ABSTRACT

Gregg A. Howe. AN in vivo ASSAY FOR 2μ PLASMID RECOMBINATION. (Under the direction of Dr. Carlo V. Bruschi) Department of Biology, July 1987. The 2μ plasmid of Saccharomyces cerevisiae encodes a site-specific recombinase called FLP which catalyzes efficient recombination between two 599 base pair inverted repeats located on the plasmid. The purpose of this study was to develop an in vivo assay for phenotypic detection of FLP-mediated recombination. A tester plasmid was constructed such that the yeast LEU2 and ADE8 genes are flanked on either side by directly oriented copies of the 2μ plasmid repeat sequences. FLP-mediated recombination between these sequences in vivo produces two circular DNA molecules, one containing LEU2 and the 2μ origin of replication and the other containing ADE8. The loss of the ADE8-containing circle during subsequent cell divisions, as a result of its inability to replicate autonomously, is detected in this assay as a phenotypic shift from red colonies to white colonies. This system was used to establish that recombination between 2μ plasmid repeat sequences occurs at frequencies close to 100% in FLP+ cells. Interestingly, in flp- cells recombination is not fully abolished but rather continues at a frequency of about 15%. Experiments demonstrating that these residual events do not require the FLP recognition sequence suggest that they are mediated by a chromosomally-encoded homologous recombinase. In addition to

observing the expected phenotypes, several distinct classes of red and white variegated colonies also occurred. An examination of the recombination products in these cells showed that these patterns are the manifestation of intra- as well as intermolecular recombination. Quantitative measurements of these products indicate that intramolecular recombination is more frequent then intermolecular recombination in vivo. A simple model explaining these findings is proposed.

AN in vivo ASSAY

FOR

2μ PLASMID RECOMBINATION

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2μ PLASMID RECOMBINATION

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INTRODUCTION

Site-specific recombination is an important class of genetic exchange in organisms ranging from viruses to man. The molecular mechanism of this type of recombination is different from that of homologous recombination primarily with respect to DNA substrate requirement. Whereas homologous recombination requires large tracks of non-specific DNA sequence homology between partner molecules, site-specific recombination events involve very short but specifically defined regions of DNA homology. This mechanistic distinction reflects differences in the types of functions performed by homologous and site-specific recombination systems. The former type of recombination is most often associated with the random exchange of genetic markers during meiosis. As a result, homologous recombination is capable of providing the cell with an almost infinite genetic variability. Site-specific DNA rearrangements, on the other hand, provide a variety of organisms with a convenient means to regulate the performance of defined--and most often essential--biological tasks. The list of functions attributable to site-specific recombination has grown to include the integration and excision of the bacteriophage lambda genome from its host's chromosome (Nash, 1981), the control of host range for bacteriophages Mu (Kamp et al., 1978) and P1 (Iida et al., 1982), the resolution of cointegrate structures generated during the transposition of Tn3 and related transposons

(Kostrikin et al., 1981; Arthur and Sherratt, 1979; Reed, 1981), phase variation of Salmonella flagellar antigens (Zieg, 1977; Simon et al., 1980), the regulation of genes controlling nitrogen fixation in Anabaena heterocysts (Golden et al., 1985), and the production of a diversity of immunoglobulins (Bernard et al., 1978) and T-cell receptors in vertebrate somatic cells (Clark et al., 1984).

Perhaps the best understood eukaryotic site-specific recombination system is the one associated with the 2μ plasmid of Saccharomyces cerevisiae. A 6318 base-pair (bp), double-stranded circular DNA species, this plasmid is found in most strains of S. cerevisiae at 60-100 copies per diploid cell (Clark-Walker and Miklos, 1974). DNA sequencing of the 2μ plasmid revealed the presence of two precise 599 bp inverted repeat sequences which divide the plasmid into a large and a small unique sequence region (Figure 1; Hartley and Donelson, 1980). Several loci have been mapped to the unique regions and three of them, REP1, REP2, and REP3, define a plasmid partitioning system responsible for allocating equal numbers of plasmid molecules to mother and daughter cells during cell division (Jayaram et al., 1983a; Kikuchi, 1983; Jayaram et al., 1985; Cashmore et al., 1986). Another 2μ plasmid gene, called FLP, encodes a site-specific recombinase which acts on the inverted repeats to catalyze the inversion of one unique region relative to the other (Broach and Hicks, 1980). Consequently, in yeast cells there are found equal

amounts of two isomers of the plasmid, designated form A and form B (Beggs, 1978). In addition to catalyzing DNA inversion between inverted repeats, FLP can also promote deletion, or "cut-out", between artificially constructed direct repeats (Vetter et al., 1983; Jayaram et al.,1983b) . It has also been shown that FLP can mediate intermolecular recombination between 2μ plasmid repeats located on different DNA molecules (Cox, 1983; Jayaram et al., 1983).

The dissection of the molecular details of the 2μ plasmid recombination system has been a topic of intense interest. Intitial in vivo experiments indicated that the minimal sequence required for FLP-mediated recombination was a region less than 65 bp located within the 599 bp inverted repeats (Broach et al., 1982). DNase I footprinting experiments done with purified FLP protein revealed a protected region of about 50 bp (Andrews et al., 1985). Recent in vitro studies have precisely defined this minimal sequence as consisting of three 13 bp repeat sequences surrounding an 8 bp core region (Figure 2; Andrews et al., 1985; Gronostajski and Sadowski, 1985a; Senecoff et al., 1895; Proteau et al., 1986). These experiments have now been extended to the analysis of specific FLP-DNA interactions. Upon binding to its recognition sequence, FLP makes an 8 bp staggered cut and remains bound to the 3' termini of each break via a phosphotyrosyl linkage (Andrews et al., 1985; Gronostajski and Sadowski, 1985b). In vivo evidence has recently emerged that strand exchange occurs

within the 8 bp core region and proceeds through a heteroduplex intermediate. (McLeod et al., 1986).

Although much is known about the molecular mechanism of 2μ plasmid recombination, the biological contribution of this plasmid to the yeast cell, if any, remains unknown: there is no obvious phenotypic difference between strains harboring 2μ plasmid and strains lacking it (called [cir+] and [cir0] strains, respectively). Indeed, several studies have found that [cir0] strains have a small but measurable growth rate advantage over their [cir+] counterparts (Futcher and Cox, 1983; Mead et al, 1986). A recent proposal by Futcher (1986) suggests that FLP-mediated DNA inversion is responsible for the amplification of 2μ plasmid copy number by transiently changing the 2μ mode of replication from theta to double rolling circle. The results of an elegant series of experiments by Volkert and Broach (1986) confirm this proposal. This finding supports the assertion that the 2μ plasmid, making no known positive contribution to its host and devoted solely to its own replication and survival, represents what has been termed "selfish" DNA (Doolittle and Sapienza, 1980; Orgel and Crick, 1980).

The cryptic nature of the 2μ plasmid makes its detection in yeast cells impossible without a direct examination of DNA content. Furthermore, in vivo assays for 2μ plasmid recombination have often relied upon detecting changes in DNA restriction patterns generated by interconversion between the

plasmid's two isomeric forms. Such a method for detecting 2μ plasmid recombination would be inadequate as a means for 1), the isolation of either cis- or trans-acting flp- mutant plasmids 2), studying the kinetics of FLP-mediated recombination in vivo or 3), assessing the possible involvement of chromosomally-encoded functions in FLP-mediated recombination. As a prelude to addressing these questions we have designed a biological assay for phenotypic detection of 2μ plasmid recombination. In this system, a tester plasmid was constructed which is divided in half by direct repeats of the 599 bp 2μ repeat sequence. One half of the plasmid contains the yeast LEU2 gene, the 2μ plasmid origin of replication, and sequences necessary for maintenance and selection in E. coli. The other half of the plasmid contains the yeast ADE8 gene. Upon using this construct to transform an appropriate strain (leu2 ade1 ade2 ade8 [cir+]) to LEU+ , FLP protein, provided in trans by the endogenous 2μ plasmid, will recombine the direct repeats to produce two circular molecules. Since the recombination product harboring ADE8 lacks its own origin of replication, it is expected to be lost during cell division. Thus, the ratio of LEU+ ade8 cells to LEU+ ADE8 cells provides a measure of FLP-mediated recombination in vivo.

The most salient feature of this assay is its incorporation of the ADE8 gene in a modification of the red-white system known as the Roman effect (Roman, 1957).

Briefly, the purine precursor P-ribosylamino imidazole, a red pigment, readily accumulates in yeast cells carrying the ade1 and ade2 mutations. These mutations therefore impart a red phenotype to yeast colonies which in the wild-type case would be white. In the biosynthesis of purine nucleotides, another mutation, ade8, produces a metabolic block which precedes the ade1 ade2 block. Consequently, the presence or absence of the ade8 mutation in an ade1 ade2 genetic background can be detected as a white or red colony, respectively. In this assay, the FLP-mediated recombinational excision of ADE8 from the tester plasmid and its concomitant loss permits the phenotypic expression of the otherwise recessive ade8 located in the chromosome. This event should be heralded by a phenotypic shift from red colonies (ade1 ade2 ADE8) to white colonies (ade1 ade2 ade8).

The results of the experiments presented here confirm the reliability of this assay as a means to visually detect 2μ plasmid recombination. We found that in FLP+ cells 2μ plasmid recombination occurs at a frequency of 99.8%. An unexpected finding was that in flp- cells this efficiency is reduced by only about 5-fold and that the remaining residual activity is independent of the FLP recognition sequence. This finding supports the idea that there are other cellular recombinases capable of recombining the 2μ plasmid repeat sequences at significant frequency. In addition to the expected colony phenotypes we also observed several distinct classes of red

and white variegated phenotypes which are the manifestation of multiple recombination events. These results are presented and discussed in terms of the dynamics between intramolecular and intermolecular recombination.

MATERIALS AND METHODS

Media. Defined media (COM) contained 2% glucose, 0.17% ammonium sulfate, 0.5% yeast nitrogen base, 300 ug/ml threonine, 100 ug/ml leucine, 50 ug/ml arginine, lysine, phenylalanine, tryptophan, and tyrosine, 20 ug/ml uracil, histidine, and methionine, and 10 ug/ml adenine sulfate. 1.5% agar was added for solid media. Leucine minus medium (LMM) is COM without leucine. Adenine minus medium (AMM) is COM without adenine. YPD is a complex media consisting of 2% glucose, 2% polypeptone, and 1% yeast extract. E. coli strains were grown in LB (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.5, plus 1.5% agar for solid media). Ampicillin was added to 100 ug/ml for selection of recombinant plasmids.

Strains. The yeast strain FAS20 (ade1 ade2 ade8 leu2 ura3 trp1 can1 [cir+]) was used as a recipient for transformation experiments and was constructed as follows. A364A (a ade1 ade2 ura1 his7 tyr1 gal1 lys2 [cir+]) was crossed with DBY746 (his1 leu2 ura3 trp1 can1 [cir+]) and the resulting diploid was sporulated. A haploid clone, designated B-X3H42 (ade1 ade2 leu2 trp1 ura3 his can1 [cir+]) was selected by random spore analysis. B-X3H42 was crossed with

31-1-17B (a ade8 arg4 trp1 ura3 CUP1 [cir+]) and after sporulation of the dipliod, FAS20 was obtained by selection of random haploids.

An isogenic [cir0] derivative of FAS20 was created by the method of Dobson et al. (1980). Briefly, strain FAS20 was transformed to leucine prototrophy with the LEU2-containing hybrid plasmid pJDB219 (Beggs, 1978). A single FAS20(pJDB219) transformant was grown for about 25 generations in LMM, diluted into YPD, and grown for another 10 generations. Several leu- segregants were obtained and screened for the loss of 2μ plasmid by DNA hybridization with a 2μ plasmid probe (pJDB219). One leu- segregant, designated FAS21, was found that did not contain 2μ plasmid sequences.

Escherichia coli strain WA921 (F- hsdK res mod thr leu met) was used as the host strain for all plasmids described in this work with the following exceptions. GM272 (dam-3 dcm-6 hsdS21 lacY1 tsx-78 supE44 galK2 galT22 mtl-1 metB1) was used for the preparation of plasmids which required restriction digestion with BclI. C600 (F- thi-1 leuB6 lacY1 tonA21 supE44) was used for the recovery of plasmid pBH15.

