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The FLP protein of the yeast 2-μm plasmid: Expression of a eukaryotic genetic recombination system in *Escherichia coli*

(gene expression/site-specific recombination/gene cloning)

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ABSTRACT **The *FLP* gene of the yeast 2-μm plasmid is involved in a site-specific recombination event that results in the inversion of a set of sequences within the plasmid. This gene has been cloned and expressed in *Escherichia coli*. Expression of the *FLP* gene results in efficient recombination within the bacterial cell, which is specific for plasmids containing at least one 2-μm plasmid recombination site. This work demonstrates that (*i*) FLP protein is actively involved in 2-μm plasmid recombination; (*ii*) no other factors specific to yeast are required for the reaction; (*iii*) FLP protein acts efficiently in *trans*; (*iv*) FLP protein will promote site-specific insertion and deletion reactions in addition to the inversion reaction; and (*v*) FLP-promoted recombination is not dependent upon any DNA structural features unique to yeast chromatin.**

The yeast 2-μm plasmid is a circular DNA molecule of 6,318 base pairs (bp) present at about 70 copies per cell in most yeast strains (1). Its sequence has been determined (2), and it has been used in a variety of ways as a yeast cloning vector (3). A prominent feature of the sequence is the presence of a 599-bp inverted repeat. The two copies of this sequence are present on opposite sides of the circle. Recombination between these repeats, mediated by a system encoded within the plasmid, serves to invert a segment of the plasmid sequence relative to the remainder. This results in two forms of the plasmid, A and B (Fig. 1), which are found in equal concentrations in yeast cells (1).

This recombination event has been shown to be site specific. The part of the repeat that is required for recombination has been narrowed to a region of 65 bp spanning an *Xba* I restriction site (4). A 4-bp deletion within the *Xba* I site abolishes recombination, so that this site is clearly part of the required sequence (4). This system is thus analogous to the site-specific DNA inversions, deletions, and insertions that occur in a number of prokaryotic systems (5–8). No cellular function has been defined for 2-μm plasmid circle recombination. This system, however, offers an opportunity to examine a eukaryotic recombination event.

The 2-μm plasmid contains a number of open reading frames (1, 2). One of these, designated *FLP*, has been implicated in the recombination process (9, 10). It has not been determined whether other yeast factors are necessary, although the other genes on the plasmid are not required (4). The FLP gene product may carry out the reaction by itself or play an auxiliary role as part of a larger complex. In any case, this gene provides the obvious starting point for an analysis of the system. To provide a potentially rich source of the FLP protein, it seemed desirable to clone and express the *FLP* gene in *Escherichia coli*. This has been accomplished and has permitted an analysis of the properties of 2-μm plasmid recombination in an *in vivo* system in the absence of other yeast proteins.

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MATERIALS AND METHODS

Enzymes and Plasmid DNA. *E. coli* DNA polymerase I and T4 DNA ligase were gifts from S. Scherer of this department. Nuclease S1 was purchased from Sigma. Restriction endonucleases were purchased from New England BioLabs. The plasmid 82-6B was a gift of V. Zakian of the Fred Hutchinson Cancer Research Center (Seattle, WA). The plasmid pCQV2 (11), constructed by C. Queen of the Massachusetts Institute of Technology, was provided by J. Flynn of this department. The plasmid pXF3 (12) was provided by P. Southern of this department.

Miscellaneous Methods. Isolation of DNA fragments from agarose gels, restriction digests, DNA ligations, and transformation of bacterial cells with plasmid DNA were carried out by procedures described in detail elsewhere (12, 13). Rapid screening of transformed colonies was carried out by using the rapid plasmid isolation from small cultures described by Davis *et al.* (13). For more detailed analysis, including the results presented in Figs. 3–6, plasmid DNA was purified by banding twice in cesium chloride gradients containing ethidium bromide as described (12). In every case this DNA was obtained from cells grown with selection for all appropriate drug resistances. Agarose gel electrophoresis was carried out by using 0.8% agarose in a Tris acetate buffer system as described (13).

