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Identification of the Crossover Site during *FLP*-Mediated Recombination in the *Saccharomyces cerevisiae* Plasmid 2μm Circle

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The *FLP* protein of the *Saccharomyces cerevisiae* plasmid 2μm circle catalyzes site-specific recombination between two repeated segments present on the plasmid. In this paper we present results of experiments we performed to define more precisely the features of the *FLP* recognition target site, which we propose to designate *FRT*, and to determine the actual recombination crossover point in vivo. We found that essential sequences for the recombination event are limited to an 8-base-pair core sequence and two 13-base-pair repeated units immediately flanking it. This is the region identified as the *FLP* binding site in vitro and at which *FLP* protein promotes specific single-strand cleavages (B. J. Andrews, G. A. Proteau, L. G. Beatty, and P. D. Sadowski, Cell 40:795–803, 1985; J. F. Senecoff, R. C. Bruckner, and M. M. Cox, Proc. Natl. Acad. Sci. USA 82:7270–7274, 1985). Mutations within the core domain can be suppressed by the presence of the identical mutation in the chromatid with which it recombines. However, mutations outside the core are not similarly suppressed. We found that strand exchange during *FLP* recombination occurs most of the time within the core region, proceeding through a heteroduplex intermediate. Finally, we found that most *FLP*-mediated events are reciprocal exchanges and that *FLP*-catalyzed gene conversions occur at low frequency. The low level of gene conversion associated with *FLP* recombination suggests that it proceeds by a breakage-joining reaction and that the two events are concerted.

The 2μm circle plasmid of the yeast *Saccharomyces cerevisiae* encodes a specialized recombination system. This system consists of two homologous sites within two 599-base-pair (bp) segments of identical sequence, present in inverted orientation within the plasmid, and an enzyme encoded in the plasmid gene *FLP* that catalyzes recombination between these two sites (2–4). This recombination system provides a mechanism for plasmid amplification, apparently by inducing conversion of the mode of plasmid replication from theta to rolling circle through inversion of the relative orientation of the two replication forks at the ends of a replication bubble (6a, 24; A. Murray and J. Szostak, personal communication). Recombination systems of this type are a consistent feature of circular, double-stranded yeast plasmids (21, 22).

Site-specific recombination events have been documented in both procaryotic and eucaryotic cells, and in most cases the rearrangements resulting from these events have profound consequences for the biology of the organism. The *FLP* system provides a well-defined model system for eucaryotic site-specific recombination, and its experimental accessibility has prompted extensive in vivo and in vitro studies on the mechanism of the *FLP* reaction (1b, 3, 6, 6b, 12, 19, 23). Despite this extensive analysis, several fundamental questions regarding *FLP* recombination remain unanswered. These include identification of the specific site of recombination within the repeats, the specific sequences recognized by the enzyme, and the mode of strand exchange during the reaction. In addition, the relationship between recent observations on the properties of *FLP* protein in vitro and the actual recombination reaction promoted by *FLP* in vivo has not been clarified.

In this paper we present results of experiments we performed to define more precisely the features of the *FLP* recognition site and to determine the actual recombinational crossover point in vivo. We found that sequences essential for the recombination event are limited to an 8-bp core sequence and two 13-bp repeated units immediately flanking it. This is the region identified as the *FLP* binding site in vitro and at which *FLP* protein promotes specific single-strand cleavages (1, 19). In addition, we found that strand exchange during *FLP* recombination occurs most of the time within the core region but that the actual site of crossing over either occurs randomly within the core or proceeds through a heteroduplex intermediate. Finally, we found that although most *FLP*-mediated events are reciprocal exchanges, *FLP* can apparently also catalyze gene conversions at low frequency. These results are discussed in terms of possible models for *FLP*-mediated recombination.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* strains used in this study included DH1 (*recA1 thi-1 supE44 relA1 gyrA96 endA1 hsdR17* [*hsdR hsdM*$^+$]), provided by D. Hanahan; HB101 (F$^-$ *hsdS20* [*hsdR hsdM*] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*), provided by D. Shortle; MutD (F$^-$ *mutD5 metE lacZ trpA*), provided by D. Oliver; BD1528 (F′ *met hsdR hsdM supE supF ung-1 nadB7*), provided by B. Duncan through D. Shortle; and BW310 (*ung-1 hisB* [*hsdR*$^+$ *hsdM*$^+$]), obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. Yeast strain MM1 (*MATa leu2-3 leu2-112 ura3-52 trp1 ade1 gal2 HIS3::FLP* [cir^0]) has been described previously (12). Strain FVY2-6B (*MATα leu2-3 leu2-112 ura3-52 trp1 his3 GAL*$^+$ [cir^0]) was constructed by a genetic cross.

The precise structure and construction of plasmid pMMD2 has been previously described (12). Its salient features are indicated in Fig. 1. Plasmids designated pWP are identical to

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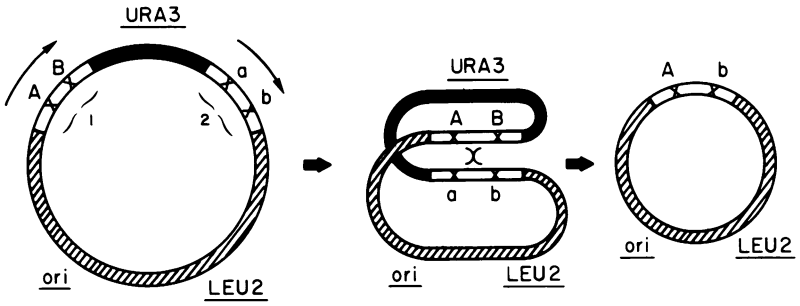

FIG. 1. *FLP*-mediated recombination of plasmid pMMD2. Plasmid pMMD2 (left), whose precise structure has been previously described (12), contains two complete repeat segments from the 2μm circle (unfilled regions) in direct orientation, bracketing in one half of the molecule (hatched region) the yeast *LEU2* gene, the 2μm origin of replication, and *bla* and *ori* sequences from pBR322. The repeats bracket the yeast *URA3* gene in the other half of the molecule (filled region). The plasmid shown represents a derivative of pMMD2 that contains two point mutations, a and b, in the right-hand repeat unit. The other repeat carries the wild-type alleles of these mutations. In vivo *FLP*-mediated recombination between the two repeats (middle figure) causes excision of the *URA3* gene, which does not persist in the cell, to yield the plasmid on the right. If, as drawn, crossing over occurs between sites a and b, then the recombinant plasmid will carry the wild-type allele A and the mutant allele b. Regions 1 and 2 are discussed in the text.

plasmid pMMD2 except that each of them carries a small (50- to 100-bp) deletion across the *Xba*I site in the 2μm circle repeat nearer the *LEU2* gene in the plasmid. An *Xho*I octanucleotide linker is inserted at the site of each deletion. The precise endpoints of the deletions in those plasmids used in this study were determined by sequencing by chemical cleavage procedures (11) and are identified in Fig. 2. Plasmid pINV1 was constructed by inserting the *Bam*HI-to-*Stu*I restriction fragment from plasmid pMMD2 that encompasses the mutated *FRT* site into *Bam*HI-*Stu*I-digested plasmid CV20 DNA (4).

