# Fine Structure Recombinational Analysis of Cloned Genes Using Yeast Transformation

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ABSTRACT

We describe a general method for analyzing the genetic fine structure of plasmid-borne genes in yeast. Previously we had reported that a linearized plasmid is efficiently rescued by recombination with a homologous restriction fragment when these are co-introduced by DNA-mediated transformation of yeast. Here, we show that a mutation can be localized to a small DNA interval when members of a deletion series of wild-type restriction fragments are used in the rescue of a linearized mutant plasmid. The resolution of this method is to at least 30 base pairs and is limited by the loss of a wild-type marker with proximity to a free DNA end. As a means for establishing the nonidentity of two mutations, we determined the resolution of two-point crosses with a mutant linearized plasmid and a mutant homologous restriction fragment. Recombination between mutations separated by as little as 100 base pairs was detected. Moreover, the results indicate that exchange within a marked interval results primarily from one of two single crossovers that repair the linearized plasmid. These approaches to mapping the genetic fine structure of plasmids should join existing methods in a robust approach to the mutational analysis of gene structure in yeast.

RECOMBINANT DNA technology currently applied to the yeast, *Saccharomyces cerevisiae*, includes convenient methods that facilitate a mutational analysis of gene structure and function (reviewed in BOTSTEIN and DAVIS 1982). To isolate new mutations, a cloned gene residing on a plasmid can be mutagenized and introduced via transformation into an appropriate yeast host to screen for altered gene function. The plasmid of a transformant displaying a mutant phenotype can be returned to an *Escherichia coli* host, permitting isolation of the mutant gene for direct analysis and further manipulation.

This basic methodology lacks a simple, sensitive method for mapping to a high resolution a mutation on a plasmid in yeast. Even with the use of *in vitro* mutagenesis, new mutations need to be localized unambiguously to the gene of interest, to be distinguished from silent or modifying lesions, and to be localized to DNA segments small enough to be convenient for sequence analysis. Furthermore, localizing genetic regions of substantial importance to gene function is of immediate interest in a mutational analysis.

To devise an approach to the high resolution mapping of plasmids, we utilized the known property of free DNA ends as efficient substrates for homologous recombination in yeast (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; reviewed in ORR-WEAVER and SZOSTAK 1985). We have reported (KUNES, BOTSTEIN and FOX 1984, 1985) that a plasmid DNA broken in a sequence absent from the yeast genome is efficiently rescued by recombination with a homologous restriction fragment included during yeast transformation. Here, we describe some features of recombination with these substrates and utilize this reaction in two approaches to plasmid fine structure analysis, two-point heteroallelic crosses and deletion mapping.

## MATERIALS AND METHODS

**Strains and media:** Yeast strains DBY1226 (*MATα his4-519 met8-1 leu2-3,112 ura3-3*) and DBY1227 (*MATα his4-519 met8-1 leu2-3,112 ura3-18*) were constructed in this laboratory by standard methods (SHERMAN, FINK and LAWRENCE 1979). Yeast strain DBY2052 (*MATα lys2-801 leu2-3,112 ura3-52 hxk1::LEU2 hxk2-202*) is a derivative of DBY1315 constructed by disruption of *HXK1* with the cloned *LEU2* gene (RATZKIN and CARBON 1977; H. MA and D. BOTSTEIN, unpublished data) and deletion of the *HXK2* locus. The *hxk2* deletion allele, *hxk2-202*, is a derivative of the cloned *HXK2* gene (see below) constructed *in vitro* by removing the *HXK2* coding region from between the flanking sites *Sac*I and *Xba*I (see pRB313 in Figure 5 for the positions of these sites). The genomic *HXK2* locus was replaced with this deletion allele by recombination (see BOTSTEIN and DAVIS 1982 for details), yielding strain DBY2052. As a result of the presence of the *hxk1* and *hxk2* mutations, this strain cannot grow on fructose as a sole carbon source, but can grow on glucose due to the presence of glucokinase.

Yeast was grown in YEPD (complete) or SD (minimal) medium (SHERMAN, FINK and LAWRENCE 1979). When appropriate, 2% fructose was substituted for 2% glucose as carbon source. *E. coli* was grown in LB (complete) medium

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(MILLER 1972) containing, when appropriate, 100 μg ampicillin/ml (Sigma).

**Plasmids:** Plasmids pRB28, pRB29 and pRB30 (Figure 1) were constructed by FALCO *et al.* (1982). Plasmid pSK265 (Figure 3) is a derivative of pRB30 constructed by removing the *Bam*HI, *Bgl*II fragment of 5′ *URA3* flanking sequence, and incorporating a *Sac*I-linker (Collaborative Research, Waltham, Massachusetts) in the recircularized product. Plasmid pRB45 and its derivative plasmids containing *URA3′lacZ* gene fusions (pRB70, pRB72, pRB73, pRB74, pRB75, pRB76, pRB79, pRB80, pRB81 and pRB82) were constructed by ROSE and BOTSTEIN (1983). Plasmid pRB313 (Figure 2) is a derivative of the yeast vector YCp50 (C. MANN and R. W. DAVIS, personal communication) containing a 3.8-kb yeast *Eco*RI fragment bearing the *HXK2* gene, isolated by STACHELEK *et al.* (1986). This plasmid complements the chromosomal deletion allele of *HXK2* described above, restoring the ability of strain DBY2052 to utilize fructose as a sole carbon source. Mutant derivatives of pRB313 lacking *HXK2* complementing activity were isolated after transformation of strain DBY2052 with plasmid DNA mutagenized by amplification in an *E. coli mutD* host (ERLICH and COX 1980). The resulting mutations were mapped to small DNA intervals within the cloned *HXK2* gene (see Figure 2) by the deletion method described in the text. A derivative of pRB313 with a known *hxk2* mutation, pRB430 (*hxk2-430*), was constructed *in vitro* by deleting 4 base pairs (bp) at the *HXK2 Kpn*I site (nucleotide 266–269, see Figure 2). The 3.8 kb *HXK2 Eco*RI fragment was also subcloned in the vector YIp5 (BOTSTEIN *et al.* 1979) to construct pRB309. Deletion series of *HXK2* were generated by *Bal*31 partial digestion of pRB309 linearized at either the *Sac*I site or *Xba*I site flanking *HXK2*, with incorporation of a *Bgl*II-linker (Collaborative Research) in the recirculation of the linear molecules. The resulting deletions were shown to extend into the *HXK2* coding sequence to various points as determined by restriction analysis, ending at the site of the *Bgl*II-linker inserted during the construction.

