± 2.34	ND	0.65 ± 1.35	0.06 ± 0.05	NA	NA	NA
SL1	Unique	−	1.63 ± 0.49	0.85 ± 0.35	0.51 ± 0.27	0.13 ± 0.21	NA	NA	NA
(*M26*)	DNA	+	3800 ± 730	4200 ± 970	10.37 ± 3.09	0.21 ± 0.18	NA	NA	2000 ± 630
SL2	Unique	−	2.65 ± 1.42	0.77 ± 0.34	0.54 ± 0.70	0.01 ± 0.01	NA	NA	NA
(*M375*)	DNA	+	5700 ± 700	4200 ± 737	1.84 ± 0.90	0.15 ± 0.17	NA	NA	50 ± 90
SL3	Duplicated	−	2.50 ± 0.42	1.12 ± 0.37	0.62 ± 0.42	0.03 ± 0.02	NA	NA	NA
(*M26*)	DNA	+	4900 ± 660	2300 ± 560	16.2 ± 50.0	1300 ± 670	1000 ± 250	100 ± 80	440 ± 110
SL4	Duplicated	−	2.37 ± 0.76	0.73 ± 0.52	0.58 ± 0.47	0.13 ± 0.39	NA	NA	NA
(*M375*)	DNA	+	4900 ± 730	2200 ± 500	4.75 ± 17.1	1100 ± 530	500 ± 230	120 ± 90	1200 ± 370

Recombinant frequencies (per 10^4 viable cells ± SD) were obtained as described in MATERIALS AND METHODS. −, HO expression repressed; +, HO expression induced; ND, not determined; NA, not applicable.

90% of the resulting colonies were recombinants. This high recombinant frequency allowed the analysis of all (unselected) cells arising from a duplication strain following DSB induction. This permitted an unbiased analysis of all the different fates of the recombination substrate.

The spectrum of DSB-induced recombinants depended on whether the DSB was introduced within duplicated or unique DNA: When the DSB was situated within unique DNA between the *ade6* heteroalleles, over 99.8% of DSB-induced recombinants were deletion type in which the single copy of *ade6* was either wild type (Ade$^+$ Ura$^-$) or one of the heteroalleles (*ade6-L469 ura*$^-$ or *ade6-M26/M375 ura*$^-$). With these strains, the relative proportions of recombinants that arose spontaneously differed markedly from those that arose after DSB induction (Table 3). When the DSB was located in duplicated DNA in the left-hand *ade6-L469* heteroallele, most (average 77%) were deletion-type recombinants in which the single copy of *ade6* was exclusively either wild type or the uncut heteroallele. The remaining recombinants were conversion-types (average 23%). In most of these the copy of *ade6* receiving the DSB was the recipient of genetic information, which is a prediction of the DSB/gap-repair model for recombination (SZOSTAK *et al.* 1983). Several different types of conversion-type recombinants were observed depending on the length of the conversion tract: those in which only the *MATa* site was lost, those that coconverted both the *MATa* site and the *L469* mutation to wild type, and those that converted all the information of the left-hand heteroallele to that of the right-hand *ade6-M26/M375* heteroallele.

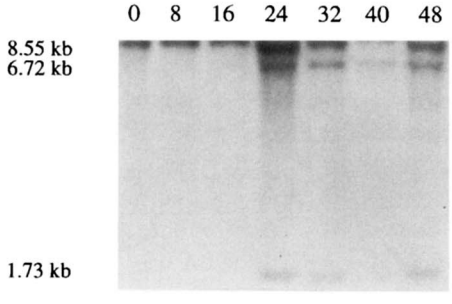

FIGURE 7.—Induction of DSBs by HO in SL2. DNA from cells induced for HO expression was extracted, digested with *Xho*I and analyzed as described in MATERIALS AND METHODS. The unrecombined substrate not cut by HO produced a fragment of 8.55 kb (Figure 2) and that cut by HO produced fragments of 6.72 and 1.73 kb.

Table 3 shows the proportions of the different classes of scorable spontaneous and DSB-induced recombinants obtained. For SL3 and SL4, the proportion of spontaneous and DSB-induced recombinants are comparable, except for the difference of which heteroallele, the left-hand *ade6-L469* or the right-hand *ade6-M26/M375,* becomes wild type in conversion-type recombinants. These results suggest that spontaneous recombinants could arise due to spontaneous DSBs within duplicated DNA in the right-hand *ade6* heteroallele. The reason for the biased conversion of the right-hand *ade6* heteroallele to wild type during spontaneous recombination is unknown (SCHUCHERT and KOHLI 1988).