Plasmid Constructions. As shown in Figure 3, plasmid YEp13 was used as a base on which to construct all plasmids used in this study. YEp13 contains a full length copy of pBR322 with the 2241 bp EcoRI fragment of 2μ plasmid (form B) inserted at the EcoR1 site. Located at the PstI site within the 2μ moiety is a 4.0 Kb yeast fragment containing the LEU2

gene.

pBH15. YEp13 was digested with HindIII followed by ligation with T4 ligase. A product containing the 120 bp HindIII- HindIII deletion was identified by restriction analysis after recovery in E coli. This plasmid, called pBH14, was digested with BamHI and PvuII. After purification of the larger 8.9 Kb fragment from a agarose gel, the 3'recessed BamHI termini were filled-in with Klenow fragment and dNTPs. Ligation of the resulting blunt ends restores the BamHI site and the desired product, called pBH14A, was recovered in E. coli. The LEU2-containing EcoRI fragment of pBH14A was deleted by treating pBH14A with EcoRI followed by ligation. This derivative was recovered in E. coli and is called YpB. YpB was next cut with HpaI followed by ligation. A product containing the 1.4 Kb HpaI-HpaI deletion, called YpC, was recovered in E. coli. Next, the LEU2-containing EcoRI fragment deleted from pBH14A was reinserted into the EcoRI site of YpC. This was achieved by using a ligated mixture of EcoRI cut YpC and EcoRI cut YEp13 to transform E. coli C600 to leucine prototrophy (the yeast LEU2 complements the leuB of C600) and ampicillin resistance. A plasmid called pBH15 was identified that contained the correct EcoRI-generated fragment in the original orientation. The purpose of constructing plasmids YpB and YpC was to create a unique BclI site in the 2μ region as well as to delete the nonessential HpaI-HpaI fragment.

pBH15A. A 3.8 Kb BamHI fragment containing the yeast ADE8 gene was previously isolated from a genomic library cloned into the plasmid YRp16 (Fogel et al., 1982). This fragment was purified from a BamHI digest of YRp16 and inserted into the BclI site of pBH15. A product containing the ADE8 insert was identified by restriction mapping and is called pBH15A. The ability of pBH15A to complement an ade8-18 mutation was confirmed by using it to transform yeast strain 31-1-17B to adenine prototrophy.

pBHD and pBHI. The construction of plasmids carrying the 2μ plasmid repeats in both direct and inverted orientation was as follows. An 860 bp Sau3A fragment containing a copy of the 2μ plasmid repeat was isolated from plasmid pJDB219. After purification on a 1.7% agarose gel, this fragment was ligated into dephosphorylated BamHI-ends of pBH15A. As judged by restriction analysis, this strategy permitted the isolation of products that carry the 2μ plasmid repeats in both direct and inverted orientation, called pBHD and pBHI, respectively.

pBHDL. Plasmid pBHDL was constructed by first dephosphorylating the ends of HindIII cut pBHD. This fragment was ligated to HindIII cut lambda DNA and the mixture was recovered in E. coli. pBHDL was identified as a pBHD plasmid containing the 2322 bp lambda HindIII insert and the orientation of this insert was determined by restriction mapping. pBHDL lacks the 533 bp HindIII fragment originally contained on pBHD. This deletion does not affect the

components of the 2μ plasmid recombination system.

pBHDX. Plasmid pBHDX was derived by cutting pBHD with XbaI and treating 3 ug of the resulting two fragments with 3 units of S1 nuclease for 30 min at 37C. This mixture was ligated and retreated with XbaI prior to transformation of E. coli. A 12.4 Kb plasmid product was recovered that lacked both the original XbaI sites and was called pBHDX. Other than the deletions at the XbaI sites, pBHDX is identical to pBHD. The sizes of these deletions as determined from restriction mapping were about 30 bp in one of the repeat sequences and 50 bp in the other repeat sequence.

DNA Transformation. Yeast strains were transformed by growing cells in 100 ml YPD media at 30C in a 500 ml baffled flask with vigorous shaking. Cells were harvested in late log phase by centrifugation at 1000xg for 5 min, washed once with 10 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8), and resuspended in 20 ml CATE (10 mM CaCl2 in TE buffer). This suspension was transferred to a 250 ml flask and incubated at 30C with gentle shaking for 30 min. The cells were then centrifuged as before and resuspended in TE buffer to a density of 5 x 10-9 cells/ml. Aliqouts of 0.1 ml were distributed to prechilled glass tubes (17x100 mm) and incubated statically with 5 ug plasmid DNA for 10 min at 4C. Samples were heat shocked for 5 min at 42C, returned to ice for 10 min , and 1 ml of 40% polyethylene glycol (v/v, PEG-4000 in TE buffer) was added. After a 45 min incubation

at 25C, cells were centrifuged and resuspended in 1 ml SOS (1 M sorbitol, 10 mM CaCl2, 33% liquid YPD media). This suspension was incubated statically for 20 min at 25C prior to plating 0.1 ml aliquots onto selective media. Plates were incubated for 5 days at 30C and then for 1 day at 4C before scoring the transformant phenotypes. E. coli strains were transformed as described by Mandel and Higa (1970).

DNA Preparations. Plasmid DNA from yeast transformants was prepared by inoculating individual colonies into 100 ml LMM. After growth to stationary phase (approximately 10 generations), cells were centrifuged at 2000 x g and resuspended in 3.5 ml 1 M sorbitol, 0.1 M EDTA, 1.4 mM ß-mercaptoethanol, pH 8.0. After adding 7.5 ul zymolyase (1 mg/ml), the cell suspension was incubated at 37C for 30 min. The spheroplasts were centrifuged as above and the supernatant discarded. The following were added in the order given: 10 ul diethylpyrocarbonate, 3.2 ml TE buffer, 0.32 ml 0.5 M EDTA, 0.16 ml 2 M Tris base, 0.32 ml 10% SDS, and 1 ml 5 M potasium acetate. This mixture was incubated on ice for 1 hr and centrifuged at 12000 x g for 30 min at 4C. The supernatant was poured into a 30 ml Corex tube and mixed with 2.2 volumes of 95% ethanol. The DNA precipitate was collected by centrifugation at 8000 x g and dried in a vacuum oven at 50C for 15 min. The DNA pellet was taken up in 1 ml TE buffer, extracted 3x with phenol: chloroform: isoamyl alcohol (24: 24: 1), and ethanol precipitated. The final pellet was

collected by centrifugation at 11,000 x g, washed once with 70% ethanol, and after drying, resuspended in 0.1 ml TE buffer containing 40 ug/ml RNase. Plasmid DNA was isolated from E. coli as specified by Birnboim and Doly (1979) and purified on a two-step CsCl gradient as described by Garger et al (1983).

DNA Hybridization. DNA from agarose gels was transferred to nitrocellulose filter paper for hybridization by a modification of the Southern procedure described by Wahl et al. (1979). 32P-labeled plasmid probes were prepared using a nick-translation kit purchased from Bethesda Research Laboratories, Inc. Hybridization conditions used were those described by Wahl et al. (1979) except that dextran sulfate was omitted from the hybridization buffer. Hybridized filters were exposed to Kodak XAR-2 X-ray film with Dupont Lightning Plus intensifying screens.

Miscellaneous Methods. General nucleic acid procedures and agarose and polyacrylamide gel electrophoresis were as described by Maniatis et al. (1982). Restriction enzymes and lambda DNA were purchased from International Biotechnologies, Inc. S1 nuclease and calf intestinal phosphatase were purchased from Bethesda Research Laboratories, Inc. Unless otherwise specified, the reaction conditions used were those recommended by the supplier.

RESULTS

I. Phenotypic Analysis of Transformants

As an experimental control for the Roman effect the normally white strain FAS20 (ade1 ade2 ade8 leu2 [cir+]) was transformed to leucine prototrophy with plasmid pBH15A. This plasmid, which contains the yeast LEU2 and ADE8 genes, is not expected to undergo intramolecular recombination since it contains only a single copy of the 2μ repeat sequence (Figure 3). In this experiment, the emergence of red transformant colonies demonstrates complementation of the chromosomal ade8 allele by the ADE8 gene on the plasmid and hence the phenotypic alteration from white to red (Table 1 and Plate 1B).

Plasmid pBHD is identical to pBH15A except that it contains two 2μ repeat sequences in direct orientation (Figure 3). This plasmid was transformed into strain FAS20 by selecting for leucine prototrophs. It is expected that pBHD will readily recombine to produce two circular products as shown in Figure 4. One product, designated pRL, contains the LEU2 gene and the 2μ origin of replication and can thus be propagated in yeast. The other recombination product, pIA, contains the ADE8 gene but cannot replicate autonomously. The resulting loss of pIA during cell division is expected to restore the white phenotype. The actual phenotypes observed in this experiment were divided into two categoriesare as shown in Table 1: those which were completely white (white,

Plate 1A) and those which were mostly white but contained tiny red streaks and specks (red speck, Plate 1D).

To insure that the phenotypes associated with pBHD transformants are dependent on the orientation of the repeat sequences in pBHD, FAS20 was also transformed with plasmid pBHI. pBHI is identical to pBHD except that the repeat sequences are inversely oriented with respect to one another. In this case, recombination is expected to invert the pIA moiety with respect to the pRL moiety. Thus, the maintenance of ADE8 should impart a red phenotype to FAS20(pBHI) transformants. The major phenotypic class observed in these experiments was a phenotype called red star (Table 1 and Plate 1E).

In the above experiments, FLP function is provided in trans by the endogenous 2μ plasmid of FAS20. The phenotypic profiles of a flp- strain were also scored by using FAS21, an isogenic [cir0] derivative of FAS20, as a recipient for each of the tester plasmids. In these cases, red colonies are expected due to the absence of recombination. The predominant phenotype observed in this experiment was pink rather than red (Table 1 and Plate1E).

II. Intramolecular Recombination of pBHD Produces White and Red Speck Phenotypes.

Greater than 99% of FAS20(pBHD) transformant colonies were either completely white or mostly white with red specks (Table

1 and Plate 1). As noted previously, the phenotypic shift from red to white upon transformation of FAS20 with pBHD is expected to be due to intramolecular recombination between the direct repeats of pBHD. To verify this, a white FAS20(pBHD) transformant was grown to stationary phase (eight to ten generations) in luecineless medium (LMM). Total DNA was prepared from this culture and examined by Southern hybridization (Figure 5). The major DNA species having homology with the pBR322 probe is a pBHD deletion product whose structure is consistent with that of pRL (see Figure 4). To confirm this result, the DNA from eight individual white pBHD transformants was extracted and used to transform E. coli to ampicillin resistance. In all eight cases the pRL recombination product was recovered as judged by restriction analysis (Figure 6). Identical results were obtained using DNA prepared from a red speck FAS20(pBHD) transformant (data not shown). DNA from a red pBH15A transformant was also examined by Southern analysis (Figure 5). In this case, the only detectable plasmid species was the parental pBH15A molecule. These experiments demonstrate that in the FLP+ background of FAS20, recombination between the direct repeats of plasmid pBHD causes a phenotypic shift from red to white or red speck.

III. Intermolecular Recombination Occurs Between the Resolution Products of pBHD and Endogenous 2μ Plasmid.

A. pRL/2μ Recombination. Several bands on the autoradiograph in Figure 5 (arrows) indicate that in addition to pRL, other recombination products are formed in FAS20 after transformation with pBHD. Since these products contain pBR322 sequences, we reasoned that they could be recovered in E. coli if the pBR322 origin of replication and the ß-lactamase gene were maintained. To test this possibility, DNA from a white FAS20(pBHD) transformant was used to transform E. coli. The plasmid content of 58 ampicillin resistant clones was screened by restriction mapping. 55 of the clones contained pRL (50 monomers and 5 dimers). The remaining 3 clones harbored a 13.3 Kb plasmid consisting of a full length copy of pRL joined within the 599 bp repeat to a full length copy of 2μ plasmid. This structure, called pRL/2μ, was recovered in two isomeric forms designated pRL/2μA and pRL/2μB. Restriction mapping showed that these isomers differed from one another with respect to the orientation of the 2μ plasmid large unique region.

