Plasmid construction by homologous recombination in yeast

(Saccharomyces cerevisiae; transformation; plasmid recombination; YCp50 derivatives; YEpl420 [previously called p72] 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, YEpl, and YEplp) 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

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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; $R_{or}$, resistance; SD, yeast synthetic minimal medium with dextrose; SDS, sodium dodecyl sulfate; Tc or Tet, tetracycline; ::, novel joint.

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-μm 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
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 (MATa 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), his3-4200 (Struhl, 1985a), lys2-8120 (Simchen et al., 1985), leu2-3,2-112 (Botstein et al., 1979) and trp1-d901 (Hieter et al., 1985). The plasmids YEp7, 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 YEp5 (Botstein et al., 1979), with all junction sites destroyed. The yeast episomal plasmid YEp420, previously called pJ2, was constructed by blunt-end ligation into the PvuII site of YEp5 the 1.58-kb HpaI-HindIII fragment of the yeast 2-μm circle (form B) (Broach, 1983; Hartley and Donelson, 1980) containing the putative replication origin. The plasmid 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 polynucleotide 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 3.3-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 μg Ap/ml or 15 μg 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 μl 0.1 M EDTA pH 8.0 in 1.5-ml microfuge tubes. To each tube was added 100 μl of zymolyase (zymolyase-100; 2 mg/ml; Seikagaku Kogyo Co., Ltd., 2-9 Nihonbashihoncho, Chuo-ku, Tokyo, 103 Japan), followed by incubation at 37°C for 1–2 h; 90 μl 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 μl 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, AaI; Av, AviI; B, BamHI; Ba, Ball; Be, BstEII; Bn, BssHII; Bw, BxI; Bs, BsmI; Bt, BstXI; C, ClaI; D, DraIII; E, SpeI; F, Spal; G, BglII; H, HindIII; J, NruI; K, KpnI; L, BglII; M, Smal; Ms, MatII; N, NcoI; Nd, NdeI; Nt, NheI; O, NotI; P, PstI; Pv, PvuII; Q, HpaI; R, EcoRI; S, SalI; Sc, SacI; Sn, SmalII; Sp, Spal; St, SstII; Sy, SstI; T, ThI; U, PvuI; V, BglII; W, AvaIII(VsiI); X, XbaI; Y, AaI; Z, XmaIII(EagI). The solid black regions are pBR322 sequences, the stippled ones are yeast 2-μm sequences, and other regions are indicated explicitly on the maps.
Fig. 2. Restriction maps of the plasmids providing homologous DNA fragments for the recombinatorial 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 BamHI, ClaI, EcoRI 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-μm and pBR322 DNA are not known (Struhl et al., 1979). Restriction analysis indicates that the ClaI and HindIII sites of pBR322 were lost, but the AatI and EcoRV 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 cells are then washed with buffer A and resuspended in buffer B. They are mixed by pipetting up and down. The cells are then lysed by treatment with lysozyme (1 mg/ml) on ice for 15 min (2–5 min). Lysis is confirmed by mixing buffer C, and the cell lysate is recovered by centrifugation.

The pellets are washed with buffer A and dissolved in buffer B. The pH is then adjusted to 8.0 by incrementally adding 0.1 M NaOH. The solution is then mixed with gentle agitation. The supernatants were recovered by centrifugation. The plasmids, 1 μg per lane, are then transformed into E. coli DH5α.