It appears that DSB-induced mitotic intrachromosomal recombination in *S. pombe* is very similar to that in *S. cerevisiae,* rather than to that in mammalian cells. Although *S. pombe* (GOEDECKE *et al.* 1994) and *S. cerevisiae* (ORR-WEAVER and SZOSTAK 1983; SCHIESTL *et al.* 1993) do have activities to promote nonhomologous end-joining, in the presence of homologous sequences, homologous recombination events were predominant. In mammalian cells nonhomologous end-joining events predominate over homologous recombination events even in the presence of homologous sequences (reviewed by ROTH and WILSON 1988). Also, whereas unequal sister chromatid exchanges are common

TABLE 3

Proportion of scorable spontaneous and DSB-induced recombinants

Duplication	Location of *Mata*	Deletion type (Ura$^-$)				Conversion type (Ura$^+$)			
		Ade$^+$		Ade$^-$		*M26/M375* → *ade6*$^+$		*L469* → *ade6*$^+$	
		Spontaneous	Induced	Spontaneous	Induced	Spontaneous	Induced	Spontaneous	Induced
SL1 (*M26*)	Unique DNA	52 ± 16[a]	47 ± 9	27 ± 11	52 ± 12	16 ± 9	<1 ± <1	5 ± 7	<1 ± <1
SL2 (*M375*)	Unique DNA	67 ± 36	58 ± 7	19 ± 9	42 ± 7	14 ± 18	<1 ± <1	<1 ± <1	<1 ± <1
SL3 (*M26*)	Duplicated DNA	59 ± 10	58 ± 8	26 ± 9	27 ± 7	14 ± 10	<1 ± <1	<1 ± <1	15 ± 8
SL4 (*M375*)	Duplicated DNA	62 ± 20	60 ± 9	19 ± 14	27 ± 6	15 ± 12	<1 ± <1	3 ± 10	14 ± 7

[a] Percentage of the total types of recombinants specified in this table, from data in Table 2.

with intrachromosomal recombination substrates in mammalian cells (BOLLAG and LISKAY 1991), with *S. pombe* and *S. cerevisiae* unequal sister chromatid interactions appear to be rare (JACKSON and FINK 1981; NICKOLOFF *et al.* 1989; THOMAS and ROTHSTEIN 1989). In this study no *ade6* triplications, which are diagnostic of unequal sister chromatid exchanges, were observed.

Studies in *S. cerevisiae* with intrachromosomal or extrachromosomal substrates, in which the DSB was made in unique DNA, also showed that the vast majority of recombinants were due to deletion-type events (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989; SUGAWARA and HABER 1992). Similarly, in *S. cerevisiae* substrates in which the DSB was made in one of two copies of the repeated gene, the same types of conversion- and deletion-type recombinants were induced (NICKOLOFF *et al.* 1986, 1989; RAY *et al.* 1988; RUDIN *et al.* 1989; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992). Although the proportions of conversion- and deletion-type recombinants were substrate specific in *S. cerevisiae*, they were the same for spontaneous and DSB-induced events. For example, in one study using duplications of the *ade4* gene (RAY *et al.* 1988) over 90% of the spontaneous and DSB-induced recombinants were conversion type. In separate studies using duplications of the *ura3* gene (NICKOLOFF *et al.* 1986, 1989), spontaneous and DSB-induced deletion-type events outnumbered conversion-type events. The basis for this substrate specificity is not currently known.

DSB-induced recombinants could arise via the SSA and DSB/gap-repair recombination pathways: As discussed earlier, it has been suggested that there are two competing pathways for the repair of a DSB produced between directly repeated sequences in *S. cerevisiae* (RUDIN and HABER 1988; RUDIN *et al.* 1989; FISHMAN-LOBELL and HABER 1992; FISHMAN-LOBELL *et al.* 1992; SUGAWARA and HABER 1992), one based on the SSA model (LIN *et al.* 1990a) and the other on the DSB/gap-repair model (SZOSTAK *et al.* 1983).

