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TRANSFORMATION IN YEAST: DEVELOPMENT OF A HYBRID CLONING VECTOR AND ISOLATION OF THE *CAN1* GENE

(*Saccharomyces cerevisiae;* plasmid vector; arginine permease)

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SUMMARY

We have constructed a plasmid, YEp13, which when used in conjunction with transformation in yeast is a suitable vector for isolating specific yeast genes. The plasmid consists of pBR322, the *LEU2* gene of yeast, and a DNA fragment containing a yeast origin of replication from 2μ circle. We have demonstrated the utility of this cloning system by isolating the yeast gene encoding the arginine permease, *CAN1*, from a pool of random yeast DNA fragments inserted into YEp13.

INTRODUCTION

A detailed analysis of eukaryotic regulation is predicated upon the isolation of DNA fragments containing specific genes of interest. While recombinant DNA technology provides a means of cloning random fragments of genomic DNA, the current limitation in isolating specific genes is the identification of an appropriate clone amongst a bank of random clones. Investigators who have isolated specific genes from the yeast, *Saccharomyces cerevisiae*, have relied on two general techniques: (1) expression of yeast DNA in *E. coli*, as manifested either by complementation of *E. coli* mutations or by production of immunologically cross-reactive material (Struhl et al., 1976; Ratzkin and Carbon, 1977; Clarke et al., 1979); and (2) hybridization with specific RNA or DNA probes to banks of random yeast clones (Beckman et al., 1977; St. John and Davis, 1979; Petes et al., 1978). However, both these techniques have substantial inherent limitations.

The demonstration of yeast transformation with cloned DNA by Hinnen et al. (1978) has afforded the possibility of a third and, perhaps, more general technique for isolating specific yeast genes and possibly higher-eukaryotic genes; namely, identification by expression and complementation directly in yeast.

Beggs (1978) has shown that this technique is feasible by isolating de novo the *LEU2* gene of yeast from a random pool of yeast DNA containing clones through transformation of a *leu2*$^-$ strain of yeast. In this paper we describe an improved vector for cloning specific genes by transformation of yeast similar to several plasmids described by Struhl et al. (1979). In addition, we demonstrate the utility of this system by describing the isolation of a yeast gene, the locus encoding the arginine permease of yeast, *CAN1*, which could only have been obtained by selection in yeast.

MATERIALS AND METHODS

(a) Strains and media

Yeast strains used in this study include strain DC5 (a *leu2*-3 *leu2*-112 *his3 can1*-11), strain DC85 (**a** *leu2*$^-$ *his4 cry1*), strain DC55 (**a** *arg4 leu2*-3 *leu2*-112 *lys2 try CAN1*) and strain DC76 (α *arg4 leu2*-3 *leu2*-112 *lys2 ade6 can1*-11). The *leu2*-3 *leu2*-112 allele present in these strains was constructed by Hinnen et al. (1978), and has a reversion frequency to Leu$^+$ of less than $1/10^{12}$ (no revertants observed). Synthetic medium (SC) consists of 0.67% yeast nitrogen base (Difco), 2% glucose, 2% Difco agar, and amino acid, purine, and pyrimidine supplements as described (Whelan et al., 1979). YEPD medium contains 1% yeast extract, 2% Bactopeptone and 2% glucose. Regeneration agar for yeast transformation consists of SC plus 1 M sorbitol and 1% YEPD broth with appropriate amino acids deleted.

Plasmids were propagated in *E. coli* strain C600 (*leuB thr pro*) grown in Luria Broth (Miller, 1972) supplemented with ampicillin (100 μg/ml) or tetracycline (25 μg/ml) as required or on M9TP (1XM9 salts (Miller, 1972), 1% glucose, 1 mM $MgCl_2$, 20 μg/ml thiamine, 100 μg/ml threonine, and 10 μg/ml proline). *E. coli* strains were grown in M9CAA (1XM9 salts, 1% casamino acids, 1% glucose, 1 mM $MgCl_2$, and 20 μg/ml thiamine) for large-scale plasmid preparations.

