Efficient Targeted Integration at *leu1-32* and *ura4-294* in *Schizosaccharomyces pombe*

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ABSTRACT

Homologous integration into the fission yeast *Schizosaccharomyces pombe* has not been well characterized. In this study, we have examined integration of plasmids carrying the *leu1*$^+$ and *ura4*$^+$ genes into their chromosomal loci. Genomic DNA blot analysis demonstrated that the majority of transformants have one or more copies of the plasmid vector integrated via homologous recombination with a much smaller fraction of gene conversion to *leu1*$^+$ or *ura4*$^+$. Non-homologous recombination events were not observed for either gene. We describe the construction of generally useful *leu1*$^+$ and *ura4*$^+$ plasmids for targeted integration at the *leu1-32* and *ura4-294* loci of *S. pombe*.

TARGETED integration by homologous recombination is a standard tool of *Saccharomyces cerevisiae* yeast genetics and is rapidly becoming a standard procedure in the yeast *Schizosaccharomyces pombe* as well. In *S. pombe*, however, integration by homologous recombination has presented problems less familiar to *S. cerevisiae* geneticists (RUSSELL and NURSE 1986b; RUSSELL 1989). Many integrating vectors currently available for use in *S. pombe* are based on complementation of *S. pombe* mutations with *S. cerevisiae* genes. In single copy, such complementation may be inefficient, favoring multiple integrations. Many single-copy integrations into *S. pombe* utilizing the *S. cerevisiae* marker gene *URA3* fail to complement *ura4*. (RUSSELL 1989). The *S. cerevisiae* marker gene *LEU2* can complement *S. pombe leu1* mutations, but often the majority of transformants are double or triple tandem integrants, which can dramatically affect cellular phenotype (RUSSELL and NURSE 1986a, 1987).

Very few studies on integration into *S. pombe* have been published. GRIMM and KOHLI (1988) analyzed integration at the *ura4* locus. They examined six stable Ura$^+$ transformants receiving linearized *ura4*-containing plasmid DNA into a *ura4-294* strain and found that five of these transformants had undergone gene conversion events. The other transformant contained a wild-type hybridizing fragment, as well as an additional band the size of which was inconsistent with simple homologous integration at *ura4*. Genetic analysis revealed that the *ura*$^+$ information was located at the *ura4* locus. Thus, as the authors state, it is likely that this transformant also arose via gene conversion, and the additional hybridizing band was due to integration of a truncated fragment. They also examined integration of linearized plasmid into a *ura4-D6* strain. This deletion eliminates the restriction site that was used to linearize within the *ura4* plasmid sequence, but retains homology to plasmid sequences at the 5′- and 3′-regions of the *ura4* gene. Six stable Ura$^+$ transformants were analyzed by genomic blot analysis. Four of the transformants contained the expected single or multiple integrations at the *ura4* locus; the other two contained multimers integrated at other locations in the genome. Thus, *S. pombe*, like *S. cerevisiae*, tends to favor homologous recombination over non-homologous recombination when homology exists between the chromosome and the transforming DNA. However, in this study, gene conversion appeared to be the predominant event when a *ura4-294* containing strain was examined. GRALLERT *et al.* have published a thorough study of one-step gene disruption at the *suc1* locus, using *ura4* as the selectable marker. They found that 75–90% of the stable *ura*$^+$ transformants contained the expected disruption, indicating a high rate of homologous integration.

We have undertaken a study involving integration at the *leu1-32* locus and find only homologous integration events with a low rate of gene conversion. The same results were obtained in a study of integration at the *ura4-294* locus: we obtain a high frequency of single homologous insertions at the desired loci. The frequency of gene convertants obtained at both loci is similar to that reported for integration in *S. cerevisiae*. As *ura4-294* and *leu1-32* are common auxotrophic markers in *S. pombe* strains, we have constructed convenient plasmids for targeting integration to these loci.

MATERIALS AND METHODS

Yeast strains and plasmids: *S. pombe* strains used for integrative transformation are listed in Table 1. The integrative plasmids are described in Table 2 and are diagrammed in Figure 1. Plasmids were prepared by the boiling mini-prep method (HOLMES and QUIGLEY 1981) or by Qiagen columns (Qiagen Inc., Chatsworth California). Plasmid pJK4 was constructed by ligating the 1.8-kb *Hin*dIII fragment of *ura4* into the *Hin*dIII site of pBSII(KS$^+$). Plasmid pJK13 was constructed

TABLE 1

***S. pombe* strains used in this study**

Strain name	Genotype
BP34	*ura4-294 leu1-32 ade6-m210 h⁻*
BP427	*ura4D-18 leu1-32 ade6-m210 h⁻*
JKpX2-8D	*leu1-32 his5-303 h⁺*
JKp163	*leu1-32 ade5-36 h⁺*
JKp167	*leu1-32 ade6-250 h⁺*