Recombinant DNA methods were performed essentially as described by MANIATIS, FRITSCH and SAMBROOK (1982).

**DNA preparation and restriction enzyme cleavage:** Plasmid DNA was isolated from *E. coli* by a modification (RAMBACH and HOGNESS 1977) of the method of CLEWELL and HELINSKI (1969). Plasmid DNA was further purified by banding once in a CsCl/ethidium bromide density gradient (RADLOFF, BAUER and VINOGRAD, 1967). Supercoiled plasmid DNA was stored in TE buffer (10 mM Tris, pH 8.0, 1 mM $Na_2EDTA$) at 4° or −20°. Yeast DNA was isolated by the method of HOLM *et al.* (1986) from cultures grown in liquid SD medium with selection for plasmid maintenance. Chicken erythrocyte DNA was obtained from Calbiochem-Behring (La Jolla, California). After dissolution in TE, it was sonicated to an average molecular weight of $5 \times 10^5$, phenol/chloroform (1:1, v/v, equilibrated to pH 8.0) extracted three times, precipitated in ethanol three times and stored in TE buffer at 4°.

Restriction enzymes were used according to the recommendations of the manufacturer (New England Biolabs, Beverly, Massachusetts).

**Yeast transformation:** Yeast transformation by the spheroplasting method was performed essentially as described by HINNEN, HICKS and FINK (1978) with the exception that STC buffer (1 M sorbitol, 10 mM Tris, pH 7.5, 10 mM $CaCl_2$) was substituted for 1 M sorbitol in the third wash after treatment with glusulase (Dupont Pharmaceuticals, Wilmington, Delaware). The final suspension contained spheroplasts at a concentration of $1 \times 10^9$/ml, of which typically 10% were colony-forming units in regeneration agar with complete supplements. In an individual transformation, 100 μl of spheroplast suspension was added to 10 μl of TE buffer containing plasmid DNA and 10 μg sonicated chicken erythrocyte (carrier) DNA, prepared as described above. Plating in regeneration agar on SD selective medium was followed by incubation at 30° for 4–5 days. Selection for $Leu^+$ transformants was made in the presence of a growth limiting quantity (1 μg/ml) of leucine. Under these conditions, with a plasmid containing both the *URA3* and *LEU2* genes (pRB28), a similar yield was obtained with selection for either $Ura^+$ or $Leu^+$ transformants.

Yeast transformation by the alkali cation method of ITO *et al.* (1983) was performed as modified by KUO and CAMPBELL (1983) using 0.1 M lithium acetate. Each transformation included 50 μg carrier DNA.

## RESULTS

**Two-point heteroallelic crosses in a plasmid-borne *URA3* gene:** Previously we reported (KUNES, BOTSTEIN and FOX 1985) that a high frequency of transformants results after co-transformation of yeast with a plasmid linearized in a region lacking homology with the yeast genome and a homologous restriction fragment that spans the plasmid's two-chain break. More than 95% of these transformants were shown to have restored the linearized plasmid to its parental circular form via recombination with the homologous restriction fragment.

We investigated the possible resolution of a two-point cross where a mutant restriction fragment is used in the rescue of a mutant linearized plasmid. In principle, the frequency of wild-type recombinants that result in such a cross could depend on the order of the markers with respect to the free DNA ends of the substrates. As shown in Figure 1, there must be at least two crossovers, one on each side of the break of the linearized plasmid, in order to form a viable circular product. If these were indeed single crossover events, a wild-type recombinant plasmid could be expected only with the mutant alleles proximal to the free DNA ends that flank the marked interval (see Figure 1). Thus, in the original cross depicted in Figure 1, wild-type recombinant plasmids would result with the cleavage site of the linearized plasmid on the left side of the marked interval, and double mutant plasmid recombinants would result with the cleavage on the right side. In the reciprocal cross, however, cleavage of the linearized plasmid on the right side of the marked interval, but not on the left, would result in the formation of wild-type recombinants. With the plasmid cleaved within the marked interval (not shown), wild-type recombinants would be observed in both the original and reciprocal crosses.

These expectations were tested in a study of recombination between two homologous plasmids distinguished by the mutant allele of their resident yeast *ura3* gene. Plasmids pRB29 and pRB30 (Figure 1) are autonomous yeast plasmids containing a cloned yeast *LEU2* gene (RATZKIN and CARBON 1977) and a *ura3-*