(b) DNA preparation

Yeast DNA was isolated from appropriate strains by the method of Cryer et al. (1975) using equilibrium centrifugation in CsCl as a final purification step. Plasmid DNA was isolated from *E. coli* by standard procedures. Restriction endonuclease digestions were performed in buffer containing 6 mM Tris · HCl, pH 7.8, 6 mM $MgCl_2$ and 6 mM β-mercaptoethanol using commercial restriction enzymes (New England BioLabs). Reactions were terminated by incubation at 65°C. Ligation reactions consisted of 50 mM Tris·HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 40—100 units/ml T4 DNA ligase (New England BioLabs), and the indicated concentration of digested DNA and were performed at 19°C for 2 h or at 4°C for 16 h. DNA samples were labeled in vitro by the method of Maniatis et al. (1975). Fractionation of DNA was accomplished by electrophoresis on horizontal agarose slab gels poured and run in 40 mM Tris-acetate, pH 7.6, 1 mM EDTA, and 1.0 μg/ml ethidium

bromide. Transfer of DNA from agarose gels to nitrocellulose filters was effected by the method of Southern (1975).

Hybridization of labeled DNA to DNA immobilized on nitrocellulose filters was performed as described elsewhere (Hinnen et al., 1978).

(c) Transformations

Transformation of yeast cells with purified plasmid DNA was performed as described elsewhere (Hinnen et al., 1978; Hicks et al., 1978).

Transformation of yeast can also be accomplished by mixing yeast protoplasts with bacterial cells harboring plasmid DNA after treatment with lysozyme. Bacterial cells were grown selectively overnight either in 10 ml liquid cultures or on LB plates containing the appropriate antibiotic. $5 \cdot 10^9$ cells were suspended in 25% sucrose, 50 mM Tris pH 7.8, washed once and resuspended in the same buffer plus 1 mg/ml lysozyme. After 10 min on ice, cells were pelleted and resuspended in 50 µl of 1 M sorbitol, 10 mM Tris, pH 7.8, and 10 mM $CaCl_2$. This suspension was then used in place of the DNA solution in the standard transformation protocol.

Transformation of bacterial cells with DNA was performed using calcium shocked *E. coli* cells as described elsewhere (Petes et al., 1978).

(d) Construction of YEp13

Plasmid YEp13 was constructed in two steps as described below. pBR322 (Bolivar et al., 1977) and plasmid pMB9-B1 (pMB9 containing 2µ circle cloned at one of the 2µ circle *Eco*RI sites; Beggs, 1978) were digested with *Eco*RI, ligated, and then used to transform *E. coli* to ampicillin resistance. The resultant transformants were screened by in situ hybridization with labeled 2µ circle DNA (Grunstein and Hogness, 1976) to identify those clones containing pBR322 into which 2µ circle had been inserted. One such plasmid, CV4, was shown by subsequent restriction analysis to contain the small *Eco*RI fragment of 2µ circle, B form. Hicks et al. (1978) have shown that this fragment promotes the efficient replication in yeast of plasmids into which it has been inserted. CV4 was then partially digested with restriction endonuclease *Pst*I and incubated in the presence of T4 ligase with restriction fragments resulting from a complete *Pst*I digestion of plasmid pY*eleu10* (ColE1 carrying the complete *LEU2* gene of yeast; Ratzkin and Carbon, 1976). The ligation mixture was used to transform *E. coli* strain C600 to ampicillin resistance and the resultant transformants screened for complementation of the *leuB* mutation. The plasmid from one such Leu^+ Amp^R Tet^R clone was purified and designated YEp13, the restriction map of which is shown in Fig.1.

(e) Construction of a YEp13 yeast DNA bank

50 µg of *Bam*HI digested genomic DNA isolated from strain DC56 was incubated with 10 µg *Bam*HI digested YEp13 DNA in a 0.3 ml ligation reaction. 1 µl aliquots of this ligation mixture were used to transform *E. coli* strain C600 to ampicillin resistance. The resulting transformants were screened for

tetracycline resistance and the tetracycline-sensitive clones (which constituted 10—20% of the total number of transformants) retained. In this manner 6600 *amp*^{R} *tet*^{S} clones were obtained and stored individually. 33 cultures, each consisting of cells from 200 separate clones, were grown and used as sources of plasmid DNA.

RESULTS

Fig.1 shows a diagram of the plasmid YEp13, on which are indicated the relevant restriction sites. It consists of the entire bacterial plasmid pBR322, the small *Eco*RI restriction fragment of the B form of the yeast plasmid 2μ circle, and a *Pst*I restriction fragment containing the entire *LEU2* gene of yeast, derived from plasmid pY*eleu10*. There are several features of this plasmid which make it a highly suitable vehicle for cloning by transformation in yeast. These features are discussed separately below.