TABLE 2

DNA plasmids used in this study

Plasmid name	Description
pJK4	Plasmid for integration at *ura4*. This plasmid has the 1.8-kb *Hin*dIII fragment of *ura4* in the *Hin*dIII site of pBS(KS⁺) as shown in Figure 1
pJK13	Plasmid containing the *leu1⁺* gene inserted in the *Sma*I site of pJK4 (Figure 1). pJK13 derivatives include plasmids with a *ura4::lacZ* fusion (see MATERIALS AND METHODS)
pJK142	A modification of pBSII(SK⁺), described in the text
pJK148	Plasmid for integration at *leu1*, illustrated in Figure 1
pJK210	Plasmid for integration at *ura4*, illustrated in Figure 1
pCG1	1.8-kb *ura4 Hin*dIII fragment in pUC8 (a gift of JURG KOHLI)

by first filling in the ends of the 2.1-kb *Cla*I fragment of *leu1* and ligating the fragment into the *Sma*I site of pJK4. Then 210 bp of the upstream region of *ura4* were deleted to generate pJK13. Derivitives of pJK13 were made by exonuclease III digestion of the *ura4* promoter starting at the *Hin*cII site (see Figure 1). Thus, "pJK13 derivatives" refers to a series of promoter deletions of the *ura4* gene. *lacZ* fusions of several of these promoter deletions were made by fusing the *lacZ* gene in frame with the *ura4* gene at the *Stu*I site. Three integrants of each promoter deletion and its corresponding *lacZ* fusion were tested, totaling 154 integrants. Plasmid pJK142 was constructed by first ligating the polylinker containing *Pvu*I fragment of pBSII(SK⁺) to the *Pvu*I fragment of pBS(M13⁺) containing the unique *Nde*I site (Figure 1). This backbone is the same as that used in constructing the pRS series of integrating vectors commonly used in *S. cerevisiae* (SIKORSKI and HIETER 1989). To construct pJK148, a 2.1-kb *Cla*I fragment of the *S. pombe leu1⁺* gene from plasmid pYK311 (from CHARLIE HOFFMAN; (KIKUCHI *et al.* 1988)) was inserted at the unique *Nde*I site of pJK142 by filling in the restriction sites, and subsequent blunt end ligation. Note that a *Cla*I site was fortuitously regenerated at the 3′ end of the *leu1⁺* gene during this cloning (see Figure 1). pJK210 was constructed by inserting the 1.8-kb *Hin*dIII fragment of *ura4* into the unique *Nde*I site of pJK142. This was done by filling in the restriction sites and ligating the blunt ends.

Growth media and transformations: *S. pombe* strains were grown on YEC (5 g yeast extract, 2 g casaminoacids/liter) 2% agar plates supplemented with 250 μg/ml uracil and adenine. SC-ura and SC-leu plates were prepared as described by ROSE *et al.* 1990).

S. pombe strains were transformed as follows. A colony was inoculated into 10 ml YEC and grown overnight at 30° to an OD_{600} of 0.8–1.2. The cells were pelleted (4 min, 1800 rpm), and washed in 5 ml H_2O, followed by a wash in 5 ml LiAc/TE (0.1 M lithium acetate/10 mM Tris-HCl, pH 7.6/1 mM EDTA). The cells were then resuspended in 0.01 volume LiAc/TE or, if they were to be frozen, LiAc/TE containing 10% glycerol. For freezing, cells were aliquoted (100 μl) and placed at −80°. Cells prepared in this manner maintain high competency for integrative transformation for about 3 weeks.

The transformation procedure is based on the dimethyl sulfoxide (DMSO)-enhanced protocol of HILL *et al.* (1991). A 20-μg sample of boiled herring sperm carrier DNA (2 μl of 10 mg/ml) and the transforming DNA (up to 10 μl volume) were added to 100 μl of cells and incubated for 10 min at room temperature. Then 260 μl of LiAc/40% PEG_{4000}/TE were added, and the cells were incubated for 30–45 min at 30°. Filter-sterilized DMSO (43 μl) was added, and the cells were heat shocked for 5 min at 42°. Cells were then pelleted, resuspended in 500 μl H_2O, and plated onto the selective medium, either SC-ura or SC-leu. Plates were incubated for 3–4 days at 30°.

Genomic DNA blot analysis: Genomic DNA minipreps were prepared as described (LEVIN *et al.* 1990). DNA from colony purified Leu⁺ or Ura⁺ transformants were digested, electrophoresed, blotted, and probed using standard techniques (MANIATIS *et al.* 1982). Genomic DNA from Leu⁺ transformants was digested with *Cla*I, *Bam*HI or *Xba*I; DNA from Ura⁺ transformants was digested with *Eco*RI. The *leu1* probe was random hexamer labeled DNA (FEINBERG and VOGELSTEIN 1983) from the 5′ *Eco*RV fragment of the *S. pombe leu1⁺* gene (KIKUCHI *et al.* 1988). The *ura4* probe was a 1.8-kb *Hin*dIII fragment containing the *ura4* gene (GRIMM *et al.* 1988). Washes were done in 0.2 × SSC, 0.1% sodium dodecyl sulfate at 65°.

RESULTS

Integration at *leu1-32*: Two different *leu1⁺* containing plasmids were used for studying targeted integration to *leu1-32* (Table 2 and Figure 1). For integration, the plasmids were linearized with *Nru*I or *Nde*I and transformed using the LiAc technique described in Materials and methods. Efficiencies of 500–1000 transformants per μg of DNA were obtained when plasmids were linearized with either *Nru*I or *Nde*I.