Isolation of *FRT* site mutations. To isolate pMMD2 mutants by bisulfite mutagenesis, we first generated heteroduplex molecules between pMMD2 DNA and DNA from a selected pWP plasmid. We used slightly different protocols in isolating recombination-deficient and recombination-proficient mutations. To obtain recombination-deficient mutants, we recovered plasmid pMMD2 DNA from a modification-competent (*hsdM*$^+$) *E. coli* strain and digested 5 μg of it with *Bam*HI. *Bam*HI cuts at a single site in pMMD2, well removed from the 2μm circle repeats. We recovered pWP-2A plasmid DNA from a modification-deficient (*hsdM*) *E. coli* strain and digested 5 μg of it with *Xho*I. We mixed the two DNA samples in 1.0 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 M NaCl–1 mM EDTA, heated the sample to 90°C for 5 min, and then incubated it at 63°C for at least 60 min. This process yielded two species of heteroduplex molecules as well as both parental, double-stranded, linear species. Both heteroduplex species were essentially identical to pMMD2 except for a single-strand nick at the *Bam*HI site and a gap in the other strand at the site corresponding to the deletion in plasmid pWP-2A. This gap exposes the recombination site as single-stranded DNA. The two heteroduplex species differed only in which strand was exposed at the single-strand gap. We treated the heteroduplexed DNA sample with 1 M sodium bisulfite at 37°C for 30 min as described previously (20) to obtain approximately 1% deamination of the exposed cytosine residues. We transformed the treated DNA into strain BW130 (*hisB ung-1 hsdR*$^+$), a process which excludes linear DNA species as well as any contaminating uncut parental pWP-2A DNA. We pooled the approximately 400 transformants into groups of 10 each, isolated plasmid DNA from the pooled cultures, and then used each sample to transform yeast strain MM1 to uracil prototrophy by the lithium acetate method (9). We obtained numerous Ura$^+$ transformants with each DNA sample, but retained only one transformant from each. We recovered the mutant plasmid by preparing yeast DNA from the transformants and using it to transform *E. coli* DH1 to ampicillin resistance.

To prepare mutated derivatives of plasmid pMMD2 that still retained recombination proficiency, we used the above procedure except for the following modifications. First, we used plasmids pWP-12A and pWP-3A to generate heteroduplexes with plasmid pMMD2 DNA. Second, plasmid pMMD2 DNA was obtained from a modification-deficient strain, and plasmid pWP-12A and pWP-3A DNA samples were obtained from a modification-proficient strain. Third, we treated the heteroduplexed samples with 3 M sodium bisulfite for 4 to 8 h. Finally, we tested each initial *E. coli* transformant individually for its ability to transform strain MM1 to uracil prototrophy. Those that were able to transform strain MM1 to Leu$^+$ but not to Ura$^+$ were retained.

We sequenced all the bisulfite-generated mutant plasmids by primer extension procedures (18) after subcloning the appropriate 2μm circle repeat onto M13mp18 bacteriophage. We subcloned the anticipated mutated repeat as a *Bam*HI-to-*Hin*dIII fragment, and we subcloned the presumed residual wild-type repeat from each mutant plasmid as a *Pst*I-to-*Hin*dIII fragment. We then used a synthetic hexadecameric oligonucleotide complementary to a sequence 80 bp 3′ to the *Xba*I site in the repeat as a primer for the sequencing reactions. M. Zoller at Cold Spring Harbor Laboratory synthesized the oligonucleotide for us on an Applied Biosystems model 280 DNA synthesizer by the phosphoramidite method. The repeats of plasmid Mu5T2 were sequenced by chemical cleavage procedures (11), after digesting the plasmid with *Xba*I, labeling the 5′ ends with [γ-^{32}P]ATP and T4 kinase, and isolating the appropriate fragments subsequent to additional restriction digestion.

Identification of the *FLP* crossover site. The procedure for identifying the crossover site during *FLP*-mediated recombination, using both Frt$^+$ and Frt$^-$ mutant plasmids, is described in Results. The sequences of recovered, recombined plasmids were determined by primer extension (18) as described above, after subcloning a *Pst*I-to-*Bam*HI fragment spanning residual 2μm circle repeat onto M13mp18 DNA.

Construction of homozygous mutant plasmids. Plasmids Mu5T2-2, 545-2, 624B-2, and 613B-2 are identical in overall structure to pMMD2 but carry the respective mutation indicated in Fig. 2 at both *FRT* sites within the plasmid. We

constructed them by plasmid-targeted, gap-repair transformation (15) on the basis of the following rationale. The *URA3*-containing *Xba*I fragment from pMMD2 (Fig. 1 and 2: single *Xba*I sites lie in the middle of the 2μm repeats of pMMD2), if circularized, would be identical to the species excised from pMMD2 by *FLP*-mediated recombination. Therefore, homologous recombination between the 2μm circle repeat on a deleted derivative of pMMD2 and the 2μm circle repeat of the circularized *Xba*I fragment would reconstruct the pMMD2 plasmid. Since the linear fragment is equivalent to such a circularized molecule cleaved at the *Xba*I site, it is an appropriate substrate to promote double-strand break-mediated homologous recombination between the repeat on a deleted plasmid and the repeat spanning the *Xba*I site of the fragment (17). In addition, even if the region around the *Xba*I site is removed by limited exonuclease treatment, the same enhancement and targeting of recombination is achieved (15). However, in this case, gap repair recreates both intact repeats, using the intact repeat of the resident plasmid as a template. In this fashion, the reconstructed plasmid carries at both repeats whatever mutation was originally present in the single repeat on the resident deleted plasmid.

Following the above rationale, we transformed strain MM1 to leucine prototrophy with plasmids Mu5T2, 545, 624B, and 613B and obtained single Ura$^-$ transformants (see Table 1). We recovered in *E. coli* plasmids present in several such transformants obtained with each parent plasmid. Restriction analysis of the recovered plasmids confirmed that they derived from the parent plasmids by *FLP*-mediated deletion of the *URA3* sequences lying between the *FRT* sites. We assessed whether the recovered plasmids had retained or lost the mutation originally present in the single mutant *FRT* site on the parent plasmid by restriction or sequence analysis as described above and in Fig. 3. We then used one deleted plasmid that was obtained from each parent and that retained the original mutation to transform strain FVY2-6B (*leu2 ura3 his3* Flp$^-$ [cir^0]) to leucine prototrophy. We then transformed the Leu$^+$ transformants to uracil prototrophy using an *Xba*I restriction fragment from pMMD2 spanning the *URA3* gene. Before transformation, we incubated the *Xba*I fragment with sufficient BAL 31 to remove an average of 50 bp from each end. Ura$^+$ transformants could have arisen by integrative recombination into the resident plasmid, directed by homology of the free ends of the transforming fragment (17), or by conversion of the chromosomal *ura3* allele. We identified the former class by its mitotic segregation of the *URA3* marker and recovered the reconstructed plasmids in *E. coli*. We confirmed the structure of the plasmid by restriction analysis and the presence of the expected mutation at both sites by restriction analysis or sequence analysis.