Strains. Recombinant plasmids in all cases were recovered in the *E. coli* strain HB101 (*recA*$^-$) (12). The plasmid pCQV2 was maintained in *E. coli* C600 (*recA*$^+$) (12).

Plasmid Constructions. Plasmids constructed in this study are illustrated in Fig. 2.

FLP substrates. The plasmid 82-6B was isolated by J. E. Donelson from a library of random yeast DNA fragments cloned in the *E. coli* plasmid pMB9. It contains about 1.5 copies of the yeast 2-μm plasmid sequence (2). A full-length copy of the A form of the 2-μm plasmid was obtained by isolating the smaller of the two fragments generated by *Pst* I cleavage of plasmid 82-6B. This was inserted into the *Pst* I site of pXF3 to produce the plasmid pMMC1. The plasmid pXF3 contains no *Ava* I site. The plasmid pMMC1 was digested with *Ava* I and religated, and a clone was selected in which the short 1.5-kilobase (kb) fragment had been reinserted in the opposite orientation. This was designated pMMC3 and contains the two 2-μm plasmid repeats in direct orientation. The plasmid pMMC10 was con-

Abbreviations: bp, base pair(s); phage λp_R, bacteriophage λ rightward promoter; kb, kilobase(s).

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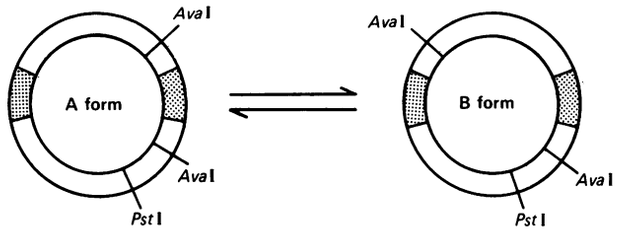


FIG. 1. FLP-promoted recombinational inversion within the yeast 2-μm plasmid. The inverted repeats are identified as the shaded regions.

structed by digestion of 10 μg of pMMC3 DNA with *Xba* I, isolation of the larger of the two fragments produced, and circularization of that fragment with T4 DNA ligase.

FLP expression. The plasmid pCQV2 (11) contains sequences from pBR322, including the replication origin and the region encoding ampicillin resistance. The plasmid also contains sequences from bacteriophage λ, including the temperature-sensitive repressor *c*I857, the phage λ rightward promoter (λp_R), and sequences downstream up to and including the initiation codon of the *cro* gene. The pCQV2 fragment between the *Sph* I and *Pvu* II sites was replaced with the *Sph* I–*Pvu* II fragment from pMMC1, which spans the *FLP* gene and includes one complete 2-μm plasmid repeat (Fig. 2). This generated a new plasmid, pMMC4, in which the *FLP* gene was present in the proper orientation relative to λp_R, but was about 100 bp downstream from the normal *cro* start site. To remove this 100 bp, 3 μg of pMMC4 DNA was cleaved with *Sph* I and *Bam*HI, treated with 0.2 unit of nuclease S1, and recircularized with T4 DNA ligase. Analysis of the products of this procedure is described in *Results*.

RESULTS

Expression of the FLP Protein in *E. coli*. The sequences after the *cro* initiation codon in pCQV2 were reconstructed so that a *Bam*HI site is present. Cleavage with *Bam*HI and removal of the four-nucleotide overhang leaves a blunt end immediately after the *cro* ATG.

The *FLP* gene has a cleavage site for *Sph* I (used in the construction of pMMC4) located near its initiation codon (2). Cleavage with *Sph* I and removal of the four-nucleotide overhang leaves a blunt end, which eliminates only the initiation codon. Thus, blunt-end ligation to the prepared *Bam*HI site in pCQV2 should result in a construction in which the *FLP* gene is positioned exactly as the *cro* gene normally is positioned relative to phage λp_R and the cro ribosome binding site.