(c) Transformation

Yeast transformation was carried out essentially by the method (Itaya et al., 1981) and 1 fl. was the minimum transformation efficiency. The DNA restriction map

Fig. 3. Introduces restriction sites in intervals I and II, and the cloning orientation in interval III. (e) A his3 mutation is introduced for His" transformants. Restriction enzymes are PvuII, BglII, ClaI, EcoRI, and NdeI.
1–2 h. The samples were centrifuged in a microfuge for 15 min (12000 × 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 μl 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

![Diagram](image-url)

**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) + *SalI*], 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* + *PvuI*, 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*; E, *EcoRI*; F, *SphI*; M, *SmaI*; O, *XhoI*; P, *PvuII*; R, *EcoRI*; S, *SalI*; U, *PvuI*. 
(1) Simple integrations

A DNA fragment to be inserted into the recombinant plasmid must span the regions of overlap between the YEp21 fragment and the yeast chromosome. The homology with the chromosomal region itself is not required. After linearization by restriction endonucleases, the DNA fragment is introduced into yeast by transformation as described above. The YEp21 integration vector is not used in these experiments. The overlapping regions of the YEp21 and the yeast chromosome resulted in a linearized YEp21 fragment of the form shown above. Ten of these integrants, each obtained from a single colony of a strain without uracil, were isolated using antibiotics. As a result of this recombination, the 12.8 kb YEp21 fragment was inserted into the yeast chromosome. The YEp21 fragment is 12.8 kb and contains the LYS2 gene. It was recovered in the manner described above and was used to prepare DNA for the conversion of the strain. The DNA was transformed into the yeast strain with the resulting Leu+ transformants, and the latter into Leu- cells, containing the recombinant plasmid. The resulting transformants were recovered on the plate below.

(2) Substitution integrations

It is often desirable to substitute a yeast gene with a bacterial gene. This can be achieved by recombination between a yeast chromosomal gene and the YEp21 plasmid. In the example depicted, a yeast chromosome containing the LYS2 gene at the SmaI site was transformed into a ura3 strain with the linearized YEp21 that contained the SmaI insert. As a result, the yeast chromosome, containing the LYS2 gene, was substituted for the yeast chromosome not containing the LYS2 gene.

For the positions of other sites, see the map of YEp21 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.

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, BamHI; C, ClaI; P, PstI; R, EcoRI; S, SalI; X, XbaI. 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 3, 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 YE271 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 structure, 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>
<thead>
<tr>
<th>New plasmid a</th>
<th>Linearized plasmid</th>
<th>Homologous fragment</th>
<th>Selection c</th>
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<td></td>
<td>Plasmid b</td>
<td>Digested with c</td>
<td>Plasmid d</td>
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<tr>
<td>YCp401</td>
<td>YCp50</td>
<td>EcoRI, BamHI</td>
<td>pRB306</td>
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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.
<table>
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TABLE II
New plasmids and their features