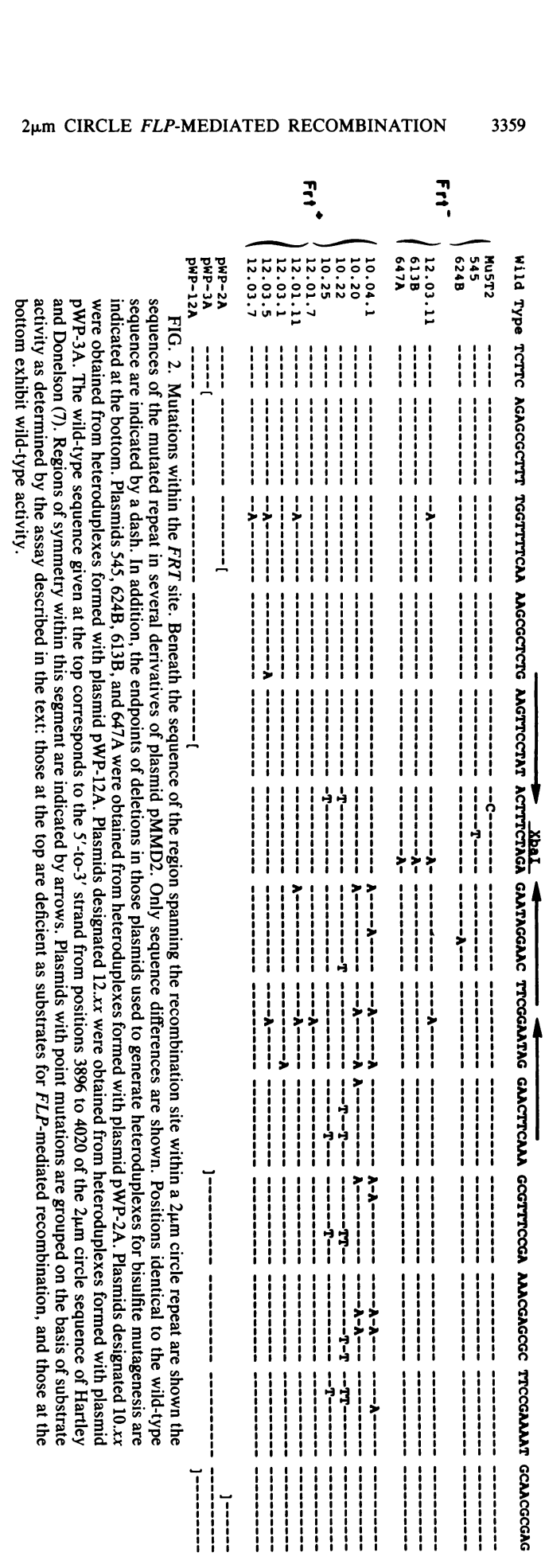

FIG. 2. Mutations within the *FRT* site. Beneath the sequence of the region spanning the recombination site within a 2μm circle repeat are shown the sequences of the mutated repeat in several derivatives of plasmid pMMD2. Only sequence differences are shown. Positions identical to the wild-type sequence are indicated by a dash. In addition, the endpoints of deletions in those plasmids used to generate heteroduplexes for bisulfite mutagenesis are indicated at the bottom. Plasmids 545, 624B, 613B, and 647A were obtained from heteroduplexes formed with plasmid pWP-2A. Plasmids designated 10.*xx* were obtained from heteroduplexes formed with plasmid pWP-3A. The wild-type sequence given at the top corresponds to the 5′-to-3′ strand from positions 3896 to 4020 of the 2μm circle sequence of Hartley and Donelson (7). Regions of symmetry within this segment are indicated by arrows. Plasmids with point mutations are grouped on the basis of substrate activity as determined by the assay described in the text: those at the top are deficient as substrates for *FLP*-mediated recombination, and those at the bottom exhibit wild-type activity.

RESULTS

Assay for *FLP*-mediated recombination. To examine various aspects of *FLP*-mediated recombination in vivo, we used an assay we previously developed (12). The assay is based on the ability of *FLP* to promote excision of sequences lying between two *FRT* sites in direct orientation. Plasmid pMMD2, whose structure is diagrammed in Fig. 1, contains two separate repeat regions from 2μm circle present in direct, rather than inverted, orientation. These repeats bracket the yeast *URA3* gene in one half of the molecule and the yeast *LEU2* gene and the 2μm circle origin of replication in the other. Plasmid pMMD2 is capable of transforming a

strain. If the recipient strain carries a functional *FLP* gene, then excision of the *URA3* gene from plasmid pMMD2 occurs readily in vivo, as diagrammed in Fig. 1. We previously showed that this recombination event occurs with such a high efficiency that no Ura$^+$ prototrophs are recovered from transformation of pMMD2 into an Flp$^+$ strain (12). On the other hand, if the *FLP* recombination system is inactive, either because the recipient strain lacks a functional *FLP* gene or because the plasmid carries a *cis*-active defect within one or both of the *FRT* sites, then the plasmid will transform the strain to uracil prototrophy at high frequency. Thus, the appearance of Leu$^+$ Ura$^-$ transformants from plasmid pMMD2 signals an intact recombination system, while Leu$^+$ Ura$^+$ transformants herald a defect in the recombination system. The recipient strain for pMMD2 in all the experiments described previously and in this report contains a single copy of the *FLP* gene integrated adjacent to the chromosomal *HIS3* locus and is devoid of endogenous 2μm circle plasmid.

Point mutations in the *FRT* site that block recombination. We selected and characterized point mutations within a 2μm circle repeat that substantially reduce the efficiency with which the repeat serves as a substrate for *FLP*-mediated recombination. We accomplished this by mutagenizing plasmid pMMD2 and then transforming the pooled, mutagenized plasmids into a *leu2 ura3* [cir^0] strain that contains a single, integrated copy of the *FLP* gene. By isolating Ura$^+$ transformants we selected those plasmids deficient in the recombination system. We recovered plasmids from individual Ura$^+$ transformants and retained those that were able to retransform the same strain to Ura$^+$ at high frequency.

We used two different mutagenesis protocols to generate Frt$^-$ mutations in plasmid pMMD2. In the first regimen, plasmid pMMD2 was propagated in and recovered from a strain of *E. coli* that carries a mutation in the *mutD* gene. Mutations arise in this strain with a frequency of 10^{-4} per base pair per generation and include transversions as well as transitions (5). Using pMMD2 DNA isolated from strain MutD we obtained 33 Frt$^-$ pMMD2 mutants from approximately 5×10^4 Leu$^+$ transformants. Two of these mutant plasmids were recovered, and the regions spanning both *FRT* sites in both plasmids were sequenced. Both plasmids contained the same single transition mutation. One of the plasmids was retained and designated Mu5T2 (Fig. 2, line 1). Since the remaining 31 mutants were not independently derived, we did not examine them.

Our second approach was to use the single-strand-specific deaminating reagent, sodium bisulfite, in conjunction with pMMD2 plasmid containing a 50- to 100-bp single-strand gap. The gap spanned that part of one repeat encompassing those sequences necessary for initiation of recombination. In these experiments, we treated the gapped plasmid with sodium bisulfite so that only approximately 1% of the exposed C residues were deaminated. As a consequence, most of the plasmid molecules retained the wild-type sequence and a few acquired a single base-pair change in the exposed region. The mutagenized plasmids were recovered in *E. coli*, pooled in groups of 10, and then used to transform the *leu2 ura3* Flp$^+$ yeast strain to Leu$^+$. Those transformants that were also Ura$^+$ were retained as candidates for containing Frt$^-$ plasmids. By this procedure, we obtained 4 independent plasmids deficient in recombination (plasmids 545, 624B, 613B, and 647A in Fig. 2) from a total of 80 Leu$^+$ transformants.

The sequence of the mutated repeat segment in each of the *frt* plasmids is shown in Fig. 2 (the first five sequences). For all the mutants, only one repeat contained a base-pair change. The other repeat in each plasmid retained the wild-type sequence. In addition, each mutant contained only a single-base-pair change within 200 bp spanning the recombination site. Plasmid Mu5T2, recovered after *mutD* mutagenesis, contains a T · A-to-C · G transition within the core region at the junction between the core and the flanking dyad repeat. Plasmids 545, 613B, 624B, and 647A were obtained after bisulfite mutagenesis, and each contains a transition mutation that could have arisen by deamination of a C residue. The mutations in plasmids 613B and 647A are identical and lie within the core region, abolishing the *Xba*I site. Similarly, the mutation in plasmid 545 also abolishes the *Xba*I site in the core region. The mutation in plasmid 624B lies within the 13-bp repeat flanking the right side of the core, 7 bp from the core-repeat junction.

TABLE 1. Residual recombination activity of Frt$^-$ plasmids[a]

Plasmid	No. of colonies with the following phenotype:		% Ura$^-$
	Leu$^+$ Ura$^+$	Leu$^+$ Ura$^-$	
Mu5T2	650	15	2.5
545	750	150	17
624B	600	30	5
613B	100[b]	900	90
12.03.11	60[b]	500	89

[a] Each of the listed plasmids was used to transform strain MM1 to Leu$^+$ by the method of Ito et al. (9). After 3 days the transformants were replica plated directly to SD-minus-uracil plates, and the numbers of Ura$^+$ and Ura$^-$ colonies were determined after 2 days of incubation at 30°C. The percentage of Ura$^-$ colonies among the transformants is shown in the third column.