Purified pCQV2 DNA (Fig. 3) showed a normal gel pattern consisting primarily of supercoiled monomers. The lower of the two minor bands in Fig. 3 reflects contamination by the relaxed monomers present in small amounts in all such preparations. The other minor band probably represents a small number of supercoiled dimers generated by homologous recombination. Strain C600, from which the pCQV2 DNA was obtained, is *recA*$^+$.

Purified pMMC4 DNA also showed a normal gel pattern (Fig. 3) except for the presence of a minor band migrating at approximately the position expected for supercoiled tetramers. A possible explanation for this band will be presented in the *Discussion*.

To place the *FLP* gene in the position normally occupied by the *cro* gene relative to phage λp_R, pMMC4 DNA was treated as described. The DNA was recovered in HB101, and the plasmid DNA from 30 of the resulting amp^R colonies was subjected to a rapid screening. In each case the recovered plasmids appeared to be slightly shorter than pMMC4, as expected (not shown). In six cases, however, only a small fraction of the DNA appeared as monomers, the remainder migrating as a series of larger bands. The patterns were identical in all six cases. One of these was designated pMMC6 and used for further study.

The plasmid pMMC6 was purified from cells grown at 30°C, and the DNA gel pattern (Fig. 3) was consistent with a series of multimeric species, with dimers and tetramers most prominent. This result might be expected if the *FLP* gene were expressed and if the FLP protein actively promoted recombination involving the recombination site within the 2-μm plasmid repeat inherited by pMMC6 from pMMC4.

This explanation for the observed gel pattern is supported by

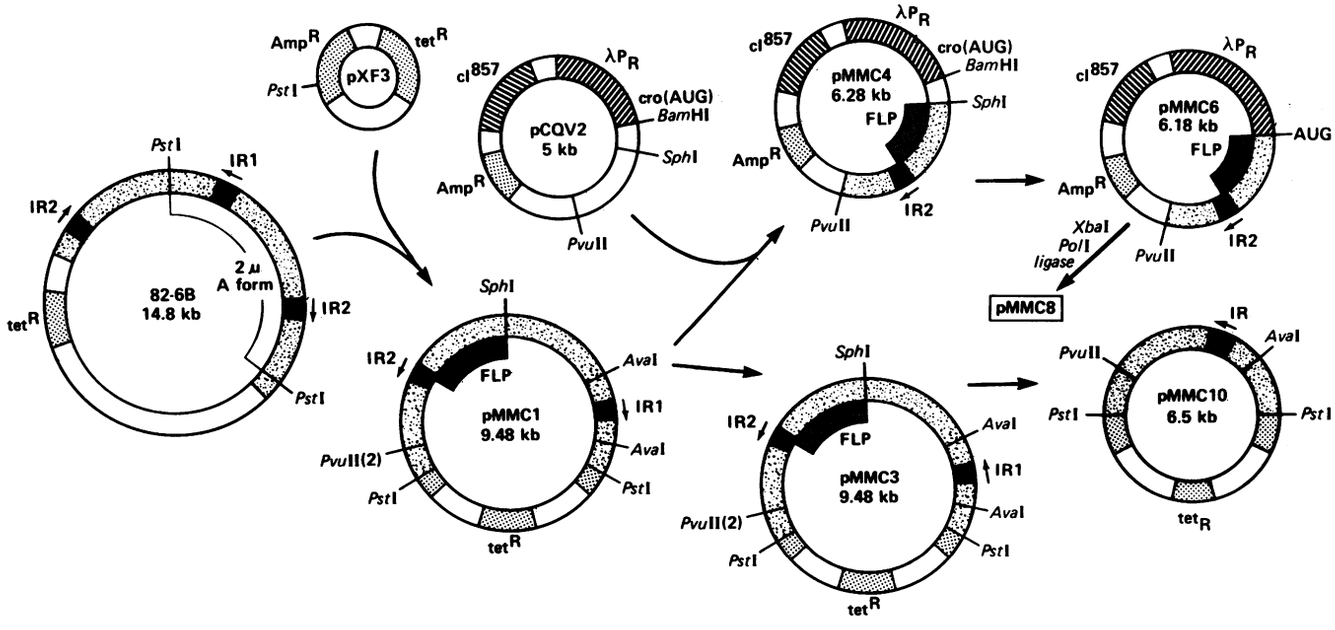


FIG. 2. Construction of recombinant plasmids used in this work. The relative size of the plasmids and the position and size of various sequences are approximate. Sequences derived from different sources (bacteriophage λ, the 2-μm plasmid, etc.) are shaded differently. IR, inverted repeats (■) from the 2-μm plasmid.