(1) Efficient transformation. YEp13 transforms appropriate *leu2*$^{-}$ yeast strains to Leu^{+} at an efficiency of 10^{3} to 10^{4} colonies per μg of DNA. This efficiency permits one to screen the equivalent of the entire yeast genome starting with only a few micrograms of an appropriate pool of random yeast DNA fragments inserted into the plasmid. An additional benefit of this efficiency is that purification of the plasmid is not a prerequisite for transformation of yeast: we obtain Leu^{+} transformants by mixing yeast spheroplasts di-

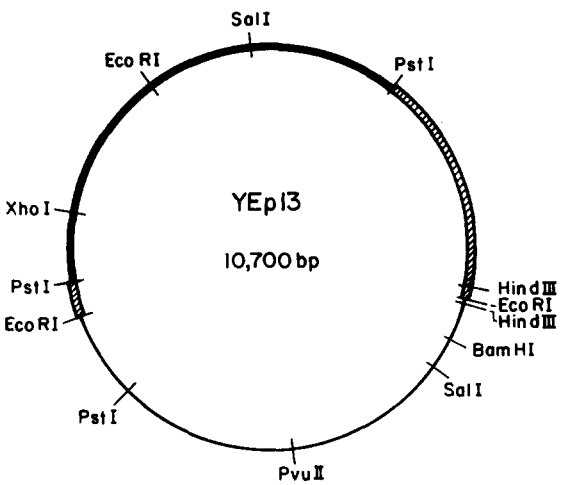


Fig. 1. Schematic drawing of plasmid YEp13. The location of restriction sites on plasmid YEp13, constructed as described in MATERIALS AND METHODS, was determined by single and multiple restriction digestions of the purified plasmid. Fragment sizes were determined by comparison of migration distances with those of pBR322 restriction DNA fragments of known size after electrophoresis on agarose gels. The thin line in the schematic indicates pBR322 sequences; the heavy line, sequences derived from plasmid pY*eleu10*; and the hatched line, 2μ circle sequences.

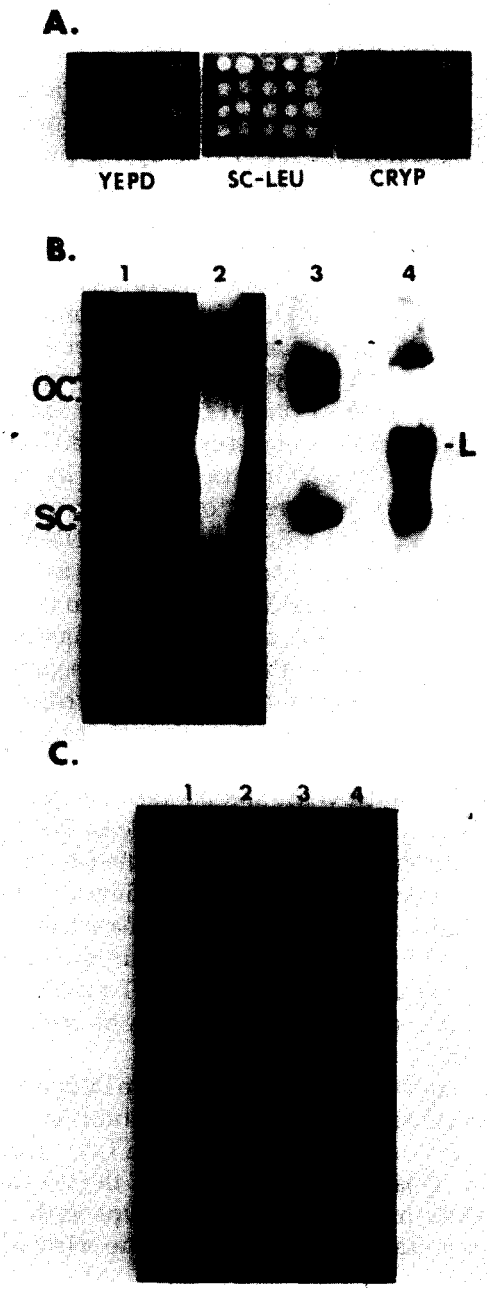
rectly with bacterial protoplasts harboring the plasmid as described in MATERIALS AND METHODS. These yeast transformants are in all respects identical to those obtained by transformation with purified DNA.