[b] Present as Ura$^+$ papillations at the site of replication.

None of the single-base-pair mutations completely abolishes *FLP* recombination. That is, although all the plasmids examined gave Leu$^+$ Ura$^+$ transformants at high frequency, we could recover Leu$^+$ Ura$^-$ subclones from these Leu$^+$ Ura$^+$ transformants in every case. To obtain a estimate of the relative residual recombination activity of the various *frt* plasmids, we determined the proportion of Ura$^-$ clones among approximately 1,000 Leu$^+$ tranformants obtained with each mutant plasmid. These results are presented in Table 1. As is evident, all the plasmids gave rise, at a measurable frequency, to Ura$^-$ clones immediately upon transformation. As shown below, these clones arose by excision of the *URA3* gene from the plasmid. For plasmids Mu5T2, 545, and 624B, this frequency is relatively low, although plasmids Mu5T2 and 624B consistently exhibit a tighter Frt$^-$ phenotype than does plasmid 545. On the other hand, most of the transformants obtained with plasmid 613B have completely lost the *URA3* gene by the time the transformants have grown into colonies. In addition, those clones that retain the *URA3* marker immediately after transformation lose it at such a rapid rate during mitotic growth that only a small proportion of the cells within a clone are Ura$^+$. Thus, although plasmid 613B exhibits a significantly reduced recombination activity compared with that of the parent pMMD2 plasmid, the mutation in plasmid 613B yields a very leaky Frt$^-$ phenotype.

Point mutations in the *FRT* site that do not affect recombination. As a complementary procedure for characterizing essential elements of the *FRT* site, we isolated and identified mutations around this region that do not measurably inhibit recombination. We treated plasmid pMMD2 DNA, containing a 70-bp single-strand gap across one of the *FRT* sites, with sufficient sodium bisulfite to induce an average of five

deaminations per gapped molecule. We recovered individual mutant plasmids in a *ung E. coli* strain and then tested each recovered plasmid for its ability to undergo *FLP*-mediated recombination. Those that were capable of transforming our *ura3 leu2 FLP* strain to Leu$^+$ but incapable of transforming it to Ura$^+$ were scored as phenotypically Frt$^+$. We obtained 10 mutant plasmids that were phenotypically Frt$^+$ from 56 plasmids derived from two separate heteroduplexes.

The sequence of the mutated *FRT* recombination site for each of Frt$^+$ plasmids is shown in Fig. 2 (plasmids designated 10.*xx* or 12.*xx*). Each plasmid carries several transition mutations within that region of the plasmid that existed as a single-strand gap during treatment with bisulfite. For all the plasmids, the nature of the transitions is consistent with mutagenesis having occurring by deamination of several C residues on only one strand in the gapped region. There appears to be no bias as to which single strand was mutagenized.

As is evident from the sequences of the mutated plasmids, the *FLP* recombination region can sustain multiple base-pair changes without measurably diminishing recombination activity. With one exception, which we discuss below, none of these phenotypically wild-type plasmids carries any of the mutations we showed in the previous section could diminish *FLP* recombination. Thus, to a first approximation, the base-pair changes that do not affect recombination activity and those that do affect activity constitute mutually exclusive sets. The exception is plasmid 12.03.11, which carries the G · C-to-A · T transition present in the nominally Frt$^-$ plasmids 613B and 647A. However, as shown in Table 1, measurement of the frequency of loss of the *URA3* marker reveals that all three of these plasmids display the same phenotype intermediate between Frt$^+$ and Frt$^-$, regardless of the criteria used in their selection. Finally, in the collection of mutants obtained by all the various procedures, we found representatives of transitions at every G · C base pair but two in the region extending from the core through the two repeats on the right. Assuming that the site is symmetrical, we can account for every G · C base pair within the recombination region.

Recombination is normal between two *frt* sites carrying the identical core mutation. In the previously described mutant analysis, the recombination reaction examined was that between a wild-type site and one carrying a mutation. We were also interested in examining the relative efficiency of recombination between two sites each carrying the identical mutation. As described in Materials and Methods, we constructed pMMD2 derivatives in which each of the *frt* mutations previously identified was present in both repeats on the plasmid. We assessed the efficiency with which these plasmids underwent intramolecular recombination in a slightly different manner from that previously described. Strain FVY2-6B containing each of the doubly mutant plasmids, as well as each of the analogously singly mutant plasmids previously examined, was mass mated in liquid to strain MM1. After incubation to allow diploid formation, cells were plated on solid medium that permitted only diploid, *LEU2* plasmid-bearing cells to grow.

We determined the degree to which plasmids originally present in one parent strain underwent recombination catalyzed by the *FLP* gene product delivered by the second parent strain. This was accomplished simply by replica plating the diploid colonies to uracil-free medium to determine the proportion of colonies that had retained or lost the *URA3* marker. These data are presented in Table 2. As is evident, the frequency of occurrence of Ura$^-$ colonies for the singly mutant plasmids is significantly lower by this assay than by that used previously to yield the data reported in Table 1. This is not surprising since each of the plasmid-bearing parental cells that mates with an MM1 cell delivers a large number of plasmids into the zygote, all of which eventually must have to undergo recombination to yield a Ura$^-$ colony. In contrast, direct transformation of strain MM1 introduces only a small number of plasmids per cell, which increases the probability that all plasmids present in the cell undergo recombination before cell division.

TABLE 2. Efficiency of *FLP*-mediated recombination between identically mutated repeats

Mutation[a]	Recombination proficiency (% Ura$^-$ colonies)[b]	
	Mutant × wild type	Mutant × mutant
Mu5T2	1	98
545	5	100
624B	1	1
613B	27	100

[a] Mutations are those listed in Fig. 2. Plasmids with one mutant repeat and one wild-type repeat (mutant × wild type) have the same designation as the corresponding mutation and are described in Materials and Methods and the text. Plasmids with the indicated mutation present in both repeats (mutant × mutant) are designated by the mutation number with a -2 suffix. Their constructions are described in Materials and Methods.

[b] Derivatives of plasmid pMMD2 containing the indicated mutation in either one or both of the plasmid *FRT* sites were used to transform strain FVY2-6B to leucine prototrophy. Individual transformants were grown in SD-minus-leucine medium to 5 × 10^6 cells per ml, sonicated briefly, and mixed with an equal number of sonicated MM1 cells that had been pregrown in SD medium. Cells were pelleted and incubated at 30°C in the same tube without decanting the culture medium. After 5 h, cells were suspended, and appropriate dilutions of the suspension were plated on solid SD medium lacking leucine, adenine, and histidine to select for plasmid-bearing diploids. After 3 days of incubation at 30°C, colonies were replicated to SD minus uracil. The percentage of Ura$^-$ colonies was determined after 2 days of incubation.

In some but not all cases, recombination efficiency is substantially higher in those plasmids in which the same mutation is present at both interacting repeats than it is in the corresponding plasmid carrying the mutation in only one repeat. Specifically, plasmids 545-2, 613B-2, and Mu5T2-2 exhibit recombination levels equivalent to that of wild-type pMMD2 by this assay, whereas the parental plasmids, 545, 613B, and Mu5T2, exhibit diminished recombinational capacity. In contrast, plasmid 624B-2 shows the same reduced level of recombination as does the corresponding plasmid having only one mutant repeat. Thus, all the *frt* mutations that lie within the core region can be suppressed the presence of the equivalent mutation within the second interacting chromatid.