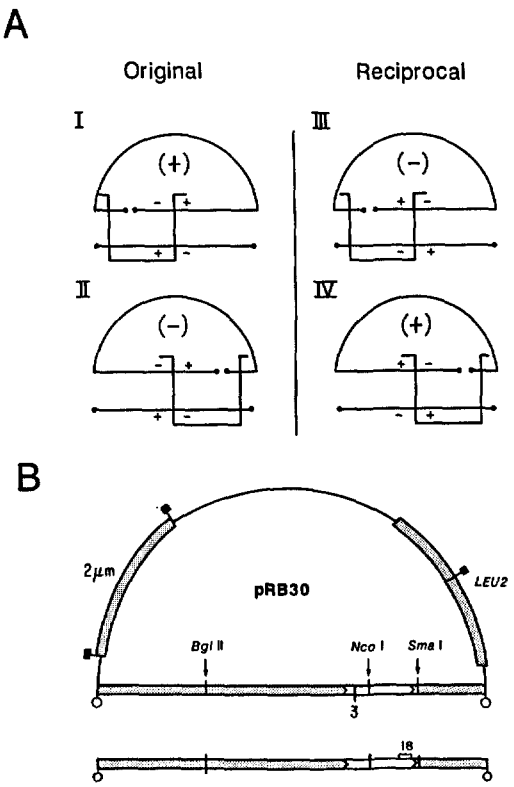


FIGURE 1.—Formation of a *URA3* recombinant plasmid by two single crossovers between a linearized plasmid and homologous restriction fragment. A, As indicated by the brackets, at least two crossovers, one on each side of the break in the linearized plasmid, are required to yield a circular product. In the original cross, with the plasmid linearized on the left of the marked interval (I), wild-type recombinants are formed by a single crossover that falls within the marked interval. With the plasmid linearized on the right of the marked interval (II), a double mutant circular product is formed by a single crossover within the marked interval; no wild-type recombinants are expected. In the reciprocal cross, a wild-type circular product is formed with the plasmid linearized on the right side of the marked interval (IV), but not with cleavage on the left side (III). The crosses which yield wild-type recombinants are those with the two mutant alleles proximal to the free DNA ends that flank the marked interval. B, Plasmids pRB28 (*URA3*), pRB29 (*ura3-18*), and pRB30 (*ura3-3*) were constructed by FALCO, ROSE and BOTSTEIN (1983). These plasmids are derivatives of pBR322 (BOLIVAR *et al.* 1977) containing the cloned yeast *LEU2* gene (RATZKIN and CARBON 1977) and a yeast 2 μm circle segment that renders them autonomous in yeast (BEGGS 1978; BROACH and HICKS 1980). The mutation *ura3-3* (am) has been located at nucleotide 245 (ROSE, GRISAFI and BOTSTEIN, 1983) in the *URA3* coding sequence (open box between arrowheads). The mutation *ura3-18* (am) has been mapped (FALCO, ROSE and BOTSTEIN 1983; LOSSON and LACROUTE 1979) to the region indicated by a bracket between nucleotides 880 and 1030. The plasmids have a unique *Bgl*II cleavage site about 2.0 kb to the left of the *ura3-3, ura3-18* interval, a unique *Nco*I site within the interval (nucleotide 432 of the *URA3* sequence), and a unique *Sma*I site to the right of the interval (nucleotide 1106 of the *URA3* sequence). Shown below the *ura3-3* plasmid is the 5.5-kb *ura3-18* *Bam*HI fragment that spans the two-chain break of the linearized plasmids. Restriction sites are denoted as follows: O, *Bam*HI; ■, *Eco*RI.

*3* (am) gene insert or a *ura3-18* (am) gene insert, respectively. Each plasmid has a unique site for cleavage by *Bgl*II, *Sma*I, and *Nco*I located, respectively, on the left, on the right, or inside, of the *ura3-3, ura3-18* marked interval (Figure 1). The 5.5-kb *Bam*HI fragment generated by *Bam*HI, *Eco*RI cleavage of these plasmids spans the two-chain break of the linearized plasmids.

The original cross was performed by transforming the *ura-3-3 leu2* strain DBY1226 with the *ura3-3* plasmid in linearized form and the *ura3-18* plasmid in fragmented (*Bam*HI, *Eco*RI-cleaved) form. Separate addition of the *ura3-3* plasmid linearized within the marked interval, or the fragmented *ura3-18* plasmid resulted in few, or no, $Ura^+$ transformants (Table 1). Co-transformation with these two DNAs resulted in a high frequency of transformants on either $Ura^+$ or $Leu^+$ selection. Of the $Leu^+$ transformants, 30% displayed the $Ura^+$ phenotype. All of 18 $Ura^+$ transformants tested were found to be $Leu^+$; both phenotypes were unstable, and co-segregated. These 18 transformants were further shown to harbor a plasmid of the parental structure (data not shown). On co-transformation with the *ura3-3* plasmid linearized on the left of the marked interval and the fragmented *ura3-18* plasmid, 12% of the resulting $Leu^+$ transformants were found to be $Ura^+$. However, with the *ura3-3* plasmid linearized on the right side of the marked interval, a significantly smaller fraction, 2.6%, were found to be $Ura^+$. Were *URA3* recombinants to arise solely via single exchange events, no *URA3* recombinants would be expected in this latter cross. The results are thus consistent with the formation of *URA3* recombinants primarily, but not exclusively, by a single crossover required for the repair of the linearized plasmid.

The reciprocal cross was performed by transforming the *ura3-18 leu2* strain DBY1227 with the *ura3-18* plasmid in linearized form and the *ura3-3* plasmid in fragmented form. On addition of the fragmented *ura3-3* plasmid with the *ura3-18* plasmid linearized within the marked interval, the frequency of *URA3* recombinants differed only modestly from that in the original cross (Table 1). However, relative to the outcome in the original cross, the *URA3* recombinant frequency was decreased about fourfold with the *ura3-18* plasmid linearized on the left of the marked interval and increased more than sixfold with the *ura3-18* plasmid linearized on the right of the marked interval. This outcome, in agreement with the expectations outlined above, clearly demonstrates that the resolution of a two-point cross depends on the order of the markers with respect to the free DNA ends of the substrates.