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<td>Clal, HindIII, Sall, SmaI, Spdl, XbaI</td>
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<td>LEU2</td>
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<td>YCp408</td>
<td>HIS3 URA3</td>
<td>Aaal, Apal, BglII, BspMI, BstXI, Clal, EcoRI, EcoRV, HpaI, NcoI, NotI, PswI, Sall, SmaI, Spdh, XhoI, XmaI (EagI)</td>
</tr>
<tr>
<td>YCp409</td>
<td>LEU2</td>
<td>Aaal, BamHII, BglII, BspMI, BstEI, DraIII, EcoRV, HindIII, NdeI, NotI, PswI, Sall, Spdh, XhoI, XmaI (EagI)</td>
</tr>
<tr>
<td>YCp410</td>
<td>TRP1</td>
<td>Aaal, BamHII, BglII, BspMI, BstEI, DraIII, EcoRV, HindIII, HpaI, KpnI, MsrII, NdeI, NotI, PswI, Sall, Spdh, XhoI, XmaI (EagI)</td>
</tr>
<tr>
<td>YCp411</td>
<td>TRP1 URA3</td>
<td>Aaal, Apal, BamHII, BglII, BspMI, BstXI, Clal, EcoRI, EcoRV, HindIII, HpaI, KpnI, MsrII, NdeI, NotI, PswI, Sall, Spdh, XhoI, XmaI (EagI)</td>
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<tr>
<td>YEp425</td>
<td>LYS2 URA3</td>
<td>Clal, HindIII, HpaI, Sall, SmaI, Spdh, XhoI</td>
</tr>
<tr>
<td>YEp426</td>
<td>LYS2</td>
<td>Clal, HindIII, HpaI, Sall, SmaI, Spdh, XhoI</td>
</tr>
<tr>
<td>YEp427</td>
<td>TRP1</td>
<td>Aaal, BamHII, BglII, BspMI, BstXI, Clal, DraIII, MsrII, NdeI, NotI, PswI, Sall, ScaI, Spdl, SphI, Stul, ThIIIII, XmaI (EagI)</td>
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<td>YEp428</td>
<td>TRP1 URA3</td>
<td>Aaal, Apal, BamHII, BglII, BspMI, BstXI, Clal, MsrII, NdeI, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, ThIIIII, XmaI (EagI)</td>
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<td>URA3</td>
<td>Aaal, Apal, BamHII, BglII, BspMI, BstXI, Clal, EcoRI, EcoRV, HindIII, NcoI, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, ThIIIII, XmaI (EagI)</td>
</tr>
<tr>
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<td>TRP1</td>
<td>Aaal, BamHII, BglII, BspMI, BstXI, EcoRI, EcoRV, HindIII, MsrII, NotI, PswI, Sall, ScaI, Spdl, SphI, Stul, ThIIIII, XhoI</td>
</tr>
<tr>
<td>YEp433</td>
<td>TRP1 URA3</td>
<td>Aaal, Apal, BamHII, BglII, BspMI, BstXI, EcoRI, EcoRV, HindIII, MsrII, NdeI, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, ThIIIII, XmaI (EagI)</td>
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<tr>
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<td>LEU2</td>
<td>Aaal, BamHII, BglII, BspMI, BstXI, EcoRI, EcoRV, HindIII, MsrII, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, ThIIIII, XmaI (EagI)</td>
</tr>
<tr>
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<td>LEU2 URA3</td>
<td>Aaal, Apal, BamHII, BglII, BspMI, BstXI, Clal, DraIII, EcoRV, HpaI, KpnI, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, XbaI, XhoI, XmaI (EagI)</td>
</tr>
<tr>
<td>YEp436</td>
<td>LEU2-d URA3</td>
<td>Aaal, BamHII, BglII, BspMI, BstXI, Clal, HpaI, KpnI, NcoI, NotI, PswI, Sall, SmaI, Spdl, SphI, Stul, XbaI, XhoI, XmaI (EagI)</td>
</tr>
<tr>
<td>YEp437</td>
<td>LEU2-d HIS3</td>
<td>Aaal, BamHII, BglII, BspMI, BstXI, Clal, HpaI, KpnI, NotI, Sall, SmaI, Spdl, SphI, Stul, XbaI, XhoI, XmaI (EagI)</td>
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<td>YR5441</td>
<td>TRP1 HIS3</td>
<td>Aaal, BglII, BspMI, BstII, Clal, DraIII, KpnI, MsrII, NdeI, NotI, PswI, Sall, ScaI, Spdl, SphI, Stul, ThIIIII, XbaI, XhoI</td>
</tr>
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<td>TRP1 LEU2</td>
<td>Aaal, BglII, BspMI, BstII, Clal, DraIII, HpaI, KpnI, MsrII, NdeI, NotI, PswI, Sall, ScaI, Spdl, SphI, Stul, XbaI, XmaI (EagI)</td>
</tr>
</tbody>
</table>
In a similar manner, we constructed derivatives of several yeast episomal plasmids (YPE; autonomous due to the presence of a portion of the yeast plasmid 2-μm circle) and the TRP1 ARG1 containing the yeast-replicating plasmid YEp7 (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-μm 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-μm plasmid, YEp420, linearized by cleavage with EcoRI, BamHI, or Sall. 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 Sall 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 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 Sall-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). The His+ transformants were then subjected to Southern analysis (Fig. 2). The His+ transformants were indistinctly...
As shown in Fig. 5, the HIS3 gene was cloned into YEp420, YEp421, or YEp422. The HIS3 insert on these linearized plasmids must incorporate HindIII, or SalI. Location of the HIS3 insert on the linearized plasmids, respectively, is shown outside of the plasmids. In each case, recombination generated products with HIS3 insert via HindIII and SalI, respectively.