Identification of *FLP*-promoted crossover site in vivo. From previous studies we know that *FLP*-mediated recombination between 2μm circle repeats is a reciprocal event that is initiated within a 65-bp region spanning the *Xba*I site (3). We used our mutated pMMD2 plasmids to identify whether crossing over during recombination occurs at a specific site, and if so, where that site is located. The rationale for the experimental approach is apparent from the diagram in Fig. 1. One repeat unit (designated repeat 2 in Fig. 1) in each mutant derivative of pMMD2 carries one or more base-pair changes, labeled a and b in the figure. The other repeat carries markers A and B, the wild-type alleles of the base-pair changes present in repeat 2. If a marker mutation in repeat 2 lies to the left of the crossover point as it is drawn in Fig. 1, then the mutation will be excised by *FLP* recombination and the wild-type allele of that site will be present in the residual plasmid product of the recombination event. On

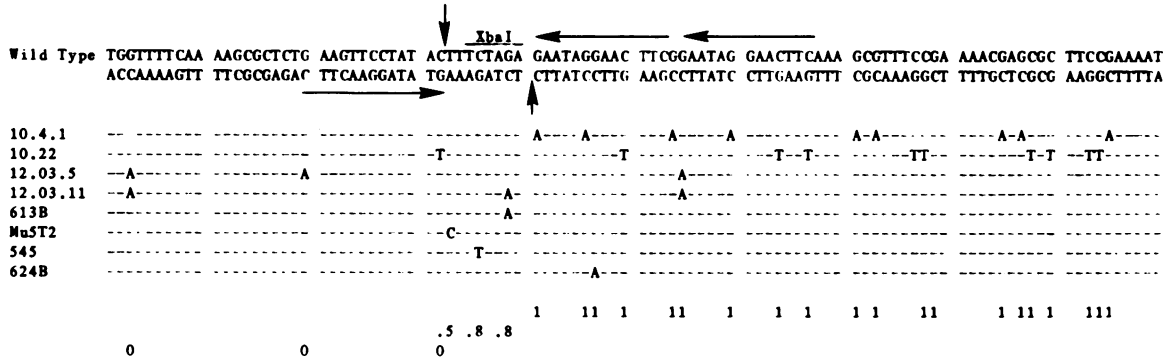

FIG. 3. Identification of the crossover site during *FLP*-mediated recombination. The sequences of the marked recombination site in those pMMD2 mutant plasmids used to determine the *FLP* crossover site are indicated below the wild-type sequence of this region. Each plasmid was transformed into the Flp$^+$ yeast strain MM1 and allowed to undergo excisional recombination as diagrammed in Fig. 1. At least 10 independent recombined plasmids derived from each starting plasmid were recovered and sequenced. The numbers at the bottom of the figure denote the fraction of the time the marker directly above it was retained in those recombined plasmids derived from plasmids originally carrying that marker. The position and orientation of the 13-bp repeated element within the recombination site are indicated by horizontal arrows, and the sites of single-strand cleavage induced by purified *FLP* protein in vitro are indicated by vertical arrows.

the other hand, if a marker mutation lies to the right of the recombination crossover point, then it will be retained in the final product. That is, retention or loss of a particular marker mutation after *FLP*-mediated recombination signals that the crossover lies to the left or right, respectively, of the site of the mutation. Thus, in the hypothetical case in Fig. 1, the presence of A and b alleles in the recombined plasmid indicates that crossing over occurred between the positions of mutations a and b.

We used both Frt$^+$ and Frt$^-$ plasmids in our evaluation of the crossover site. In the case of Frt$^+$ plasmids, we obtained several hundred transformants with each mutant plasmid and pooled all the transformants for each mutant. We recovered recombined plasmids from the pooled transformants by isolating DNA and using it to transform *E. coli* to ampicillin resistance. We pooled all the *E. coli* transformants (more than 100 transformants), prepared plasmid DNA from the pooled culture, and subcloned the repeat region from the plasmid DNA into an M13 vector. We then sequenced the subcloned repeat from 10 of the recovered M13 recombinant phages. Since large numbers of colonies were pooled at each step of the recovery procedure, each M13 subclone we sequenced represents the product of an independent recombination event that occurred during propagation of the marked plasmid in yeasts. For Frt$^-$ plasmids, we obtained 10 to 20 independent yeast transformants with each plasmid. We then identified a Ura$^-$ subclone from each transformant, recovered the plasmid from each subclone by transforming *E. coli* with isolated DNA, and then recloned into M13 and sequenced the repeat from one such plasmid from each subclone.

The results of our analysis are presented in Fig. 3. Beneath the sequence of the wild-type *FRT* site are the sequences of the marked plasmids used in the experiment. Below each base-pair marker examined, we indicate the fraction of the time that particular marker appeared in the product of recombination of the pMMD2 plasmid derivative initially bearing that marker. As is evident, all the markers to the left of the core region are excised during the recombination event, indicating that the crossover site lies to the right of the rightmost of these markers. In addition, all the markers to the right of the core region are always retained after recombination. Thus, crossing over during recombination occurs to the left of the leftmost of these markers. Finally, markers within the core region are sometimes retained and sometimes lost after recombination. We can conclude that for the most part *FLP*-mediated recombination induces strand exchange within the core region of the *FRT* site but that the particular site of crossing over within the core region is ambiguous. Possible explanations for this ambiguity within the core region are presented in the Discussion.

These results demonstrate that, most of the time, *FLP*-mediated crossing over occurs within the core region. To obtain a measure of the extent to which recombinational crossing over is confined to the core region, we took advantage of plasmid derivative 10.22, in which one of the marker mutations has created a new restriction site, *Dra*I, 20 bp 3′ of the core region. We obtained several hundred transformants of strain MM1 with plasmid 10.22. Since plasmid 10.22 is phenotypically wild type with regard to recombination proficiency, all the transformants contain only recombined plasmids. We pooled all the transformants, prepared DNA from the pooled culture, and digested it with *Dra*I. We fractionated the digested DNA by electrophoresis on an agarose gel, transferred the fractionated DNA to nitrocellulose, and probed it with labeled 2μm circle repeat sequence DNA. For most of the recombinants, crossing over would have occurred at the core region, thereby retaining the *Dra*I site. Plasmids that have lost the *Dra*I site represent those in which strand exchange occurred to the right of the core region. Thus, the proportion of DNA that is not cut at the position corresponding to the *Dra*I restriction site provides measure of crossing over outside the core region. As is evident from the results shown in Fig. 4, less than 1% of the DNA lost the new *Dra*I site. Thus, crossing over outside the core region upon *FLP*-mediated recombination probably occurs no more often than 1% of the time.

***FLP* promotes a small but detectable level of gene conversion.** To determine the extent to which *FLP* can induce gene conversions around the *FRT* site, we again took advantage of plasmid 10.22. We constructed plasmid pINV1 (Fig. 5), which carries two 2μm circle repeats present in inverted orientation. These repeats bracket pBR322 plus *LEU2* in one half of the molecule and *URA3* sequences in the other. We obtained one of the repeats from plasmid 10.22, and so it carries a *Dra*I site adjacent to the *Xba*I site in *FRT*. We acquired the other repeat from wild-type 2μm circle, and accordingly, it has no *Dra*I site at that position. We intro-