**Two-marker recombination in a plasmid-borne *HXK2* gene:** The plasmid cleavages utilized in the two-point crosses above generate free DNA ends with homology to the chromosomal *URA3* locus. To rule out the possibility that recombination with the chro-

**TABLE 1**

**Two-marker recombination in a plasmid-borne *URA3* gene: frequency of *URA3* recombinants**

| Cross in Figure 1 | Linear plasmid | Fragmented plasmid | Frequency | | Ura$^+$ among Leu$^+$ transformants (%) |
|---|---|---|---|---|---|
| | | | Ura$^+$ | Leu$^+$ | |
| | 3 ·*Nco*I | None | 0 | $2.2 \times 10^3$ | ND |
| | None | 18 | $1.0 \times 10^1$ | 0 | ND |
| I | 3 ·*Bgl*II | 18 | $6.0 \times 10^3$ | $5.0 \times 10^4$ | 12.0 |
| | 3 ·*Nco*I | 18 | $1.4 \times 10^4$ | $5.0 \times 10^4$ | 30.0 |
| II | 3 ·*Sma*I | 18 | $1.4 \times 10^3$ | $2.6 \times 10^4$ | 2.6 |
| III | 18 ·*Bgl*II | 3 | $4.8 \times 10^2$ | $3.8 \times 10^4$ | 3.2 |
| | 18 ·*Nco*I | 3 | $2.4 \times 10^3$ | $2.2 \times 10^4$ | 20.0 |
| IV | 18 ·*Sma*I | 3 | $2.4 \times 10^3$ | $2.2 \times 10^4$ | 17.0 |

The plasmid crosses illustrated in Figure 1 (I, II, III, or IV) are indicated in the table. Plasmids pRB30 (*ura3-3*) and pRB29 (*ura3-18*), described in Figure 1, are denoted as "3," and "18," respectively. The *ura3-3 leu2-3,112* strain DBY1226 was the host for crosses involving linearized pRB30 (*ura3-3*), and the *ura3-18 leu2-3,112* strain DBY1227 was the host for crosses involving linearized pRB29 (*ura3-18*). Hence, the formation of *URA3* recombinants via plasmid integration was prevented. Transformation was carried out by the spheroplasting method with DNA mixtures containing 10 μg sonicated carrier DNA, and when indicated, 100 ng of a linearized (*Bgl*II-, *Nco*I- or *Sma*I-digested) plasmid DNA and 4 μg of a fragmented (*Bam*HI-, *Eco*RI-digested) plasmid DNA. The frequency of Ura$^+$ transformants and Leu$^+$ transformants was determined by direct selection. For each cross, approximately 500 Leu$^+$ transformants were scored for the Ura$^+$ phenotype.

mosomal *URA3* locus contributed to the outcome of those crosses, a similar analysis was carried out with the cloned gene for yeast hexokinase II, *HXK2*, in a strain where the chromosomal locus had been deleted.

Two-marker crosses were performed by co-transforming the *hxk2* deletion strain DBY2052 with one mutant plasmid linearized on the left of the marked interval and a heteroallelic mutant plasmid in fragmented form (see Figure 2). In each case where the cross was performed with both dispositions of markers, a four- to 20-fold higher frequency of *HXK2* recombinants occurred with the mutant alleles proximal to the free DNA ends that flank the marked interval (Table 2), consistent with the result of the preceding analysis of two-marker recombination in the *URA3* gene. Furthermore, with this disposition of markers, the frequency of *HXK2* recombinants increased with the size of the marked interval, from 0.9 to 20.4%, a feature that is clearly expected. The yield of *HXK2* recombinants in the reciprocal crosses, with one exception, showed no dependence on the size of the marked interval, yielding approximately 1% *HXK2* recombinants in each case. The one exception was in the case of the smallest interval, the cross of *hxk2-518* with *hxk2-519*, which resulted in 0.2% *HXK2* recombinants. The result suggests that, if multiple exchange accounts for the low frequency of *HXK2* recombinants detected in the reciprocal crosses, multiple events are insensitive to the size of the marked interval, except at very short distances.

**Marker loss due to the proximity of a two-chain break:** We describe below a second approach to plasmid fine structure analysis that is based on an assay for marker rescue. If, as indicated above, single crossovers that repair a linearized plasmid often occur at sites distant from the molecule's original free DNA ends, a genetic marker near a DNA end would often be lost. This would diminish the marker rescue frequency. The expectation was tested by examining the effect of the cleavage position of the linearized plasmid on the rescue of a marker residing on a homologous restriction fragment. We would expect that with the cleavage site of the linearized plasmid close to the resident marker, an increased proportion of the transformants would display the restriction fragment's allele. The *ura3-18* plasmid (Figure 1) was linearized with either *Bgl*II, *Nco*I, or *Sma*I, as described above, and introduced along with the fragmented *URA3* plasmid into the *ura3-18 leu2* strain DBY1227. As shown in Table 3, the fraction of Leu$^+$ transformants displaying the Ura$^+$ phenotype was highest (96%) with the *ura3-18* plasmid cleaved at the site nearest to the mutation, and lowest (58%) when linearized at the site furthest from *ura3-18*. In the reciprocal experiment, with the *URA3* plasmid in linearized form and the *ura3-18* plasmid fragmented, the inverse result is obtained, with the greatest yield of Ura$^+$ transformants occurring with the *URA3* plasmid linearized at the site furthest from the wild-type marker. As a control, a co-transformation was performed with a linearized plasmid and homologous restriction fragment that were both wild-type *URA3*. The observation that 99% of the Leu$^+$ transformants displayed the Ura$^+$ phenotype indicates that the chromosomal marker, *ura3-18*, is rarely contributed to the repaired plasmid. These observations are extended in the experiments which follow.