The production of pRL/2μ cointegrates is most easily explained by one of the two pathways shown in Figure 7. In either case it would be expected that an equilibrium would be established between the intramolecular recombination of the direct repeats on pRL/2μ and the intermolecular recombination between pRL and 2μ plasmid. In attempting to understand the dynamics of these molecular interactions, quantitative measurements of the relative amounts of pRL and pRL/2μ isomers

were made by densitometry scanning of the autoradiographs of Southern blots such as the one shown in Figure 5. The restriction pattern generated by PstI (Figure 5, lane H) is particularly useful for this purpose because it discriminates pRL, pRL/2μA, and pRL2μB (but not the other two possible pRL/2μ isomers) when probed with pBR322. It was determined from this experiment that the relative amounts of pRL, pRL/2μA, and pRL/2μB in FAS20(pBHD) transformants grown for 32 generations are 1.000, 0.147, and 0.145, respectively (Figure 8). This indicates that about 30% of the population of pRL molecules had participated in intermolecular recombination with endogenous 2μ plasmid. The same experiment performed with DNA prepared from cells harvested 29 generations after transformation showed identical proportions of these plasmid species. This fact suggests that these measurements represent an established equilibrium between inter- and intramolecular recombination and that this equilibrium favors the accumulation of products derived from intramolecular recombination.

B. pIA/2μ Recombination. The observation that pRL readily recombines with endogenous 2μ plasmid suggested that pIA, the nonreplicating resolution product of pBHD, could undergo similar recombination events. Indeed, the red speck phenotype displayed by the majority of FAS20(pBHD) transformants (Table 1 and Plate 1D) indicates that the ADE8

gene is maintained within a small percentage of cells in a colony. This would be expected if the ADE8-pIA moiety, after its deletion from pBHD, is rescued from mitotic loss by recombining with the endogenous 2μ plasmid. It is worth noting here that the red speck phenotype is extremely unstable. This was determined by streaking individual red speck transformants onto LMM to isolate single colonies. Inspection under a stereoscope showed that only 2% of these colonies retained the red speck phenotype while the remaining colonies were completely white. This instability would be expected due to the continual re-excision of pIA from pIA/2μ and its subsequent loss during the growth of the colony. Indeed, such instability would be accenuated if, as is the case for pRL-2μ recombination, intramolecular recombination is favored over intermolecular recombination.

The direct detection of pIA sequences was attempted in Southern hybridization experiments. To facilitate these experiments, a pIA-specific hybridization target was provided by cloning a 2.3 Kb HindIII fragment of phage lambda DNA into the HinDIII site of pBHD The resulting plasmid is called pBHDL (See Materials and Methods). The phenotypic profile of FAS20(pBHDL) transformants is 64% red speck and 36% white (n=500) and is therefore very similar to the profile observed for FAS20(pBHD) transformants (Table 1). In these experiments, a single pBHDL red speck transformant was grown for 10 generations in LMM. Total DNA was extracted and

examined by Southern hybridization using the total lambda genome as a pIA-specific probe. In three separate experiments, lambda sequences were not detected even after prolonged exposure of the autoradiographs. Similar experiments using the same yeast DNA against a pBR322 probe revealed the expected pRL and pRL/2μ structures (data not shown).

The instability of the red speck phenotype as well as the inability to detect pIA sequences in hybridization experiments suggested that pIA suffered a high rate of mitotic loss under conditions that did not select for the presence of ADE8. We therefore repeated these experiments in a strain that allowed direct selection of ADE8 function. Strain 31-1-17B (relevant genotype: ade8 [cir+]) was transformed with pBHDL and the transformants were selected on adenine minus media (AMM) for about 23 generations. To determine the effect of relaxing the selective pressure on ADE8, parallel cultures of COM and AMM media were inoculated with cells from a single transformant clone. After 10 generations, DNA was purified from an equal amount of cells from both cultures and examined by Southern hybridization using the pIA-specific lambda probe. The results in Figure 9 show that the lambda target sequences have been maintained as the 2.3 Kb HindIII fragment which was cloned into the parental pBHDL vector. Furthermore, these sequences are part of a 7.3 Kb XbaI fragment which corresponds to full length pIA. The EcoRI digest demonstrates that pIA

sequences have recombined with endogenous 2μ plasmid to produce pIA/2μA (4.1 and 3.0 Kb bands) and pIA/2μB (3.0 and 2.4 Kb bands). It cannot be determined from this experiment whether the 4.3 Kb EcoRI band corresponds to a parental pBHDL fragment (4343 bp) or circular, unintegrated pIA (4388 bp). However, failure to detect either parental pBHD or pBHDL in previous experiments using pBR322 probes (Figure 5 and data not shown) suggests that this band was derived from unintegrated circular pIA. Also shown in Figure 9 is that the maintenance of pIA is dependent upon the direct selection of ADE8. Cells that were supplemented with adenine contained about 5-fold less pIA than did the culture grown in adenine deficient media. Possible models considering the formation of the pIA/2μ intermediate are shown in Figure 7.

IV. The Red Star Phenotype

The major phenotype associated with FAS20(pBHI) transformants is a red and white variegated pattern called the red star phenotype (Table 1 and Plate 1E). To characterize the molecular events underlying this intriguing mixture of red and white, the DNA from 500 pooled red star colonies was returned to E. coli and the plasmid content of eight ampicillin resistant clones was examined by restriction mapping (Figure 10). All eight clones harbored a 10.2 Kb plasmid which was cut twice with XbaI to generate a 7.1 Kb fragment and a 3.1 Kb fragment. Fine mapping of these

fragments identified them as pRL (7.1 Kb) and the 3.1 Kb XbaI fragment of 2μ plasmid containing the Small Unique Region (SUR). Furthermore, these hybrid plasmids were recovered in E. coli in two isomeric forms (pRL/2μSURA and pRL/2μSURB) which are distinguishable by the orientation of pRL with respect to the 2μ DNA.

A model explaining the production of pRL/2μSUR structures is shown in Figure 11. As indicated, the first step in the production of pRL/2μSUR cointegrates must have been a recombination event between pBHDI and the 2μ plasmid. This would generate a structure carrying two pairs of direct repeats. Whereas recombination between one pair of direct repeats would produce the "parental" pBHI and 2μ products, recombination between the other pair of direct repeats would generate the pRL/2μSUR and pIA/2μLUR "recombinant" products. Subsequent isomerization of these products could then occur through FLP-mediated inversion between indirect repeats. This model accounts for the variegated apppearance of the red star phenotype and in essence is due to the transfer of ADE8 from a LEU2-containing selected replicon (pBHI or pBHI/2μ) to an unselected replicon (pIA/2μLUR).

V. The Pink Phenotype.

Strain FAS21, which lacks the endogenous 2μ plasmid, produced mostly pink instead of red colonies when transformed with either of the pBH plasmids (Table 1). Close examination

of the pink colonies (Plate 1C) shows that the P-ribosylamino pigment is heterogeneously distributed across the surface of the colony. This observation suggests that the cell population is heterogeneous with regard to the expression of ADE8. To determine if this effect is due to an increased rate of plasmid loss, colonies were tested for their percentage of cells that had retained the transforming plasmid under selective conditions (Table 2). These results show that for strain FAS21, only 1-5% of the cells within a colony retained the input plasmid after about 23 generations on selective media. This high rate of plasmid instability was observed for all plasmids and colony phenotypes tested. In comparison to strain FAS20, this represents a 10 to 50 fold increase in the rate of mitotic loss of the plasmid. This result is consistent with the observation that FAS21 LEU+ transformants have a much slower growth rate under selective conditions (LMM) then do FAS20 LEU+ transformants. Under nonselective conditions the growth rate of these two strains are equal (data not shown). We believe that plasmid instability in strain FAS21 reflects the fact that this strain, being [cir0], does not contain the 2μ-encoded REP plasmid partitioning system. In strain FAS20 these functions are provided in trans by endogenous 2μ plasmid.

VI. FLP-independent Recombination Occurs Readily Between the Repeat Sequences of pBHD and pBHDL.

Using the [cir0] derivative FAS21 it was possible to determine if the FLP recognition sequences were capable of recombination in the absence of FLP. This question was addressed by transforming FAS21 to leucine prototrophy with pBHD and screening for the presence of white colonies. As shown in Table 1, about 18% of these transformants gave rise to white colonies. These experiments were repeated using plasmid pBHDL to transform the same host strain in two independent experiments. In these cases, the proportion of white colonies / white colonies plus pink colonies was 28.9% and 49.7% in a total number of 412 and 788 colonies scored, respectively. Southern analysis of DNA prepared from a white FAS21(pBHDL) transformant showed that recombination between the 2μ direct repeats of pBHDL had occurred (Fig. 12, lane D). This DNA was used to transform E. coli to ampicillin resistance and the plasmid content of 15 such transformants was characterized by restriction analysis. The expected pRL recombination product was recovered in all cases (data not shown). Interestingly, DNA prepared from a pink FAS21(pBHDL) transformant contained a mixture of parent (pBHDL) and product (pRL) plasmids as demonstrated by Southern hybridization (Fig. 12, lane C). This was confirmed by recovery of both pBHDL and pRL in E. coli (data not shown).

The production of the pRL recombination product in FAS21 suggests the presence of a chromosomally-encoded function capable of recombining the 599 bp 2μ repeats . It was

therefore of interest to analyze whether or not such a function specifically recognizes the FLP recognition target sequence, designated FRT by McLeod et al. (1986). To determine this, an frt- mutant plasmid called pBHDX was constructed which contains internal FLP-site deletions within both of the pBHD repeat sequences (see materials and methods). This type of deletion is known to abolish FLP-mediated recombination in vivo (Broach et al., 1982). pBHDX was used to transform both FAS20 and FAS21 and the phenotypes of the resulting LEU+ transformants were scored as shown in Table 3a. The interpretation of this data presented in Table 3b conveys the point that the FLP recombination system consists of two components: the trans-acting FLP protein and a cis-acting FRT site. This experiment shows that the destruction of either one or both of these components does not fully abolish recombination activity as detected by this assay. This finding indicates that the observed 10%-20% residual recombination arose via a FLP-independent pathway.

DISCUSSION

The experiments described here provide a method for the detection of 2μ plasmid recombination in vivo, similar to an assay developed by McLeod et. al. (1984). McLeod used a tester plasmid essentially identical to pBHD except that the URA3 gene was used in place of the ADE8 gene. After

transformation of a leu2 ura3 (FLP+ or flp-) strain with this plasmid, LEU+ transformants were screened, by replica plating onto uracil-less media, for the maintenance or loss of the URA3 gene initially present on the tester plasmid. Thus, they used the ratio of LEU+ ura- colonies: LEU+ URA+ colonies as a measure of recombination. Our use of the red-white system obviates the need for a negative selection step to detect recombination events. The major advantage of this is that it allows direct visual detection of recombination events which occur during the growth of individual colonies, in issue addressed in greater detail below.

The major phenotypes observed in this assay have been examined at the molecular level in order to determine the accuracy and limitations of the system. The characterization of the major phenotypes associated with FAS20(pBHD) transformants (white and red speck) showed that these phenotypes were the direct result of a recombination event between the direct repeats of the test plasmid. That these phenotypes occur at a frequency of 99.8% is an indication that FLP-mediated recombination is very efficient. The in vivo measurements made by McLeod et al. (1984) report this frequency to be >99.9%. It is worth noting that in using our assay to detect FLP-mediated recombination there are several possible sources of error. The first relates to the small proportion of white colonies (2%-3%) observed in the

phenotypic analysis of pBH15A transformants of both FAS20 and FAS21 (Table 1). Although these colonies were not investigated, they are likely to be due in part to mutation of ADE8 during the transformation process as has been previously reported by Clancy et al. (1984). A second source of false positives is the possibility that there exists a FLP-independent recombination pathway capable of recombining the test plasmid. This issue is addressed in greater detail below. Another source of error that initially concerned us was the possibility that the integration of of pIA into endogenous 2μ plasmid (i.e., pIA/2μ) would produce false negatives. Although we have shown that this event does occur (Figure 9), it apparently manifests itself as a red speck phenotype rather than a red phenotype (Plate 1B and 1D).

Very little is known about the dynamics of FLP-mediated inter- and intramolecular recombination in vivo. As a preliminary means to address this question we measured the proportions of free pRL and pRL/2μ intermolecular recombination products in FAS20(pBHD) transformants under steady state conditions (Figure 8). We found that 30% of the pRL population existed as pRL/2μ intermediates. This finding is in general agreement with the fact that about 20% of all 2μ plasmid molecules exist in dimeric and higher multimeric forms (Petes and Williamson, 1975; Guerineau et al. 1976). We interpret this data as an indication that intramolecular

recombination is favored over intermolecular recombination in vivo. If these two types of recombination were equally efficient then the ratio of free pRL to pRL/2μ would be expected to be close to 1.0. In the same experiment we also showed that pRL/2μ isomers A and B were present in equal amounts. This has been shown to be the case for the A and B isomers of the 2μ plasmid (Beggs, 1978).