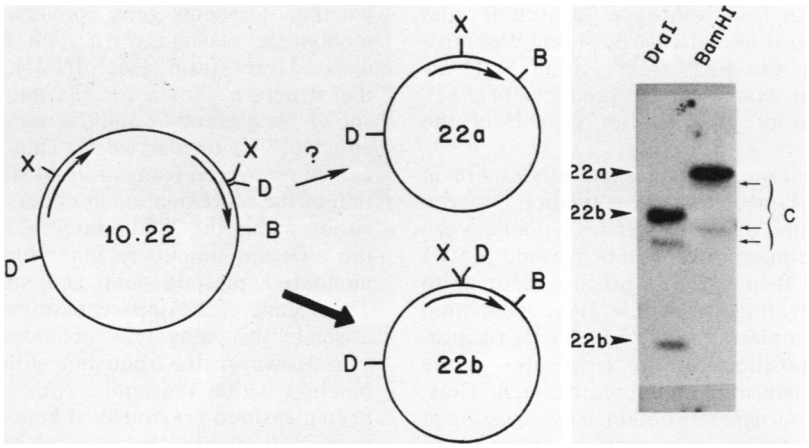

FIG. 4. Level of *FLP*-mediated crossing over outside the core. Plasmid pMMD2 derivative 10.22, which carries a novel *Dra*I site immediately to the right of the crossover point in the marked repeat (Fig. 2), yields primarily plasmid 22b upon *FLP*-mediated excisional recombination. However, if crossing over were to occur to the right of the *Dra*I site, then plasmid 22a would be generated. The extent to which this occurs was measured by transforming plasmid 10.22 into the *FLP*$^+$ strain MM1. Genomic DNA was isolated from a pooled culture of several hundred independent transformants. A 3-μg sample of the DNA was digested with the indicated enzymes, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with labeled pMMD2 DNA. The positions of fragments in the *Dra*I digest derived from recombinant plasmid 22b are noted, as is the expected position of the *Dra*I fragment from plasmid 22a. The positions of chromosomal fragments in the two digests are indicated by the C. Restriction sites: X, *Xba*I; D, *Dra*I; B, *Bam*HI.

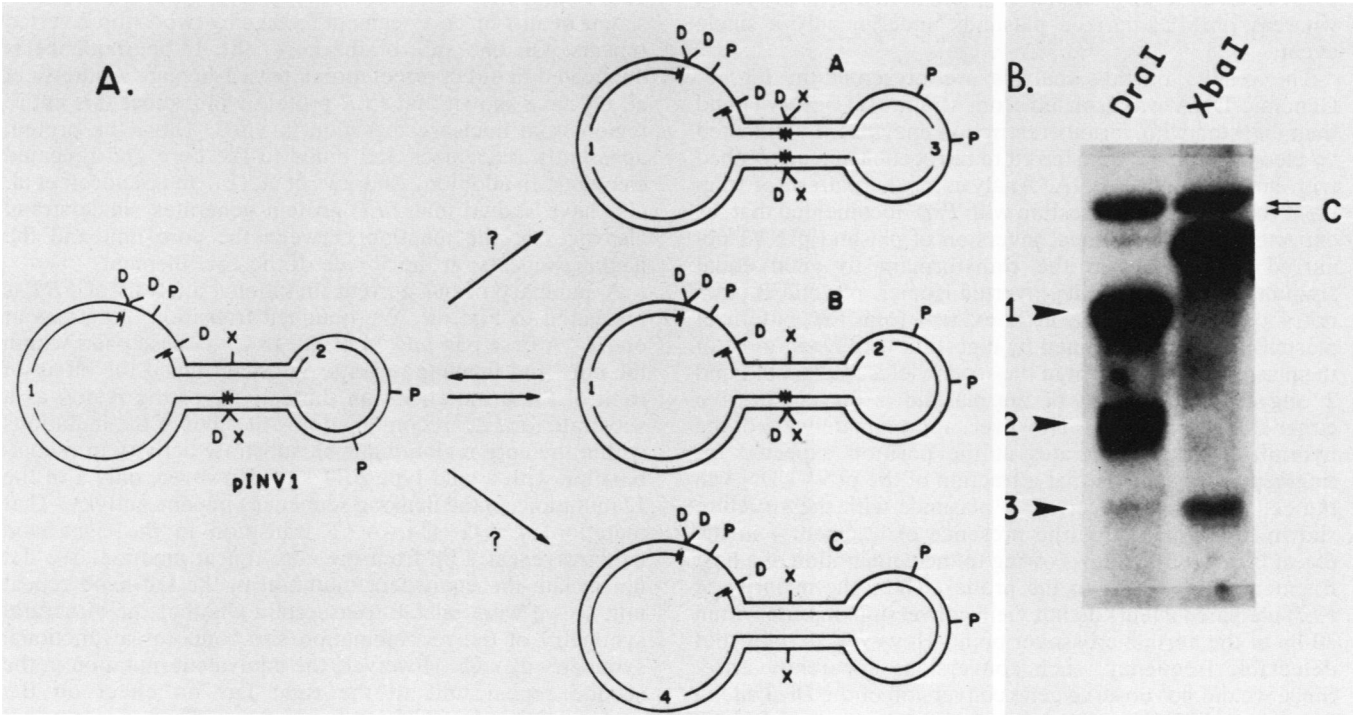

FIG. 5. *FLP*-induced gene conversion. (A) Potential products of *FLP*-mediated recombination of plasmid pINV1. At the right a diagram of the structure of plasmid pINV1 is shown, indicating the relative positions of *Dra*I (D), *Pst*I (P), and *Xba*I (X) restriction sites. The sources of the two larger *Dra*I restriction fragments, labeled 1 and 2, are indicated. *FLP*-mediated inversion of pINV1 yields plasmid B on the right, assuming that recombination is strictly reciprocal between the *FRT* sites located at the *Xba*I restriction sites (Fig. 3). As is evident, the *Dra*I restriction fragments are unchanged by this event. On the other hand, if sequences around *FRT* undergo conversion during recombination, then plasmids A and C could arise. These plasmids would yield novel *Dra*I restriction fragments, labeled 3 and 4. (B) Recombination products of plasmid pINV1. DNA was isolated from strain MM1 transformed with plasmid pINV1. Samples (3 μg) were digested with the indicated restriction enzymes, fractionated on a 0.7% agarose gel, transferred to nitrocellulose, and probed with labeled pINV1 DNA. The positions of fragments in the *Dra*I digest derived from plasmids pINV and B are shown (1 and 2), as is the expected position of the novel fragment derived from plasmid A (3). The positions of chromosomal fragments in the two digests are indicated by the C.

acquired the other repeat from wild-type 2μm circle, and accordingly, it has no *Dra*I site at that position. We introduced this plasmid into the *FLP*$^+$ [cir^0] strain MM1 by transformation and then examined the products of *FLP*-mediated recombination of pINV1 after growth of the transformant.

Since the 2μm circle repeats in plasmid pINV1 are in inverted orientation, *FLP*-mediated recombination between the repeats yields inversion of the sequences lying between them. This results in the interconversion of plasmid pINV1 and the plasmid labeled B in Fig. 5, a process identical to that obtained in vivo with authentic 2circle. If recombination between the *FRT* sites on plasmid pINV1 is strictly reciprocal, then the relative positions of the *Dra*I sites in the plasmid will remain unchanged upon interconversion. Thus, in this case, the pattern of fragments obtained by digestion of the mixture of plasmids present in a transformant of strain MM1 would be identical to that obtained by digestion of plasmid pINV1 itself. On the other hand, if recombination is associated with gene conversion of one or the other repeat, then plasmids labeled A and C would be generated from pINV1 in the transformed strain. Digestion of these plasmids with *Dra*I would yield novel plasmid fragments, which we have designated 3 and 4 in Fig. 5. Thus, the extent to which fragments 3 and 4 are present in *Dra*I digests of DNA isolated from strain MM1[pINV1] is a measure of the extent to which *FLP* recombination causes gene conversion of sequences adjacent to *FRT*. It should be noted that this experiment differs from the one described above, since pINV1 can undergo multiple recombination events in yeasts, whereas pMMD2-derived plasmids undergo only a single event.

