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Plasmid construction by homologous recombination in yeast

(*Saccharomyces cerevisiae*; transformation; plasmid recombination; YCp50 derivatives; YEp420 [previously called β 72] derivatives)

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SUMMARY

We describe a convenient method for constructing new plasmids that relies on interchanging parts of plasmids by homologous recombination in *Saccharomyces cerevisiae*. A circular recombinant plasmid of a desired structure is regenerated after transformation of yeast with a linearized plasmid and a DNA restriction fragment containing appropriate homology to serve as a substrate for recombinational repair. The free ends of the input DNA molecules need not be homologous in order for efficient recombination between internal homologous regions to occur. The method is particularly useful for incorporating into or removing from plasmids selectable markers, centromere or replication elements, or particular alleles of a gene of interest. Plasmids constructed in yeast can subsequently be recovered in an *Escherichia coli* host. Using this method, we have constructed an extended series of new yeast centromere, episomal and replicating (YCp, YEp, and YRp) plasmids containing, in various combinations, the selectable yeast markers *LEU2*, *HIS3*, *LYS2*, *URA3* and *TRP1*.

INTRODUCTION

The molecular analysis of gene function in the yeast *S. cerevisiae* has been aided tremendously by the development of methods for the manipulation of

genes using recombinant DNA technology (Botstein and Davis, 1982). During the process of analysing a particular cloned gene, one often finds it necessary to change the plasmid's selectable marker, or to move the cloned gene to a different plasmid; for example, from a low-copy-number centromere plasmid to a multicopy 2- μ m plasmid or the reverse. Also, genetic analyses often require introducing many new alleles of a cloned gene onto a particular plasmid for subsequent studies of function. These objectives are normally attained through the use of in vitro techniques, which can be complex and tedious.

Methods that rely on homologous recombination

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Abbreviations: Ac, acetate; Amp or Ap, ampicillin; bp, base pair(s); kb, kilobases or 1000 bp; $P_{gal1-10}$, promoter region between the yeast *GAL1* and *GAL10* genes; R^r or r^r , resistance; SD, yeast synthetic minimal medium with dextrose; SDS, sodium dodecyl sulfate; Tc or Tet, tetracycline; ::, novel joint.

in yeast following transformation (Hinnen et al., 1978) have been employed in manipulating the chromosomal loci of cloned genes (see, for example, Winston et al., 1983). The power and applicability of these methods has been greatly expanded by the use in transformation of plasmid DNA containing double strand breaks, which are highly reactive for homologous recombination in yeast (Orr-Weaver et al., 1981; 1983). Here we extend these methods to the manipulation of plasmid-borne genes. We describe a fast and reliable method for plasmid construction that is based on the efficient repair of a linearized plasmid by recombination with a homologous DNA restriction fragment during yeast transformation (Kunes et al., 1985). The method is demonstrated by constructing a number of new yeast vectors, using the common pBR322 (Bolivar et al., 1977) backbone of the existing array of yeast vectors (Botstein et al., 1979; Broach, 1983) to provide homologous regions for recombination.

MATERIALS AND METHODS

(a) Strains, plasmids and media

The yeast strains used in this study are all derived from S288C (*MAT α SUC2 gal2*). Strains bearing the following markers were used in transformation with a plasmid containing the corresponding wild-type clone: *ura3-52* (Carlson et al., 1984), *his3- Δ 200* (Struhl, 1985a), *lys2- Δ 120* (Simchen et al., 1985), *leu2-3,2-112* (Botstein et al., 1979) and *trp1- Δ 901* (Hieter et al., 1985). The plasmids YRp7, YEp6 and YEp21 (Botstein et al., 1979), and pSI4 (Broach, 1983) have been described. The yeast centromere plasmid YCp50 (C. Mann, personal communication) contains the 1.75-kb *PvuII-EcoRI* fragment of *CEN4* (Mann and Davis, 1986) and the 0.84-kb *EcoRI-HindIII* fragment of *ARS1* (Tschumper and Carbon, 1980) blunt-end ligated into the *PvuII* site of YIp5 (Botstein et al., 1979), with all junction sites destroyed. The yeast episomal plasmid YEp420, previously called β 72, was constructed by blunt-end ligation into the *PvuII* site of YIp5 the 1.58-kb *HpaI-HindIII* fragment of the yeast 2- μ m circle (form B) (Broach, 1983; Hartley and Donelson, 1980) containing the putative replication origin. The plas-

mid pGM65 (provided by G. Maine, Biotechnica International Inc., Cambridge, MA) contains a 0.85-kb *EcoRI-BamHI* fragment of the *GAL10-GAL1* promoter region (Yocum et al., 1984) inserted between the *EcoRI* and *BamHI* sites of YEp420, with the *GAL1* promoter at the *BamHI* end and the *GAL10* promoter at the *EcoRI* end. The *BamHI* site is from a *BamHI*-linker that was ligated to the *GAL* promoter sequence just upstream from the *GAL1* start codon. Plasmid pPL7 (J. Mullins, personal communication) is a derivative of pBR322 with a 90-bp polylinker fragment inserted between the *EcoRI* and *BamHI* sites. Plasmids pRB315 and pRB328 are derivatives of pPL7 containing the 0.82-kb *EcoRI-PstI* fragment bearing the *TRP1* gene (lacking *ARS1*; Tschumper and Carbon, 1980) and the 1.77-kb *BamHI* fragment with the *HIS3* gene (Struhl, 1985b), respectively, as described in Schatz et al. (1986). Plasmid pRB506 is a derivative of pPL7 with the 5.3-kb *ClaI-XbaI* fragment containing the *LYS2* gene (see map in Barnes and Thorner, 1986) cloned between the *ClaI* and *XbaI* sites. The maps of plasmids YCp50, YEp420, YRp7, pGM65, and pSI4 are shown in Fig. 1, and those of YEp21, YEp6, pPL7, pRB315, pRB328, pRB506 are shown in Fig. 2.

Yeast was grown in SD medium (Sherman et al., 1979) supplemented with 0.01% of all amino acids, uracil, and adenine, except that the appropriate amino acid or base was absent when selection was applied. *E. coli* was grown in LB medium in the presence of 50 μ g Ap/ml or 15 μ g Tc/ml, when appropriate.

(b) DNA manipulations

Yeast DNA was isolated by a modified version of the procedure described by Winston et al. (1983). Yeasts were harvested from 10 ml stationary phase cultures and resuspended in 400 μ l 0.1 M EDTA pH 8.0 in 1.5-ml microfuge tubes. To each tube was added 100 μ l of zymolyase (zymolyase-100; 2 mg/ml; Seikagaku Kogyo Co., Ltd., 2-9 Nihonbashi-honcho, Chuo-ku, Tokyo, 103 Japan), followed by incubation at 37°C for 1–2 h; 90 μ l of 6% SDS 0.7 M Tris base was added to each tube with mixing by inversion. The tubes were then incubated at 65°C for 30 min. After the addition of 80 μ l 5 M KAc and gentle mixing, the tubes were incubated on ice for