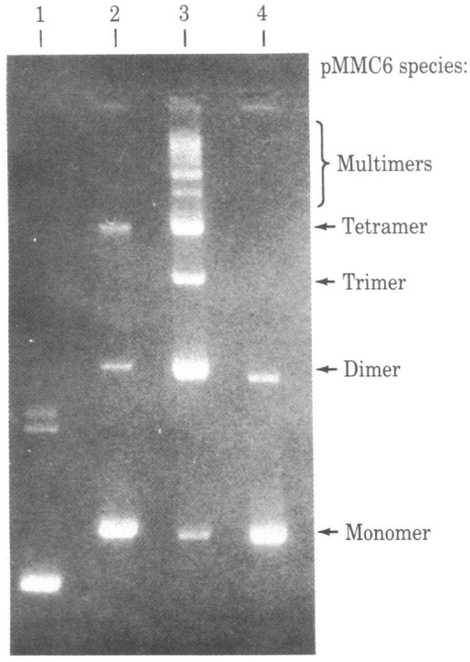


FIG. 3. Expression of FLP protein in *E. coli*. Agarose gel electrophoresis of plasmid DNAs was carried out as described. Lanes: 1, pCQV2; 2, pMMC4; 3, pMMC6; 4, pMMC8.

several additional observations.

(*i*) Cleavage of pMMC6 DNA with *Pvu* II, which should cleave both pMMC4 and pMMC6 only once, resulted in a single band (Fig. 4). Thus, all of the bands are derived from a single DNA species and are not artifacts of the purification procedure.

(*ii*) Monomeric pMMC6 DNA was isolated from agarose gels as described by Dretzen *et al.* (14). The recovered DNA yielded a single band on agarose gels that migrated in the position of supercoiled pMMC6 monomers. In addition, cleavage of the DNA with *Pvu* II yielded the single band expected for cleavage of pMMC6 DNA. Aliquots of a single culture of HB101 were

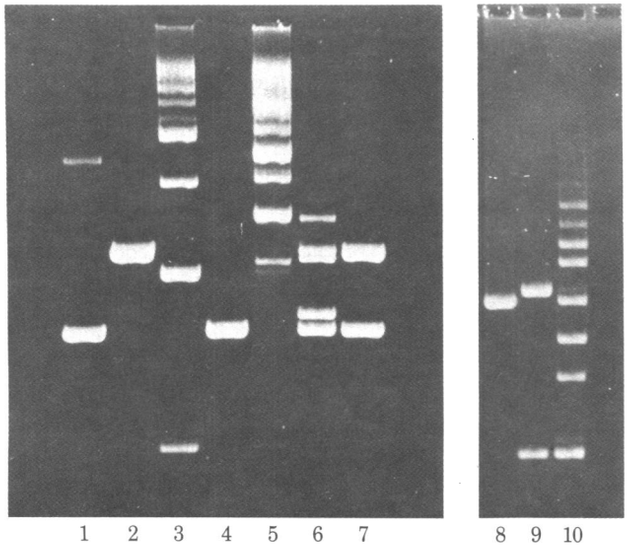


FIG. 4. Intermolecular recombination promoted by FLP protein. Agarose gel electrophoresis and restriction digests were carried out as described. Lanes: 1, pMMC3 DNA; 2, *Pvu* II-digested pMMC3 DNA; 3, pMMC6 DNA; 4, *Pvu* II-digested pMMC6 DNA; 5, pMMC6/3 DNA; 6, *Pvu* II-digested pMMC6/3 DNA; 7, *Pvu* II-digested pMMC3 and pMMC6 DNAs; 8, *Pst* I-digested pMMC6 DNA; 9, *Pst* I-digested pMMC3 DNA; 10, *Pst* I-digested pMMC6/3 DNA.