Numerous Leu⁺ transformants receiving pJK13 (Table 2 and Figure 1) and pJK13 derivatives (Figure 1 and as described in MATERIALS AND METHODS) were assessed by genomic DNA blot analysis. A diagram of possible homologous integration events is shown in Figure 2A, and a representative blot is shown in Figure 2B. Of 154 Leu⁺ transformants screened, 87% represented plasmid integrations at *leu1*, 13% were *leu1⁺* convertants, and none integrated elsewhere in the genome (Table 3). Thus, all transformants targeted the correct locus. The relatively low percentage of gene conversion events is comparable to that reported for integration at the *LEU2* and *HIS3* loci in *S. cerevisiae* (ORR-WEAVER *et al.* 1981).

Multiple (tandem) integrants occurred at a frequency of 20%. Of the 30 multiple integrations, 8 had integrated three or more plasmid molecules in tandem. As indicated in Table 3, there was one complex homology-dependent integration at *leu1-32*. This integrant, shown in Figure 2B, lane 3, is unique among the 154 events we studied. We have classified it as homology-dependent

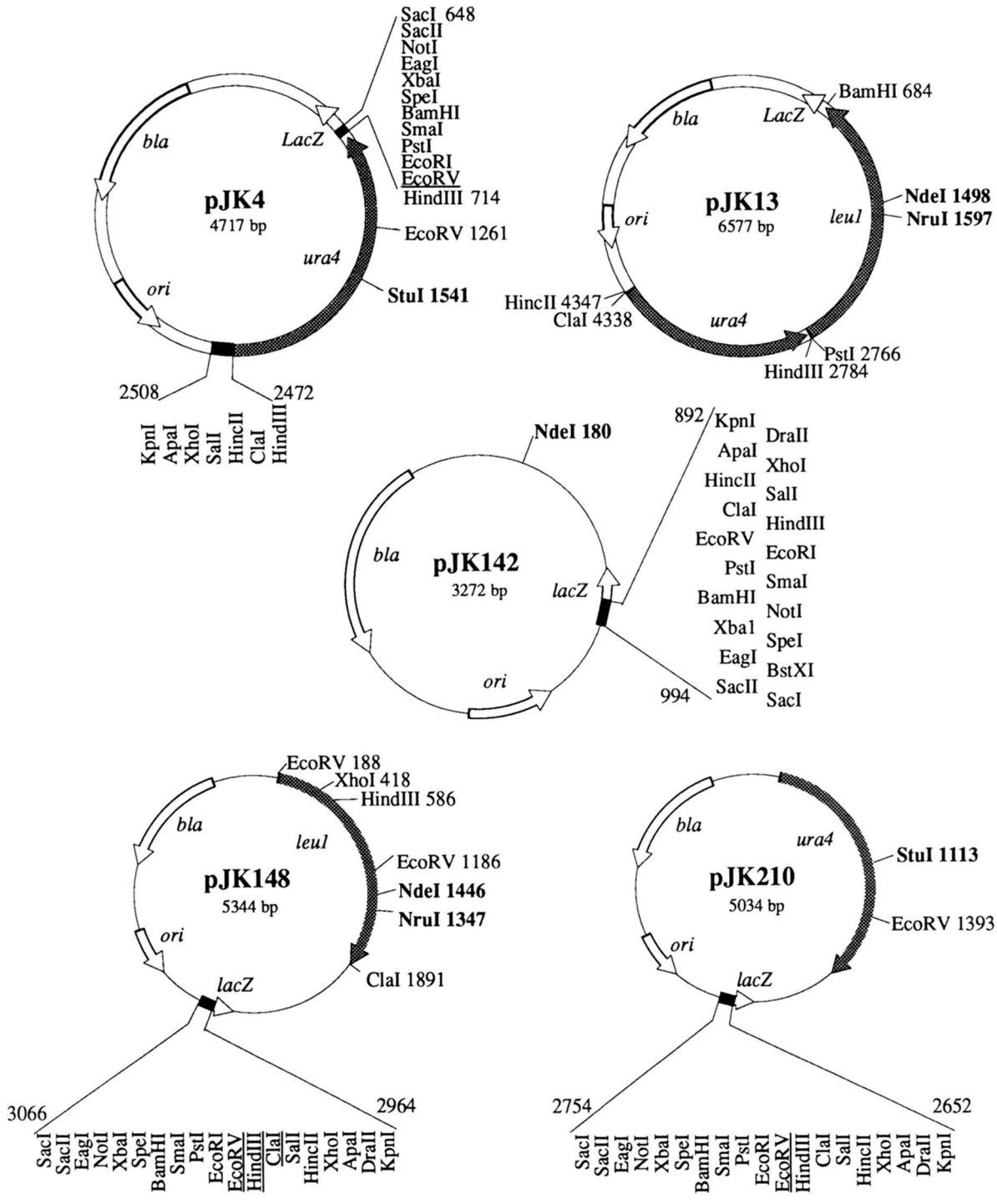


FIGURE 1.—Plasmid maps of *S. pombe* integrating vectors. Sites in bold print are unique to the plasmid and have been used for linearization prior to homologous integration. Details of construction are given in MATERIALS AND METHODS. Derivatives of pJK13 referred to in the text indicate exonuclease III deletions of the *ura4* gene promoter beginning at the *Hinc*II site. pJK142 is the backbone plasmid for the construction of pJK148 and pJK210. The *Nde*I site shown in bold is the position at which the selectable marker genes *leu1* and *ura4* were inserted to generate pJK148 and pJK210 respectively. Selected restriction sites within the *leu1*$^+$ gene, the *ura4*$^+$ gene and the plasmid polylinker are shown. Underlined sites in the polylinker are non-unique sites. Other unique sites in the *leu1* gene of pJK148 are: *Sty*I, 371; *Eco*NI, 568; *Eco*47III, 1177; *Bsm*I, 1260; *Bsu*36I, 1267; *Spl*I, 1296; *Tth*3I, 1331; *Sna*BI, 1974. Other unique sites in the *ura4* gene of pJK210 are: *Bsm*I, 260; *Bsg*I, 628; *Pfl*MI, 930; *Bsu*36I, 956; *Bbs*I, 1170; *Avr*II, 1578. Other labeled genes are *bla*, bacterial β-lactamase gene conferring ampicillin resistance; *ori*, bacterial origin of replication; *lacZ*, β-galactosidase gene allowing for blue/white color selection in *E. coli*. The plasmids pJK142, pJK148 and pJK210 are available as a kit from the American Type Culture Collection (12301 Parklawn Dr., Rockville, Maryland 20852), accession number 86958. Sequence files for the plasmids are available from in the Genome Sequence Database with the following accession numbers: pJK148, L25927; pJK210, L25928.