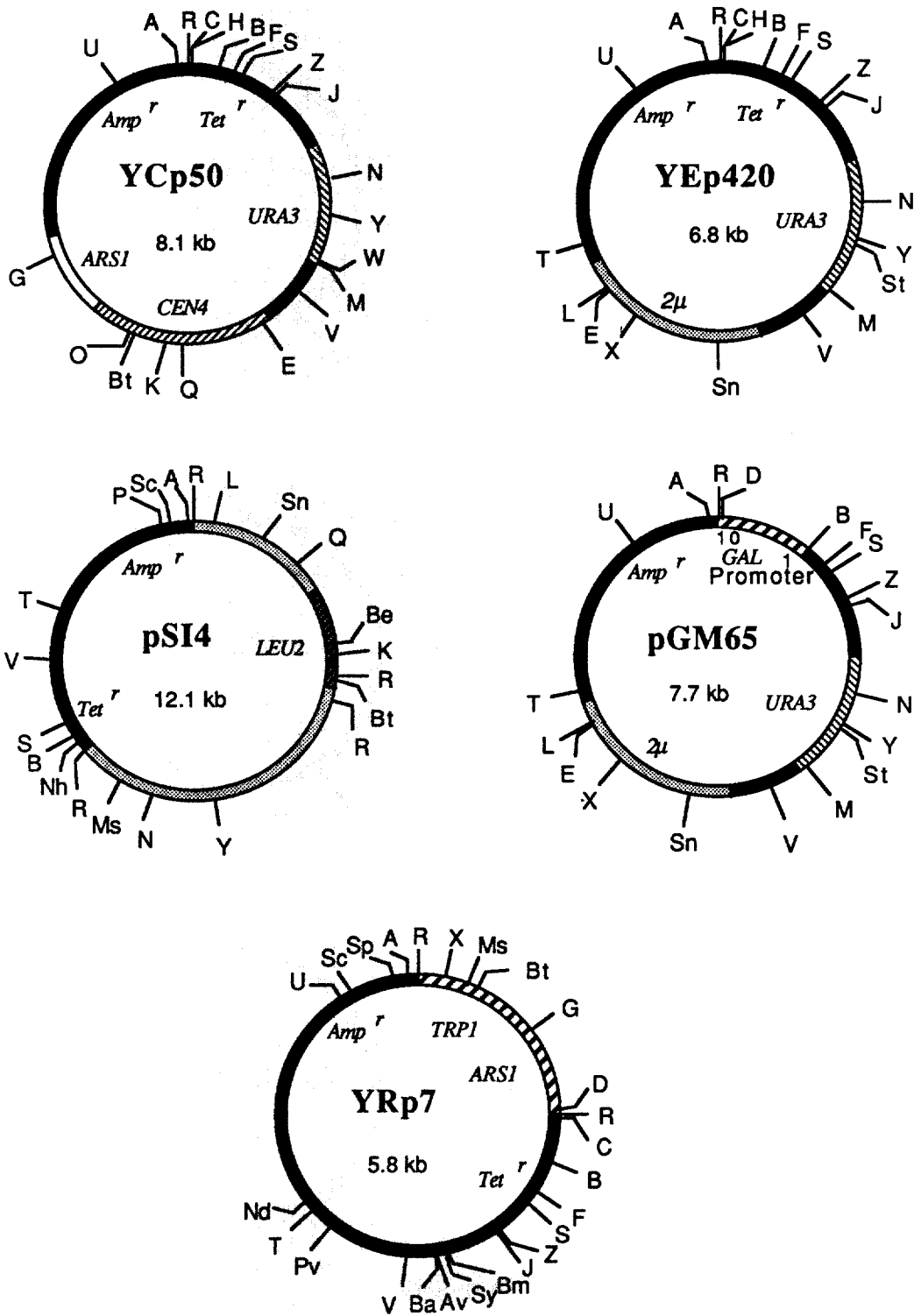


Fig. 1. Restriction maps of the plasmids used to generate linearized plasmids for vector construction by recombination. The restriction site positions are derived from the published sequences of the various components (see MATERIALS AND METHODS, section a). For each plasmid depicted, the lengths of differently shaded regions are proportional to their actual lengths. Only the *EcoRI* and known unique sites are shown. Restriction enzymes: A, *AatI*; Av, *AvaI*; B, *BamHI*; Ba, *BalI*; Be, *BstEI*; Bh, *BssHIII*; Bm, *BsmI*; Bs, *BspMI*; Bt, *BstXI*; C, *ClaI*; D, *DraIII*; E, *SpeI*; F, *SphI*; G, *BglII*; H, *HindIII*; J, *NruI*; K, *KpnI*; L, *BclI*; M, *SmaI*; Ms, *MstII*; N, *NcoI*; Nd, *NdeI*; Nh, *NheI*; O, *XhoI*; P, *PstI*; Pv, *PvuII*; Q, *HpaI*; R, *EcoRI*; S, *SalI*; Sc, *ScaI*; Sn, *SnaBI*; Sp, *SspI*; St, *StuI*; Sy, *StyI*; T, *Thi111I*; U, *PvuI*; V, *BspMII*; W, *AvaIII(NsiI)*; X, *XbaI*; Y, *ApaI*; Z, *XmaIII(EagI)*. The solid black regions are pBR322 sequences, the stippled ones are yeast 2- μ sequences, and other regions are indicated explicitly on the maps.

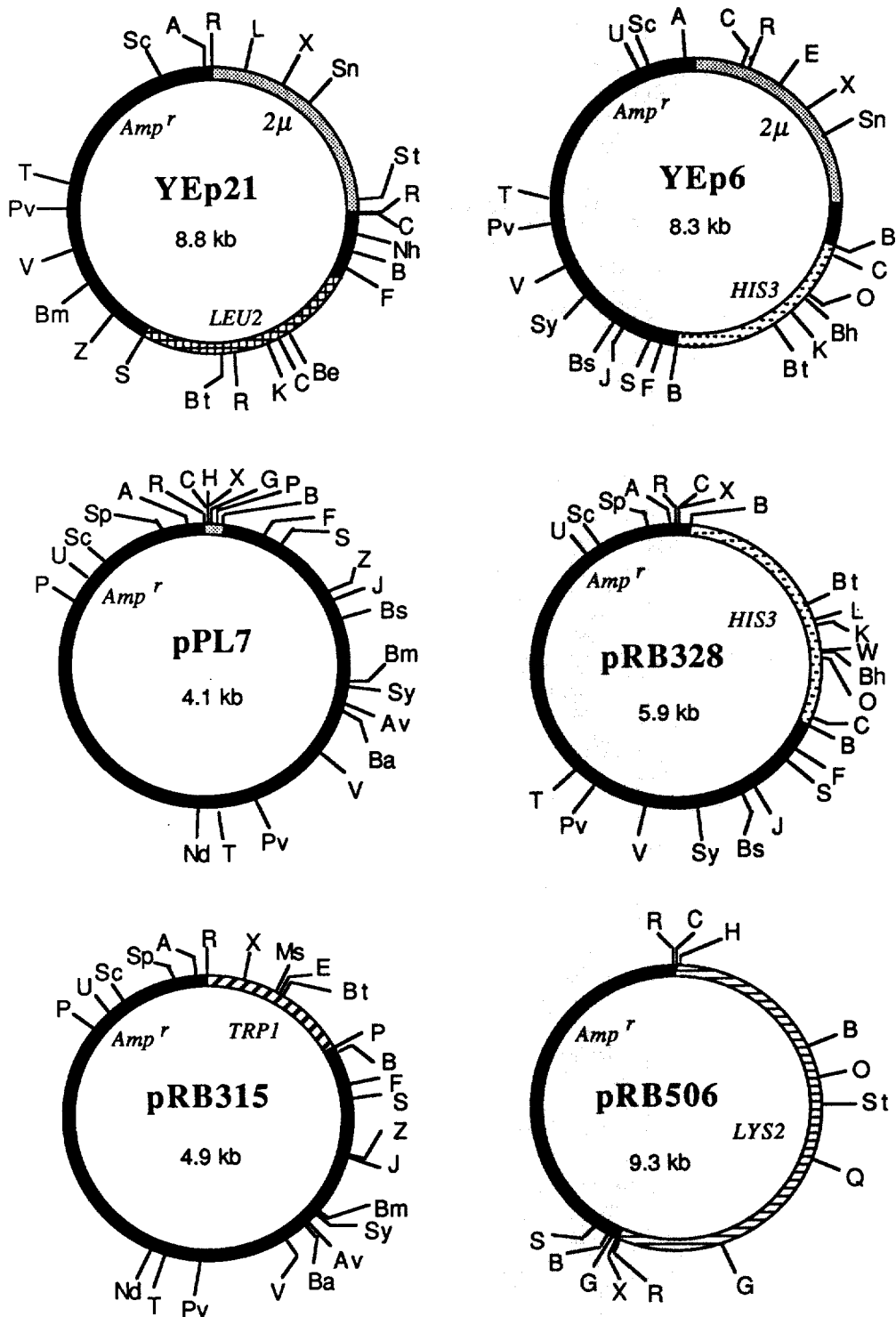


Fig. 2. Restriction maps of the plasmids providing homologous DNA fragments for the recombinational repair of linearized plasmids. Restriction sites are deduced from known sequences when available (see MATERIALS AND METHODS, section a). Within each plasmid, the lengths of differently shaded regions are proportional to their actual lengths. See legend of Fig. 1 for restriction site codes. Only the *Bam*HI, *Cla*I, *Eco*RI and known unique sites are shown. During the construction of YEp6, a portion of pBR322 was deleted, so that the precise position of the joints between 2- μ m and pBR322 DNA are not known (Struhl et al., 1979). Restriction analysis indicates that the *Cla*I and *Hind*III sites of pBR322 were lost, but the *Aat*I and *Eco*RV sites are still present. The information on pRB506 is not complete because the sequence of the *LYS2* fragment is not available. The solid black regions are pBR322 sequences.

1–2 h. The samples were centrifuged in a microfuge for 15 min ($12\,000 \times g$). The resulting supernatants were transferred to new tubes, filled with ethanol and mixed by gentle inversion. The precipitate was recovered by centrifugation in a microfuge for 15 s. The pellets were rinsed with cold ethanol, air-dried, and dissolved in $400 \mu\text{l}$ 10 mM Tris 1.0 mM EDTA pH 8.0 by incubation at room temperature for 1–2 h with gentle agitation. The samples were then centrifuged in the microfuge for 15 min and the supernatants were transferred to new tubes. To recover plasmids, 1% of the DNA solution was used to transform *E. coli* (Davis et al., 1982).

(c) Transformation of yeast

Yeast transformation was performed by a modified version (Kuo and Campbell, 1983) of the LiAc method (Ito et al., 1983). Typically, to obtain a few thousand transformants, a DNA mixture containing 0.05–0.2 μg of a linearized plasmid, 0.5–1.0 μg of a DNA restriction fragment, and 50 μg of sonicated

chicken erythrocyte DNA (Calbiochem-Behring, La Jolla, CA) was added to about 2×10^8 competent yeast cells.