In SL1 and SL2, with the DSB induced within unique DNA, the lack of two homologous ends would preclude DSB/gap-repair as a pathway for recombination, so that the SSA pathway would predominate, giving rise to mostly deletion-type events. This is what is seen, and the SSA model shown in Figure 8 can explain the different types of DSB-induced Ade$^+$ Ura$^-$ and Ade$^-$ Ura$^-$ (*ade6-L469* or *ade6-M26/M375*) recombinants obtained with substrates SL1 and SL2. The *ade6* repeat unit is 1887 bp in length, the *L469* point mutation in the left-hand heteroallele is at bp 1648 and the *M26* and *M375* mutations in the right-hand heteroallele are at bp 317 and 314, respectively. Our results suggest that the single-strand 5′-to-3′ exonuclease digestion exposes extensive complementary homologous single strands in the two *ade6* repeats. According to the model, digestion on the lower strand could extend beyond the position of *M26*$^+$/*M375*$^+$ (bp 317/314) in the left-hand *ade6* gene and on the upper strand either before (Figure 8, SL2: a) or beyond (Figure 8, SL2: b) the position of *L469*$^+$ (bp 1648) in the right-hand copy of *ade6*. Following annealing of the complementary strands and cutting of the 3′ single-strand tails, two types of hDNA are formed in which either only the *M26/M375* site is hybrid (Figure 8, SL2: e), or both the *M26/M375* and the *L469* sites are hybrid (Figure 8, SL2: f). Mismatch correction of hDNA results in either Ade$^+$ Ura$^-$ or Ade$^-$ Ura$^-$ recombinants. The absence of Ade$^-$ Ura$^-$ recombinants that retain both mutations (*L469* and *M26/M375*) suggests that either the upper or lower single strands of the hDNA covering the entire *ade6* locus are subject to mismatch repair.

With the DSB induced within duplicated DNA, the two pathways could compete, in which case recombination products derived from the SSA and DSB/gap-repair models should be seen. The DSB/gap-repair model can account for all the DSB-induced recombinants obtained from SL3 and SL4. As described earlier, following DSB-induction within the *ade6-L469* heteroallele, three main types of convertants were obtained. The fact that the *ade6-M26/M375-pUC8-ura4*$^+$*-ade6-M26/M375* convertants were obtained shows that the conversion tract can extend 1.36 kb to the left of the DSB. The DSB/gap repair model (Figure 9) could account for both the deletion and conversion recombinants derived