The results of this analysis are presented in Fig. 5. Genomic DNA was isolated from strain MM1[pINV1] and then digested with various restriction enzymes, fractionated by electrophoresis, transferred to nitrocellulose, and probed with labeled pINV1 DNA. Analysis of the pattern of fragments obtained after digestion with *Pst*I documented that, as expected, recombinational inversion of plasmid pINV1 occurred extensively in the transformant to yield equal amounts of pINV1 and its inverted isomer, plasmid B (data not shown). In addition, as is evident from the pattern of plasmid fragments obtained by digestion with *Dra*I, most of the plasmid DNA present in the strain yields fragments 1 and 2, suggesting that most of the plasmid is present in vivo either as pINV1 or as B. However, approximately 2% of the hybridizable DNA migrates at the position expected for fragment 3, suggesting that a fraction of the pINV1 DNA in the cell has been converted to plasmids with the structure shown for A in Fig. 5 (the presence of fragment 4 in the digest is difficult to assess owing to the comigration of a host fragment homologous to the probe). Thus, the majority of *FLP*-mediated events do not yield conversion of a site within 30-bp of the normal crossover point. However, at a low but detectable frequency, such conversions apparently arise. Since we did not observe gene conversion of the *Dra*I site in the repeat of pINV1 when it is propagated in a strain lacking *FLP* (data not shown), we conclude that this low level of apparent gene conversion is a consequence of *FLP*-mediated recombination.

Certain dimers arising by *FLP*-mediated recombination between two plasmid pINV1 momomers would yield fragments 3 and 4 upon *Dra*I digestion. Nonetheless, *FLP* resolution of such dimers would yield only plasmids pINV1 and B. To test whether fragment 3 seen in Southern analysis of total DNA is a consequence of such dimer formation or whether it reflects gene conversion events, we recovered monomeric plasmids in a *recA E. coli* strain from DNA isolated from strain MM1[pINV1]. Of 40 such plasmids 2 had the structure shown for plasmid A, 1 had the structure shown for plasmid C, and the remainder had the structure of either pINV1 or plasmid B. Thus, true converted plasmids can be recovered from strain MM1[pINV1]. Finally, resolution of the aforementioned dimers by homologous recombination within the 280-bp interval between the *Dra*I site and the adjacent unique region within pINV1 would yield a monomeric plasmid identical in structure to plasmid A or C. Thus, some of the apparent convertant plasmids could have arisen by this pathway, which does not involve gene conversion. However, the frequency of homologous mitotic recombination within the entire 2μm circle inverted repeat has been measured previously at between 10^{-4} and 10^{-5} (3). We suspect, therefore, that such events do not contribute significantly in generating the apparent convertant plasmids.

DISCUSSION

The *FLP* recombination target site, *FRT*. The sequence of DNA spanning the site of recombination in 2μm circle, which we propose to designate the *FLP* recombination target, or *FRT*, site, is presented in Fig. 6A. We previously showed that initiation of recombination occurs within a 65-bp region in the middle of the inverted repeats present on 2μm circle plasmid and that all the sequences necessary for initiation of recombination are restricted to this region (3). This region encompasses a domain of dyad symmetry consisting of an 8-bp core segment flanked by two 13-bp inverted repeats. On one side of the core, the 13-bp sequence is duplicated in direct orientation after a 1-bp gap. Andrews et al. (1) have shown that *FLP* protein will protect this entire region from nuclease digestion in vitro. Thus, the protein apparently recognizes and binds to the core and repeated elements. In addition, Andrews et al. (1a) and Senecoff et al. (19) have shown that *FLP* protein generates single-strand cleavages at the junction between the core unit and the flanking repeats, at the 5′ side of the core element.

A summary of our current mutational analysis of *FRT* is presented in Fig. 6B. We obtained transition mutations at one T · A base pair and 14 of the 18 C · G base pairs within the core and flanking repeats. We determined the effect of each of these mutations on the activity of the region as a substrate for *FLP* recombination. All three of the mutations within the core region diminish substrate activity in recombination with a wild-type *FRT* site. However, only 1 of the 12 mutations in the flanking sequences impairs activity. That mutation is a G · C-to-A · T transition in the right-hand flanking repeat 7 bp from the core-repeat junction. We did not isolate the equivalent mutation in the left-hand repeat unit so we were unable to ascertain whether the structural symmetry of the recombination site connotes a functional symmetry as well. However, the equivalent mutation in the second repeat unit to the right has no effect on the recombinational proficiency of the site. This suggests that this most distal repeat element either is not directly involved in recombination or is involved in recombination in a manner different from the repeat units immediately flanking the core.

The results from our mutational analysis are reasonably compatible with those obtained by Sadowski and his colleagues and by Cox and his colleagues on the in vitro properties of *FLP* protein (1, 1b, 6, 6b, 19, 23). Gronostajski and Sadowski (in press) find that the minimum double-strand

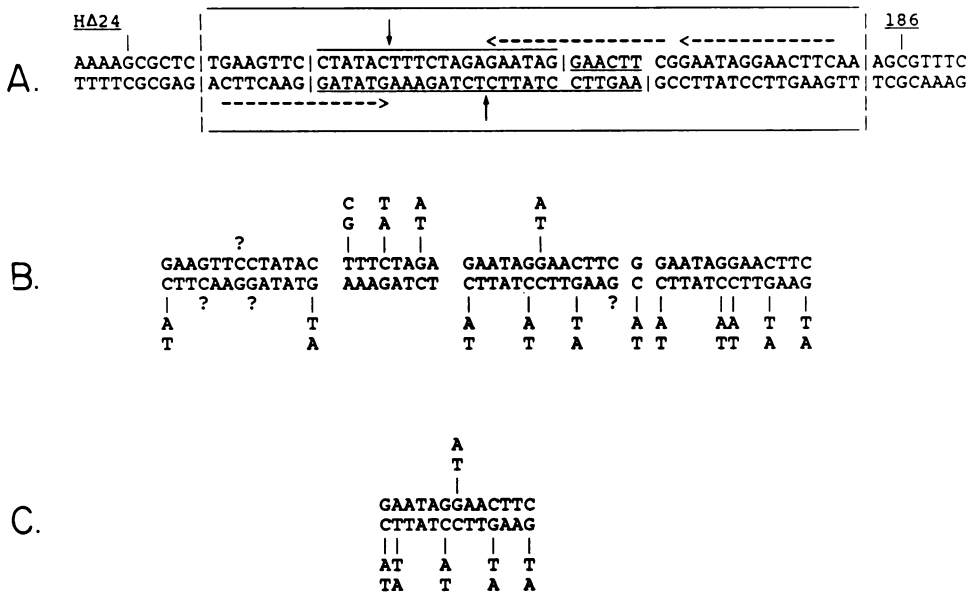

FIG. 6. Essential elements of the *FLP* Recombination Target Site, *FRT*. (A) The *FRT* site. Superimposed on the wild-type sequence are the positions of the deletion (HΔ24) and insertion (186) that define the outer boundaries of the site, the location and orientation of the repeated 13-bp sequence (horizontal arrows) within the site, the positions of single-strand cleavages generated by purified *FLP* protein in vitro as determined by Andrews et al. (1a) (vertical arrows), the region protected from nuclease digestion by purified *FLP* protein as determined by Andrews et al. (1a) (outer box), and the minimal substrate region as determined by Gronostajski and Sadowski (in press) (inner box). (B) Summary of the effects of *FRT* point mutations. Above and below the sequence of *FRT*, segmented to emphasize the core region and flanking 13-bp repeats, are shown the base-pair changes that were analyzed in this study. Base-pair changes shown above the sequence are those that abolish or reduce the ability of the site to serve as a substrate for *FLP*-mediated recombination. Base-pair changes shown below the sequence do not diminish substrate activity of the site, at least as determined by the in vivo assay used in this study. Those G · C base pairs for which mutations were not recovered are indicated by question marks. (C) Consensus mutational analysis of the flanking repeat. Assuming that the two 13-bp repeats immediately flanking the core participate in *FLP* recombination in identical ways, a consensus mutational analysis can be derived by pooling information of the effects of mutations in either repeats and taking into account the single polymorphism between them. This summary of effects of single base-pair changes on the activity of a repeat is superimposed on the sequence of the repeat, using the same convention as in panel B to indicate the consequence of each mutation. The orientation of the repeat is the same as those to the right of the core in panels A and B.