(2) Replication in yeast and bacteria. YEp13 can replicate efficiently as an intact plasmid in both yeast and *E. coli.* This ability is conferred in the former case by the presence of the 2μ circle DNA and in the latter case by the presence of pBR322 sequences. The ability of YEp13 to replicate in yeast is demonstrated by several criteria. First, the *LEU*$^+$ determinant on YEp13 segregates as a non-Mendelian element in genetic crosses (Fig.2a). That is, YEp13 is distributed to all four meiotic products and is therefore not maintained by stable integration into a chromosome. Second, uncut DNA isolated from a *leu2*$^-$ yeast strain transformed to Leu$^+$ with YEp13, can transform *E. coli* to resistance to ampicillin and tetracycline at an efficiency of approx. 10—20 bacterial transformants per μg yeast DNA. Plasmids isolated from these bacterial transformants have identical sizes and restriction patterns as YEp13 DNA used initially to transform the yeast strain (Fig.2c). Third, as described in paragraph (3) below the plasmid can be lost by mitotic segregation. Fourth, uncut DNA isolated from a *leu2*$^-$ strain transformed with YEp13 contains a DNA species with homology to pBR322 which migrates at the same position as purified YEp13 DNA (Fig.2b). DNA homologous to pBR322 is found also in the region of the gel at which host chromosomal DNA migrates. This may indicate that in vivo some of the YEp13 molecules are integrated into the genome, as suggested by Struhl et al. (1979). However, by the criteria described above, YEp13 can be considered functionally to be an independently replicating plasmid.

The bifunctional replication of YEp13 in both yeast and bacteria facilitates the isolation of specific yeast genes. A bank of clones of random yeast DNA fragments inserted into YEp13 can be constructed and then amplified by *E. coli.* This pool of DNA can then be used to transform yeast, selecting for complementation of a particular gene of interest. The plasmid in the yeast transformant can then be isolated and amplified by extracting DNA from the yeast clone and transforming *E. coli*, selecting for ampicillin resistance. Thus the DNA fragment corresponding to the yeast gene complemented can be recovered directly from the yeast transformant.

(3) Mitotic segregation in yeast. Although the replication of YEp13 is efficient in yeast, one can obtain Leu$^-$ segregants of a *leu2*$^-$ strain harboring YEp13 by growth of the strain on leucine containing media. These Leu$^-$ segregants arise at approx. 1% per generation. This is a useful feature of the vector since it allows one to confirm the association of a selected phenotype with the plasmid, by demonstrating the concomitant loss of Leu$^+$ and the selected phenotype following this mitotic segregation.

(4) Availability of cloning sites. YEp13 retains intact the ampicillin and tetracycline resistance genes of pBR322, and contains a single *Bam*HI restriction site in the *TET*^{R} gene. Consequently, using standard ligation procedures, fragments generated either by *Bam*HI or *Bgl*II restriction digestion (since


A.
YEPD
SC-LEU
CRYP
B.
1
2
3
4
OC
-L
SC
C.
1
2
3
4

*Bam*HI and *Bgl*II digestion of DNA yield the same four base overlap) can be inserted at this site. Alternatively, DNA can be inserted following random shear or digestion by some other enzyme using poly(A):poly(T) tailing techniques. Plasmids containing inserts can be isolated in *E. coli* by transformation to ampicillin resistance and screening for tetracycline sensitivity. In addition, retention of the intact ampicillin resistance gene permits recovery in *E. coli* of the plasmid from specific transformed yeast clones, as described above.

(5) Facilitated transformation with LEU2. The presence of the *LEU2* gene on YEp13 enhances its potential as a cloning vector in two ways. First, the selection of a specific gene by transformation of an appropriate yeast strain with YEp13 containing random DNA insertions can be performed as a double selection, requiring complementation of both *leu2*$^-$ and the gene of interest. This obviates the need for a nonreverting mutation in the gene one wishes to complement. That is, since 10^4 Leu$^+$ transformants from a pool of YEp13 DNA containing random DNA insertions are sufficient to ensure a high probability of introducing any one particular region of the yeast genome into at least one clone, complementation of a mutation whose reversion frequency is no higher than 1 in 10^5 can be readily accomplished. Second, there is no requirement for a direct selection for complementation of the gene of interest. One needs only a scorable phenotypic difference between the recipient strain and the desired transformant. One can select 10^4 Leu$^+$ transformants and screen these by replating on appropriate media to score for a desired change in the phenotype of the strain.