RESULTS AND DISCUSSION

(a) Introduction of new selectable markers to plasmids

The characterization of a cloned yeast gene often calls for the introduction of new selectable markers to the gene-carrying plasmid. For example, many gene clones are isolated from plasmid libraries that bear a particular selectable marker, such as the yeast *URA3* gene, that may not suffice in subsequent studies. The introduction of a new selectable marker can be accomplished by using a DNA fragment containing the new marker to repair by recombination a linearized plasmid bearing the cloned gene. Three basic ways, in which this reaction can be

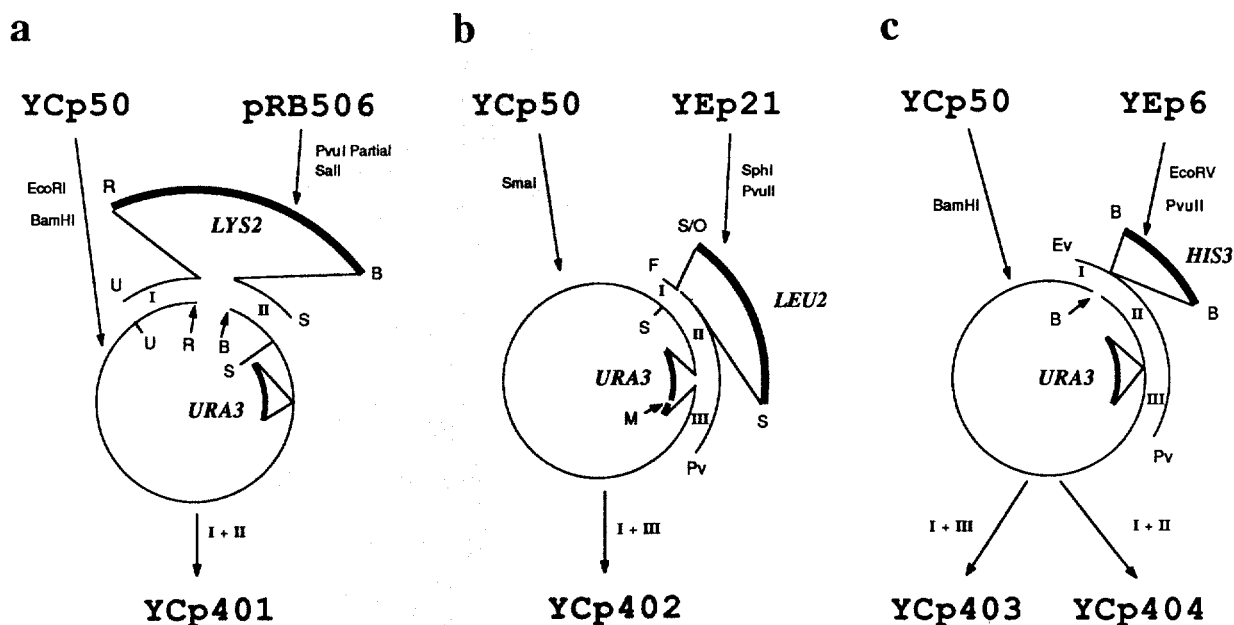


Fig. 3. Introduction of new selectable markers to a plasmid by recombination in yeast. (a) A *lys2* strain was transformed with YCp50 cut with *EcoRI* + *BamHI* and pRB506 [cut with *PvuI* (partial) + *SallI*], applying selection for *Lys*⁺ transformants. Recombination events in intervals I and II generate YCp401. (b) A *leu2* strain was transformed with YCp50 cut with *SmaI* and YEp21 cut with *SphI* + *PvuII*, applying selection for *Leu*⁺ transformants. The formation of a *LEU2* carrying plasmid requires recombination events in intervals I and III. (c) A *his3* strain was transformed with a mixture of YCp50 cut with *BamHI* and YEp6 cut with *EcoRV* + *PvuII*, applying selection for *His*⁺ transformants. Recombination events in homologous intervals I and III produce YCp403, while recombination events in intervals I and II produce YCp404. Restriction enzyme codes: B, *BamHI*; Ev, *EcoRV*; F, *SphI*; M, *SmaI*; O, *XhoI*; Pv, *PvuII*; R, *EcoRI*; S, *SallI*; U, *PvuI*.

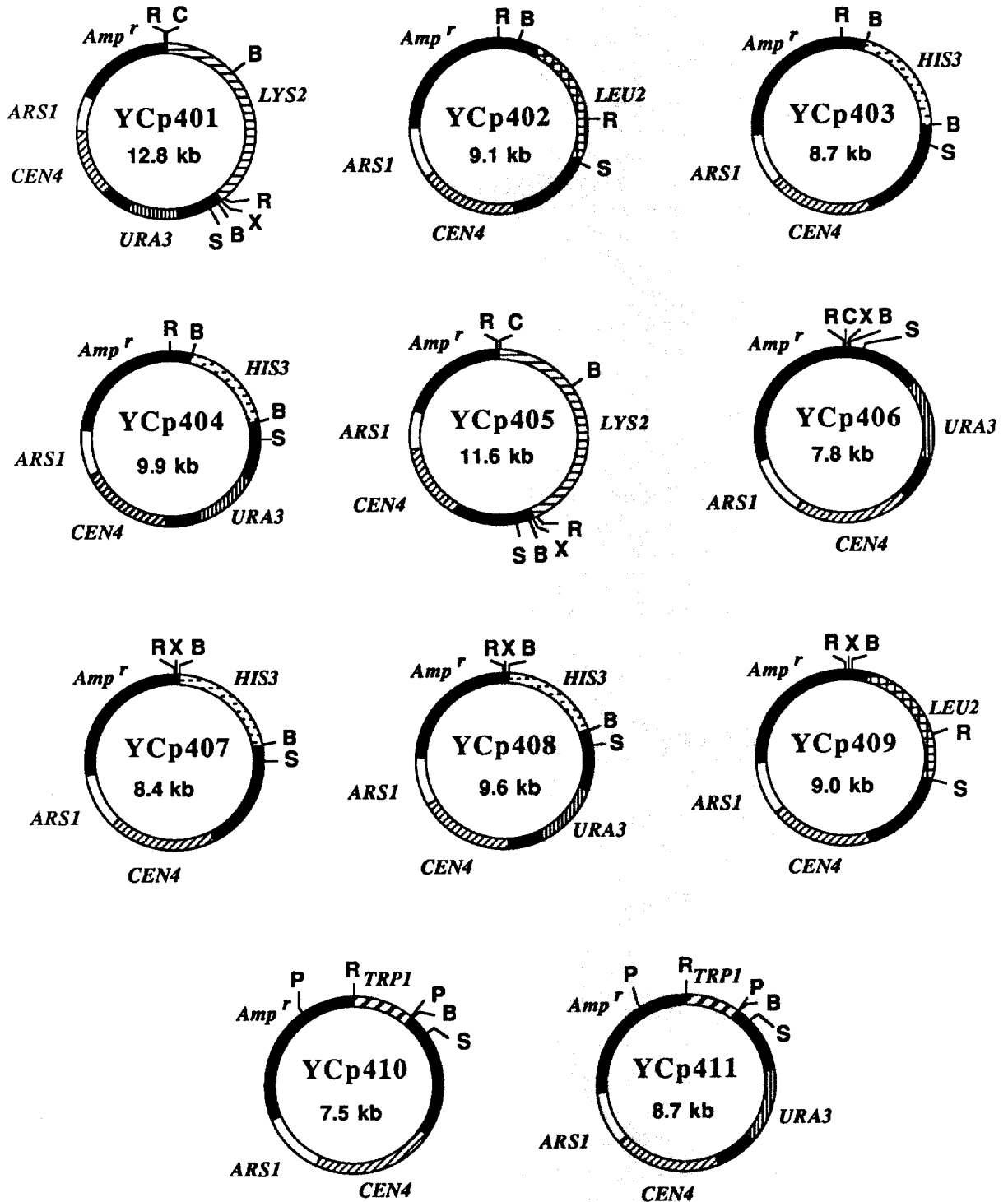


Fig. 4. Restriction maps of YCp50 derivatives constructed by recombination in yeast. For each plasmid depicted, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B, *Bam*HI; C, *Cl*AI; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I. For the positions of other sites, see the map of YCp50 in Fig. 1 and those of plasmids providing the various DNA fragments in Fig. 2. The yeast sequences are indicated and the solid black regions are from pBR322.

employed to introduce new markers to a plasmid, have been developed.