**Deletion mapping of cloned genes by a marker rescue assay:** One approach to mapping a mutation on a plasmid is to assay for rescue of the wild-type allele from each member of a nested deletion set of restriction fragments derived from the wild-type gene (Figure 3). Since many phenotypes are not amenable to a direct selection, the most generally applicable

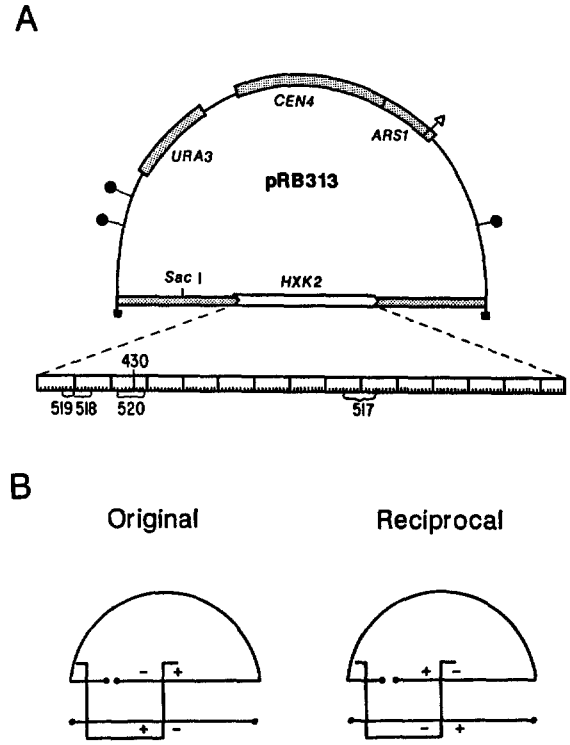


FIGURE 2.—Two-point heteroallelic crosses in a plasmid-borne *HXK2* gene. A, Plasmid pRB313 is a derivative of the yeast *CEN4 ARS1* vector YCp50 containing the *HXK2* gene on a 3.8-kb yeast *Eco*RI DNA fragment isolated by STACHELEK *et al.* (1986). The expanded scale of the *HXK2* coding region (1470 base pairs) shows the approximate map positions of mutations in derivatives of pRB313 lacking *HXK2* complementing activity in yeast. The mutants were localized by the deletion mapping method described in the text (see Figure 5) to the regions indicated by brackets, with the exception of *hxk2-430*, which is a 4-bp deletion (nucleotides 266–269) introduced at the *HXK2 Kpn*I site. Restriction sites are denoted as follows: ■, EcoRI; ●, *Bgl*I; Δ, *Bgl*II. B, Two-marker crosses with the *hxk2* derivatives of pRB313 were performed with a mutant plasmid linearized to the left of the marked interval by *Sac*I cleavage and a heteroallelic *hxk2* plasmid fragmented by digestion with *Eco*RI, *Bgl*I, and *Bgl*II. The *hxk2 Eco*RI fragment generated by the latter cleavage spans the break of the *Sac*I-linearized plasmid. The original cross, with the mutant alleles proximal to the free DNA ends that flank the marked interval, and the reciprocal cross, are shown.

approach would be to select for a second functional gene on the linearized mutant plasmid, and screen the resulting transformants for rescue of the wild-type allele. A mutation could be unambiguously localized between the deletion endpoints of any two restriction fragments provided that wild-type information located very close to the molecular end of a restriction fragment is included in the plasmid product often enough to be detected.

This approach was tested by mapping a mutation, *ura3-3*, that has been localized by DNA sequence analysis (ROSE, GRISAFI and BOTSTEIN 1983). A nested deletion set of the wild-type *URA3* gene was constructed by ROSE and BOTSTEIN (1983) in the form of a group of plasmids bearing *URA3'lacZ* gene fusions. These plasmids differ in the amount of 3′ *URA3*

**TABLE 2**

**Two-marker recombination in a plasmid-borne *HXK2* gene: frequency of *HXK2* recombinants**

| Cross in Figure 2 | Linearized plasmid marker | Fragmented plasmid marker | Hxk$^+$ among Ura$^+$ transformants (%) |
|---|---|---|---|
| Original | 519 | 518 | 0.9 |
| Reciprocal | 518 | 519 | 0.2 |
| Original | 518 | 520 | 4.2 |
| Original | 518 | 430 | 5.6 |
| Reciprocal | 430 | 518 | 1.0 |
| Original | 430 | 517 | 12.2 |
| Reciprocal | 517 | 430 | 1.1 |
| Original | 518 | 517 | 20.4 |
| Reciprocal | 517 | 518 | 1.0 |

Two-marker crosses were carried out by transforming the *ura3 hxk2-202* strain DBY2052 by the alkali cation method with DNA mixtures containing 50 μg of sonicated carrier DNA, 100 ng of a *Sac*I-linearized *hxk2* mutant plasmid and 4 μg of a second mutant plasmid fragmented by cleavage with *Eco*RI, *Bgl*I, and *Bgl*II. The recombination substrates generated by these cleavages and the disposition of markers in each cross, original or reciprocal, are illustrated in Figure 2. For each cross, Ura$^+$ transformants occurred at a high frequency on SD medium, of which approximately 1000 were screened for the Hxk$^+$ phenotype.

coding sequence removed at the site of joining to *'lacZ*, and can be cleaved at this junction to generate a fragment that contains a fraction of the *URA3* gene, and that extends to a constant endpoint (Figure 3). The *URA3* deletion restriction fragments are homologous to the autonomous *ura3-3, LEU2* plasmid pSK265 (Figure 3), and span the two-chain break resulting from cleavage of pSK265 at its unique *Sac*I site.

Transformation of the *ura3-3 leu2* strain, DBY1226, by separate addition of *Sac*I-linearized pSK265 or the *URA3* deletion fragments produced few, if any, Ura$^+$ transformants (not shown). The combination of linearized pSK265 with any of the *URA3* deletion fragments resulted in a high frequency of Leu$^+$ transformants. With the presence of those

**TABLE 3**

**Effect of linearized plasmid cleavage site position on rescue of a restriction fragment marker[a]**

| Linear plasmid allele[b] | Fragmented plasmid allele | Ura$^+$ among Leu$^+$ transformants |
|---|---|---|
| + ·*Nco*I | + | 99 |
| 18 ·*Bgl*II | + | 58 |
| 18 ·*Nco*I | + | 80 |
| 18 ·*Sma*I | + | 96 |
| + ·*Bgl*I | 18 | 76 |
| + ·*Nco*I | 18 | 37 |
| + ·*Sma*I | 18 | 21 |

[a] See the legend to Table 1 for experimental details.
[b] Plasmids are indicated as "+" for pRB28 (*URA3*) and "18" for pRB29 (*ura3-18*).