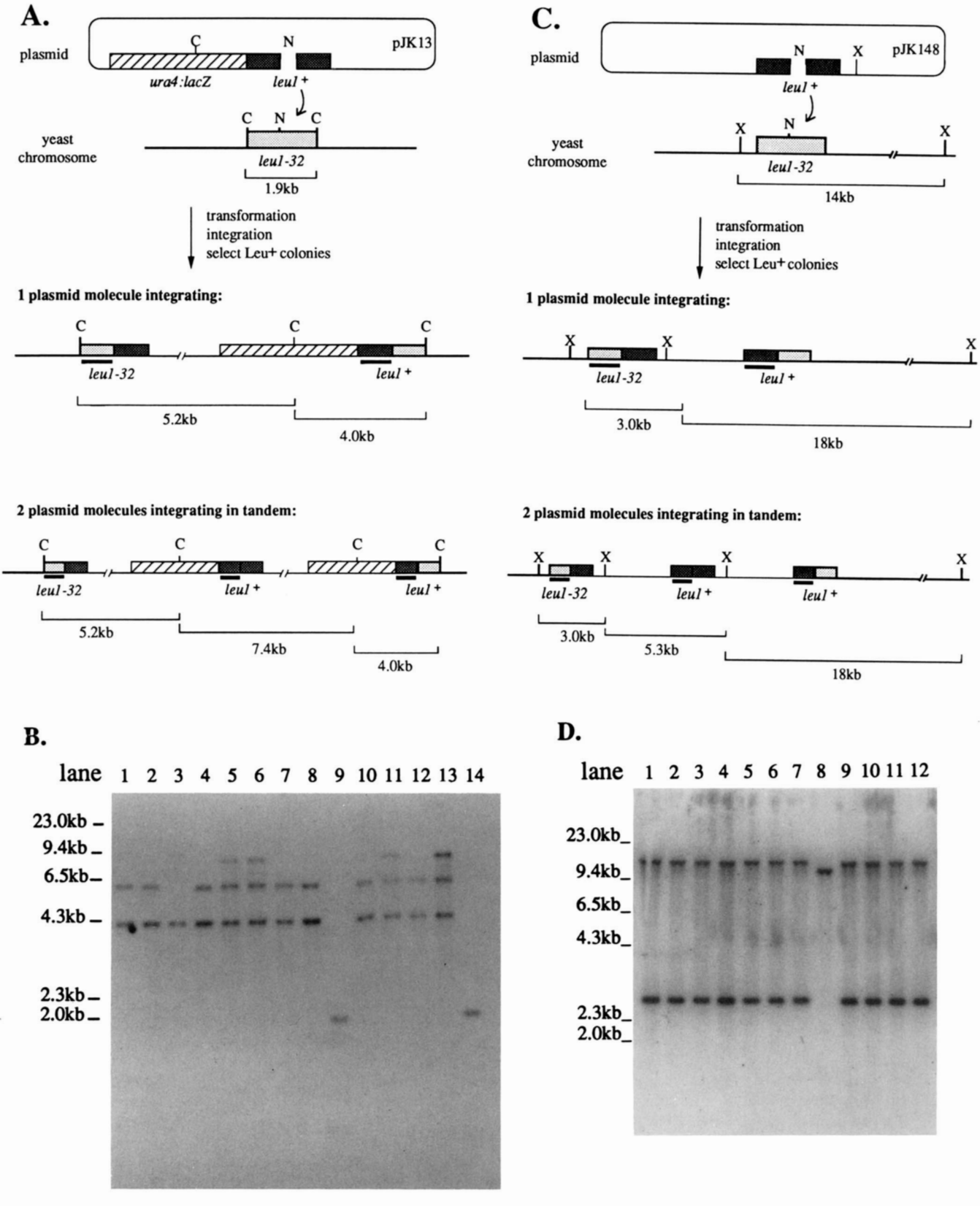


FIGURE 2.—Integration at *leu1-32*. (A) Diagram of integration events observed with a pJK13 derivative containing a *ura4:lacZ* fusion. The thick bars indicate the extent of the probe used for genomic DNA blot analysis. The single letter restriction enzyme designations are: C, *Cla*I; N, *Nru*I. *Cla*I fragments hybridizing to the probe are bracketed, and their respective sizes given in kilobase pairs. This diagram assumes that the *leu1-32* mutation is in the 5′ region of the gene; its actual location is unknown. Plasmid DNA is shown as a thin line and genomic DNA as a thick line. The hatched boxes represent plasmid insert sequence (*ura4::lacZ*), the lightly stippled boxes represent genomic *leu1* sequence, and the darkly stippled boxes represent plasmid *leu1* sequence.

TABLE 3

Homologous recombination at *leu1-32* in *S. pombe*

Transformation event	Events	Percent
Single integrations at *leu1-32*	103	67
Multiple integrations at *leu1-32*[a]	30	20
Complex homology-dependent integration at *leu1-32*[b]	1	0.7
Integrations not at *leu1-32*	0	
Convertants to *leu1*$^+$	20	13

Vector pJK13 (Table 2) was integrated as described. Data are the results of genomic DNA blot analysis of 154 Leu$^+$ transformants of strain BP427.

[a] Of the multiple integrants, six contained three tandem plasmid copies, and two had greater than three copies, as assessed by the intensity of the unit length plasmid band (see Figure 2). The remainder were double integrants.

[b] See Figure 2B, lane 3, and DISCUSSION in text.

because the 1.9-kb *leu1-32* fragment is clearly absent, and the 4.0-kb band expected of homologous integration is present. However, both the 5.2- and 7.4-kb bands expected of a tandem integration are missing, and a new band of 8.3-kb is present. This suggests that the transformant underwent a multiple integration followed by a deletion that included the *Cla*I site between the 7.4- and 5.2-kb fragments. Alternatively, the transformant could be a single integrant with a deletion event which included the 5′ *Cla*I site of the genomic *leu1-32* gene (see Figure 2A).