(1) Simple insertion

A DNA fragment that can serve as a substrate for the recombinational repair of a linearized plasmid must span the break of the plasmid, and contain two regions of overlapping homology where recombination can occur to generate a circular product. As shown in Fig. 3a, a marker located on a DNA fragment between these two regions of overlapping homology would necessarily be incorporated into this repair product. In the example shown in Fig. 3a, the yeast *URA3 CEN4 ARS1* vector YCp50 was linearized by *EcoRI* + *BamHI* cleavage and introduced into yeast along with the *LYS2*-containing fragment of a *SalI* + partial *PvuI* digest of pRB506. The *LYS2* insert lies between the two regions of overlapping homology labeled I and II. *Lys*⁺ selection resulted in a high frequency of transformants. Ten of these transformants were tested for growth without uracil and found to be *Ura*⁺, indicating that the recombinant plasmids had retained the original YCp50 marker, *URA3*, as expected. The resulting *LYS2 URA3 CEN4 ARS1* plasmid, subsequently recovered in *E. coli*, was named YCp401 (Fig. 4). It is worth noting that this product presumably could have been recovered by selecting for transformants for the original plasmid marker, *URA3*, instead of *LYS2*, since the repair event necessarily incorporates the latter into the product. The possibility of recovering the recombinant plasmid without selection for the fragment-borne gene is explored in section b, below.

(2) Substitution of a new marker for the original marker

It is often desirable to delete the original selectable marker while simultaneously introducing a new marker. This can be accomplished by using a DNA fragment that contains the new marker to repair a linearized plasmid cut within the original marker. In the example shown in Fig. 3b, YCp50 was linearized at the *SmaI* site in *URA3*, and introduced to a *leu2 ura3* strain along with the *SphI*, *PvuII* fragment of YEp21 that contains a *LEU2* gene insert. All of the resulting *Leu*⁺ transformants examined had lost the *URA3* gene. Plasmids from four of the yeast transformants were recovered in *E. coli* and shown by restriction analysis (not shown) to have the expected struc-

ture, designated YCp402 (Fig. 4). This outcome was as expected, because for a circular product containing the *LEU2* gene to form, recombination must occur in the overlapping intervals I and III (Fig. 3b). Consequently, the *SmaI*-generated *URA3* ends are lost. It is worth noting that the overlapping homology of interval I is only 89 bp long, indicating that a region of homology between the linearized plasmid and the DNA fragment can be very short and still sufficient for homologous recombination. Furthermore, while repair of *SmaI*-linearized YCp50 by recombination with the chromosomal *URA3* locus is a possibility, products that had retained *URA3* were not recovered by the *Leu*⁺ selection. The circular products of recombination with the chromosome presumably do not efficiently recombine with and incorporate the fragment-borne *LEU2* gene.

(3) Insertion or substitution

When introducing a new marker to a plasmid, one can recover products with and without the original plasmid marker if the plasmid is linearized outside of the original marker and the DNA fragment spans both the plasmid's break and the original marker's position. The construction depicted in Fig. 3c illustrates this case. Plasmid YCp50, linearized by *BamHI* cleavage, was introduced to a *his3 ura3* strain along with the YEp6 *EcoRV-PvuII* fragment bearing a *HIS3* gene insert. Repair of the linearized YCp50 requires a crossover within the overlapping homologous interval I, and in either homologous interval II or III (Fig. 3c). A crossover in interval II would retain the original *URA3* marker of YCp50 in the product, while a crossover in interval III would exclude *URA3* from the product. As expected, a high frequency of *His*⁺ transformants resulted, containing either of these two possible plasmid products, designated YCp403 and YCp404 (Fig. 4). Of 100 *His*⁺ transformants tested, 98 were *Ura*⁺. Thus recombination between the linearized plasmid and DNA fragment in interval III was rare, an observation that might bear on the recombination mechanism.

Through the use of the three basic strategies outlined above, we constructed an extended series of plasmid derivatives of YCp50 containing in several combinations the generally useful selectable markers *HIS3*, *LEU2*, *LYS2*, *TRP1* and *URA3*. These plasmids and their derivatives are described in Table I. Their structures are shown in Fig. 4.

TABLE I

New plasmids and their derivations

New plasmid ^a	Linearized plasmid		Homologous fragment		Selection ^f
	Plasmid ^b	Digested with ^c	Plasmid ^d	Digested with ^c	
YCp401	YCp50	<i>EcoRI</i> , <i>BamHI</i>	pRB506	<i>PvuI</i> , <i>SalI</i>	Lys ⁺
YCp402	YCp50	<i>SmaI</i>	YEp21	<i>SphI</i> , <i>PvuII</i>	Leu ⁺
YCp403, YCp404	YCp50	<i>BamHI</i>	YEp6	<i>EcoRV</i> , <i>PvuI</i> , <i>PvuII</i>	His ⁺
YCp405	YCp401	<i>SalI</i>	pBR322	<i>EcoRI</i> , <i>PvuII</i>	Lys ⁺
YCp406	YCp50	<i>EcoRI</i> , <i>BamHI</i>	pPL7	<i>PvuI</i> , <i>SalI</i>	Ura ⁺
YCp407, YCp408	YCp50	<i>EcoRI</i> , <i>BamHI</i>	pRB328	<i>PvuI</i> , <i>PvuII</i>	His ⁺
YCp409	YCp402	<i>EcoRI</i> , <i>BamHI</i>	pPL7	<i>PvuI</i> , <i>SalI</i>	Leu ⁺
YCp410, YCp411	YCp50	<i>EcoRI</i> , <i>SalI</i>	pRB315	<i>PvuI</i> , <i>PvuII</i>	Trp ⁺
YEp421, YEp422	YEp420	<i>BamHI</i>	YEp6	<i>EcoRV</i> , <i>PvuI</i> , <i>PvuII</i>	His ⁺
YEp423, YEp424	YEp420	<i>SalI</i>	YEp21	<i>HindIII</i> , <i>PstI</i> , <i>PvuII</i>	Leu ⁺
YEp425	YEp420	<i>EcoRI</i> , <i>BamHI</i>	pRB506	<i>PvuI</i> , <i>SalI</i>	Lys ⁺
YEp426	YEp425	<i>SalI</i>	pBR322	<i>BamHI</i> , <i>PvuII</i>	Lys ⁺
YEp427, YEp428	YEp420	<i>EcoRI</i> , <i>BamHI</i>	YRp7	<i>PvuI</i> , <i>PvuII</i>	Trp ⁺
YEp429	YEp420	<i>EcoRI</i> , <i>BamHI</i>	pPL7	<i>PvuI</i> , <i>SalI</i>	Ura ⁺
YEp430, YEp431	YEp420	<i>EcoRI</i> , <i>BamHI</i>	pRB328	<i>PvuI</i> , <i>PvuII</i>	His ⁺
YEp432, YEp433	YEp420	<i>EcoRI</i> , <i>BamHI</i>	pRB315	<i>PvuI</i> , <i>PvuII</i>	Trp ⁺
YEp434, YEp435	pGM65	<i>SalI</i>	YEp21	<i>HindIII</i> , <i>PstI</i> , <i>PvuII</i>	Leu ⁺
YEp436	pSI4	<i>BamHI</i> , <i>SalI</i>	YIp5	<i>EcoRI</i> , <i>PvuII</i>	Ura ⁺
YEp437	pSI4	<i>BamHI</i> , <i>SalI</i>	YEp6	<i>EcoRV</i> , <i>PvuI</i> , <i>PvuII</i>	His ⁺
YRp441	YRp7	<i>BamHI</i>	YEp6	<i>EcoRV</i> , <i>PvuI</i> , <i>PvuII</i>	His ⁺
YRp442	YRp7	<i>SalI</i>	YEp21	<i>HindIII</i> , <i>PstI</i> , <i>PvuII</i>	Leu ⁺

^a Each new plasmid was constructed by homologous recombination in yeast between the indicated linearized plasmid and homologous DNA fragments. The restriction maps of these plasmids are shown in Figs. 4, 6 and 7.

^b Plasmids used to generate the linearized plasmid substrates for homologous recombination in yeast. Their restriction maps are shown in Figs. 1, 4 and 6.

^c The restriction enzyme(s) used to generate the linearized plasmid.

^d Plasmids that provided the homologous DNA fragments for recombination with the linearized plasmid. Their restriction maps are shown in Figs. 1 and 2, except for pBR322.

^e The restriction enzymes used to generate the homologous DNA fragments.

^f Selection for transformants was applied on SD medium lacking the corresponding nutrient.

(Footnotes for Table II, p. 209)

^a The new yeast vectors constructed in this study. Their restriction maps are shown in Figs. 4, 6 and 7. Table I shows their derivations.

^b The selectable yeast markers residing on the indicated plasmid. In addition, the presence of the *GAL1-GAL10* promoter region is also indicated when applicable.

^c The known unique restriction sites of the vectors. The underlined sites are not in the coding or promoter regions of yeast genes, not in the *CEN4*, *ARS1* and 2- μ m sequences, nor in the Amp^R gene. They may be used for cloning purposes. The information is compiled from the maps of pBR322 and YIp5 and the reported sequences of *URA3* (Rose et al., 1984), *HIS3* (Struhl, 1985b), *LEU2* (Andreadis et al., 1982; 1984), *CEN4* (Mann and Davis, 1986), *TRP1* *ARS1* (Tschumper and Carbon, 1980), the *GAL1-GAL10* promoter (Yocum et al., 1984) and the 2- μ m circle (Hartley and Donelson, 1980). The *LYS2* restriction map was provided by Dr. G. Simchen. A restriction map of the *LYS2* locus is also available in Barnes and Thorner, 1986.