The observed preference for intramolecular recombination over intermolecular recombination in vivo could be explained by a model which assumes that FLP-mediated recombination is rate-limited by the random collision of any two FRT (FLP recognition target sequence) sites and that FLP has no topological preference for either inter- or intramolecular recombination but rather, given the appropriate substrate, would perform either of these reactions with equal efficiency. A primary result predicted by this model is that FLP-mediated recombination is inversely proportional to the distance between FRT sites. This prediction has recently been substantiated for intramolecular recombination in vitro by Gronostajski and Sadowski (1985c) who have also demonstrated that this inverse proportionality is linear over a range from 3.4 Kb to 0.4 Kb. We propose that this distance effect could also modulate the relative frequencies of inter- and intramolecular recombination in vivo through fluctuations in plasmid copy number. For example, an increase in copy number would tend to increase the frequency of intermolecular

events as a result of increasing the concentration of FRT sites. Our finding of 30% intermolecular recombination is thus an indication that, under steady state conditions of plasmid copy number, FRT sites located on the same molecule (i.e., the direct repeats of pRL/2μ) are in closer proximity to one another than are the FRT sites on separate molecules (i.e., pRL and 2μ). A second result predicted by this model is that the relative frequency of intramolecular recombination is greatest when copy numbers are low. Indirect support for this has come from Gronostajski and Sadowski (1985c) who have found that intramolecular recombination favors intermolecular recombination in vitro when reaction mixtures contained relatively low concentrations of FLP. Interestingly, this prediction complements Futcher's model for 2μ plasmid amplification which would presumably require a high absolute frequency of FLP-mediated DNA inversion during periods of reduced copy number (Futcher, 1986).

In addition to making physical measurements of recombination intermediates, this study also describes a method to visually monitor the dynamics between inter- and intramolecular recombination in vivo. The use of the red-white system has enabled us to phenotypically detect at least two intermolecular recombination events which occur during the growth of individual colonies. This is illustrated by the red speck phenotype of FAS20(pBHD)

transformants (Plate 1D) and the red star phenotype of FAS20(pBHI) transformants (Plate 1E). Characterization of the recombination products found in these colonies suggested that they arose by a two-step pathway involving both intra- and intermolecular recombination events (Figure 7 and Figure 11). A more precise definition of the kinetics of inter- and intramolecular recombination in vivo will require the analysis of recombination products at time points immediately following transformation. Such experiments would be facilitated by a means of controlling FLP expression, possibly by construcing an inducible FLP promoter as described by McLeod et al. (1984).

One of the most surprising findings of this study was the residual recombination activity of pBHD and pBHDL in the flp- strain FAS21 (Table 1). Analysis of white FAS21(pBHD) and FAS21(pBHDL) transformants confirmed that they contained the pRL recombination product shown to be produced in the FLP+ strain FAS20 (Figure 12). The use of an frt- mutant plasmid (pBHDX) demonstrated that these residual events were independent of the FLP recombination system (Table 3). These data suggest the presence of a chromosomally- encoded function capable of recombining the 2μ plasmid repeats at high frequency. This hypothesis is supported by the finding that pink FAS21(pBHD) and FAS21(pBHDL) transformants often, but not always, segregate white colonies upon subcloning (data not shown). This agrees with fact that pink pBHDL

transformants contain both pBHDL and pRL (Figure 12, lane C) while white pBHDL transformants contain only pRL (Figure 12, lane D). Thus it would appear that the observed FLP-independent recombination is a process ongoing in the yeast cell. An alternative explaination, however, is that these events occurred in the RecA+ E. coli host routinely used for the growth of plasmid DNA. The idea that the DNA used for transformation experiments contained a mixture of pBHD (or pBHDL) and pRL could account for two observations which remain unexplained. First, the frequency of white FAS21(pBHDL) colonies was 2-fold to 4-fold higher than the frequency of white FAS21(pBHD) colonies. Second, these residual recombination events seem to be manifested as a white phenotype and never a red speck phenotype (Table 3a). The use of a RecA+ E. coli host was not forseen as a problem because extensive restriction and Southern analysis of pBHD and pBHDL DNA prepared fron this strain never suggested the occurence of E. coli- mediated plasmid recombination (Figure 4; Figure 5, lanes A, C, G; Figure 12, lane A). However, conclusive experiments using plasmid DNA prepared from a recA- strain are needed to resolve this issue.

BIBLIOGRAPHY

Andrews, B.J., Proteau, G.A., Beatty, L. G., and Sadowski, P. D. (1985). The FLP recombinase of the 2μ circle of yeast: Interaction with its target sequence. Cell 40, 795-803.

Arthur, A., and Sherratt, D. (1979). Dissection of the transposition process: a transposon-encoded site-specific recombination system. Mol. Gen. Genet. 175, 267-274.

Beggs, J. D. (1978). Transformation of yeast by a replicating hybrid plasmid. Nature 275, 104-109.

Bernard, O., Hozumi, N., and Tonegawa, S. (1978). Sequences of mouse immunoglobulin light chain genes before and after somatic changes. Cell 15, 1133-1144.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7, 1513-1523.

Broach, J. R., and Hicks, J. B. (1980). Replication and recombination functions associated with the yeast plasmid, 2μ circle. Cell 21, 501-508.

Broach, J. R., Guarascio, V. R., and Jayaram, M. (1982). Recombination within the yeast plasmid 2μ circle is site-specific. Cell 29, 227-234.

Cashmore, A. M., Albury, M. S., Hadfield, C., and Meacock, P. A. (1986). Genetic analysis of partitioning functions encoded by the 2μm circle of Saccharomyces cerevisiae. Mol. Gen. Genet. 203, 154-162.

Clancy, S., Mann, C., Davis, R. W., and Calos, M. P. (1985). Deletion of plasmid sequences during Saccharomyces cerevisiae transformation. J. Bact. 159, 1065-1067.

Clark, S. P., Yoshikai, S., Taylor, G., Siu, G., Hood, L., and Mak, T. W. (1984). Indentification of a diversity segment of human T-cell receptor ß-chain and comparison with the analogous murine element. Nature 311, 387-389.

Clark-Walker, G. D., and Miklos, G. L. G. (1974). Localization and quantification of circular DNA in yeast. Eur. J. Biochem. 41, 359-365.

Cox, M. (1983). The FLP protein of the yeast 2μm plasmid: expression of a eukaryotic genetic recombination system in Escherichia coli. Proc. Natl. Acad. Sci. USA 80, 4223-4227.

Dobson, M. J., Futcher, A. B., and Cox, B. S. (1980). Loss of 2μm DNA from Saccharomyces cerevisiae transformed with the chimeric plasmid pJDB219. Curr. Genet. 2, 210-215.

Doolittle, W. F., and Sapienza, C. (1980). Selfish genes, the phenotype paradigm and genome evolution. Nature 284, 601-603.

Fogel, S., Choi, D., Kilgore, D., and Lusnak, K. (1982). The molecular genetics of non-tandem duplications at ADE8 in Yeast. Rec. Adv. Yeast Mol. Biol. 1, 269-288.

Futcher, A.B. (1986). Copy number amplification of the 2μm circle plasmid of Saccharomyces cerevisiae. J. Theor. Biol. 119, 197-204.

Garger, S. J., Griffith, O. M., and Grill, L. K. (1983). Rapid purification of plasmid DNA by a single centrifugation in a two step cesium chloride-ethidium bromide gradient. Biochem. Biophys. Res. Comm. 117, 835-842.

Golden, J. W., Robinson, S. J., and Haselkorn, R. (1985). Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium Anabaena. Nature 314, 419-423.

Gronostajski, R. M., and Sadowski, P.D. (1985a). Determination of DNA sequences essential for FLP-mediated recombination by a novel method. J. Biol. Chem. 260, 12320-12327.

Gronostajski, R. M., and Sadowski, P. D. (1985b). The FLP recombinase of the Saccharomyces cerevisiae 2μm plasmid attaches covalently to DNA via a phosphotyrosyl linkage. Mol. Cell. Biol. 5, 3274-3279.

Gronostajski, R. M., and Sadowski, P. D. (1985c). The FLP protein of the 2μ plasmid of yeast. J. Biol. Chem. 260, 12328-12335.

Guerineau, M., Grandchaamp, C., and Slonimski, P. (1976). Circular DNA of a yeast episome with two inverted repeats: Strucural analysis by a restriction enzyme and electron microscopy. Proc. Natl. Acad. Sci. USA 73, 3030.

Hartley, J. L., and Donelson. (1980). Nucleotide sequence of the yeast plasmid. Nature 286, 860-864.

Iida, S., Meyer, J., Kennedy, K., and Arber, W. (1982). A site-specific, conservative recombination system carried by bacteriophage P1. EMBO J. 1, 1445-1453.

Jayaram, M., Li, Y. Y., and Broach, J. R. (1983a). The yeast plasmid 2 micron circle encodes components required for its high copy propagation. Cell 34, 95-104.

Jayaram, M., Li, Y. Y., McLeod, M. M., and Broach, J. R. (1983b). Analysis of the site-specific recombination associated with the yeast plasmid 2 micron circle. In Mechanisms of DNA Replication and Recombination, N.R. Cozzarelli, ed. (New York: Alan R. Liss), pp. 685-694.

Jayaram, M., Sutton, A., and Broach, J. R. (1985). Properties of REP3: a cis-acting locus required for stable propagation of the Saccharomyces cerevisiae plasmid 2μm circle. Mol. Cell. Biol. 5, 2466-2475.

Kamp, D., Kahmann, R., Zipser, D., Broker, T. R., and Chow, L. T. (1978). Inversion of the G DNA segment of phage Mu controls phage infectivity. Nature 271, 577-580.

Kikuchi, Y. (1983). Yeast plasmid requires a cis-acting locus and two plasmid proteins for its stable maintenance. Cell 35, 487-493.

Kostriken, R., Morita, C., and Heffron, F. (1981). Transposition of Tn3 encodes a site-specific recombination system: identification of essential sequences, genes, and actual site of recombination. Proc. Natl. Acad. Sci. USA 78, 4041-4045.

Mandel, M., and Higa, A. (1970). Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53, 154.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

McLeod, M. F., Volkert, F., and Broach, J. (1984). Components of the site-specific recombination system encoded by the yeast plasmid 2- micron circle. Cold Spring Harbor Symp. Quant. Biol. 49, 779-787.

McLeod, M., Craft, S., and Broach, J.R. (1986). Identification of the crossover site during FLP-mediated recombination in the yeast plasmid 2 micron circle. Mol. Cell. Biol. 6, 3357-3367.

Nash, H. A. (1981). Integration and excision of bacteriophage lambda: the mechanism of conservative site-specific recombination. Ann. Rev. Genet. 15, 143-167.

Orgel, L. E., and Crick, F. H. C. (1980). The ultimate parasite. Nature 284, 604-607.

Petes, T. D., and Williamson, D. H. (1975). Replicating circular DNA molecules in yeast. Cell 4, 249.

Proteau, G., Sidenberg, D., and Sadowski, P. (1986). The minimal duplex DNA sequence required for site-specific recombination promoted by the FLP protein in yeast in vitro. Nucl. Acids Res. 14, 4787-4802.

Reed, R. R. (1981). Transposition-mediated site-specific recombination: a defined in vitro system. Cell 25, 713-719.

Roman, H. (1957). Studies of gene mutation in Saccharomyces. Cold Spring Harbor Symp. Quant. Biol. 21, 175.

Senecoff, J. F., Bruckner, R. F., and Cox, M. M. (1985). Proc. Natl. Acad. Sci. USA 82, 7270-7274.

Simon, M., Zieg, J., Silverman, M., Mandel, G., and Doolittle, R. (1980). Phase variation: evolution of a controlling element. Science 209, 1370-1374.

Vetter, D., Andrews, B. J., and Sadowski, P. D. (1983). Site-specific recombination of yeast 2μm DNA in vitro. Proc. Natl. Acad. Sci. USA 80, 7284-7288.

Volkert, C. F., and Broach, J. R. (1986). Site-specific recombination promotes plasmid amplification in yeast. Cell 46, 541-550.

Wahl, G. M., Stern, M., and Stark, G. R. (1979). Effecient transfer of large DNA fragments from agarose gels to diazobenzloxymethal-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76, 3683.

Zieg, J., Silverman, H., Hilmen, M., and Simon, M. (1977). Recombinational switching for gene expression. Science 196, 170-172.

Figure 1

Figure 1. Schematic diagram of 2μ plasmid inversion. The large black arrows depict the 599 base pair inverted repeats. The restriction sites shown are: E, EcoRI; X, XbaI; P, PstI.