As the *leu1-32* locus of *S. pombe* proved to be a very efficient target for homologous integration, we constructed a plasmid, pJK148, especially for this purpose (Figure 1). The use of the *S. pombe leu1*$^+$ gene in the plasmid eliminates the problem of weak complementation by *S. cerevisiae* genes. In constructing plasmid pJK148 we noted that the *S. pombe leu1*$^+$ gene in this plasmid weakly complements the *Escherichia coli leuB6* mutation as previously reported (KIKUCHI *et al.* 1988). Plasmid pJK148 is generally useful as the diverse polylinker allows for easy cloning of DNA into the plasmid, the plasmid contains the *E. coli lacZa* gene for blue/white color selection of inserts, and the *leu1*$^+$ gene contains several restriction sites unique to the plasmid allowing linearization within *leu1*$^+$ prior to integration.

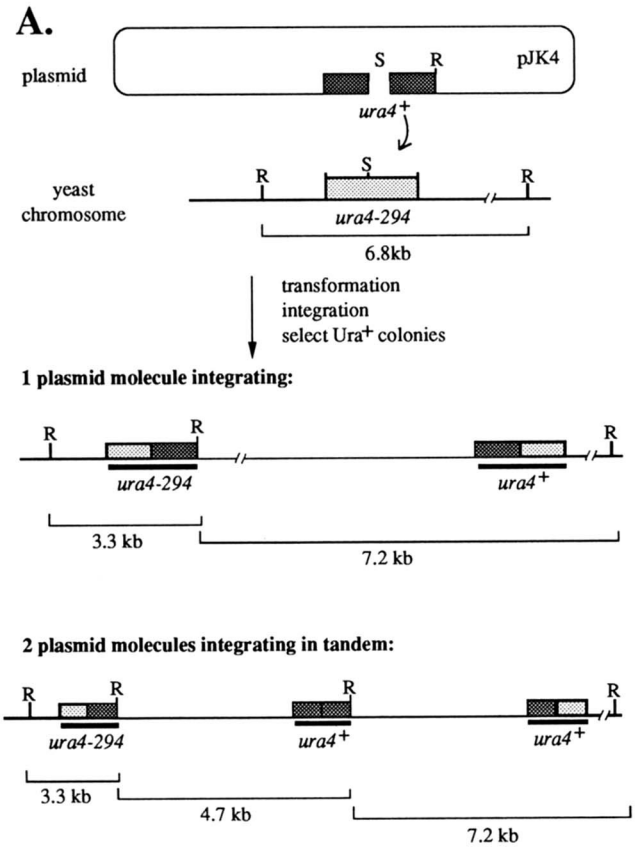
Twenty-five Leu$^+$ transformants receiving pJK148 and pJK148 derivatives (contain an insert in the polylinker) were analyzed and comparable results to pJK13 were obtained; 22 integrated at *leu1-32*, 3 were convertants to *leu1*$^+$ and 0 integrated elsewhere in the genome. A diagram of possible homologous integration events obtained with pJK148 is shown in Figure 2C, and a genomic blot of integrated pJK148 is shown in Figure 2D. For genomic blot analysis of pJK148 integrants, *Bam*HI or *Xba*I are suitable enzymes. Digestion with *Xba*I (Figure 2D) gives a hybridizing band of ~14 kb for untransformed strains (data not shown) or convertants to *leu1*$^+$ (lane 8), whereas a single integrant will yield bands of ~18 kb and 3 kb (lanes 1–7, and 9–12). *Bam*HI also results in a large genomic fragment of ~14 kb. Single integrants give hybridizing bands of ~18 and 4 kb (data not shown). For both enzymes, tandem integrants will produce a 5.3-kb vector band.