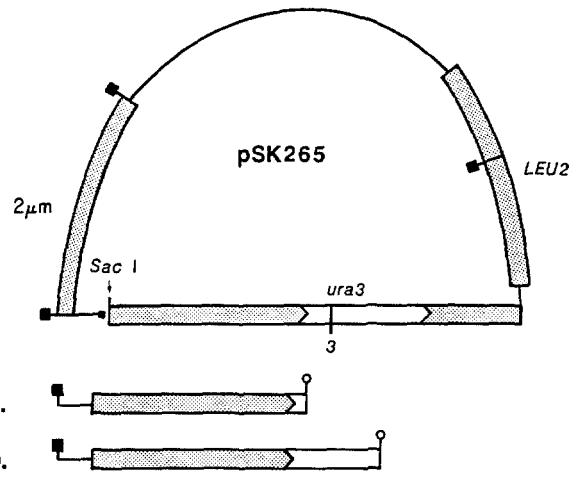


FIGURE 3.—A general method of deletion mapping for plasmid-borne genes. Plasmid pSK265, bearing a *ura3-3* gene insert (open box between arrowheads), is linearized at a *Sac*I site spanned by the homologous deletion restriction fragments, *a* and *b*. These fragments are members of a deletion set derived from the wild-type *URA3* gene constructed by ROSE and BOTSTEIN (1983) in the form of a group of plasmids bearing *URA3'lacZ* gene fusions. The *ura3-3* mutation is mapped by co-transforming yeast with the linearized mutant plasmid and each of the deletion restriction fragments, with selection for Leu$^+$ transformants. Transformants bearing the Ura$^+$ phenotype would result with fragment *b*, but not with fragment *a*, thus localizing the mutation between the endpoints of the two deletions. Restriction sites are denoted as in Figure 1.

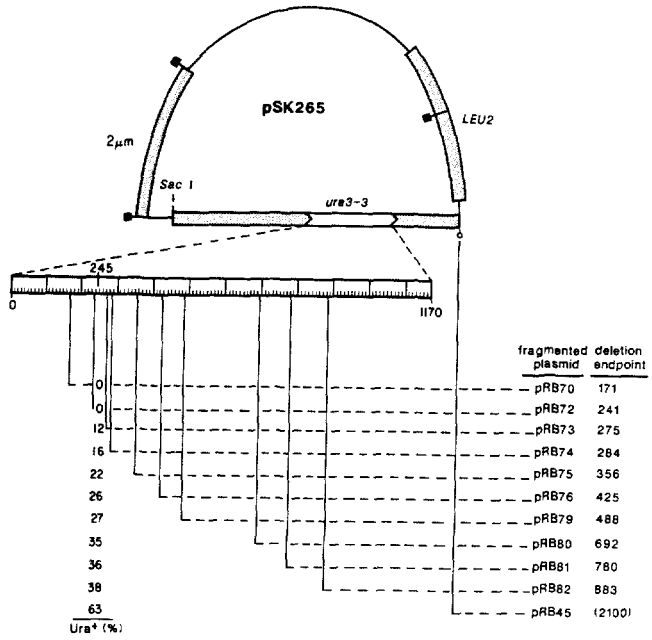


FIGURE 4.—Deletion mapping of a plasmid-borne *ura3-3* mutation. The expanded scale of the *ura3* gene (open box between arrowheads) shows the 1170-bp sequenced region and the position of the *ura3-3* amber mutation (nucleotide 245; ROSE, GRISAFI and BOTSTEIN 1983). A 3′ deletion series of the *URA3* gene was constructed by ROSE and BOTSTEIN (1983) in the form of a group of plasmids bearing *URA3'lacZ* gene fusions (see Figure 3). *Bam*HI, *Eco*RI cleavage of these plasmids generates a deletion restriction fragment that spans the break in *Sac*I-linearized pSK265. The vertical lines appending the expanded *URA3* scale and the adjoining horizontal dashed lines indicate the extent of each deletion. The nucleotide position of each deletion endpoint (ROSE and BOTSTEIN 1983) is given. Yeast strain DBY1226 (*ura3-3 leu2-3,112*) was transformed with a DNA mixture containing 100 ng of *Sac*I-linearized pSK265, 4 μg of a fragmented *URA3'lacZ* fusion plasmid, and 10 μg sonicated carrier DNA. Each transformation gave rise to about $1 \times 10^4$ Leu$^+$ transformants. The fraction of about 400 Leu$^+$ transformants that were Ura$^+$ is given in the figure column beneath the known position of *ura3-3*.

*URA3* deletion fragments expected to include the wild-type allele, more than 12% of the Leu$^+$ transformants displayed the Ura$^+$ phenotype (Figure 4). The fraction of transformants that were Ura$^+$ increased with the distance of the wild-type allele from the end of the *URA3* deletion fragments, from 12 to 63%, a feature consistent with marker loss, as described above. With the combination of linearized pSK265 and those *URA3* deletion fragments not expected to include the wild-type allele, fewer than 0.1% of the resulting Leu$^+$ transformants were found to be Ura$^+$. Thus, the *ura3* mutation is placed by the endpoints of pRB72 and pRB73 *URA3* sequence in an interval of 34 bp, consistent with the previously determined location of the *ura3-3* mutation.