^d The *HIS3* gene has an additional *ClaI* site that overlaps a *dam* methylation site. Therefore, this site will not be cut if the plasmid DNA is prepared from a *dam*⁺ *E. coli* host.

TABLE II

New plasmids and their features

Plasmid ^a	Yeast genes ^b	Unique restriction sites ^c
YCp401	LYS2 URA3	<i>Clal</i> , <i>HindIII</i> , <i>SalI</i> , <i>SmaI</i> , <i>SphI</i> , <i>XbaI</i>
YCp402	LEU2	<i>AatII</i> , <i>BamHI</i> , <i>BglII</i> , <i>BspMII</i> , <i>BstEII</i> , <i>DraIII</i> , <i>HindIII</i> , <i>NdeI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XhoI</i> , <i>XmaIII(EagI)</i>
YCp403	HIS3	<i>AatII</i> , <i>BspMII</i> , <i>BssHII</i> , <i>Clal</i> ^d , <i>DraIII</i> , <i>EcoRI</i> , <i>EcoRV</i> , <i>HpaI</i> , <i>NsiI(AvaIII)</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SpeI</i> , <i>SphI</i>
YCp404	HIS3 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>HpaI</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SpeI</i> , <i>SphI</i>
YCp405	LYS2	<i>Clal</i> , <i>HindIII</i> , <i>SalI</i> , <i>SphI</i> , <i>XbaI</i>
YCp406	URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BspMII</i> , <i>BstXI</i> , <i>Clal</i> , <i>EcoRI</i> , <i>EcoRV</i> , <i>HindIII</i> , <i>HpaI</i> , <i>KpnI</i> , <i>NcoI</i> , <i>NruI</i> , <i>NsiI(AvaIII)</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XhoI</i> , <i>XmaIII(EagI)</i>
YCp407	HIS3	<i>AatII</i> , <i>BspMII</i> , <i>BssHII</i> , <i>Clal</i> ^d , <i>DraIII</i> , <i>EcoRI</i> , <i>HpaI</i> , <i>NsiI(AvaIII)</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i>
YCp408	HIS3 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>EcoRV</i> , <i>HpaI</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i>
YCp409	LEU2	<i>AatII</i> , <i>BamHI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>DraIII</i> , <i>EcoRV</i> , <i>HindIII</i> , <i>NdeI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XhoI</i> , <i>XmaIII(EagI)</i>
YCp410	TRP1	<i>AatII</i> , <i>BamHI</i> , <i>BglII</i> , <i>BspMII</i> , <i>DraIII</i> , <i>EcoRI</i> , <i>EcoRV</i> , <i>HindIII</i> , <i>HpaI</i> , <i>KpnI</i> , <i>MstII</i> , <i>NdeI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XhoI</i> , <i>XmaIII(EagI)</i>
YCp411	TRP1 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BglII</i> , <i>BspMII</i> , <i>EcoRI</i> , <i>HindIII</i> , <i>HpaI</i> , <i>KpnI</i> , <i>MstII</i> , <i>NcoI</i> , <i>NruI</i> , <i>NsiI(AvaIII)</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XhoI</i> , <i>XmaIII(EagI)</i>
YEp421	HIS3	<i>AatII</i> , <i>BspMI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>BstXI</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>EcoRV</i> , <i>KpnI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>ScaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>Tth111I</i> , <i>XbaI</i> , <i>XhoI</i>
YEp422	HIS3 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>BstXI</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>KpnI</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>Tth111I</i> , <i>XbaI</i> , <i>XhoI</i>
YEp423	LEU2	<i>AatII</i> , <i>BamHI</i> , <i>BclI</i> , <i>BsmI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>BstXI</i> , <i>HpaI</i> , <i>HindIII</i> , <i>KpnI</i> , <i>NheI</i> , <i>NruI</i> , <i>PstI</i> , <i>PvuI</i> , <i>ScaI</i> , <i>SalI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XmaIII(EagI)</i>
YEp424	LEU2 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BclI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>BstXI</i> , <i>HpaI</i> , <i>HindIII</i> , <i>KpnI</i> , <i>NcoI</i> , <i>NheI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>XbaI</i> , <i>XmaIII(EagI)</i>
YEp425	LYS2 URA3	<i>Clal</i> , <i>HindIII</i> , <i>HpaI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SphI</i> , <i>XhoI</i>
YEp426	LYS2	<i>Clal</i> , <i>HindIII</i> , <i>HpaI</i> , <i>SalI</i> , <i>SphI</i> , <i>StuI</i> , <i>XhoI</i>
YEp427	TRP1	<i>AatII</i> , <i>BamHI</i> , <i>BclI</i> , <i>BglII</i> , <i>BsmI</i> , <i>BspMII</i> , <i>BstXI</i> , <i>Clal</i> , <i>DraIII</i> , <i>MstII</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>ScaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>StyI</i> , <i>Tth111I</i> , <i>XmaIII(EagI)</i>
YEp428	TRP1 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BclI</i> , <i>BglII</i> , <i>BspMII</i> , <i>BstXI</i> , <i>Clal</i> , <i>MstII</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>Tth111I</i> , <i>XmaIII(EagI)</i>
YEp429	URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BclI</i> , <i>BglII</i> , <i>BspMII</i> , <i>Clal</i> , <i>EcoRI</i> , <i>EcoRV</i> , <i>HindIII</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>Tth111I</i> , <i>XmaIII(EagI)</i>
YEp430	HIS3	<i>AatII</i> , <i>BspMI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>BstXI</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>KpnI</i> , <i>NheI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>ScaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StyI</i> , <i>Tth111I</i> , <i>XhoI</i>
YEp431	HIS3 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>BstXI</i> , <i>Clal</i> ^d , <i>EcoRI</i> , <i>EcoRV</i> , <i>KpnI</i> , <i>NcoI</i> , <i>NheI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>Tth111I</i> , <i>XhoI</i>
YEp432	TRP1	<i>AatII</i> , <i>BamHI</i> , <i>BclI</i> , <i>BsmI</i> , <i>BspMII</i> , <i>BstXI</i> , <i>EcoRI</i> , <i>EcoRV</i> , <i>HindIII</i> , <i>MstII</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>ScaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StyI</i> , <i>Tth111I</i> , <i>XmaIII(EagI)</i>
YEp433	TRP1 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BclI</i> , <i>BspMII</i> , <i>BstXI</i> , <i>EcoRI</i> , <i>HindIII</i> , <i>MstII</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>Tth111I</i> , <i>XmaIII(EagI)</i>
YEp434	LEU2	<i>AatII</i> , <i>BamHI</i> , <i>BclI</i> , <i>BsmI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>BstXI</i> , <i>Clal</i> , <i>DraIII</i> , <i>EcoRV</i> , <i>HpaI</i> , <i>KpnI</i> , <i>NruI</i> , <i>PstI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>XbaI</i> , <i>XmaIII(EagI)</i>
YEp435	LEU2 URA3	<i>AatII</i> , <i>ApaI</i> , <i>BamHI</i> , <i>BclI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>BstXI</i> , <i>Clal</i> , <i>HpaI</i> , <i>KpnI</i> , <i>NcoI</i> , <i>NruI</i> , <i>PvuI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i> , <i>SpeI</i> , <i>SphI</i> , <i>StuI</i> , <i>XbaI</i> , <i>XmaIII(EagI)</i>
YEp436	LEU2-d URA3	<i>AatII</i> , <i>BamHI</i> , <i>BclI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>BstXI</i> , <i>KpnI</i> , <i>MstII</i> , <i>NheI</i> , <i>SalI</i> , <i>SmaI</i> , <i>SnaBI</i>
YEp437	LEU2-d HIS3	<i>AatII</i> , <i>ApaI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>BstEII</i> , <i>MstII</i> , <i>NcoI</i> , <i>SalI</i> , <i>ScaI</i> , <i>SnaBI</i>
YRp441	TRP1 HIS3	<i>AatII</i> , <i>BclI</i> , <i>BspMII</i> , <i>BssHII</i> , <i>Clal</i> ^d , <i>DraIII</i> , <i>KpnI</i> , <i>MstII</i> , <i>NruI</i> , <i>NsiI(AvaIII)</i> , <i>PvuI</i> , <i>PvuII</i> , <i>SalI</i> , <i>ScaI</i> , <i>SphI</i> , <i>SspI</i> , <i>StuI</i> , <i>StyI</i> , <i>Tth111I</i> , <i>XbaI</i> , <i>XhoI</i>
YRp442	TRP1 LEU2	<i>AatII</i> , <i>BalI</i> , <i>BamHI</i> , <i>BglII</i> , <i>BsmI</i> , <i>BspMII</i> , <i>BstEII</i> , <i>DraIII</i> , <i>HpaI</i> , <i>KpnI</i> , <i>MstII</i> , <i>NdeI</i> , <i>NruI</i> , <i>PvuI</i> , <i>PvuII</i> , <i>SalI</i> , <i>ScaI</i> , <i>SphI</i> , <i>StuI</i> , <i>XbaI</i> , <i>XmaIII(EagI)</i>

In a similar manner, we constructed derivatives of several yeast episomal plasmids (YEp; autonomous due to the presence of a portion of the yeast plasmid 2- μ m circle) and the *TRP1 ARS1* containing the yeast-replicating plasmid YRp7 (Botstein et al., 1979; Fig. 1). These new plasmids and their derivations are listed in Table I, and their structures are shown in Figs. 6 and 7. In Table II, we have listed the yeast markers of the new plasmids, and the known unique restriction sites of each plasmid.