It should be noted that pJK13 and its derivatives were integrated into strain BP427, and pJK148 was integrated into each of the remaining strains listed in Table 1. Thus, only homology-dependent integration events were obtained using a variety of strains.

Integrations at *ura4-294*: Given the high frequency of homologous integrations at *leu1-32*, we also conducted a study of integration at *ura4-294*. The plasmid used for this study, pJK4, contains the 1.8-kb *Hin*dIII fragment of *ura4* in pBSII (Figure 1). Based on a previous report (GRIMM and KOHLI 1988), we expected to find a high percentage of gene conversion events and few homologous integration events when targeting to *ura4-294*. Surprisingly, we found mostly homologous integrations at *ura4-294*, with a relatively low percentage of gene convertants.

For integration, pJK4 was linearized with *Stu*I, and transformed as described in MATERIALS AND METHODS. A diagram showing the possible homologous integration events is shown in Figure 3A. In the initial experiment, shown in Figure 3B, twelve Ura$^+$ colonies were assessed

(B) Genomic DNA blot analysis of Leu$^+$ transformants receiving the pJK13 derivative diagrammed in (A). Genomic DNAs were digested with *Cla*I. *Hin*dIII-digested phage λDNA marker bands are indicated on the left. DNA from untransformed *S. pombe* contains a hybridizing band of 1.9 kb, lane 14. Thus, the transformant in lane 9 is a convertant to the wild-type *leu1*$^+$ gene. For single copy vector integration, the probe hybridizes to two fragments of 4.0- and 5.2-kb (lanes 1, 2, 4, 7, 8, 10 and 12). If more than one plasmid copy integrates in a tandem array, then a vector fragment of 7.4 kb is also detected, as in lanes 5, 6, 11 and 13. These integrants each appear to contain a tandem array of two copies of the vector as all bands are of the same intensity. The integrant in lane 3 has undergone a complex homology-dependent integration event (see explanation in text). (C) Diagram of integration events observed with pJK148. The thick bars indicate the extent of the probe used for genomic DNA blot analysis. The single letter restriction enzyme designations are: X, *Xba*I; N, *Nru*I. *Xba*I fragments hybridizing to the probe are bracketed, and their respective sizes given in kilobase pairs. This diagram assumes that the *leu1-32* mutation is in the 5′ region of the gene; its actual location is unknown. Plasmid DNA is shown as a thin line and genomic DNA as a thick line. The lightly stippled boxes represent genomic *leu1* sequence, and the darkly stippled boxes represent plasmid *leu1* sequence. (D) Genomic blot analysis of Leu$^+$ transformants receiving pJK148. Genomic DNAs were digested with *Xba*I. Lane 8 contains a hybridizing band of 14 kb, the size expected for a convertant to *leu1*$^+$. The remainder of the lanes contain hybridizing bands of 18 and 3.0 kb, as expected for a single homologous integration at *leu1-32*. If more than one plasmid copy integrates in a tandem array, then a vector fragment of 5.3 kb would also be detected.