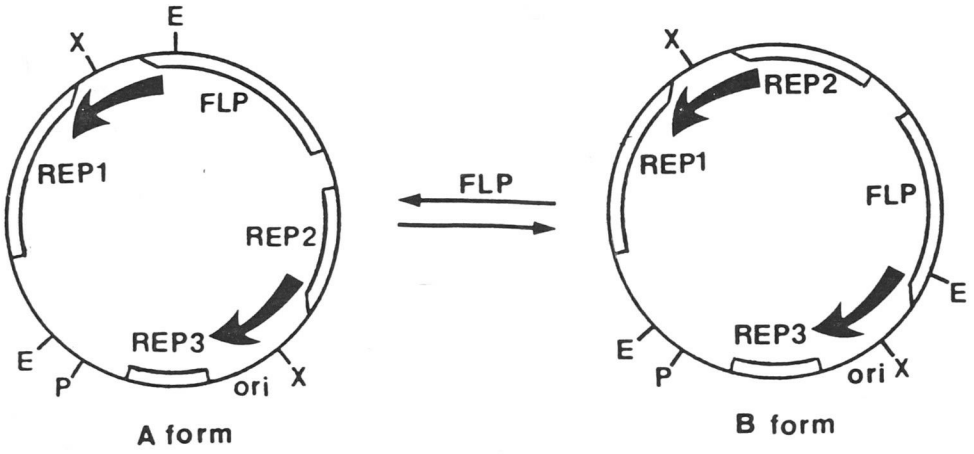
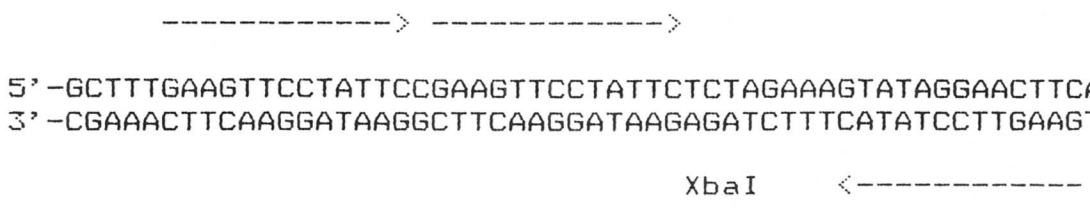

E
X
FLP
REP1
REP2
REP3
E
P
ori
X
A form
FLP
X
REP2
REP1
FLP
E
REP3
E
P
ori
X
B form

Figure 2

Figure 2. Diagram of the DNA sequence involved in FLP-mediated recombination. The important structural features include three 13 bp repeat elements shown by the horizontal arrows. The vertical arrows denote the points of FLP-induced strand cleavage. Located within the spacer region is the recognition sequence for XbaI (5'-TCTAGA-3').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5'-GCTTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAG-3'
3'-CGAAACTTCAAGGATAAGGCTTCAAGGATAAGAGATCTTTCATATCCTTGAAGTCTC-5'
XbaI
<----------

Figure 3

Figure 3. Diagram of plasmid constructions. pBR322 sequences are shown as a heavy black line, 2μ plasmid sequences as an open bar, and yeast chromosomal sequences as a single line. The stipled bars indicate the sequences inserted into the previous plasmid. The 2μ plasmid 599 base pair repeats are shown by arrows. See Materials and Methods for the details.

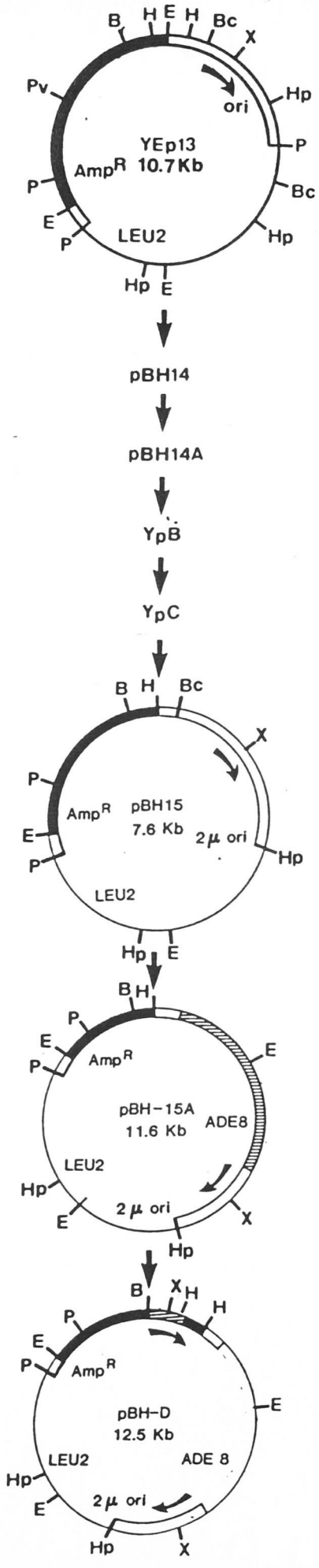

B H E H Bc X
Pv
ori
Hp
YEp13
Amp^R 10.7Kb
P
Bc
P
E
P
LEU2
Hp
Hp E
pBH14
pBH14A
YpB
YpC
B H Bc
X
P
Amp^R pBH15
7.6 Kb
2μ ori
E
P
Hp
LEU2
Hp E
B H
P
E
P
Amp^R
E
pBH-15A
11.6 Kb
ADE8
LEU2
Hp
2μ ori
E
X
Hp
B X H
H
P
E
P
Amp^R
pBH-D
12.5 Kb
E
LEU2
ADE 8
Hp
2μ ori
E
Hp
X

Figure 4

Figure 4. Schematic diagram of pBHD intramolecular recombination. pBR322 sequences are shown as a heavy black line, 2μ plasmid sequences as an open bar or a stipled bar, and yeast chromosomal sequences as a single line. The 2μ plasmid repeat sequences are shown by the arrows. The indicated restriction sites are: E, EcoRI; X, XbaI; P, PstI; Hp, HpaI; B, BamHI.

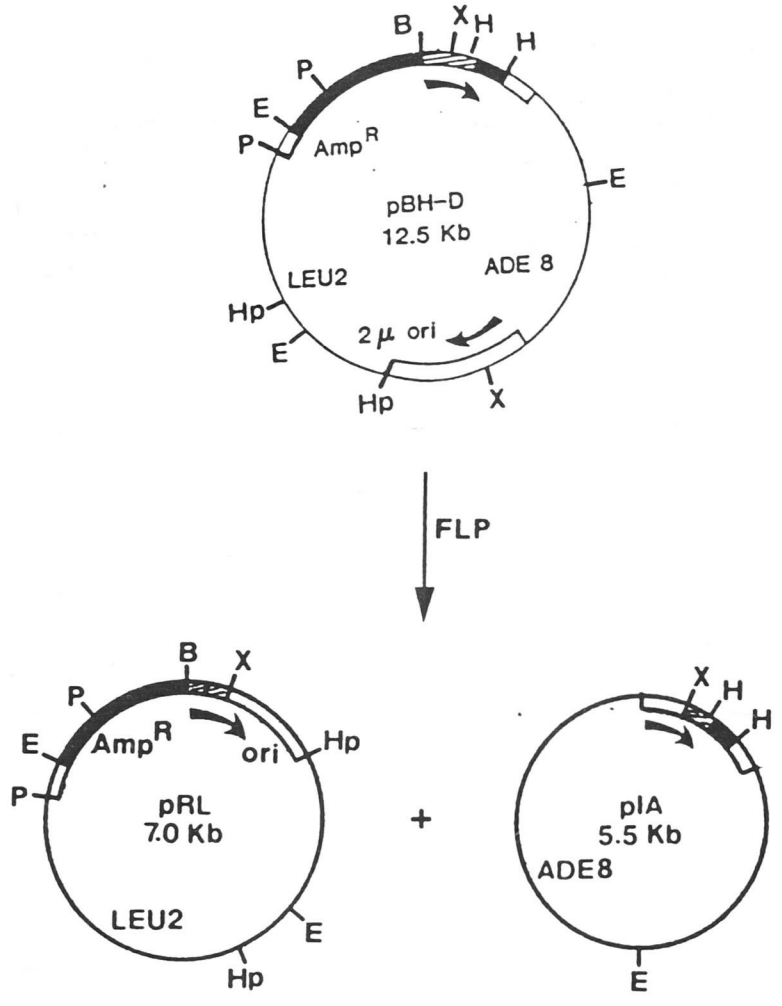

B
X
H
H
P
E
P
Amp^R
E
pBH-D
12.5 Kb
ADE 8
LEU2
Hp
E
2μ ori
Hp
X
FLP
B
X
P
E
Amp^R
ori
Hp
P
pRL
7.0 Kb
LEU2
E
Hp
+
X
H
H
pIA
5.5 Kb
ADE8
E

Figure 5

Figure 5. Southern hybridization analysis of FAS20(pBHD) and FAS20(pBH15A) transformant using a pBR322 probe. Lanes A, C, E, and G contain purified pBHD DNA treated as follows: undigested, lane A; BamHI, lane C; XbaI, lane E; PstI, lane G. Lanes B, D, F, and H contain total DNA prepared from a white FAS20(pBHD) transformant and was treated as follows: undigested, lane B; BamHI, lane D; XbaI, lane F; PstI, lane H. Lanes I, K, and M contain purified pBH15A digested with the following enzymes: BamHI, lane I; XbaI, lane K; PstI, lane M. Lanes J, L, and N contain total DNA prepared from a red FAS20(pBH15A) transformant treated as follows: BamHI, lane J; XbaI, lane L; PstI, lane N.

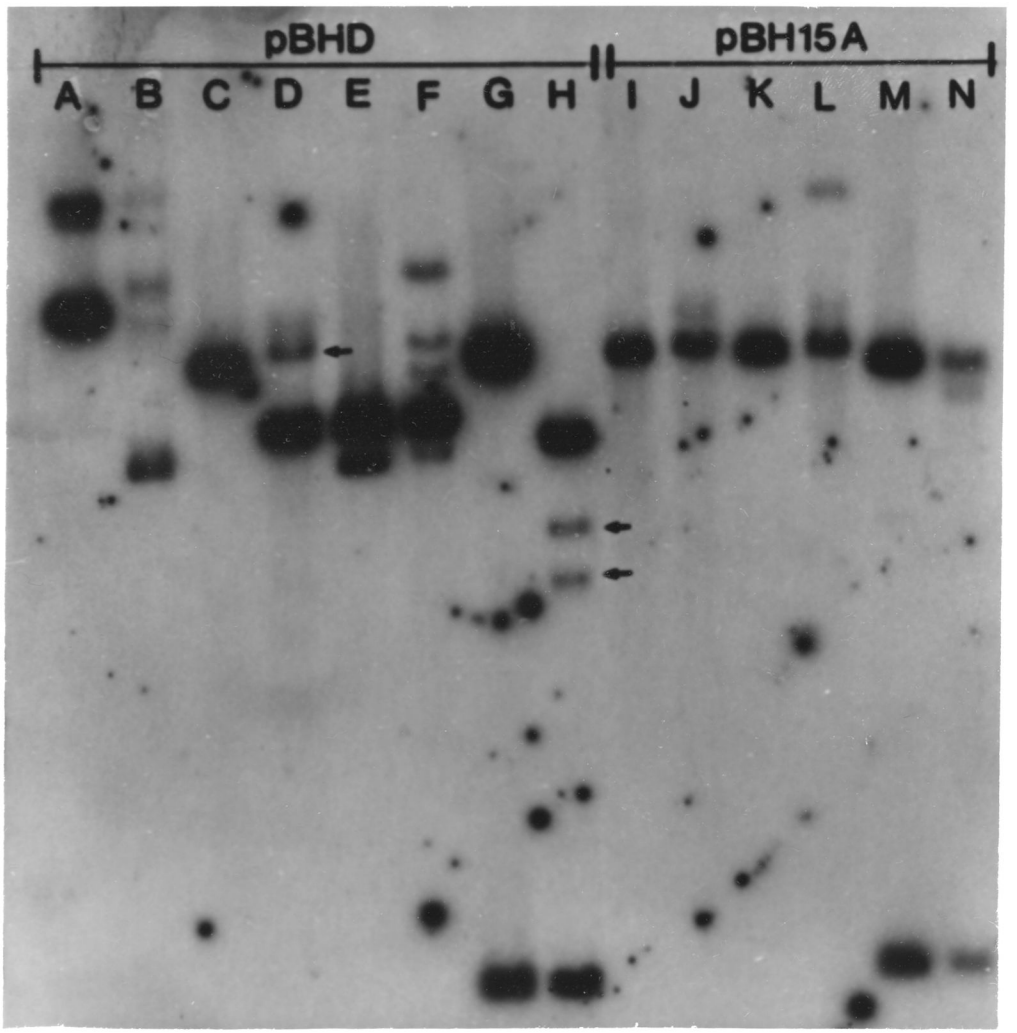

pBHD
pBH15A
A
B
C
D
E
F
G
H
I
J
K
L
M
N

Figure 6

Figure 6. Recovery of pRL in E. coli as demonstrated by restriction analysis. Total DNA prepared from a FAS20(pBHD) transformant was used to transform E. coli to ampicillin resistance. The plasmid content of one such transformant was treated as follows: undigested, lane B; BamHI, lane D; XbaI, lane F; SalI, lane H; EcoRI, lane J. Purified pBHD was run as a control after being treated as follows: undigested, lane A; BamHI, lane C; XbaI, lane E; SalI, lane G; EcoRI, lane I.