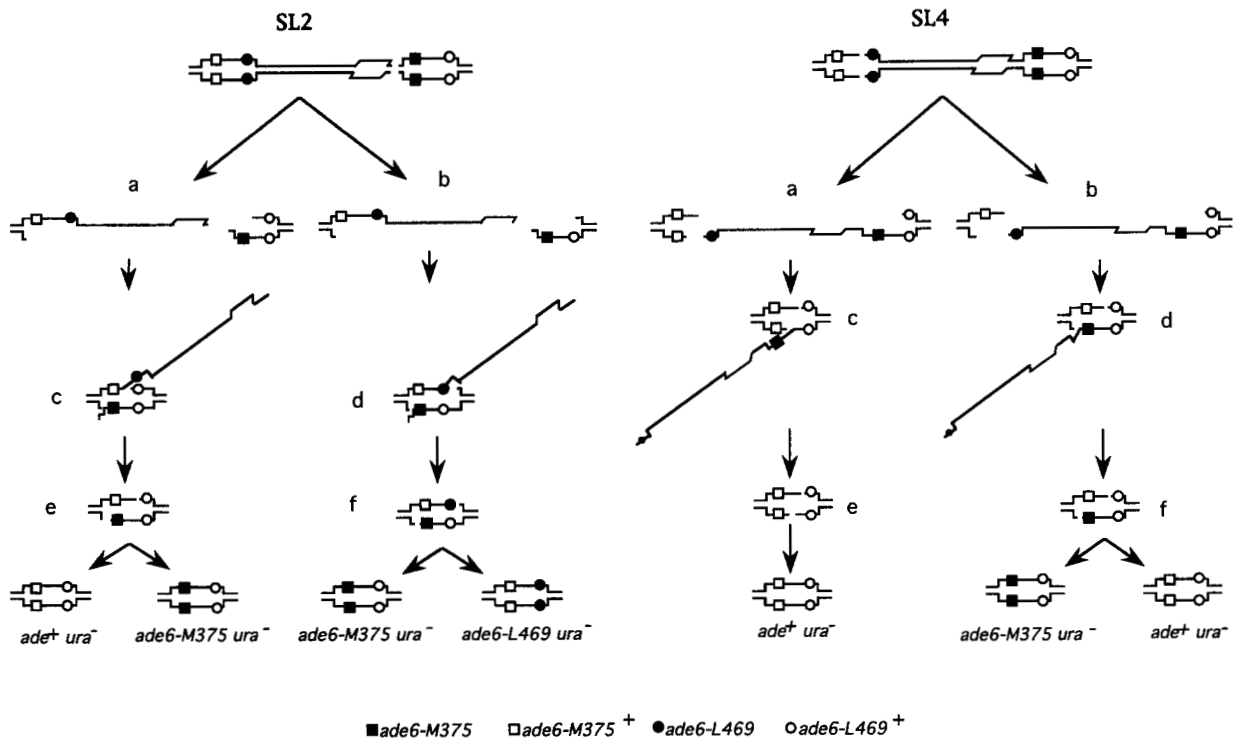

FIGURE 8.—Single-strand annealing model for DSB-induced recombination. For SL2, the DSB is made in nonhomologous DNA. A 5′-to-3′ exonuclease digests the substrate to reveal homology. In a the top strand is digested up to a point before the position of *L469*$^+$. In b the exonuclease digests past the *L469*$^+$ site. The bottom strand is digested past the *M375*$^+$ position in the left-hand heteroallele. Alternatively, the digestion of the bottom strand could stop before the positions of the *L469* mutation or the *M375*$^+$ positions (not shown). Homologous sequences align to give rise to a single copy of *ade6* with heteroduplex DNA at only the *M375* position in c or in both positions in d with associated 3′ tails, or in only the *L469* position (not shown). 3′ tails are cleaved to produce the structures shown in e and f, and mismatch repair of the heteroduplexes yields either wild-type or mutant genes (*ade6-L469* or *ade6-M375*). For SL4, analogous steps are shown. Digestion of the bottom strand to a point before (a) or after (b) the *M375*$^+$ position. In both cases, the top strand is digested to a point before (a and b) or after (not shown) the *L469*$^+$ position. Aligned sequences in which *ade6* is either wild-type (c) or has heteroduplex DNA in the *M375* position (d) with associated 3′ tails. Processing of the tails yields structures (e and f) that yield wild-type *ade6*$^+$ or *ade6-M375*.

from SL3 and SL4 only if the recombination intermediates generated are resolved greatly in favor of crossover-type events relative to noncrossover events. Only then could this model explain the substantially lower frequency of *ade6-M26/M375-pUC8-ura4*$^+$*-ade6-M26/M375* and *ade*$^+$ *ura*$^+$ convertants relative to that of *ade6-M26/M375 ura*$^-$ and *ade*$^+$ *ura*$^-$ deletions, respectively. However, the strong bias for crossover events would predict also that during the generation of convertants of *MATa* alone (the second most common class of convertants), resolution of both Holliday junctions in the DSB/gap repair model in favor of crossovers would yield some *ade6-L469/ade6-M26* and *ade6-L469/ade6-M375* double mutants. These products were never observed. Furthermore, DSB-induced deletion-type recombinants obtained with SL3 and SL4 are unlikely to have all arisen by DSB/gap repair, especially since evidence in *S. cerevisiae* suggests that gap-repaired intermediates obtained from homologous repeats in direct orientation are rarely resolved to give the crossover product (RAY *et al.* 1988; FISHMAN-LOBELL *et al.* 1992). It is more likely that the conversion-type recombinants arose by the DSB/gap repair pathway, while most of the DSB-induced deletion-type recombinants arose via the SSA pathway. The SSA model (Figure 8) can account for the deletion-type recombinants obtained with SL3 and SL4, and also provides an explanation for the absence of *ade6-L469 ura*$^-$ recombinants, since information in the left-hand *ade6* gene 3′ to the site of the DSB (which includes the *L469* mutation) is necessarily lost by exonuclease digestion.