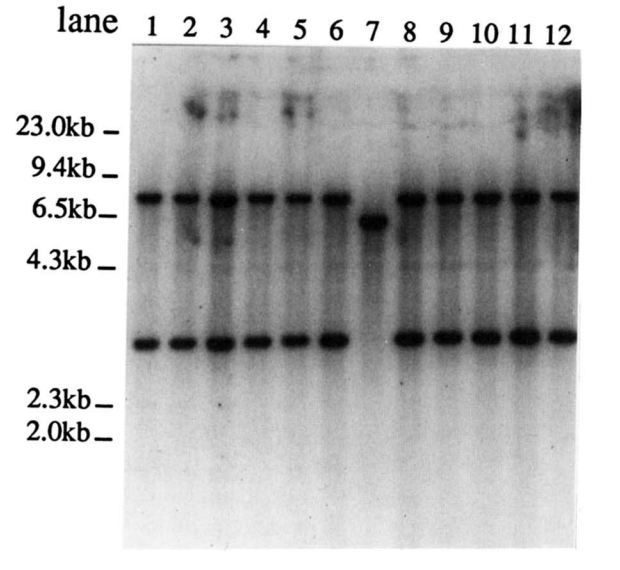


FIGURE 3.—Integration at *ura4-294*. (A) Diagram of integration events of pJK4. The thick bars indicate the extent of the probe used for genomic DNA blot analysis. The single letter restriction enzyme designations are: R, *Eco*RI; S, *Stu*I. *Eco*RI fragments hybridizing to the probe are bracketed, and their respective sizes given in kilobase pairs. This diagram assumes that the *ura4-294* mutation is in the 5′ region of the gene. Plasmid DNA is shown as a thin line and genomic DNA as a thick line. The lightly stippled boxes represent genomic *ura4* sequence, and the darkly stippled boxes represent plasmid *ura4* sequence. (B) Genomic DNA blot analysis of Ura$^+$ transformants receiving pJK4. Genomic DNAs were digested with *Eco*RI. *Hin*dIII-digested phage lambda DNA marker bands are indicated on the left. DNA from untransformed *S. pombe* contains a hybridizing band of 6.8 kb (data not shown). Thus, the transformant in lane 7 is a convertant to the wild-type *ura4*$^+$ gene. For single vector integration, the probe hybridizes to two fragments of 7.2 and 3.3 kb (lanes 1–6, 8–12). If the plasmid integrates in a tandem array, then a vector size fragment of 4.7 kb is also seen.

TABLE 4

Transformation efficiencies of *ura4*$^+$ plasmids into strain BP34

DNA (μg)	Carrier[a]	DMSO[b]	cfu/μg[c]
pJK4			
1	+	+	105
1	+	–	560
1	–	–	121
0.1	+	+	4180
0.1	+	–	300
0.1	–	–	40
pCG1			
1	+	+	54[d]
1	+	–	82
1	–	–	1
0.1	+	+	240
0.1	+	–	170
0.1	–	–	0

[a] Indicates the presence (+) or absence (–) of single-stranded carrier DNA in the transformation reaction.

[b] Indicates the presence (+) or absence (–) of DMSO in the transformation reaction.

[c] The number of colony-forming units obtained per μg of transforming plasmid DNA.

[d] The absolute frequencies in the pCG1 and pJK4 experiments cannot be compared because the cells used for pCG1 had been frozen whereas the cells used for pJK4 had not.

by genomic blot analysis. In contrast to the results of GRIMM and KOHLI (1988), 11 were single homologous integrations at *ura4-294*, and only one was a convertant to *ura4*$^+$. A difference in our study was the use of single-stranded carrier DNA and DMSO in the transformation procedure. In order to test the possible effect of transformation procedure on integrations, transformations were done in the absence of carrier DNA and/or DMSO. As shown in Table 4, these omissions dramatically reduced transformation efficiency of pJK4, especially at low concentrations of transforming DNA where only four Ura$^+$ colonies were obtained. However, genomic DNA blot analysis of these transformants revealed that low transformation efficiency had no effect on the nature of the integrations; all four of these transformants represented single-copy homology-dependent integration events (data not shown). A large number of transformants obtained under conditions using carrier only, or carrier plus DMSO, were also analyzed. Of 54 Ura$^+$ transformants, 80% were integrated at *ura4-294*, 15% were convertants, 5% were presumed diploids containing both a conversion and a homologous integration, and none were non-homologous integrants (Table 5).

TABLE 5

Homologous recombination at *ura4-294* in *S. pombe*

Transformation event	pJK4		pCG1	
	Events	Percent	Events	Percent
Single integrations at *ura4-294*	27	50	23	49
Multiple integrations at *ura4-294*	16[a]	30	18[b]	38
Diploids[c]	3	5	1	2
Integrations not at *ura4-294*	0		0	
Convertants to *ura4*$^+$	8	15	5	11

Data are the results of genomic DNA blot analysis of pJK4 and pCG1 integrated into strain BP34.

[a] Of the multiple integrants, three contained three tandem plasmid copies, and five had greater than three copies, as assessed by the intensity of the unit length plasmid band (see Figure 3). The remaining eight were double integrants.

[b] Of the multiple integrants, five contained three tandem plasmid copies, and one had greater than three copies, as assessed by the intensity of the unit length plasmid band. The remaining 12 were double integrants.

[c] These integrants were classified as diploid strains as judged by the fact that they contained both a wild-type locus and an integrated locus.

Another variable between the two studies was the integrative plasmid used. We repeated the experiment using plasmid pCG1 (kindly provided by JURG KOHLI). As seen in Table 4, this plasmid also gave a respectable transformation efficiency. Genomic blot analysis of these transformants indicated a high frequency of homologous integration at *ura4-294* (Table 5). Of 47 Ura$^+$ transformants analyzed by genomic DNA blot analysis, 87% were homologous integrations at *ura4-294*, 11% were convertants to *ura4*$^+$, 2% were presumed diploids containing both a conversion and a single homologous integration and none were non-homologous integrants. The single Ura$^+$ transformant obtained under low transformation efficiency conditions (no carrier or DMSO, Table 4) was a multiple-copy homology-dependent integration at *ura4*. The frequency of convertants to *ura4*$^+$ is similar to that obtained for integrants at *leu1-32*. It is interesting to note that for both plasmids, the highest transformation efficiencies were obtained using low amounts of transforming DNA in conjunction with single-stranded carrier DNA and DMSO.