transformed with pMMC6 monomers or pMMC4 or pCQV2 in parallel experiments. Six colonies from each transformation were subjected to rapid screening. In each case, DNA from cells transformed by pCQV2 or pMMC4 exhibited the primarily monomeric pattern shown in Fig. 3. When the same cells were transformed with pMMC6 monomers, however, the multimeric pattern of Fig. 3 was always reproduced. This pattern is thus not an artifact related to the cells used to recover the plasmid.

(*iii*) Sequences within the 2-μm plasmid repeat that are known to be required for recombination were altered in pMMC6. This is accomplished most easily by destroying the *Xba* I site as demonstrated by the work in yeast (4). In this case purified pMMC6 DNA was cleaved with *Xba* I, the resulting overhang was filled in with DNA polymerase I, and the products were recircularized with T4 DNA ligase. The only *Xba* I site in pMMC6 is the one within the 2-μm plasmid repeat. Several transformants were subjected again to rapid screening. Only supercoiled monomers were evident in all cases in which the *Xba* I site had been successfully destroyed without affecting the size of the plasmid (as determined by gel electrophoresis). One of these was designated pMMC8; purified pMMC8 DNA occupies lane 8 in Fig. 3. Destruction of the *Xba* I site clearly prevented formation of the multimeric pattern. This experiment directly implicates the *FLP* system in the generation of multimers. The end of the open reading frame that defines the *FLP* gene and the *Xba* I site are separated by 183 bp (2). Experiments described below demonstrate that whereas pMMC8 cannot participate in FLP-promoted recombination, it does express an active FLP protein.

FLP-Promoted Intermolecular Recombination. The pMMC6 multimers are most easily explained by intermolecular recombination events. It is possible, however, that the multimers could arise from intramolecular recombination within replication intermediates or "θ" structures. To detect intermolecular reactions, pMMC6 monomers and pMMC3 were used to cotransform HB101 simultaneously to amp^R and tet^R. The DNA isolated from cotransformed cells was designated pMMC6/3. Each of the plasmids alone yielded a single band when cleaved with *Pvu* II (Fig. 4). The gel pattern observed for purified pMMC6/3 DNA differed from that expected for a simple mixture of the two. When cleaved with *Pvu* II, pMMC6/3 yielded not two but at least five bands. Extensive overdigestion with *Pvu* II did not alter this pattern.

To rationalize this pattern, arbitrarily designate the recombination sites in pMMC3 as 1 and 2. The products of recombination between pMMC6 and pMMC3 should yield new *Pvu* II fragments of 9.37, 9.22, 6.29, and 6.43 kb if the pMMC3 sites are equivalent. Two more fragments of 9.48 and 6.18 kb are expected from the unreacted plasmids. At least two bands were evident in the pMMC6/3 *Pvu* II pattern for each of the bands for the cut, unreacted plasmids (Fig. 4). The presence of two bands rather than three in each case could mean that (*i*) intermolecular recombination proceeded to a point where no unreacted plasmids remained, (*ii*) the two recombination sites in pMMC3 are not equivalent, or (*iii*) the extra bands were simply not resolved on this gel. Longer gels and use of less pMMC6/3 DNA failed to reveal the additional bands (not shown).