**Bidirectional deletion mapping of uncharacterized *hxk2* mutations:** The deletion method was further demonstrated by mapping uncharacterized mutations in the *hxk2* gene. Two nested deletion sets of *HXK2*, one from each end of the gene, were constructed as described in MATERIALS AND METHODS. The mapping experiment was performed by adding the *hxk2* plasmid linearized at a site 5′ or 3′ of the *hxk2* gene along with the *HXK2* 3′ deletion fragments or the *HXK2* 5′ deletion fragments, respectively (Figure 5). With these cleavages, each member of the deletion sets produced a *HXK2* fragment that spanned the break in the linearized mutant plasmid. Figure 5 indicates the result of replica-plating the resulting Ura$^+$ transformants of DBY2052 (*ura3 hxk2-202*) to a medium on which *HXK2* function is required for growth. The resulting deletion map, constructed with deletions from both ends of the gene, is self-consistent, and accurately predicts the location of *hxk2-430*, the *hxk2* mutation constructed *in vitro* by deletion at the *HXK2 Kpn*I site. Secondly, the frequency of marker-rescue markedly decreases as the deletion endpoints approach the respective sites of the mutations from either direction, as was observed in mapping the *ura3-3* mutation above. The frequency of marker rescue is sufficient, though, to unambiguously localize the mutations to the smallest intervals attempted, those of approximately 100 bp.

## DISCUSSION

**An analysis of genetic recombination between a linearized plasmid and homologous restriction fragment:** We examined the effect of the position of the two-chain break of a linearized plasmid on two-marker recombination with a homologous restriction frag-

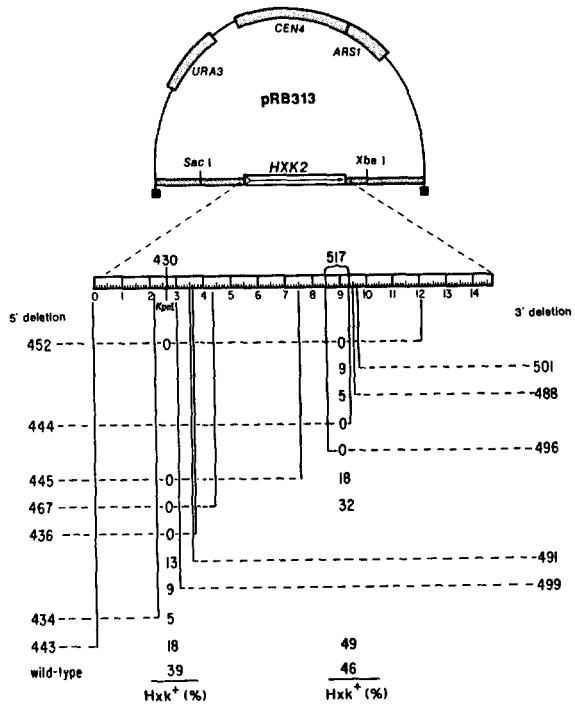


FIGURE 5.—Bidirectional deletion mapping of mutations in a plasmid-borne *hxk2* gene. Hxk$^-$ derivatives of pRB313 (Figure 2) and *HXK2* 3′ deletions and 5′ deletions were isolated as described in MATERIALS AND METHODS. Digestion of the *HXK2* deletion derivatives with *Bgl*II and *Eco*RI generates 3′ deletion fragments that span the pRB313 *Sac*I site, or 5′ deletion fragments that span the pRB313 *Xba*I site. The vertical lines appending the expanded scale of the *HXK2* coding region indicate the deletion endpoints of these fragments, as determined by restriction analysis (to an accuracy of about 25 bp in either direction). The dashed horizontal lines indicate the extent of the *HXK2* material deleted. The *ura3* strain DBY2052, in which the *HXK2* locus is deleted, was transformed by the alkali cation method with a DNA mixture containing 100 ng of an appropriately linearized *hxk2* mutant plasmid and 2 μg of a fragmented *HXK2* deletion plasmid. Approximately 1000 Ura$^+$ transformants were assayed for the Hxk$^+$ phenotype (growth with fructose as sole carbon source). The Hxk$^+$ fraction (in percentage of total transformants) recovered with each deletion fragment is given in a column beneath the apparent map position of the respective mutation.

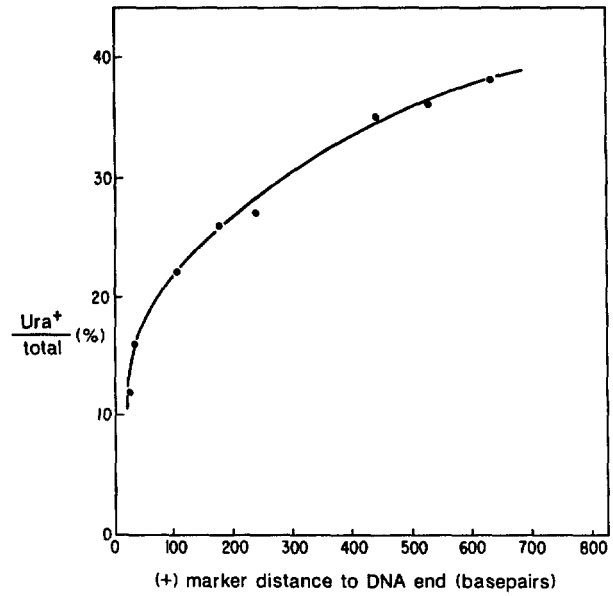


FIGURE 6.—The dependence of marker-rescue frequency on marker distance to a free DNA end. The fraction of Leu$^+$ transformants displaying the Ura$^+$ phenotype, determined in mapping the *ura3-3* mutation in pSK265 (Figure 4), is plotted as a function of the distance of the wild-type marker from the variable (*Bam*HI) end of the deletion restriction fragments. The Ura$^+$ fraction resulting with the longest wild-type fragment (63% Ura$^+$ with fragmented pRB45) is not shown in the figure.

ment. The outcome of this analysis is consistent with the formal notion that a pair of single crossovers that repair the linearized plasmid are primarily responsible for generating recombinants in the marked interval. As shown in Figure 1, with the two mutant alleles proximal to the free DNA ends that flank the marked interval, a single crossover that falls within the marked interval yields a wild-type recombinant plasmid. With the wild-type alleles proximal to these ends, a double mutant plasmid would result. This nonreciprocality was observed in the analysis of two-marker recombination in the plasmid-borne genes *URA3* and *HXK2*, with wild-type recombinants detected in the latter crosses at a reduced frequency. We therefore conclude that multiple exchange is uncommon during recombinational repair.