(b) Transfer of a cloned gene to a different plasmid

It is often desirable to move a gene from one kind of plasmid to another. A case that frequently arises is the transfer of a cloned gene onto a 2- μ m plasmid to achieve overexpression of the gene by virtue of the plasmid's high copy number. If the gene to be transferred is amenable to direct selection in yeast transformation, then the transfer can be done as described in RESULTS AND DISCUSSION, section a. This is, however, often not the case. We show here that a cloned gene on a DNA fragment can be incorporated into a plasmid by selecting the linearized plasmid's marker.

This case was demonstrated in an experiment in which *HIS3* was chosen as the fragment-borne gene

to be transferred without selection. As shown in Fig. 5, a DNA fragment containing the *HIS3* gene was used to repair the 2- μ m plasmid, YEp420, linearized by cleavage with *EcoRI*, *BamHI*, or *SalI*. With *EcoRI* or *BamHI* cleavage, the *HIS3* insert on the fragment spans the break in the linearized plasmid, and thus recombinational repair must incorporate the *HIS3* gene. On the other hand, the *SalI* site of YEp420 corresponds to a position outside of the *HIS3* insert of the fragment. As a result, recombination with the *HIS3* fragment could generate products containing or lacking the *HIS3* insert via recombination in interval I or interval II, respectively (Fig. 5).

A *ura3 his3* yeast strain was transformed with each linearized plasmid in the presence of the *HIS3*-containing fragment, with selection for the *URA3* marker of the linearized plasmid. With YEp420 linearized by either *EcoRI* or *BamHI* cleavage, nearly all (>96%) of the *Ura*⁺ transformants were *His*⁺ and contained a recombinant plasmid that had incorporated the fragment-borne *HIS3* gene. With *SalI*-linearized YEp420, the fraction of the *Ura*⁺ transformants that were *His*⁻ was several-fold larger. The increase in the *His*⁻ fraction in this case is consistent with a small portion of the repair products having enjoyed recombination in interval II

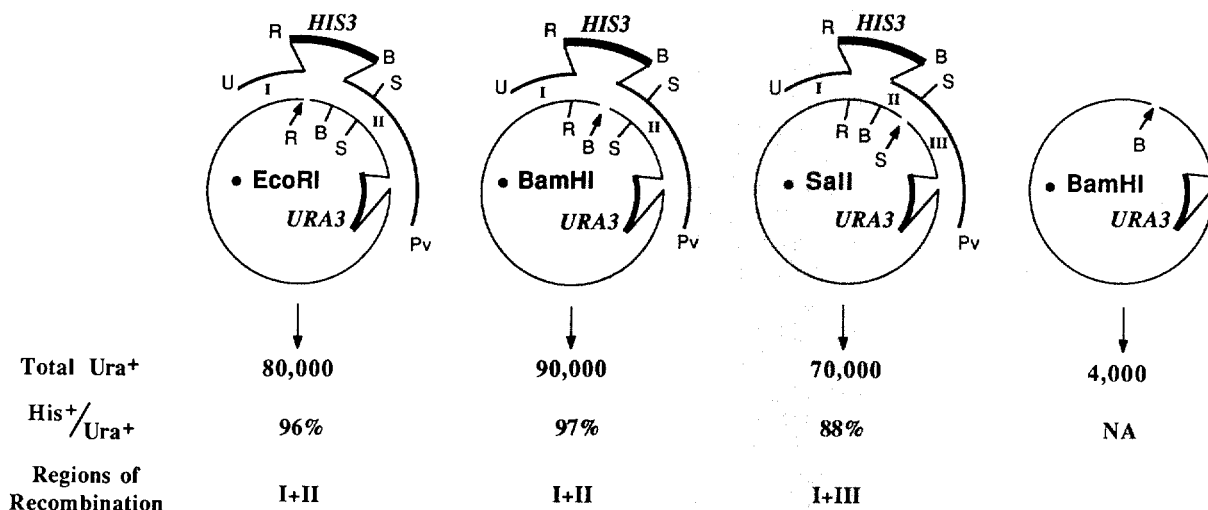


Fig. 5. Transfer of *HIS3* to a new plasmid. A *ura3 his3* strain was transformed using YEp420 (see Fig. 1 for structure) linearized at either the *EcoRI*, or *BamHI* or *SalI* site with or without (only the *BamHI* cut) the *PvuI-PvuII* fragment containing the *HIS3* gene from pRB328 (see Fig. 2 for structure). *Ura*⁺ transformants were selected on SD medium containing histidine. Total *Ura*⁺ indicates number of transformants per μ g of plasmid DNA. The *His* phenotype was scored by replica-plating more than 500 transformants from each transformation to SD medium lacking histidine. Restriction enzyme codes: B, *BamHI*; Pv, *PvuII*; R, *EcoRI*; S, *SalI*; U, *PvuI*. NA, not applicable.

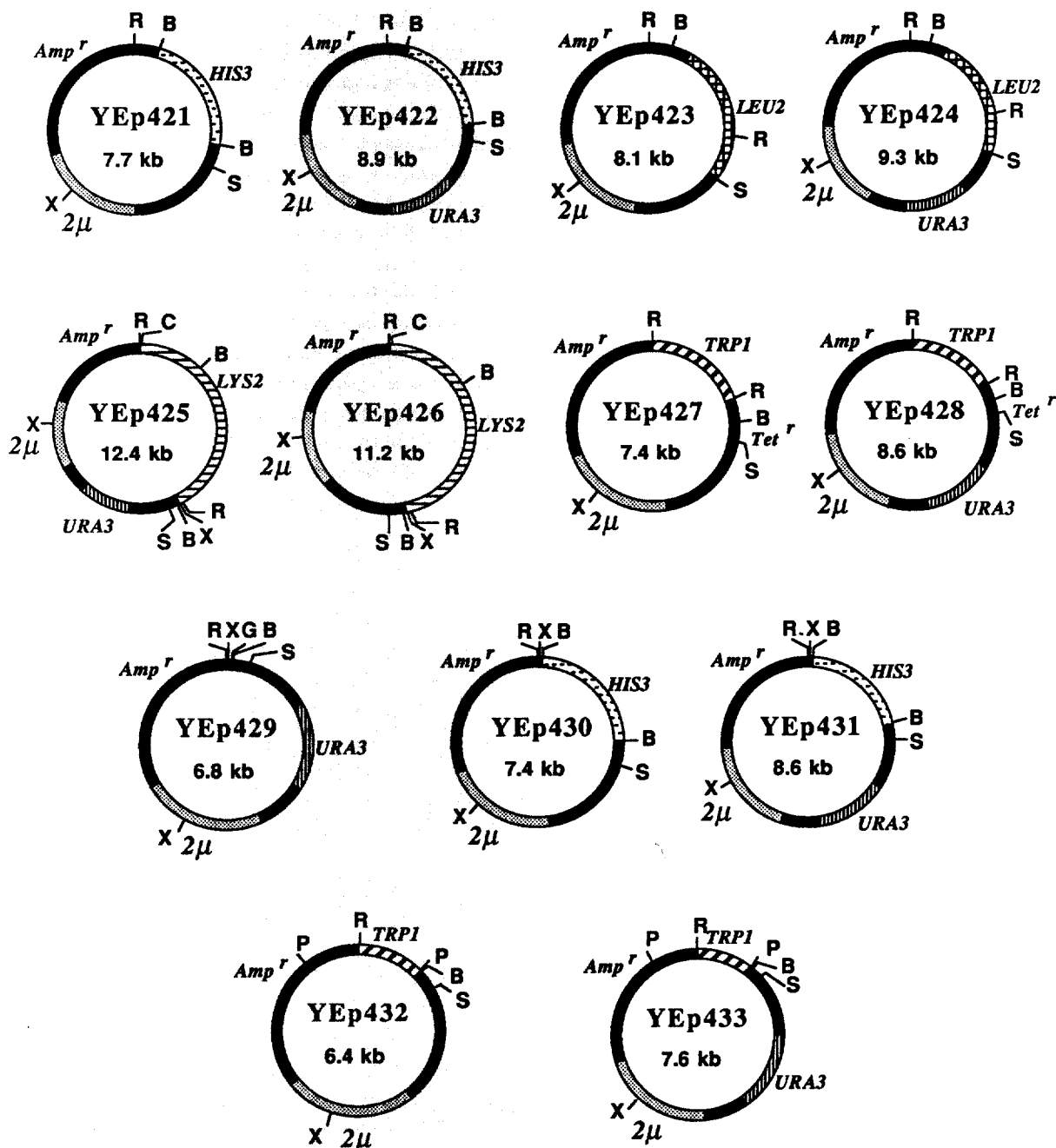


Fig. 6. Restriction maps of YEp420 derivatives with selectable markers useful for cloning in different auxotrophic backgrounds. For each plasmid depicted, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B, *Bam*HI; C, *Cla*I; G, *Bgl*II; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I. See map of YEp420 in Fig. 1 and maps of various plasmids providing fragments in Figs. 1 and 2 for positions of other sites. The yeast sequences are indicated and the solid black regions are from pBR322.