side of the core. Similarly, Senecoff et al. (19) find that the core recombination substrate encompasses the core region and most of the two flanking symmetry elements. Consistent with these observations, we found that none of the mutations in the third repeat abolishes activity. However, we also found that the seventh base pair in the right-hand repeat is essential for activity. Although the essentiality of this base pair is consistent with the apparent requirement as the minimum substrate for at least one strand of sequence spanning this base pair, the fact that the equivalent base pair in the repeat flanking the left side lies outside the minimum substrate suggests a striking assymmetry in the recombination site that belies its structural symmetry. Cox (personal communication) has determined that *FLP* protein apparently makes contact in vitro with all G residues in the two symmetry elements immediately flanking the core. We found that mutation of only the seventh residue, but of none of the others, yields a detectable diminuition of activity in vivo. Although it is possible that our assay is not sufficiently sensitive to detect some reduction in activity owing to mutation of these other sites, we can certainly conclude that the role in *FLP* protein interaction of the G · C base pair 7 bp from the core is certainly qualitatively more significant than that of other G · C residues in the symmetry elements.

Those mutations within *FRT* that demonstrably reduce activity could inhibit *FLP* function at any one of a number of steps in the reaction. Andrews et al. (1) have shown that mutation 624B in the flanking symmetry element substantially reduces the affinity of *FLP* protein for the target site. In contrast, they find that mutation 613B, within the core region, does not affect binding or cleavage of the site by *FLP* protein. Significantly, this mutation, as well as others within the core described in this report and elsewhere (19, 19a), is defective in recombination with a heterologous repeat but exhibits normal levels of recombination with repeats carrying the identical mutation. Thus, the sequence specificity of the core appears to be quite plastic. These results suggest that the core region may participate in the recombination reaction only in pairing or heteroduplex formation between the interacting chromatids during recombination and have little involvement in *FLP* recognition.

Location of the site of strand exchange during *FLP* recombination. We showed that strand exchange during *FLP* recombination occurs within the core segment of the recombination site. However, no precise double-strand exchange site within the core region could be identified by our experiments. That is, all the single base-pair markers within the core region that we examined segregated randomly after recombination. The most likely explanation is that recombination generates a heteroduplex between the two core regions participating in the reaction, as diagrammed in Fig. 7. Random repair of the resulting mismatches would yield a mixture of products, some of which would have retained and some of which would have lost the initial marker. This interpretation is consistent with the nature of the cleavages induced by *FLP* protein in vitro (1a, 19). It is also consistent

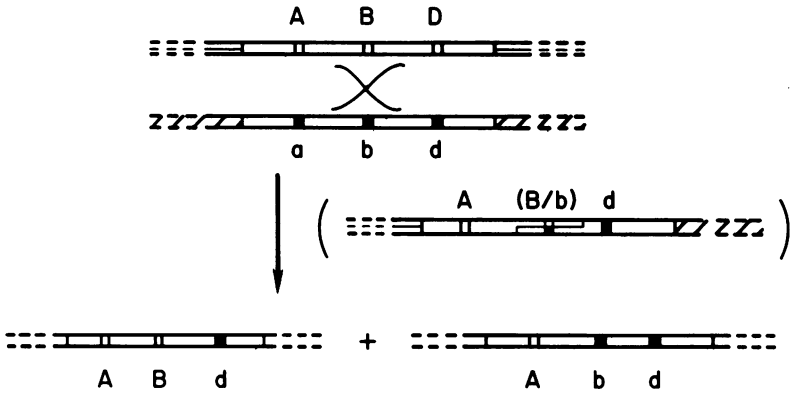

FIG. 7. Model for *FLP*-mediated crossing over. The two 2μm circle repeats present on a plasmid pMMD2 derivative are shown at the top. The lower repeat carries three marker mutations, one (b) within the core region and two (a and d) flanking it, while the upper repeat carries the wild-type alleles of these three mutations. If recombination proceeds through an intermediate in which the core region exists as a heteroduplex between strands of both parental repeats, then random repair of the mismatch or replication of the heteroduplex intermediate would yield a mixture of recombined plasmids differing in the presence or absence of the marker mutation b.

with the sites of strand exchange catalyzed by bacteriophage lambda *int* protein (13) and by bacteriophage P1 *cre* protein (8) at their respective recombination sites. Finally, if formation of such a heteroduplex is an essential intermediate step in the recombination reaction, then the inhibitory effect of mutations within the core region is readily explained.

Mechanism of *FLP*-mediated recombination. Results presented in this report suggest that *FLP* recombination proceeds through a breakage and rejoining mechanism. First, crossing over outside of the core region occurs only rarely. That is, initiation and resolution of the recombination event are restricted to a small 8-bp domain. Second, very little gene conversion is associated with *FLP*-mediated recombination. Rather, *FLP* protein almost exclusively promotes reciprocal exchanges. These results eliminate models for *FLP* recombination in which a freely diffusible Holliday structure is generated as an intermediate in the reaction. Similarly, these results diminish the likelihood that *FLP* generates a double-strand break at the *FRT* site, which would serve to initiate crossing over via gap repair using the second repeat as a template (16). Such a mechanism would be expected to entail a high level of gene conversion at the site of the double-strand break. In addition, such a mechanism would not be likely to be sensitive to inhibition by mutation within the core region, but if it was, such inhibition would certainly not be suppressible by introduction of the identical mutation at the second site.

The low level of gene conversion associated with *FLP* recombination suggests that breakage and rejoining during *FLP*-mediated recombination is concerted. That is, breakage of one strand does not appear to occur unless the second strand is able to participate in completion of the rejoining reaction. One model, but not necessarily the only model, consistent with this constraint is one in which the reaction proceeds through close association of the four strands of the two core regions, followed by strand exchange catalyzed by a topoisomerase activity of *FLP* protein. Such a mechanism has been previously proposed for *int*-catalyzed phage lambda integration (10, 14) and for *cre*-catalyzed recombination between *loxP* sites of phage P1 (8). This mechanism is also consistent with the behavior of *FLP* protein in vitro (1a, 6b, 19).

The fact that mutations that disrupt the *FRT* site are concentrated within the core region is consistent with a concerted mechanism for *FLP* recombinaiton. Such mutations would disrupt base pairing within the four-strand structure and thus inhibit formation of the requisite recombination intermediate. The fact that such mutations are suppressed by the presence of the identical mutation within the second repeat, thereby restoring homology, is also consistent with a concerted model. However, since suppression by compensating changes to restore base pairing is consistent with concerted as well as sequential mechanisms of a breakage and rejoining reaction, a stronger test of the concerted model of *FLP* recombination would be to determine the effect of mutations within the core region on the level of *FLP*-promoted gene conversion. That is, we showed that *FLP* will promote gene conversion at a low but detectable level. In a nonconcerted mechanism, base pairing between core regions of the participating strands would occur after cleavage of the site. In this case, the presence of a mutation in the core of one of the participating repeats would lead to the prolonged presistence of the double- or single-strand cleavage in one of the chromatids, which would be expected to enhance the level of gene conversions (15, 16). On the other hand, in a concerted mechanism free double-strand breaks would not be expected to arise in the absence of complete base pairing of the interacting chromatids. Thus, mutation within the core region would diminish the rate of *FLP*-promoted reciprocal recombination between the mutant repeat and a wild-type repeat without enhancing the level of gene conversion within the region. Experiments addressing this question are in progress.

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