More recent considerations have prompted a modification of the DSB/gap repair model to include extensive 5′-to-3′ exonucleolytic digestion of the DSB to create long single-stranded 3′-tails that form hDNA (WHITE and HABER 1990; SUN *et al.* 1991; NAG and PETES 1993; WU and LICHTEN 1994). These studies provided evidence for the absence of a gap and explain gene conversion by hDNA formation and mismatch repair. Thus the single-stranded DNA intermediates for SL2 and SL4 shown in Figure 8, a and b could be common intermediates for the modified DSB/gap repair and SSA pathways. Consistent with the modified DSB/gap repair and SSA models, for both HO-induced extrachromosomal and intrachromosomal events, single-stranded DNA was produced by bidirectional 5′–3′ exonucleolytic activity

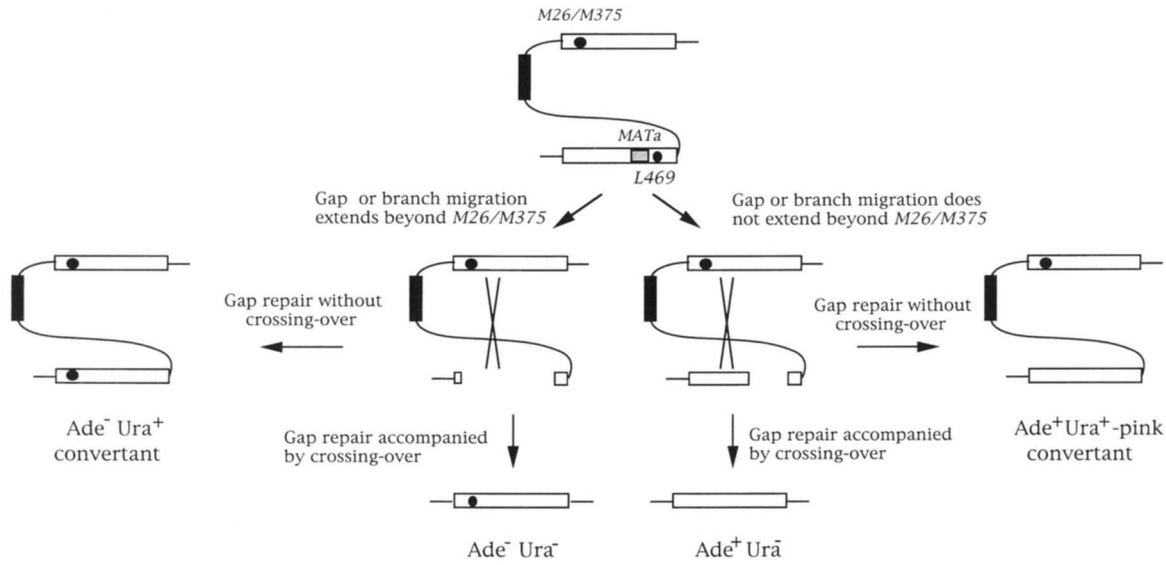

FIGURE 9.—Schematic of DSB/gap-repair model for DSB-induced recombination with *MATa* within duplicated DNA.

after DSB formation and consumed as products formed (RUDIN and HABER 1988; RUDIN *et al.* 1989; FISHMAN-LOBELL *et al.* 1992; SUGAWARA and HABER 1992).

DSB-induced half-sectored colonies provide evidence of hDNA intermediates: All models of recombination postulate the formation of hDNA intermediates. The isolation and analysis of red/white half-sectored colonies was interpreted as evidence of hDNA recombination intermediates. When uncorrected mispairs arise in duplex DNA, semiconservative DNA replication of the resultant heteroduplex segregates genetically distinct daughter cells. Half-sectored colonies from direct-repeat substrates have also been previously isolated in *S. cerevisiae* (RONNE and ROTHSTEIN 1988) and mammalian cells (BOLLAG *et al.* 1992; DENG and NICKOLOFF 1994) and interpreted as evidence of mitotic hDNA intermediates during the SSA and DSB/gap repair pathways. During the SSA mechanism, extensive single-strand DNA degradation to reveal complementary homologous regions will result in the formation of hDNA as discussed above (Figure 8). In the absence of mismatch correction, in some cases a red/white half sectored colony will result in which one half-sector will be Ade^- Ura^- and the other Ade^+ Ura^-. This was the case for all half-sectored colonies isolated from strains SL1 and SL2, and most of those isolated from SL3 and SL4. These half-sectored colonies provide compelling evidence for the formation of hDNA intermediates in the SSA pathway.

SL3 and SL4 also gave other rare types of half-sectored colonies (see RESULTS). We cannot infer from our data whether these arose from intermediates in the SSA or DSB/gap repair pathways.