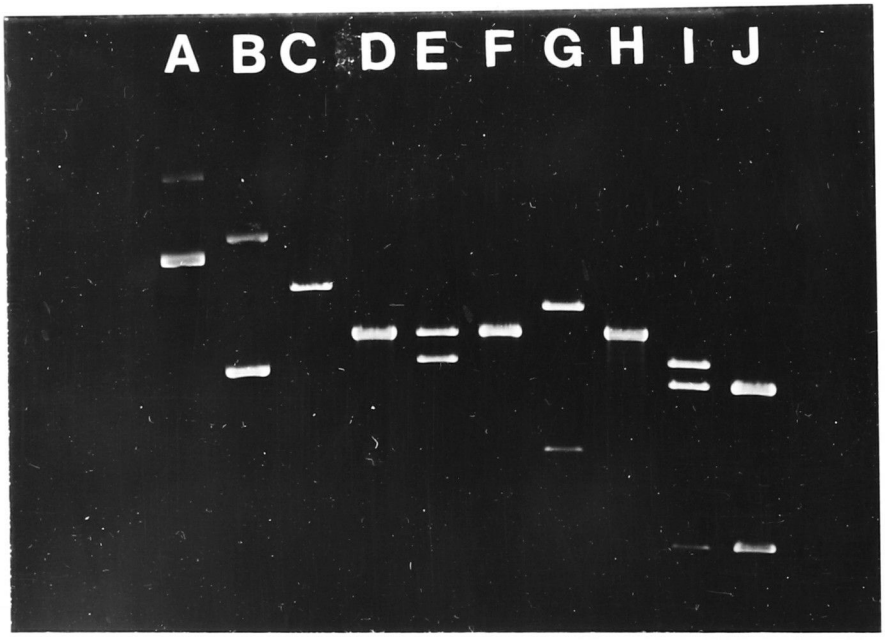

A B C D E F G H I J

Figure 7

Figure 7. Proposed two-step recombination pathways for the production of pRL/2μ and pIA/2μ. Pathway A accounts for these structures by an initial intramolecular recombination event followed by an intermolecular event . In pathway B the intermolecular recombination event precedes the intramolecular event. The filled black squares represent the 2μ plasmid origin of replication.

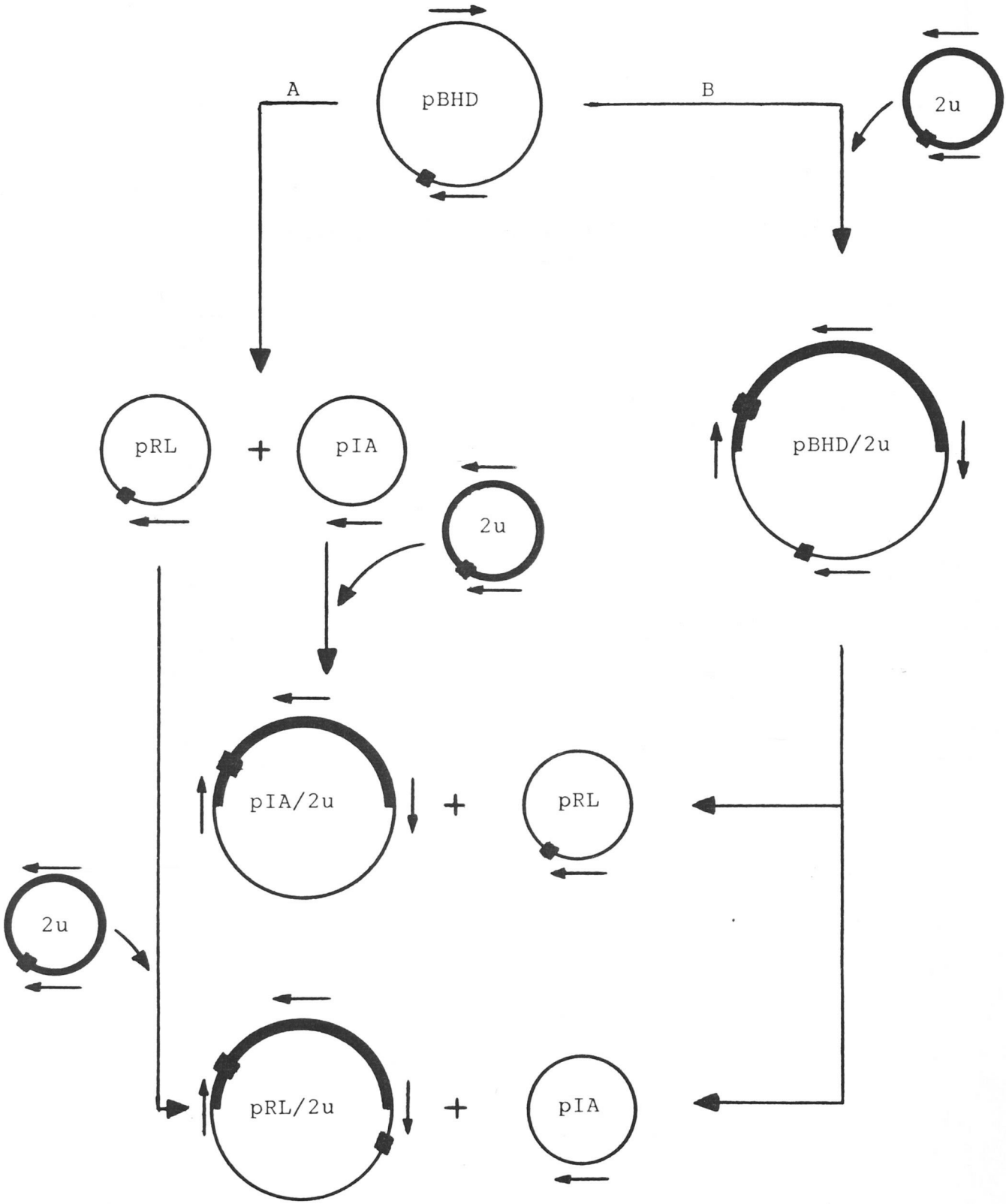
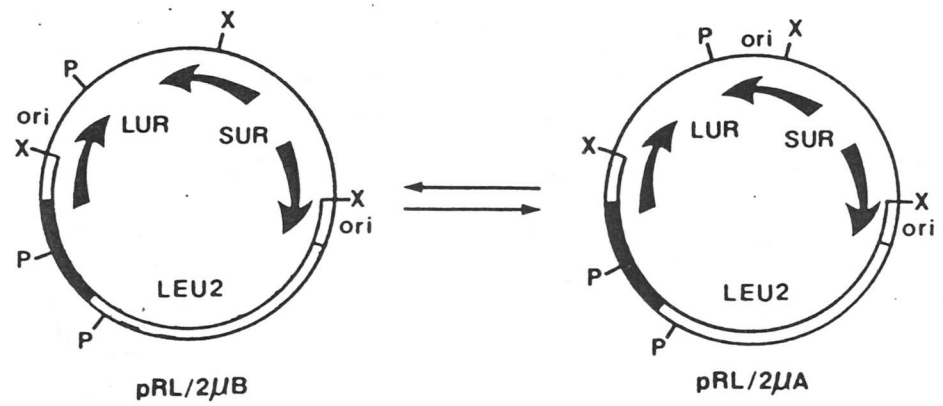

A
pBHD
B
2u
pRL
+
pIA
pBHD/2u
2u
pIA/2u
+
pRL
2u
pRL/2u
+
pIA

Figure 8

Figure 8. Densitometer scanning measurements of pRL, pRL/2μA, and pRL/2μB in a FAS2O(pBHD) transformant. This particular scan represents the top three bands in Figure 5, lane H (PstI digest). The schematic illustration of pRL/2μ isomers A and B shows that these structures are produced by the inversion of the 2μ plasmid Large Unique Region (LUR).


X
P
ori
X
LUR
SUR
X
ori
P
LEU2
P
pRL/2μB
P
ori
X
X
LUR
SUR
X
ori
P
LEU2
P
pRL/2μA

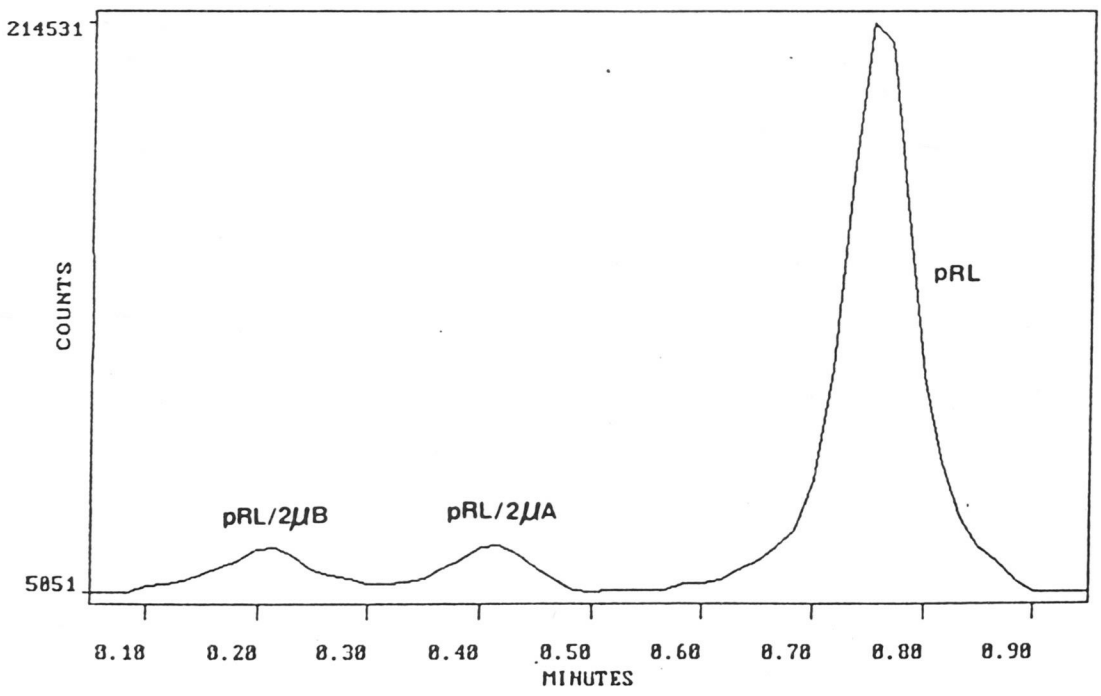

214531
COUNTS
5051
pRL/2μB
pRL/2μA
pRL
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
MINUTES

Figure 9

Figure 9. Southern analysis of 31-1-7B(pBHDL) transformants. DNA of either purified pBHDL (P), 31-1-7B(pBHDL) grown under adenine selection (+), or 31-1-7B(pBHDL) grown without adenine selection (-) was digested with the indicated enzyme and probed with lambda DNA.