Based on the above results, a plasmid was constructed for easy sub-cloning of a gene to be integrated at *ura4*. Plasmid pJK210 was constructed by inserting the 1.8-kb *Hin*dIII fragment of the *ura4* gene into the unique *Nde*I site of pJK142 (Figure 1). This plasmid, similar to pJK148, also contains a diverse polylinker allowing for easy cloning of DNA into the plasmid. The plasmid also contains the *E. coli lacZα* gene for blue/white color selection of inserts, and the *ura4*$^+$ gene contains a unique *Stu*I restriction site allowing linearization prior to integration. This plasmid has been transformed into a *ura4-294* strain and gives rise to Ura$^+$ colonies. These integrations have not been analyzed by genomic blot analysis.

DISCUSSION

Homologous integration in *S. pombe* has traditionally been thought to be a low frequency event, but few thorough analyses of integration have been published. The results reported here definitively show that homologous integration at *ura4-294* and *leu1-32* in *S. pombe* can occur at a high frequency. The frequencies of homologous integration and conversion to wild type are similar to those observed in *S. cerevisiae*; homology-dependent events are the rule, not the exception. Additionally, we never observed colonies on the control plates to which no transforming DNA had been added, indicating that the reversion rates of *ura4-294* and *leu1-32* are beyond the level of detection. These results demonstrate these loci to be excellent targets for homologous integration.

ura4-294 was previously reported to give a low frequency of homologous integration, with a high frequency of conversion to *ura4*$^+$ (GRIMM and KOHLI 1988). However, the data presented here show this locus to be as good as *leu1-32* for targeted integration. The number of events studied in the previous report was small, such that the results obtained could be a statistical aberration. In this report over 100 transformants targeted to *ura4-294* were examined, and we are confident that our data gives an accurate view of transformation events obtained. Although this locus in general gave more multiple integrants than *leu1-32*, the relative fraction of homologous integration and conversion events was the same as for *leu1-32*. The transformation procedure used in this study differs slightly from that used in previous studies; namely, we used carrier DNA and DMSO. However, even when we performed transformation experiments using the same transforming vector DNA and transformation procedure that GRIMM and KOHLI reported, homologous integration, as opposed to gene conversion, was observed. The reasons for high frequencies of gene conversions at *ura4-294* obtained in the past remain elusive. The medium used for selecting transformants was also different. However, comparison of the media shows insignificant differences, and it is thus unlikely that this could account for the different rates of gene conversion observed.

The *suc1* locus has also given conflicting results in frequencies of homologous integrations. The *suc1* locus of *S. pombe* was reported to yield a very low disruption frequency in a single-step disruption experiment (HAYLES *et al.* 1986). GRALLERT *et al.* (1993) recently studied integration at this locus with the intent of finding conditions which would improve the frequency of homologous integration events. They were able to obtain a high disruption frequency at *suc1*. Importantly, they discovered that using LiAc as opposed to spheroplasting as the transformation method gave a markedly higher number

of disruptions. This is the most likely explanation of how they were able to obtain a much higher frequency of homologous integrants as compared to previous experiments. As reported here, they also found that the amount of transforming plasmid DNA had no effect on the fraction of transformants bearing homologous integration events.

An integration system utilizing *sup3-5* suppression of the *ade6-704* nonsense mutation is also available (CARR *et al.* 1989). As *sup3-5* can suppress *ade6-704* in single copy, but is deleterious in multiple copies, single copy integrations are easily identifiable following transformation by selecting fast-growing white colonies. GRALLERT *et al.* (1993) used the *sup3-5* gene in conjunction with *ura4* selection to achieve a single-step *ura4* disruption at the *suc1* locus. The presence of the *sup3-5* gene at the 3′ end of the transforming fragment allows for selection of the desired double crossover events between the *suc1* chromosomal locus and the transforming fragment, as these integrants would all be Ura^+ Ade^-. Using this system, 21 of 22 Ura^+ Ade^- transformants were found to contain the proper disruption.

Thus, homologous integration has now been shown to occur at a very high frequency at several loci in *S. pombe*. The plasmids we have constructed should prove very useful for targeting integration of genes of interest to the *leu1* and *ura4* loci. This is particularly useful in assessing the activity of various gene constructs when needing to control for position effects. These plasmids should also be useful for targeting genomic DNA inserts to their homologous chromosomal positions (*i.e.*, for gene disruptions) in *leu1* or *ura4* auxotrophic strains. The background rate of integration at *leu1* or *ura4* in this case is unknown, but due to the lack of any homologous DNA between the *ura4* clone and the *ura4-D18* locus (in which the entire *Hin*dIII fragment containing *ura4* has been deleted from the genome (GRIMM *et al.* 1988)), neither integration at *ura4-D18* nor gene conversion should take place. Since *ura4-294 S. pombe* are resistant to the selective drug 5-fluoro-orotic acid (BOEKE *et al.* 1984), pJK210 should be useful for gene replacement by the two-step procedure. The gene being targeted for replacement would be cloned into the polylinker of pJK210. After the plasmid has been integrated at this locus, two copies of the gene of interest would be present, separated by the *ura4* gene. A recombination event occurring between the two copies removes the *ura4* gene. These colonies can be selected on medium containing 5-fluoro-orotic acid. Finally, integrating plasmids containing other *S. pombe* selectable genes can now be easily constructed by inserting those genes into the *Nde*I site of pJK142.

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