The *ade6-M26* mutation affected DSB-induced mitotic intrachromosomal recombination: The *M26* mutation creates a hot-spot for meiotic homologous recombination. (GUTZ 1971; PONTICELLI *et al.* 1988; SCHUCHERT and KOHLI 1988; SCHUCHERT *et al.* 1991; WAHLS and SMITH 1994). *M26* does not stimulate spontaneous mitotic interchromosomal (PONTICELLI *et al.* 1988) or intrachromosomal (SCHUCHERT and KOHLI 1988) recombination between *ade6* heteroalleles. However, following DSB-induction we observed several differences between duplication substrates containing *M26* compared to those containing *M375* (Table 2).

For substrates in which the DSB was made in unique DNA, the one containing the *M26* mutation (SL1) had a 1.5-fold lower frequency of Ade^+ Ura^- deletion-type recombinants, and the frequency of colonies with the unchanged substrate was increased 40-fold compared to the substrate with the *M375* mutation (SL2). It is possible that proteins that bind to the specific heptanucleotide sequence created by *M26* (WAHLS and SMITH 1994) may play a role in holding the broken chromosome together or preventing recombination by the SSA pathway. Also, for SL1 (*M26*) the frequency of DSB-induced conversion-type recombinants in which the *ade6-M26* heteroallele converted to wild-type was increased 20-fold compared to spontaneous frequencies, whereas the frequencies of spontaneous and DSB-induced conversion-type recombinants in which *ade6-L469* converted to wild-type were the same. For SL2 (*M375*), the spontaneous and DSB-induced frequencies of both of the conversion-type recombinants were the same. Thus *M26* increased the frequency of conversion-type recombinants in which the *ade6-M26* heteroallele was the recipient of wild-type information. It is interesting to note that *M26* is preferentially a recipient of genetic information in meiosis, too (GUTZ 1971).

For the substrates in which the DSB was made in duplicated DNA, the frequency of DSB-induced conversion-type recombinants, in which only the *MATa* site was removed, was slightly different (twofold higher), but the frequency of colonies with unrecombined sub-

strate was threefold lower for the substrate with *M26* (SL3) compared to the substrate with *M375* (SL4).

Thus overall, in both types of substrates, the *M26* mutation increased the frequency of DSB-induced conversion-type recombinants, but the effects were substrate dependent (SL1 *vs.* SL3) and either substantial (SL1) or subtle (SL3) compared to its effects on meiotic recombination. These effects are likely to be related to the ability of *ade6-M26* to create an initiation or resolution site for gene conversion (SCHAR and KOHLI 1994).

The increased frequency, compared to spontaneous levels, of DSB-induced conversion-type recombinants observed for SL1, in which the DSB was made in unique DNA, is interesting. Similar results were obtained in *S. cerevisiae* for recombination between repeats stimulated by a DSB in unique DNA, provided the DSB was close to one copy of the recombining alleles (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989), as is the case with our substrates. The lack of two homologous ends precludes the DSB/gap repair pathway as a mechanism for producing an increase in frequency of conversion-type recombinants in this substrate. How do DSBs in unique DNA stimulate conversion-type events? For *S. cerevisiae*, it was suggested that recombination may also occur in part by a strand invasion mechanism in which a single-stranded region forms on at least one side of the DSB (RUDIN and HABER 1988). In this model one strand invades the homologous duplex region on the opposite side and primes DNA synthesis leading to replacement of the mutant information in the duplicated DNA and resynthesis of the strand through the unique DNA. NICKOLOFF *et al.* (1989) proposed an alternative model in which the broken ends do not interact directly with the recombining regions, but instead a recombinase enters the break, searches for homology and stimulates strand exchange and hDNA formation leading to a conversion event. In both proposals, repair of the DSB is independent of the strand exchange event.

Summary: In conclusion, it appears that two pathways exist for DSB-induced mitotic intrachromosomal recombination between nontandem direct repeats in *S. pombe*. If the DSB is made within unique DNA between the repeats, the SSA pathway is predominant and the DSB is repaired by a deletion-repair event. If the DSB is made within duplicated DNA, it can be repaired by both the DSB/gap repair and SSA pathways. DSB-induced intrachromosomal mitotic recombination in *S. pombe* appears to be similar to that in *S. cerevisiae*. The detailed analysis of the different types of DSB-induced recombinants and their relative frequencies provides a strong basis for further studies and makes *S. pombe* an attractive and complementary system to help elucidate the mechanisms and genetic control of intrachromosomal recombination in eukaryotes. We are currently attempting to isolate new mutants defective in DSB-induced mitotic recombination and are determining the effects of various radiation-sensitive (*rad*) mutations.

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