(Fig. 5). The plasmids present in a total of 20 *His*⁻ *Ura*⁺ transformants from these three transformation experiments were recovered in *E. coli* and subjected to restriction analysis. These plasmids were indistinguishable from the original plasmid,

YEp420, with the original site of plasmid cleavage intact. The recovery of transformants containing these plasmids may be due to circular molecules contaminating the preparation of linearized plasmid DNA. Alternatively, these plasmids may be the pro-

ducts of recircularization of a linearized plasmid molecule by ligation after transformation, which has been observed previously (Orr-Weaver and Szostak, 1983; Suzuki et al., 1983).

We thus conclude that nearly all of the transformants recovered with selection for the marker of the linearized plasmid contain plasmids repaired by recombination with the DNA fragment. Consistent with the prominence of recombinational repair is the result of transforming with the linearized YEp420 plasmid alone. In this case, the yield (4000 per μg of DNA) of Ura^+ transformants is about 5% of the yield (80000 per μg of plasmid DNA) obtained in the presence of the *HIS3*-containing fragment. The observation that about 4% of the Ura^+ transformants obtained in the presence of the fragment were His^- is consistent with these relative yields. The

background of nonrecombinant plasmids is consistently low provided that a sufficient amount (10–20-fold in excess of the quantity of linearized plasmid DNA) of the homologous DNA fragment is present during the transformation.

The yield of approx. 1000 Ura^+ transformants with closed circular YEp420 (not shown in the figure) is several-fold less than the yield with the *Bam*HI-linearized plasmid alone, and about 80-fold less than the yield with the linearized plasmid in the presence of the *HIS3*-containing fragment. This difference in the transformation yields of closed circular DNA and linearized plasmid DNA is generally observed and may reflect a difference in the ability of yeast made competent by the LiAc method (Ito et al., 1983) to incorporate linear molecules and circular molecules. With yeast made competent by the

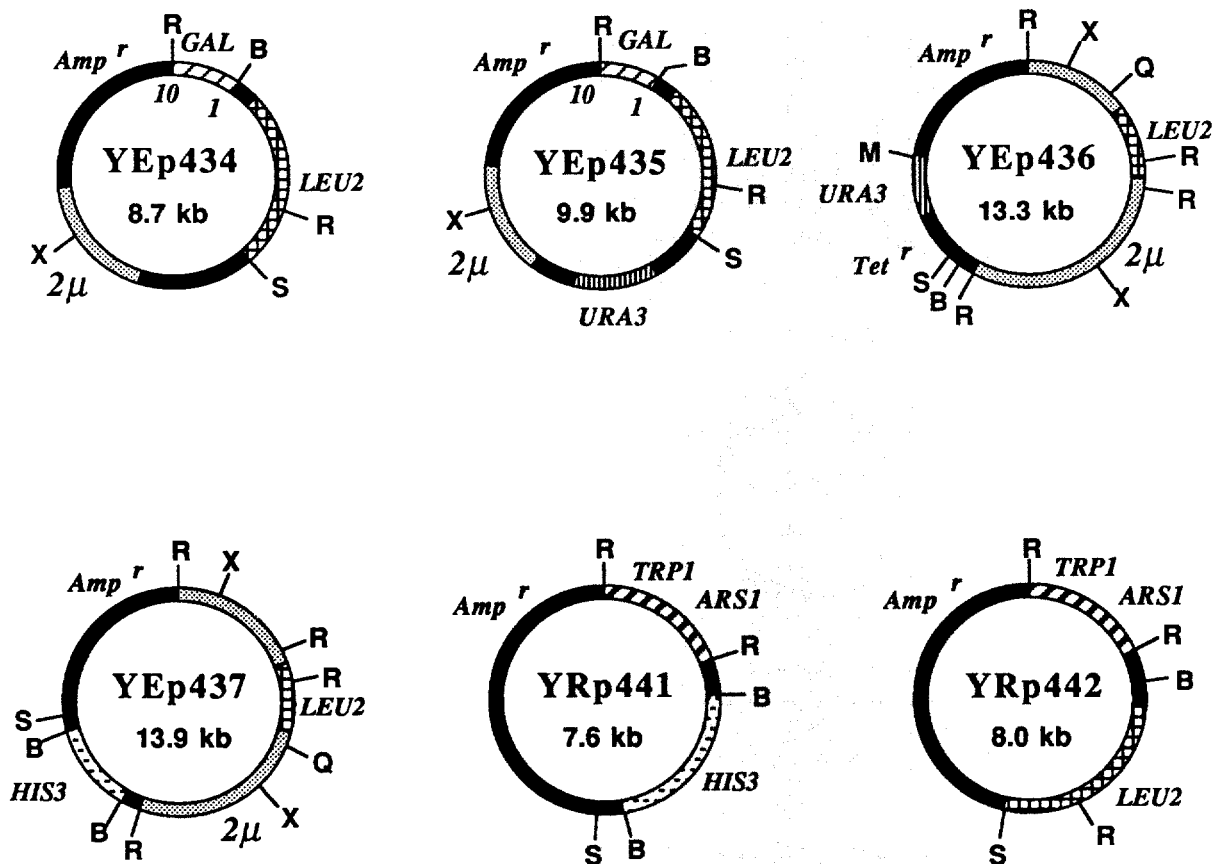


Fig. 7. Restriction maps of new vectors derived from pGM65 (for fusions to *GAL1* or *GAL10* promoters), pSI4 (high copy), and YRp7 (*ARS* plasmid). Within each plasmid, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B, *Bam*HI; Q, *Hpa*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I. See maps of parental plasmids in Figs. 1 and 2 for the positions of other sites. The solid black regions are from pBR322, and the stippled regions are from the yeast 2- μm circle plasmid.

spheroplasting method (Hinnen et al., 1978; Beggs, 1978), approximately equal transformation yields can be obtained with a closed circular plasmid DNA and a linearized plasmid in the presence of a homologous DNA fragment (Kunes et al., 1985).

(c) Introduction of new alleles to a plasmid-borne gene

Through the use of *in vitro* mutagenesis, one can obtain a large number of new alleles of a cloned gene that may then require transfer onto a plasmid useful for their characterization in yeast. This can be accomplished by using a mutant DNA fragment to repair the new plasmid in linearized form. If the gene of interest is absent from the plasmid, the transfer reaction can be carried out as described in RESULTS AND DISCUSSION, section b, by selecting for a marker on the linearized plasmid. If, on the other hand, a copy of the gene is already on the plasmid, one can introduce the new alleles by using a mutant-bearing fragment to repair a gap generated by cleaving twice within the plasmid copy. The gap removes the pre-existing allele while leaving flanking homology to serve as regions for recombination with the fragment. Such gap repair has been used to recover chromosomal mutations onto a plasmid (Orr-Weaver et al., 1983). We have found that a co-introduced DNA fragment also serves as an efficient substrate for the repair of a gapped plasmid (our unpublished observations). A specific case of introducing a new allele in which a disruption allele of the *HXK2* gene, *hxx2::URA3*, was replaced with the wild-type *HXK2* gene is shown in Fig. 8. Selection for the *HIS3* marker of the linearized plasmid allowed us to obtain the desired replacement using a fragment of *HXK2* DNA to repair the plasmid linearized by a cut at the junction between the *URA3* and *hxx2* material. It should be pointed out that it is desirable to perform this manipulation in a background where the chromosomal locus has been deleted, so that recombination with the chromosome cannot occur.