The use of this plasmid is illustrated in the following section in which we describe the isolation of the *CAN1* gene of yeast.

Fig. 2. Replication of YEp13 in yeast. (A) Strain DC5 (a *leu2*$^-$ *his3 can1*) was transformed with YEp13 to Leu$^+$ and then crossed with strain DC85 (α *leu2*$^-$ *his4 cry1*). The resultant diploid was sporulated and several asci dissected and the spores grown on YEPD. The figure shows the growth of the spore clones after replication to SC minus leucine and YEPD+ cryptopleurine. Each vertical column represents the four spores from a single ascus. Segregation of cryptopleurine resistance shows the standard 2:2 pattern characteristic of a single nuclear gene. Segregation of the Leu$^+$ determinant shows a non-Mendelian pattern associated with cytoplasmic inheritance. (B) 5 μg total uncut DNA from strain DC5 transformed to Leu$^+$ with YEp13 (lanes 2 and 4) and 0.1 μg purified YEp13 DNA (lanes 1 and 3) were fractionated on a 1% agarose gel, transferred to nitrocellulose, and probed with labeled pBR322 DNA. The ethidium bromide staining pattern of the gel is shown in lanes 1 and 2 and the autoradiogram obtained following hybridization is shown in lanes 3 and 4. The staining pattern and autoradiogram are presented on the same scale. SC denotes the positions of migration of supercoiled YEp13; OC indicates the open circular forms of the plasmid (both monomer and dimer forms of the plasmid are present in the preparation); and L indicates the position of migration of linear genomic DNA. The fourth band in lane 1 is contaminating *E. coli* DNA. (C) Ethidium bromide staining pattern of YEp13 DNA (lanes 1 and 2) and plasmid DNA recovered from strain DC5 transformed with YEp13 (lanes 3 and 4), after digestion with *Eco*RI (lanes 1 and 3) or *Pst*I (lanes 2 and 4) and fractionation on a 1% agarose gel.

Isolation and characterization of DNA containing the CAN1 *gene of yeast.*

The *CAN1* gene of *S. cerevisiae* codes for an arginine permease which, during growth on SC, is the sole transport system for arginine into the cell. This fact permits extensive genetic manipulation of the locus. *CAN1*$^+$ strains of yeast are sensitive to the arginine analogue, canavanine, since it is transported by the arginine permease into the cell and incorporated into protein with deleterious results. *can1*$^-$ strains of yeast on the other hand, are resistant to the analogue, since it is excluded from the cell by the absence *of* a functional arginine permease. Similarly, a *can1*$^-$ *arg*$^-$ strain cannot grow on SC, since it cannot transport arginine into the cell to supply its auxotrophic requirement. Such a strain will grow however, on synthetic media lacking ammonium sulfate (Whelan et al., 1979). In this case, arginine is transported into the cell through the general amino acid permease, which is relieved from repression by the absence of ammonium. A *can1*$^-$ *arg*$^-$ strain will also grow on YEPD, either for the reason cited above or through use of peptides to supply its arginine requirement. A *CAN1 arg*$^-$ strain grows perfectly well on SC. Thus, positive selections for both forward and reverse mutation of the *CAN1* locus exist.

We isolated the *CAN1* gene of yeast by transformation of strain DC76 (*arg4 can1*-11 *leu2 lys2 ade6*) to growth on SC minus leucine, using DNA from pools of clones of YEp13 into which random fragments of yeast DNA had been inserted (see MATERIALS AND METHODS). Introduction of an intact copy of either the *CAN1* gene or the *ARG4* gene carried on YEp13 would allow growth on this medium. However, since DNA used to construct the clone bank was isolated from a strain containing an *arg4* mutation, reversion of the phenotype by introduction of the *ARG4* gene is unlikely in this particular transformation.