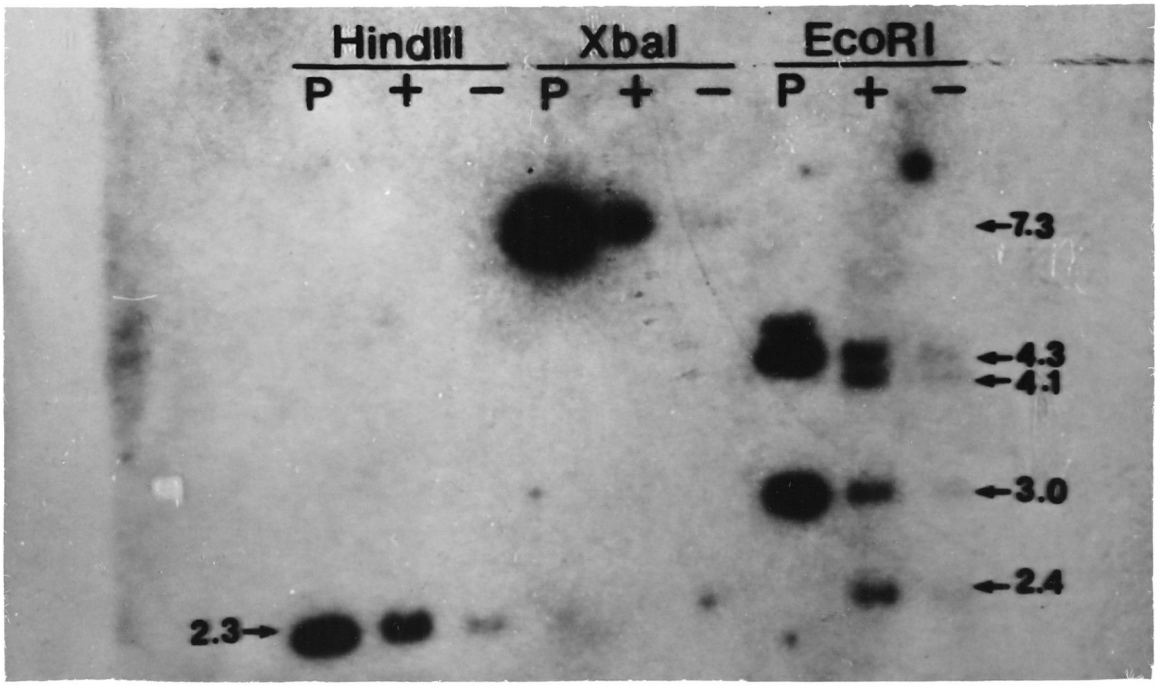

HindIII
XbaI
EcoRI
P + − P + − P + −
7.3
4.3
4.1
3.0
2.4
2.3

Figure 10

Figure 10. Restriction analysis of pRL/2μSURA and pRL/2μSURB recovered in E. coli. Purified pBHI, lanes A, D, and G; pRL/2μSURB, lanes B, E, and H; pRL/2μSURA, lanes C, F, and I. DNA in lanes A-C, D-F, and G-I were cut with XbaI, EcoRI, and HinDIII, respectively.

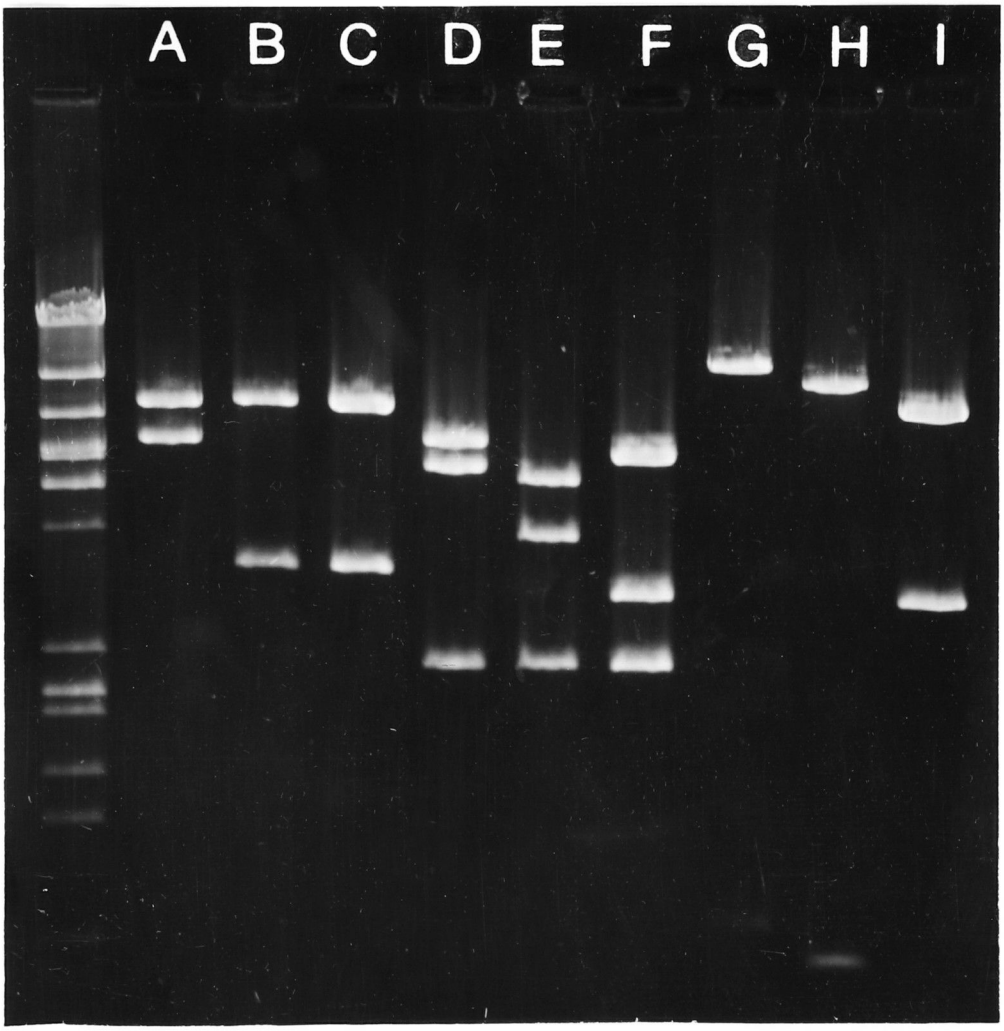

A
B
C
D
E
F
G
H
I

Figure 11

Figure 11. Proposed two-step recombination pathway for the production of pRL/2μSUR and pIA/2μLUR. The filled black squares represent the 2μ plasmid origin of replication.

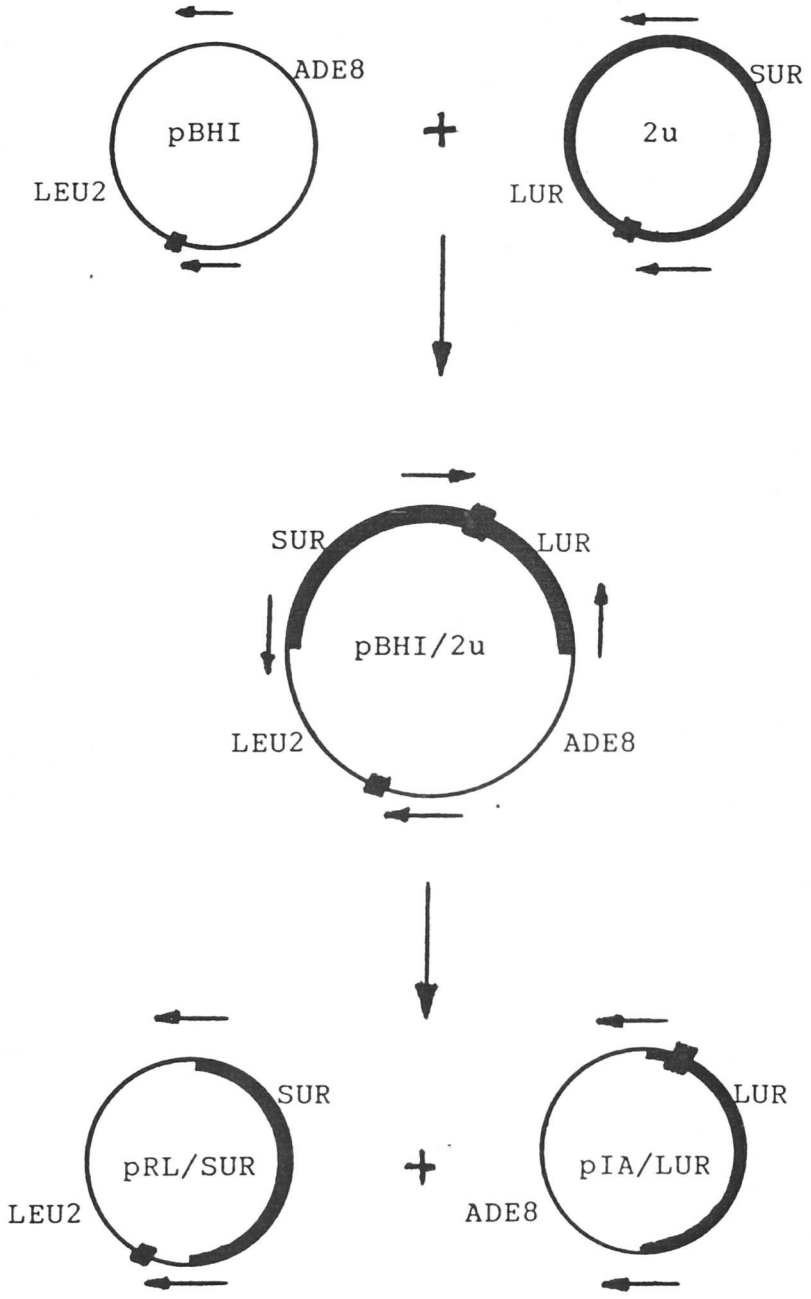

ADE8
pBHI
LEU2
+
SUR
2u
LUR
SUR
LUR
pBHI/2u
LEU2
ADE8
SUR
pRL/SUR
LEU2
+
LUR
pIA/LUR
ADE8

Figure 12

Figure 12. Southern analysis showing recombination of pBHDL in FAS21. DNA of either purified pBHD (lane A), a white FAS20(pBHD) colony (lane B), a pink FAS21(pBHDL) colony (lane C), or a white FAS21(pBHDL) colony (lane D) was cut with BamHI and probed with pBR322. Disregard lanes E, F, and G.