Each plasmid was digested with each restriction enzyme and the HIS3 gene was isolated from the URA3 gene. The HindIII digests of YEp420 and YEp421 were cleaved with HindIII and SalI, respectively. The digests were separated on a gel. The DNA from the URA3 gene in YEp420 was isolated and ligated into plasmids that had been cleaved with SalI. The ligated DNA was transformed into E. coli and the plasmid was isolated. The plasmids were used to transform yeast to Ura + and the number of transformants was determined. Each plasmid contained the HIS3 gene and was used to transform yeast to Ura + .

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, BamHI; C, ClaI; G, BglII; P, PstI; R, EcoRI; S, SalI; X, XbaI. 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.

(Yep 420), 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 YEplac420 plasmid alone. In this case, the yield (4000 per μg of DNA) of Ura + transformants is about 5% of the yield (80,000 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.

(c) Introduction of gene

Through this approach, we obtain a large number of transformants that may then be isolated for their chloramphenicol resistance for further characterization. In our system, the need to repair the natural lesion is avoided by using a mutagen to introduce the lesion of interest into the transforming DNA. The reaction conditions are those for selecting for transformants with a plasmid, using a mutagenic procedure such as UV light. The transformed cells are then plated on a medium that selects for the presence of the particular plasmid variant of interest.

RESULTS

As expected, the transformation of yeast cells with the plasmid YEplac420 using a mutagenic procedure such as UV light results in the introduction of the gene of interest. The wild-type strain containing the plasmid variant of interest allowed us to transform the yeast with a fragment of DNA, which was characterized by a mutation in the hox2 gene. The hox2 mutation is known to be desirable because it promotes the deletion of the background DNA, which cannot occur naturally.

(d) Formation of recombinant plasmids

In some cases, the formation of recombinant plasmids is achieved by the introduction of a fragment of DNA, which is characterized by a mutation in the hox2 gene. The hox2 mutation is known to be desirable because it promotes the deletion of the background DNA, which cannot occur naturally.
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 allele 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 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,
section a2, and its original rate
such circumstances
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recombination
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BamHI site
plasmid with
homologous
derived from
DNA fragment
digesting with
Nco1 + NruI
EcoRI (one
(homologous)
purified away
electrophoresis
and eluted
ends of the
nonhomologous
mants was relative to
homologous
or two nonhomologous in intermediate
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(e) Conclusions

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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, BamHI; J, NruI; K, KpnI; R, EcoRI; S, SalI.
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 Sall site, or both the Sall 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 NeoI (neither end homologous), or NeoI + NruI (one end homologous), or NeoI + 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.

ACKNOWLEDGEMENTS

We thank Greg Maine and Mark Johnston for providing plasmids. This work was supported by grants to D.B. from the National Institute of Health (Public Health Service grants GM21253 and GM18973), the American Cancer Society (MV90F), and the Biotechnology Process Engineering Center at Massachusetts Institute of Technology (grant CDR8500009 from the National Science Foundation). S.K. was supported by training grant GM07287 from the National Institute of Health. P.J.S. was supported by a Graduate Fellowship from National Science Foundation and by a Fellowship from the Whitaker Health Science Fund.

REFERENCES


Communicated by G.R. Fink.

The evidence for the plasmid pC412 element in the yeast Saccharomyces cerevisiae reveals that it contains the partial recognition sequence of another 13 direct repeats; it can integrate within the genome of yeast with attention to the recognition sequence.

INTRODUCTION

Plasmids are an important tool for genetic analysis in a wide range of species. Studies of plasmid integration sites and their mechanisms have led to the discovery of different strategies for integration.

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Abbreviations: plasmid, self-replicating extrachromosomal DNA; 
patibility group, a group of plasmids that can replicate in the same cells.