Transformation of strain DC76 with DNA from one of the pools of two hundred random YEp13 clones, but from none of the others (cf. MATERIALS AND METHODS), yielded several colonies which could grow on SC—leucine. Several genetic criteria suggest that these transformants contain the *CAN1* gene of yeast carried on YEp13. First, these transformants are unable to grow on SC minus arginine, indicating that the auxotrophic requirement for arginine is still present in the strain. Second, the ability of the transformants to grow on SC is genetically associated with the Leu$^+$ phenotype conferred by YEp13: Leu$^-$ mitotic segregants of the transformants of strain DC76 lose the ability to grow on SC. Third, the ability to grow on SC is inherited in a non-Mendelian fashion: that is, the genetic determinant responsible for growth on SC in an *arg*$^-$ *can1*$^-$ genetic background is transmitted to all four meiotic products following mating and sporulation. Finally, the plasmid displays the second phenotype associated with the *CAN1* locus. Genetic transfer of the plasmid to an Arg$^+$ *can1*$^-$ strain, which in the absence of the plasmid is resistant to canavanine, renders the strain sensitive to this analogue.

The plasmid which confers the Can$^+$ phenotype was recovered by transformation of *E. coli* to ampicillin resistance with uncut DNA isolated from the

DC76 transformant. Size analysis of plasmids thus recovered revealed two distinct size classes. However, direct transformation of strain DC76 with lysozyme-treated bacteria harboring one or the other size plasmid demonstrated that each contained DNA which would allow growth of strain DC76 on SC minus leucine. Restriction analysis of the smaller of the two plasmids (Fig.3), designated TLC-1, indicates that the plasmid consists of YEp13 plus an extra 4.6 kilobases of DNA inserted in the *Bam*HI site (one of the expected *Bam*HI sites bracketing the insert is missing, even though a detailed restriction analysis of the plasmid indicates that the entirety of YEp13 is present in TLC-1). *Arg*$^-$ *can1*$^-$ yeast strains transformed with this plasmid have the identical phenotype as the original transformant, indicating that the plasmid as isolated from *E. coli* contains DNA responsible for the selected phenotype. Restriction analysis of the larger plasmid indicates that it consists of TLC-1 into which has been inserted the entire 2μ circle genome (data not shown), a result which is discussed below.

Confirmation of the identity of the cloned DNA fragment in plasmid TLC-1 was established as described below. Since plasmid TLC-1 contains the *LEU2* gene of yeast in addition to the newly cloned fragment, we first generated a derivative plasmid by *Eco*RI digestion of TLC-1, religation at low DNA concentration, and transformation of *E. coli* to ampicillin resistance. This plasmid, TLC-1-RI, corresponds to the portion of TLC-1 between the *Eco*RI site at 8 o'clock on the map in Fig.3 and the *Eco*RI site at 5 o'clock and consists only of pBR322 and a *Bam*HI-*Eco*RI subfragment of the presumed *CAN1* region. This plasmid was used as a probe to determine the size of DNA restriction fragments in various yeast strains which were homologous to the cloned

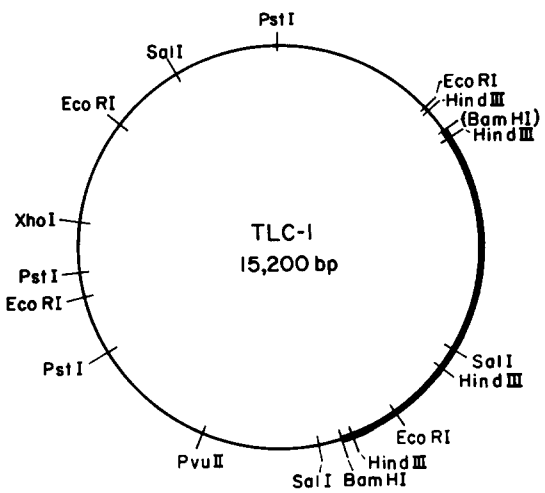


Fig. 3. Schematic drawing of plasmid TLC-1. Restriction sites on the plasmid were determined as described in the legend to Fig.1. The thin line represents YEp13 DNA sequences and the heavy line, the inserted DNA fragment containing the *CAN1* gene.