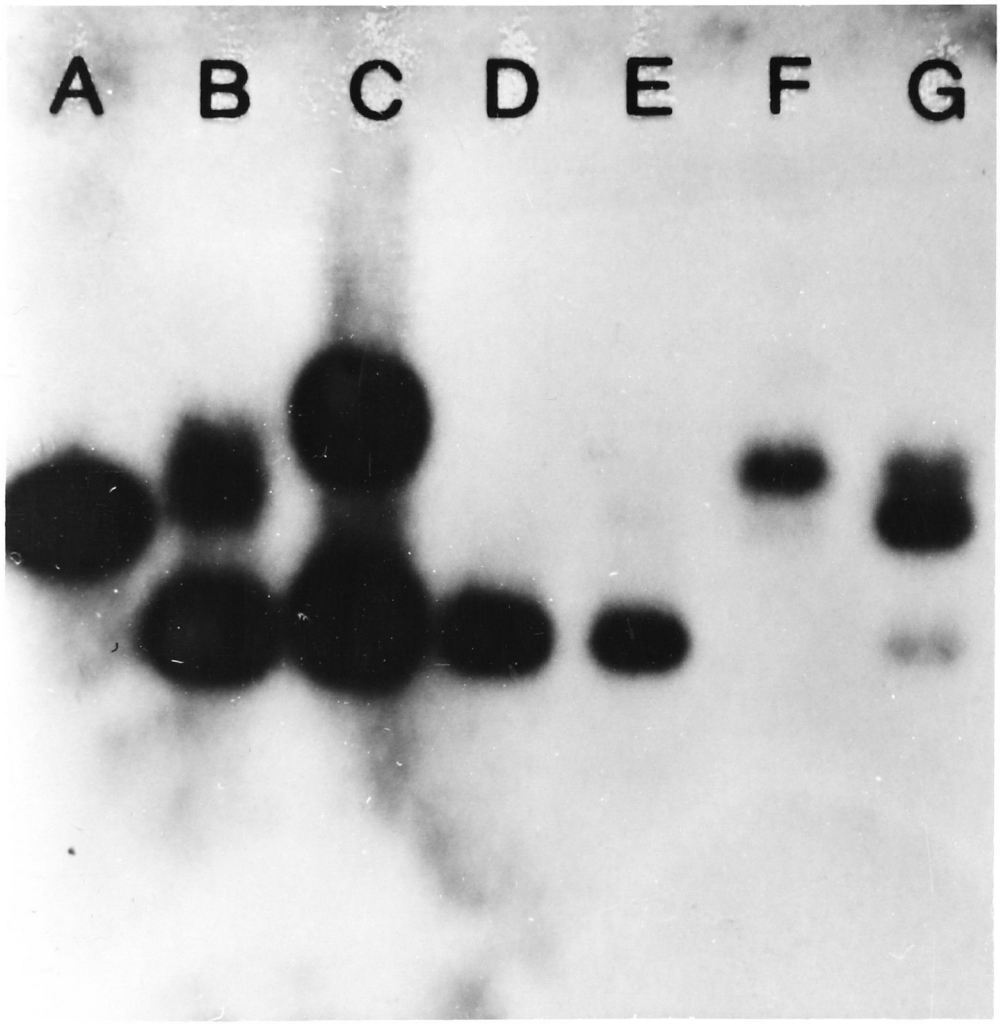
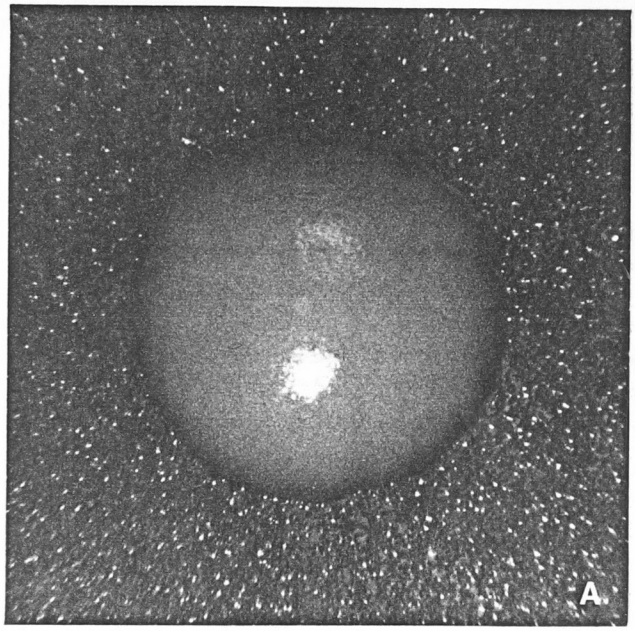
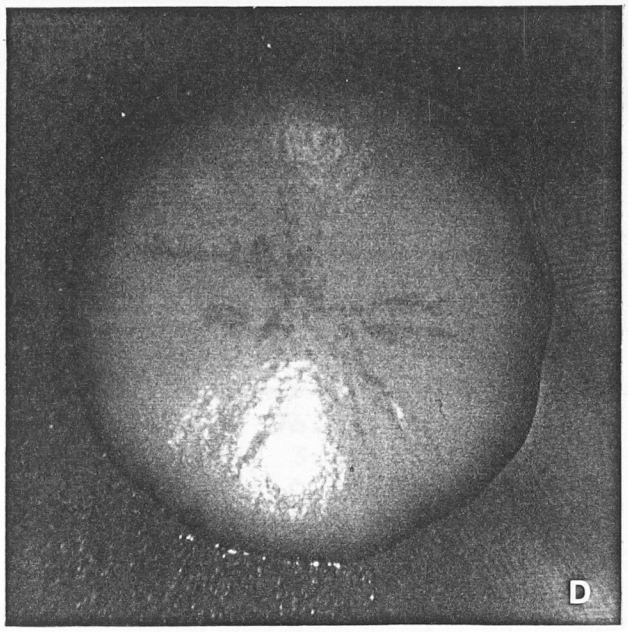
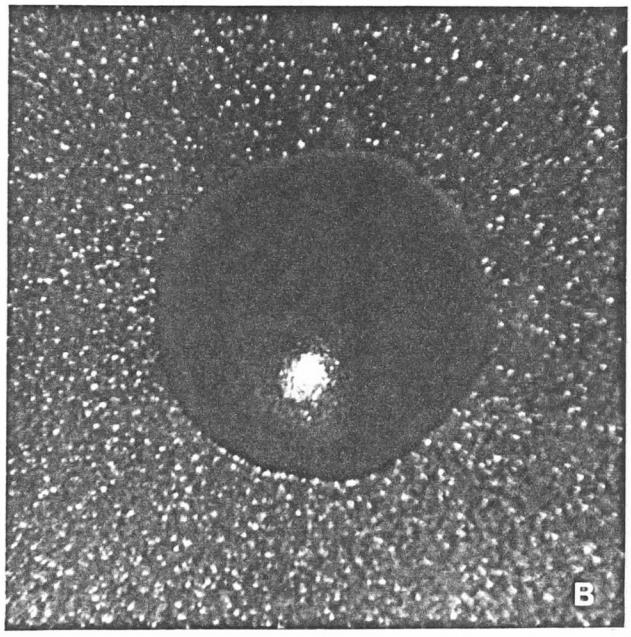

A
B
C
D
E
F
G

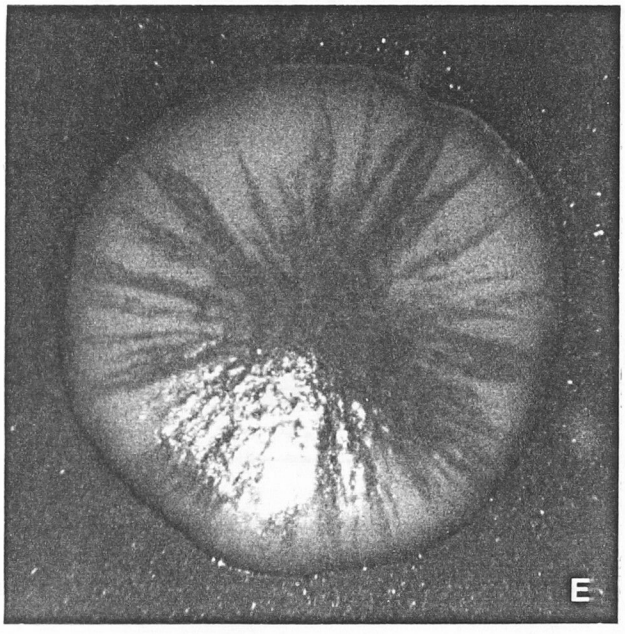
Plate 1

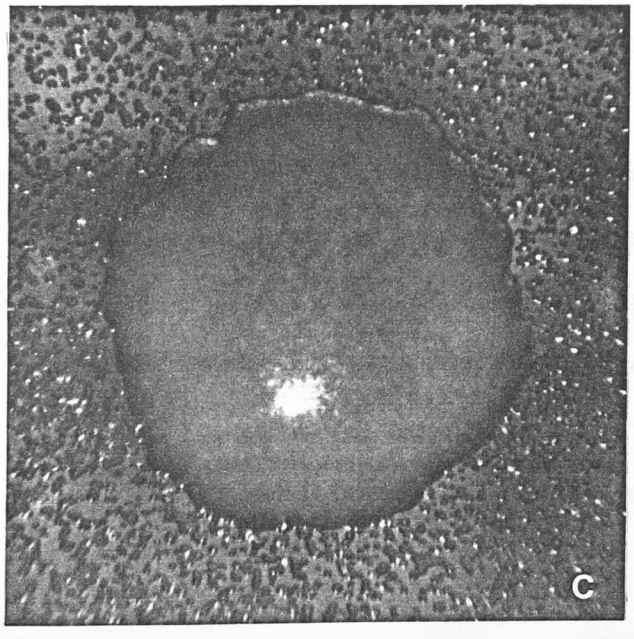
Plate 1. Illustration of the phenotypes observed in this study: white, A; red, B; pink, C; red speck, D; red star, E; sectored, F.


A


D


B


E


C

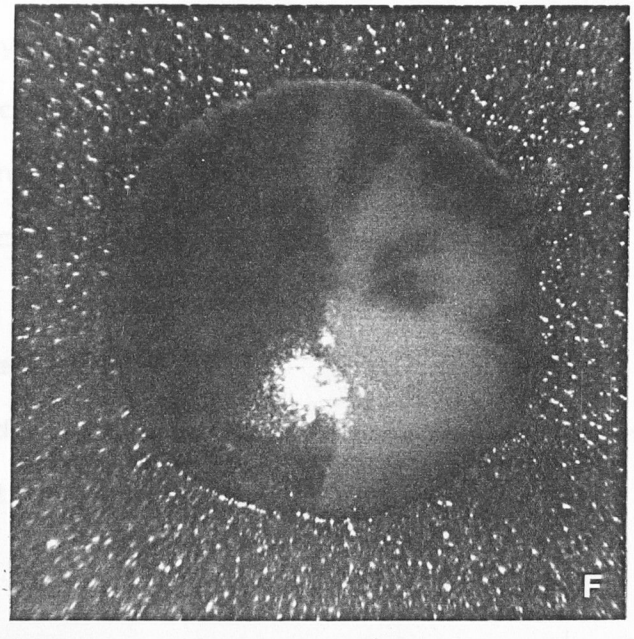

F

TABLE 1. Observed phenotypes of FAS20 and FAS21 transformed with different plasmids.

Strain	Plasmid	No. colonies	No. expts	% Phenotype ± s.d.
FAS20	pBH15A	7776	7	96.2 ± 2.9 red 3.2 ± 2.4 white 0.4 red/white
FAS20	pBHD	3284	5	69.9 ± 4.8 red speck 29.9 ± 4.9 white 0.2 red, red/white
FAS20	pBHI	2312	2	96.5 ± 1.1 red star 3.5 ± 1.1 white
FAS21	pBH15A	1534	3	95.4 ± 1.4 pink 2.2 ± 1.3 white 2.1 ± 1.2 red 0.3 red/white
FAS21	pBHD	5168	5	81.1 ± 9.3 pink 18.8 ± 9.6 white 0.6 pink/white
FAS21	pBHI	1309	2	99.5 ± 0.7 pink 0.8 white, red

Strains FAS20 [cir+] and FAS21 [cir0] were transformed to LEU+ with a plasmid carring only one 2μ plasmid repeat (pBH15A), two direct repeats (pBHD), or two inverted repeats (pBHI). The agar plates were incubated for 5 days at 30C and then refrigerated at 4C for 1 day prior to visual scoring of the phenotypes. In the case of FAS20(pBHD) transformants, a binocular dissecting scope was used to distinguish white colonies from red speck colonies. A slash (/) between phenotypes indicates those colonies that were sectored with the indicated colors. No. colonies is the total number of colonies scored for all experiments with a given strain and plasmid. The phenotypic percentages and sample

standard deviation (s.d.) were calculated by averaging the results of independent experiments (No. expts). Standard deviations are given only for those phenotypes that occured at $\geq$ 1%. See Plate 1 for an illustration of the phenotypes.

TABLE 2. Percentage of cells in a colony containing a selected plasmid.

Strain	Plasmid	Phenotype	% Cells ± s.d. with plasmid	n
FAS21	pBH15A	pink	1.1 ± 0.2	5
FAS21	pBHD	pink	1.3 ± 0.6	4
FAS21	pBHD	white	5.0 ± 4.4	4
FAS20	pBH15A	red	56.0 ± 5.0	5
FAS20	pBHDX	red	50.4 ± 1.8	2
FAS20	pBHDX	white	64.2 ± 5.7	4

Strains FAS20 [cir+] and FAS21 [cir0] were transformed to LEU+ with the indicated plasmid. After 5 days at 30C, colonies having the indicated phenotype were removed from the LMM (leucine minus media) agar plate, suspended in sterile water, and plated onto LMM and COM at appropriate dilutions. To determine the percentage of cells containing the plasmid, the number of colonies that grew on LMM was divided by the number of colonies that grew on COM. The results of n colonies tested were pooled to arrive at the mean percentage and sample standard deviation given in the table.

TABLE 3. FLP- and FRT-independent recombination activity in vivo.

a.

Strain	Plasmid	n	% Colonies with the following phenotype			
			white	red speck	red	pink
FAS20	pBHD	1112	23.5	76.3		
FAS20	pBHDX	714	20.7		79.0	
FAS21	pBHD	1559	13.1			85.8
FAS21	pBHDX	886	19.2			80.0

Table 3a. Strains FAS20 [cir+] and FAS21 [cir0] were transformed to LEU+ with the indicated plasmid. Phenotypes were scored from a total of n colonies as described in Table 1. Only those phenotypes representing >1% of the total n are listed.

b.

FLP	FRT	% Recombination
+	+	99.8
+	-	20.7
-	+	13.1
-	-	19.2

Table 3b. Strains FAS20 and FAS21 are represented as FLP+ and FLP-, respectively. Plasmids pBHD and pBHDX are represented as FRT+ and FRT-, respectively. FRT refers to the cis-acting FLP recognition target sequence. The % recombination values were derived from Table 3a and consider the white and red speck phenotypes as indicative of recombination and the red and pink phenotypes as indicative of no recombination.