An additional restriction digest was carried out, therefore, with results again shown in Fig. 4. The plasmids pMMC6 and pMMC3 have one and two *Pst* I restriction sites, respectively, with the digestion patterns shown in Fig. 4. The larger of the two pMMC3 fragments corresponds to the complete 2-μm plasmid sequence present in this plasmid and contains both recombination sites. This fragment was present only as a very minor species in the *Pst* I digest of pMMC6/3, indicating that explanation *i* above is probably correct. The largest band in the

Pvu II digest of pMMC6/3 could result from recombination between two pMMC3 molecules involving site 1 in one molecule and site 2 in the other. This would yield a hybrid dimer with *Pvu* II fragments of 12.4 and 6.4 kb. Thus, all of the observed bands are easily explained by simple, bimolecular reactions. The FLP protein expressed by pMMC6 appears to promote intermolecular recombination at some frequency, and, in fact, this type of reaction is very probable under the conditions prevailing in pMMC6/3-transformed cells at 30°C. These experiments do not preclude the possibility that intramolecular recombination may occur during replication.

Recombinational Inversion in *trans*. A similar experiment was carried out to determine if the FLP protein from pMMC6 could promote a recombinational inversion analogous to the normal FLP-promoted recombination event that occurs in yeast. The experiment also was designed to determine if the protein could act in *trans*. HB101 was cotransformed with pMMC8 and pMMC1. Plasmid DNA purified from cotransformed cells was designated pMMC8/1. Within the pMMC8/1 gel pattern, bands derived from pMMC8 were apparent (Fig. 5). This plasmid, which lacks the *Xba* I sequence required for 2-μm plasmid recombination, was recovered intact from the cotransformed cells. The normal pMMC1 bands were not present, however, but were replaced by a number of bands corresponding to larger DNA species. Cleaving pMMC8/1 with *Xba* I yielded the intact pMMC8 bands and the two fragments expected from pMMC1, demonstrating that these larger species are derived from pMMC1. The larger pMMC1 species appeared only in cells cotransformed with pMMC8; therefore, they must reflect FLP-promoted recombination. These results imply that FLP protein is expressed by pMMC8. Because pMMC8 DNA was not affected, the recombination events were clearly dependent upon an intact 2-μm plasmid recombination site. In addition, the FLP protein in this experiment must act in *trans*.

Because the *Xba* I site lies within the sequences required for recombination, this experiment will not detect an intramolecular inversion (Figs. 1 and 2). To determine if inversions had taken place, the pMMC8/1 DNA was cleaved with *Ava* I. This enzyme did not affect pMMC8 but cleaved pMMC1 (which contains one complete 2-μm plasmid sequence in the A form) into two fragments of 1,506 and 7,972 bp. Conversion of the 2-μm plasmid sequence in pMMC1 to the B form would be indicated by the presence of *Ava* I fragments of 6,098 and 3,380 bp (2) (see Figs. 1 and 2). As shown in Fig. 5, cleavage of pMMC8/1 with *Ava* I yielded the bands expected for intact pMMC8, two bands expected for pMMC1 (A form), and two new bands in the positions expected for pMMC1 (B form). This DNA concentration was chosen so that the larger bands could be clearly distinguished. The 1.5-kb band from pMMC1 (A form) was present but stained weakly compared to the others. Its presence was confirmed by using larger amounts of the cleaved DNA (not shown). The largest bands from both pMMC1 (A form) and pMMC1 (B form) appeared to be present in equal or nearly equal concentrations. This suggests an efficient inversion reaction that had proceeded to equilibrium or near equilibrium. Because the inversions are also dependent upon the presence of pMMC8, all of these events must be mediated by FLP protein from pMMC8 acting in *trans*.

Recombinational Deletion in *trans*. The third and final type of site-specific recombination is a deletion event that may occur when two sites are present on the same DNA molecule in the same orientation. To detect such an event in this system, HB101 was cotransformed with pMMC8 and pMMC3. The plasmid pMMC10, which is equivalent to one of the expected products of an FLP-promoted pMMC3 deletion, was also used in the cotransformation of HB101 with pMMC8 for control purposes. Purified DNA from the two sets of cotransformed cells was designated pMMC8/3 and pMMC8/10, respectively. The gel pattern observed with pMMC8/10 DNA again showed pMMC8 recovered intact (Fig. 6). The plasmid pMMC10, which contains one 2-μm plasmid repeat, was found as the larger DNA species resulting from FLP-promoted recombination as described above. Cleavage with *Xba* I reduced these larger species to the single band expected for pMMC10, confirming that these species are derived from pMMC10. The pattern ob-