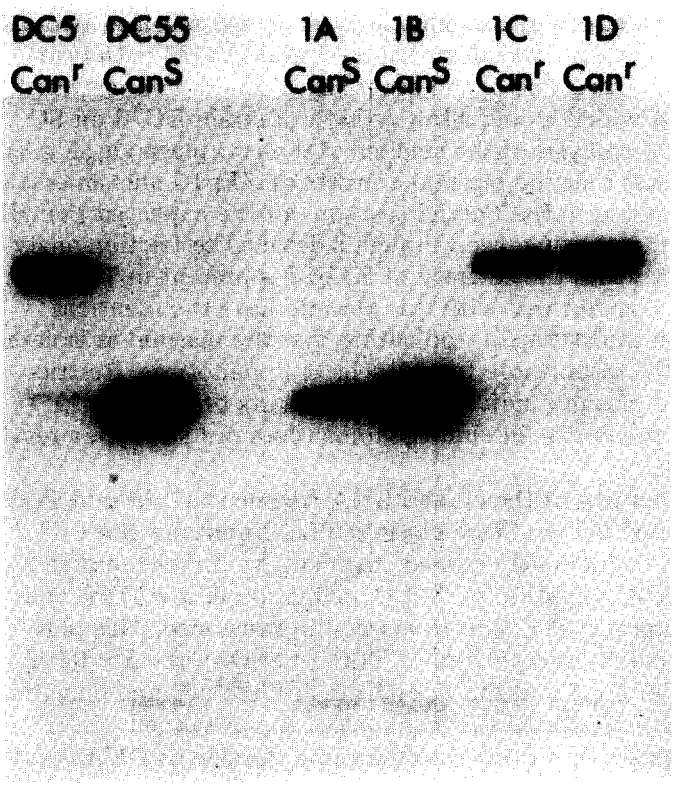


Fig. 4. Linkage of the fragment cloned in TLC-1 to the *CAN1* gene. DNA was isolated from strain DC5 (lane 1), strain DC55 (lane 2), and the four spore clones (lanes 3—6) derived from dissection of one ascus obtained after mating strains DC-5 and DC-55 and sporulation of the resultant diploid. 5 μg of each DNA were digested with *Eco*RI, fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with labeled TLC-1-RI DNA. The autoradiogram in the figure shows the size of the restriction fragment in the genome of each strain which is homologous to TLC-1-RI DNA. Strains DC5, 1C, and 1D are *can1*$^-$ (Can^r) and strains DC55, 1A, and 1B are *CAN1*$^+$ (Can^S).

DNA fragment. Figure 4 shows an autoradiogram obtained by hybridization of labeled TLC-1-RI DNA to *Eco*RI-digested yeast DNA from a *CAN1*$^+$ and a *can1*-11 strains after Southern transfer of the fractionated genomic DNA. Both strains contain a single *Eco*RI restriction fragment homologous to the yeast DNA contained on TLC-1-RI. However, the size of this fragment from the two strains is different. This fortuitous heterogeneity allowed us to correlate the cloned DNA fragment with the *CAN1* gene by standard genetic analysis in a manner similar to that described by Olsen et al. (1978) to identify the cloned SUP4 gene. The two haploid strains were mated, sporulated, and dissected, and the resultant spore clone scored for resistance to canavanine and for the size of the *Eco*RI restriction fragment homologous to TLC-1-RI. The results of the analysis of one such tetrad is shown in Figure 4. The two cana-

vanine resistant spores contain the larger restriction fragment, as did the canavanine resistant parent, while the two canavanine sensitive spores contain the smaller. Seven out of seven tetrads analyzed gave the same results, which by standard calculation, gives a greater than 99% probability that the *CAN1* gene and the restriction fragment homologous to TLC-1-RI are linked. Thus, the cloned fragment conferring the Can$^+$ phenotype in yeast, is derived from the region of the genome at which the *CAN1* gene is located. Therefore, we conclude that the cloned fragment contains the *CAN1* gene.

DISCUSSION

In this paper we have described a hybrid plasmid, YEp13, whose features make it an appropriate vector for selecting specific DNA fragments by transformation in yeast. First, random DNA fragments can be readily inserted into the plasmid and the resultant hybrid molecules selected, propagated, and amplified in *E. coli*. Second, transformation of yeast with YEp13 containing random DNA fragments is efficient. Third, random fragments cloned into YEp13 can be introduced into yeast cells by transformation to Leu$^+$ and simultaneously selected or subsequently screened for complementation of a mutation in a gene of interest. This obviates the requirement for a nonreverting mutation in the gene desired or even the requirement for a positive selection for the anticipated complementation. Fourth, the efficient replication of the plasmid in yeast allows extensive genetic analysis. Since the plasmid is transmitted to all four spores following meiosis, it can be transferred to other strains to establish cosegregation of Leu$^+$ and the selected fragment and to perform subsequent genetic tests. Finally, the cloned fragment can be recovered directly by transformation of *E. coli* to ampicillin resistance with crude DNA isolated from the yeast transformant.