(d) Formation of recombinant plasmids using substrates bearing nonhomologous free DNA ends

In some cases it may be necessary to attempt recombination between a linearized plasmid and a

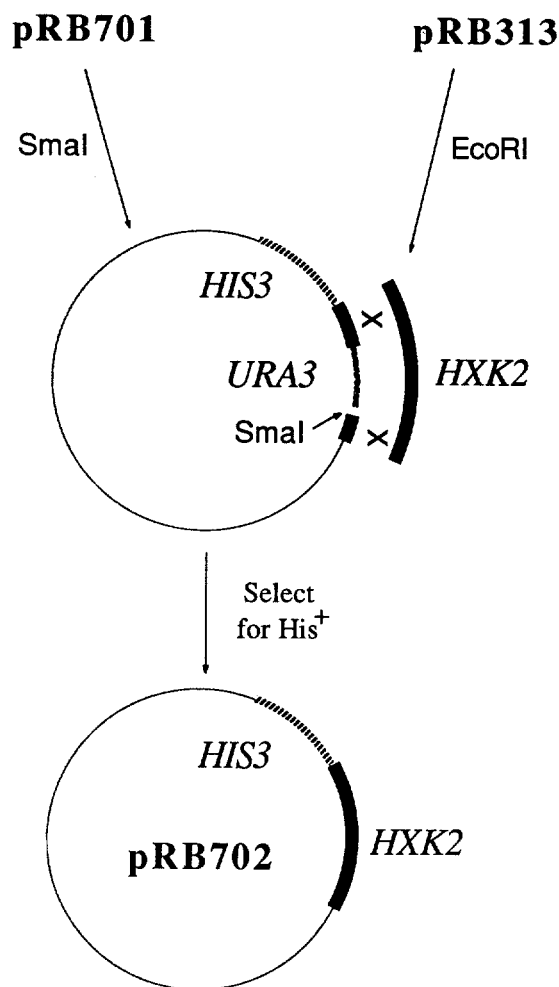


Fig. 8. Allele replacement of *HXK2* on a plasmid. A *his3 ura3 hxx1 hxx2* strain was transformed with the 3.7-kb *EcoRI* fragment of *HXK2* and pRB701 (a derivative of YEp437, see Fig. 6, with an *EcoRI* fragment of *hxx2::URA3* between *HIS3* and pBR322 at the *Bam*HI site near the *SalI* site) linearized at the unique *SalI* site at the junction of *URA3* and *hxx2* sequences. His⁺ transformants were selected on SD medium containing uracil. Transformants were transferred to SD medium without uracil and to YEP fructose medium. The Ura⁻ Hxx⁺ transformants contained the expected recombinant plasmid. Restriction analysis of plasmids recovered from yeast transformants indicates the products have the expected structure.

DNA fragment bearing nonhomologous segments at their ends, so that the required recombination events must occur within internal homologous regions. This situation would arise if appropriate restriction sites are not available to generate ends within the regions of homology between the two substrates. Another case, described in RESULTS AND DISCUSSION,

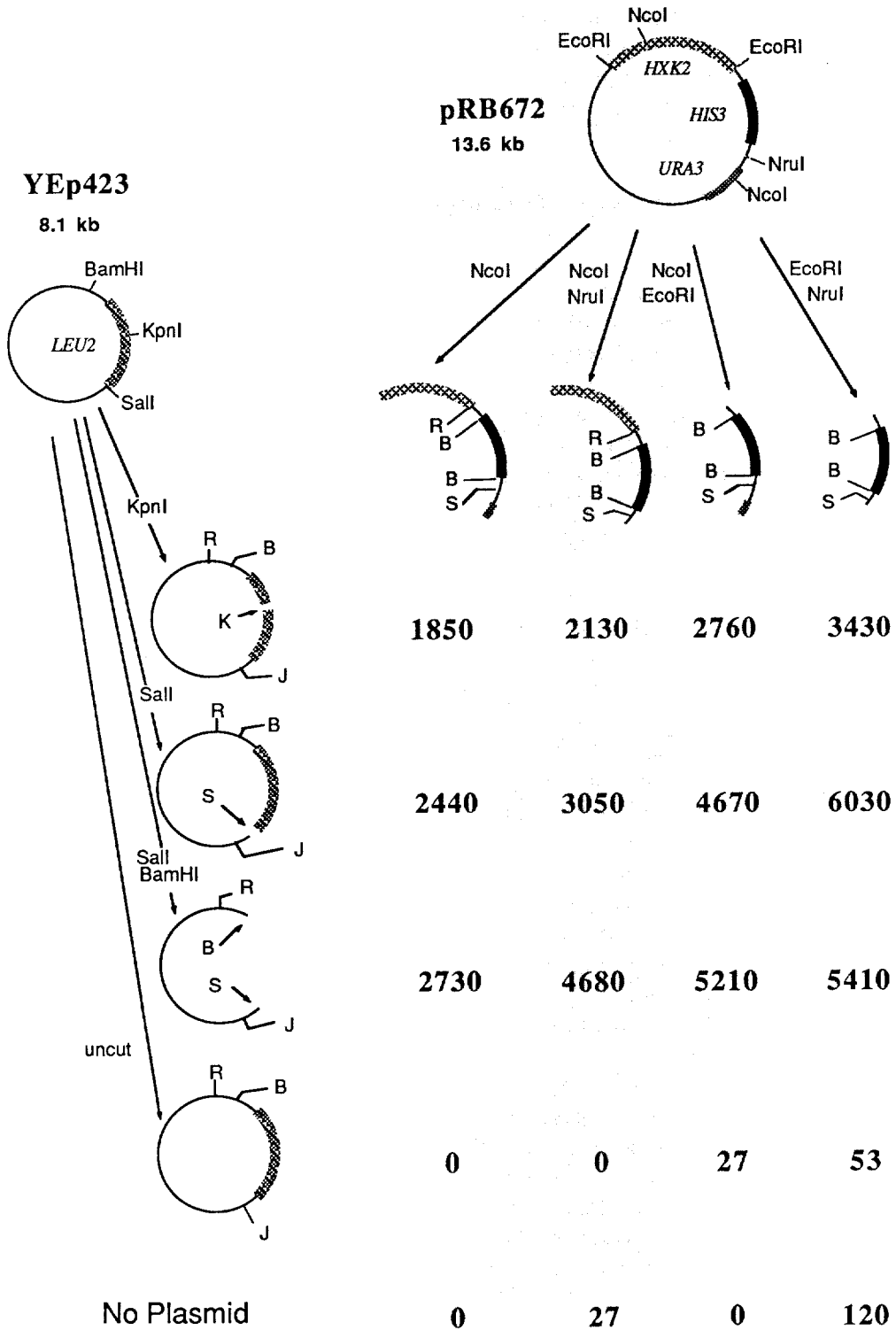


Fig. 9. Effect of nonhomologous free DNA ends on the frequency of recombinational repair of a linearized plasmid. A *his3* strain was transformed with various combinations of a linearized plasmid and a DNA fragment as indicated in the figure, applying selection for His⁺ transformants on SD medium lacking histidine. The thin-line regions on the plasmid and fragment are homologous pBR322 sequences, and the various broad-line regions are nonhomologous yeast sequences. The His⁺ frequency shown is normalized to the yield for 1 μg of plasmid DNA. Restriction enzyme codes: B, *Bam*HI; J, *Nru*I; K, *Kpn*I; R, *Eco*RI; S, *Sal*I.

section a2, arises when a plasmid is linearized within its original marker so as to facilitate its removal. In such circumstances, the nonhomologous ends might be those of the DNA fragment, or of the linearized plasmid, or possibly of both substrates. Fig. 9 illustrates an experiment designed to determine the effect of the presence of such nonhomologous ends on recombination. Plasmid YEp423 was cut at either the *KpnI* site, or the *SalI* site, or both the *SalI* and *BamHI* sites to produce, respectively, a linearized plasmid with neither end, one end or both ends homologous to a *HIS3*-containing DNA fragment derived from pRB672. Similarly, *HIS3*-containing DNA fragments were generated from pRB672 by digesting with *NcoI* (neither end homologous), or *NcoI* + *NruI* (one end homologous), or *NcoI* + *EcoRI* (one end homologous), or *EcoRI* + *NruI* (homologous ends). The substrate fragments were purified away from other plasmid fragments by electrophoresis in an agarose gel and recovered by electro-elution. As shown in Fig. 9, even with all four ends of the linearized plasmid and DNA fragment nonhomologous, the frequency of His⁺ transformants was reduced only modestly, about three-fold, relative to the case where all four ends were homologous. The remaining cases, where either one or two nonhomologous ends were present, resulted in intermediate His⁺ frequencies. These results indicate that the presence of nonhomologous DNA ends, in the size range of 0.43 to 2.3 kb, has only a modest effect on homologous recombination.

(e) Conclusions

The methods described here provide a new in vivo approach to introducing new selectable markers to plasmids, transferring nonselectable genes between different kinds of plasmids, and replacing the resident allele of a plasmid-borne gene with new ones. This method should prove a useful complement to the existing in vitro methods of plasmid construction.

With direct selection for a gene residing on the DNA fragment used in the repair of a linearized plasmid, the yeast transformants recovered may contain different products, depending on the site at which the plasmid is linearized. If the cut is outside of the plasmid's marker and the DNA fragment spans the marker's position, products that retain or

lack the original marker will both be produced. If the cut is within the plasmid's marker, the marker is not retained in the recombinant product. Because nearly all of the transformants recovered with a linearized plasmid and DNA fragment contain a product of recombination between these two DNAs, direct selection for the fragment-borne gene is not necessary. Last, we have shown that the regions of homology used for recombinational repair need not be at the ends of these DNA molecules. This observation greatly expands the possible sites at which the plasmid DNAs can be cleaved to generate reactive substrates for recombination.

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