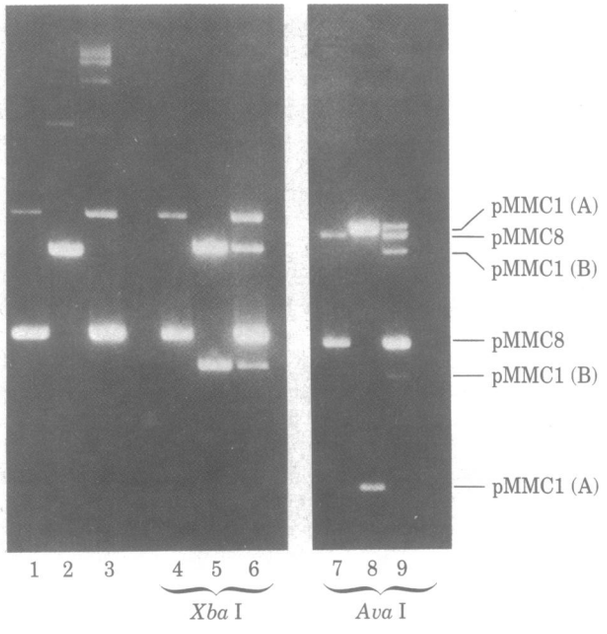


FIG. 5. FLP-promoted inversion of pMMC1 sequences. Agarose gel electrophoresis and restriction digests were as described. Lanes: 1, pMMC8 DNA; 2, pMMC1 DNA; 3, pMMC8/1 DNA; 4, *Xba* I-digested pMMC8; 5, *Xba* I-digested pMMC1 DNA; 6, *Xba* I-digested pMMC8/1 DNA; 7, *Ava* I-digested pMMC8 DNA; 8, *Ava* I-digested pMMC1 DNA; 9, *Ava* I-digested pMMC8/1 DNA. Bands in lane 9 are identified; A and B denote A and B forms of the 2-μm plasmid sequence within pMMC1.

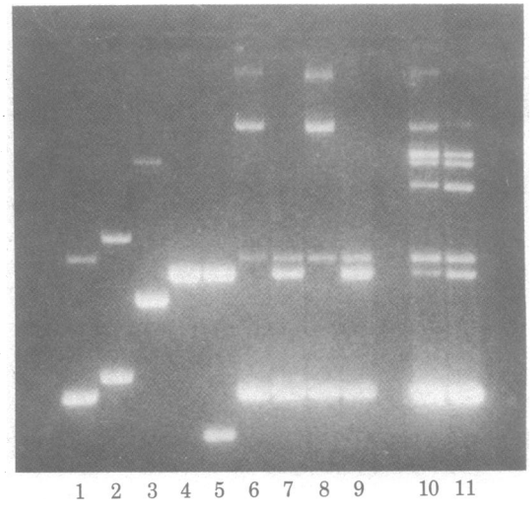


FIG. 6. FLP-promoted deletion of pMMC3 sequences. Agarose gel electrophoresis and restriction digests were carried out as described. Lanes: 1, pMMC8 DNA; 2, pMMC10 DNA; 3, pMMC3 DNA; 4, *Xba* I-digested pMMC10 DNA; 5, *Xba* I-digested pMMC3 DNA; 6, pMMC8/10 DNA; 7, *Xba* I-digested pMMC8/10 DNA; 8, pMMC8/3 DNA; 9, *Xba* I-digested pMMC8/3 DNA; 10, pMMC8/3 DNA, 0.8 μg digested for 5 min with 5 units of *Xba* I; 11, pMMC8/3 DNA, 0.8 μg digested for 10 min with 5 units of *Xba* I.

served for pMMC8/3 DNA was very similar to that observed for pMMC8/10. Cleavage of pMMC8/3 yielded not the two bands expected for pMMC3 but only the band corresponding to pMMC10. This result indicates that early after cotransformation, deletion events occurred within the pMMC3 molecules present.