We have demonstrated the viability of plasmid YEp13 used in conjunction with yeast transformation for cloning specific DNA fragments by using it to isolate the *CAN1* gene of yeast. The plasmid we recovered following selection for a Can$^+$ Leu$^+$ transformant, TLC-1, consists of YEp13 plus a 4.6 kilobase fragment of yeast DNA and confers a Can$^+$ phenotype on strains harboring it; *arg*$^-$ *can1*$^-$ strains containing TLC-1 are able to grow on SC, and *ARG*$^+$ *can1*$^-$ strains containing the plasmid are sensitive to canavanine. In addition, by tetrad analysis we have shown that a specific DNA restriction fragment of the yeast genome homologous to the cloned fragment is tightly linked to the *can1*-11 allele. Therefore, we conclude that the fragment we cloned contains the *CAN1* gene. In addition to isolating the *CAN1* gene, we have used YEp13 to clone the *HIS3* gene — which has previously been isolated by other techniques and a DNA fragment which appears to carry the mating type locus, which will be described elsewhere.

The availability of DNA containing the *CAN1* gene and of selection procedures for both forward and reverse mutations in the gene encourages the pursuit of several potentially fruitful lines of investigation. Whelan et al. (1979)

have generated an extensive fine structure map of the *CAN1* locus and have isolated deletion and/or inversions within the gene. With the use of the cloned fragment these genetic data can be evaluated in terms of the physical structure of the gene. In addition, the availability of the *CAN1* gene suggests the possibility of a further refinement of the yeast vector to facilitate cloning in yeast directly from ligation mixes. By using a cloning site within the *CAN* gene of TLC-1, or some variant of this plasmid, and applying appropriate selection procedures, only those plasmids which have incorporated an exogeneous DNA fragment would be capable of productive transformation. That is, plasmid TLC-1 cannot transform a *leu*$^-$ *can*$^-$ strain to growth on minimal media containing canavanine, since the *Leu*$^+$ transformants would carry the arginine permease and would thus be sensitive to the analogue. However, a TLC-1 derivative in which the *CAN1* gene was inactivated by insertion of a DNA fragment would give rise to Leu$^+$ Can ^{R} transformants. Thus, in a transformation under these conditions using DNA from a ligation reaction between TLC-1 and random DNA fragments, each of the resultant Leu$^+$clones would harbor a plasmid containing an exogenous piece of DNA. Finally, the arginine permease encoded by the *CAN1* gene is presumably a membrane associated protein. Thus, the cloned DNA fragment may allow sequence determination of a eukaryotic signal peptide and may offer the possibility of functional analysis of this region by appropriate genetic engineering.

In the course of these studies we have observed that several types of DNA rearrangements in our plasmids can occur at low frequency during propagation in yeast. The most common form of plasmid rearrangements occurs by insertion of the 2μ circle genome. As described in RESULTS, a larger plasmid, TLC-4, recovered in *E. coli* from a strain DC76 transformant consists of plasmid TLC-1 into which had been inserted the entire 2μ circle genome. The structure of TLC-4 suggests that the insertion occurred by homologous recombination between the inverted repeat region of the 2μ circle fragment in TLC-1 and the inverted repeat region of an A form 2μ circle in the cell, presumably by the same mechanism which catalyzes interconversion of 2μ circle. Such recombinant molecules have also been observed by Gerbaud et al. (1979), although the frequency with which we recover these molecules is lower than that reported by these workers. It should be noted that the occurrence of these aberrant plasmids is not so frequent as to impair the usefulness of YEp13 as a cloning vector, although the plasmid should be used with the *caveat* that these rearrangements can occur. On the other hand, with proper selection procedures and analyses, such alterations may provide a useful tool to generate specific plasmids in vivo which might otherwise prove difficult to construct in vitro.

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