The above analysis implies that a crossover would in some outcomes separate a marker from the viable circular product, resulting in marker loss. This was observed as the site of cleavage of the linearized plasmid was varied with respect to the site of the resident marker. With cleavage of the linearized plasmid near the site of the resident marker, an increased proportion of the resulting transformants bore the allele of the homologous restriction fragment. The effect of distance to a free DNA end on marker rescue was also observed on deletion mapping mutations in the plasmid-borne genes, *URA3* and *HXK2*. In mapping the *ura3-3* mutation, the frequency of rescue of the wild-type marker declined rapidly as the end of the *URA3* deletion fragment approached the site of the wild-type marker. A plot of this dependence (Figure 6) indicates that the half-maximal rescue frequency, 30%, occurred with the wild-type allele about 300 bp from the varied end.

These observations are reminiscent of polarized marker segregation of bacteriophage T4 terminal heterozygotes (DOERMANN and BOEHNER 1963; DOERMANN and PARMA 1967; MOSIG *et al.* 1971). Recombination between the terminally redundant phage DNA ends, which is required for phage growth (STREISINGER, EDGAR and DENHARDT 1964), results in marker loss, with markers proximal to the ends of the infecting phage lost with the greatest frequency.

Loss of genetic information from free DNA ends has also been suggested as a way to account for the outcome of several yeast DNA transactions that may involve a two-chain break. In *S. cerevisiae*, for several

*loci studied, the frequency of meiotic gene conversion* events decays for markers distributed across the locus, an observation termed polarity (see FOGEL, MORTIMER and LUSNAK 1981 for a review). SZOSTAK *et al.* (1983) have proposed that polarity originates with the creation of a two-chain break at a specific site. They suggest that double strand exonucleolytic degradation away from the site of this break erases two strands of parental information, which are replaced by copy from an intact homologue during repair. Yeast mating type switching is associated with the introduction of a two-chain break at the *MAT* locus (STRATHERN *et al.* 1982). It was suggested that exonucleolytic degradation of the resulting DNA ends removes the resident allele, leaving free DNA ends that initiate copy of an allele at either silent locus, *HML* or *HMR*. A similar model has been proposed for the transposition of an intron in the yeast mitochondrial 21S rRNA gene (ZINN and BUTOW 1985).

A consonant explanation for polarized marker loss in recombination between a linearized plasmid and a homologous restriction fragment is that exonucleolytic degradation acts on the ends of these molecules after transformation. The resulting new DNA ends would be substrates for the invasion of a homologous duplex, initiating repair that completes the recombination event. Alternatively, a recombination enzyme may enter a free DNA end and act at some distance from the end to initiate or resolve exchange, as has been proposed to account for the Chi, *cos* interaction in RecBC-mediated bacteriophage lambda recombination (SMITH *et al.* 1981; KOBAYASHI *et al.* 1982).

**The use of plasmid fine structure mapping in a mutational analysis of gene structure:** We have shown that the yield of wild-type recombinants in two-point heteroallelic crosses depends on the map orientation of the two-markers with respect to the free DNA ends of the substrates. Since this orientation would not be known with uncharacterized mutants, two crosses would need to be performed, as wild-type recombinants could result in either the original or reciprocal cross. Though the maximum resolution of this approach is not clear, we have shown that mutations may be resolved when separated by at least 100 bp (for example, in the cross between *hxk2-518* and *hxk2-519*, Table 2). With mutations that recombine, the analysis reveals the marker's order on the physical map.

In a second approach to plasmid fine structure analysis, mutations in plasmid-borne *ura3* and *hxk2* genes were localized to small DNA intervals, of less than 50 bp in some cases, by using members of a deletion set of wild-type restriction fragments in the rescue of a linearized mutant plasmid. With selection for a second, functional gene on the linearized mutant plasmid, several thousand transformants are readily screened by replica-plating to an indicator medium, enabling the detection of marker-rescue frequencies of at least 0.1%. When a direct selection for marker rescue is possible, even lower frequencies may be detected.

While the maximum resolution of the deletion mapping approach is not clear, we have used a replica-plating assay to unambiguously map mutations within a 22-bp interval (our unpublished results). On the other hand, as shown in Figure 6, the marker rescue frequency declines rapidly as the wild-type information comes to within about 40 bp from the restriction fragment end. It thus remains possible that a mutation may be located several base pairs outside of the DNA interval indicated by a deletion mapping experiment. A second consideration is the effect the mutation itself may have on the marker rescue frequency. We have shown that point mutations and very short deletions may be mapped with high resolution. Substantially larger deletion mutations are found to pose no problem with the use of this method (C. KAISER, personal communication).

This fine structure mapping methodology for cloned genes should join existing recombinant DNA technology in a robust approach to the analysis of gene structure in yeast. A gene clone, in general easily isolated from yeast, can be used to construct a null or conditional defect of the chromosomal locus that could be complemented by the wild-type clone residing on a plasmid. Screening a mutagenized population of plasmids for failure of complementation permits the isolation of a large number of mutations that are putatively in the cloned gene. The deletion mapping method described here would allow these mutations to be localized unambiguously to the gene of interest, to be distinguished from silent or modifying lesions, and to be localized to DNA segments small enough to be conveniently sequenced. Furthermore, it would immediately localize genetic regions of substantial importance to gene function, and facilitate the identification of regions of diverse function.

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