In a reaction that is the molecular equivalent of the FLP-promoted inversion described above, these events would yield two small plasmids. One of these would be equivalent to pMMC10 and retain the tet^R elements of pMMC3. The other would lack an *E. coli* replication origin and would be lost. This experiment demonstrates that the FLP protein expressed by pMMC8 can promote deletion events in *trans* as well as the inversion and insertion events already demonstrated. Two partial digests of pMMC8/3 with *Xba* I also are shown in Fig. 6, revealing a number of intermediate bands. It is possible that some of these represent catenated species. Deletion events within pMMC3 multimers might be expected to yield catenated products by the considerations of Mizuuchi *et al.* (15), adding to the complexity of the gel patterns.

DISCUSSION

The site-specific recombination system of the yeast 2-μm plasmid was successfully transferred to *E. coli*. The new *E. coli* system is identified with the yeast 2-μm plasmid system by a dependence on the presence of both the *FLP* gene (expressed) and the FLP recombination site. The evidence for this conclusion relies on observation of the recombination event itself. This event requires the recombination site from the 2-μm plasmid because (*i*) restriction analyses of *in vivo* reaction products are in all cases consistent with recombination involving only these sites, and (*ii*) destruction of sequences within the site which are known to be required for the event in yeast prevents the reaction. The requirement for expression of the *FLP* gene is implied from the observation that recombination is observed only in the presence of plasmids in which the *FLP* gene has been placed at or near the position normally occupied by the *cro* gene relative to the bacteriophage λp_R (pMMC6 or pMMC8). In the absence of pMMC6 or pMMC8, plasmids containing 2-μm plasmid recombination sites (pMMC3, pMMC1, and pMMC10) are stably maintained as unrecombined, monomeric species.

The *FLP* gene in pMMC6 or pMMC8 should be temperature inducible. All of the experiments described were carried out by using cells grown at 30°C, so that the recombination observed resulted from the basal levels of FLP protein present when λp_R was repressed by *c*I857 repressor. The amount of FLP protein present under these conditions has not yet been determined. There is potential, however, for significant amplification of the levels of FLP protein through temperature induction. Thus, the plasmids pMMC6 and pMMC8 represent a potentially highly enriched source of FLP protein. Use of *E. coli* for this purification will avoid also the protease problems common to yeast extracts (16).

The results presented here permit several additional conclusions. (*i*) FLP protein is the active agent in this recombination event. (*ii*) The FLP protein acts efficiently in *trans*. (*iii*) No other proteins or factors specific to yeast are required. It is possible that an *E. coli* protein takes the place of a required yeast function, but it is at least as likely that FLP protein acts alone. (*iv*) FLP protein can promote all three types of site-specific recombination: deletions, inversions, and insertions. This is in contrast to the resolvase system, which appears to be highly specific for deletion events (17). This versatility is not an artifact of the *E. coli* system. FLP-promoted deletion and insertion events have been observed in yeast (1, 18). In addition, multimers of the 2-μm plasmid are prevalent in populations of this DNA isolated from yeast (19). (*v*) FLP-promoted recombination does not depend upon any feature of DNA structure that is unique to yeast. In yeast, 2-μm plasmid circle DNA is packaged by a normal complement of core histones into chromatin-like nucleosomal DNA (20, 21). It is possible that histones or other yeast proteins regulate this recombination event in yeast; the *E. coli* system may owe its efficiency, in part, to their absence.

The apparent presence of tetrameric plasmid species in preparations of pMMC4 DNA may reflect a low level of expression of FLP protein in cells transformed with this plasmid. The nature of the protein produced by pMMC4 and the level at which it is present are questions that have not yet been addressed.

No statement can be made at this time about the rate of FLP-promoted recombination or the relative efficiency of the three types of recombination observed. A more complete characterization of the FLP protein and its reactions awaits the purification of the protein.

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