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Yeast/*E. coli* Shuttle Vectors with Multiple Unique Restriction Sites

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Two yeast/*E. coli* shuttle vectors have been constructed. The two vectors, YEp351 and YEp352, have the following properties: (1) they can replicate autonomously in *Saccharomyces cerevisiae* and in *E. coli*; (2) they contain the β-lactamase gene and confer ampicillin resistance to *E. coli*; (3) they contain the entire sequence of pUC18; (4) all ten restriction sites of the multiple cloning region of pUC18 including *EcoRI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI* and *HindIII* are unique in YEp352; these sites are also unique in YEp351 except for *EcoRI* and *KpnI*, which occur twice; (5) recombinant plasmids with DNA inserts in the multiple cloning region of YEp351 and YEp352 can be recognised by loss of β-galactosidase function in appropriate *E. coli* hosts; (6) YEp351 and YEp352 contain the yeast *LEU2* and *URA3* genes, respectively, allowing for selection of these auxotrophic markers in yeast and *E. coli*; (7) both plasmids are retained with high frequency in yeast grown under non-selective conditions indicative of high plasmid copy number. The above properties make the shuttle vectors suitable for construction of yeast genomic libraries and for cloning of DNA fragments defined by a large number of different restriction sites.

The two vectors have been further modified by deletion of the sequences necessary for autonomous replication in yeast. The derivative plasmids YIp351 and YIp352 can therefore be used to integrate specific sequences into yeast chromosomal DNA.

KEY WORDS — Shuttle vectors; gene cloning; *Saccharomyces*

INTRODUCTION

Complementation of mutant strains of yeast by transformation with genomic libraries provides a rapid and simple means of cloning yeast genes (Nasmyth and Reed, 1980; Broach *et al.*, 1979). Shuttle vectors containing the region of the 2 μ circle of *Saccharomyces cerevisiae* necessary to confer replicative function in yeast are often used for the construction of recombinant plasmid libraries due to their high transformation frequencies (Beggs, 1978, 1978; Struhl *et al.*, 1979; Gerbaud *et al.*, 1979). Vectors of this type also contain sequences conferring replicative function in *Eschericha coli*, the β-lactamase gene of *E. coli* to confer ampicillin resistance, and a wild type copy of a yeast gene (e.g. *LEU2, URA3, HIS3*) allowing for selection of plasmid-bearing yeast clones in a host strain containing the appropriate auxotrophic marker. Commonly used shuttle vectors capable of autonomous replication contain three or fewer unique restriction enzyme recognition sequences for use as cloning sites (Parent *et al.*, 1985; Baldari and Cesareni, 1985).

In the present communication we report two variants of a shuttle vector with a greater number of unique cloning sites. The vectors contain the entire sequence of pUC18 (Yanisch-Perron *et al.*, 1985), allowing for identification of recombinant plasmids by screening for α-complementation of the *lacZΔM15* mutation of *E. coli* (Gronenborn and Messing, 1978). We have found these vectors particularly useful for subcloning yeast genes on fragments of DNA defined by restriction enzyme recognition sequences of six base pairs. Both vectors have been modified by removal of the sequences which confer replicative function in yeast to produce integrative vectors capable of transformation only by recombination with the yeast genome.

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MATERIALS AND METHODS

Media, strains and transformations

Non-selective medium for yeast (YPD) contained 1 per cent yeast extract, 2 per cent peptone and 2 per cent glucose. Selective medium for yeast (WO) contained 0·67 per cent yeast nitrogen base minus amino acids and 2 per cent glucose supplemented as required with tryptophan, uracil, histidine, adenine and leucine at 25 μg/ml. *E. coli* was grown in LB medium (Davis *et al.*, 1980) supplemented with 40 μg/ml ampicillin when required for selection of plasmids. E medium (Davis *et al.*, 1980) supplemented as required was used for selection of specific markers in *E. coli*. Solid media contained 2 per cent or 1·5 per cent agar for growth of yeast and *E. coli*, respectively. *Saccharomyces cerevisiae* strain W303-1B (α *leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100* [cir$^+$] obtained from R. Rothstein, College of Physicians and Surgeons, Columbia University, New York, NY, U.S.A., was transformed with autonomously replicating or integrative plasmids either by the method of Beggs (1978) or by that of Ito *et al.* (1983). Transformants were selected on minimal glucose media lacking either uracil or leucine but supplemented for the other auxotrophic requirements of W303-1B. *E. coli* strain RR1 (*proA, leuB6, lacY, galK2, xyl5, mtl1, ara14, rpsL20, supE44, hsdS, λ*$^-$) was used for maintenance of plasmids and for selection of plasmids containing the yeast *LEU2* gene. *E. coli* strain MC1066 (*F*$^-$, *Δ(lac)X74, hsdR, rpsL, galU, galK, trpC9830, leuB6, pyrF::Tn5*) was used for selection of plasmids containing the yeast *URA3* gene. *E. coli* strain TB1 (*Δlac-pro, strA, ara, thi, hsdR, Φ80dlacZΔM15*) obtained from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A., was used to verify expression of the *lacZ'* fragment of pUC18 as described (Viera and Messing, 1982). All bacterial transformations were by the $CaCl_2$ procedure (Cohen *et al.*, 1972).

Miscellaneous procedures

Standard techniques were used for preparation of recombinant plasmids from *E. coli*, restriction enzyme digestions, agarose gel eletrophoresis, isolation of restriction frament from agarose gels, ligation of restriction fragments, screening of transforming DNAs, radioactive labelling of DNA by nick-translation and colony hybridisations (Maniatis *et al.*, 1982). Fragments with protruding 5' ends were converted to blunt-ended fragments using the Klenow fragment of *E. coli* DNA polymerase I (Maniatis *et al.*, 1982). Fragments with protruding 3' ends were converted to blunt-ended fragments by treatment of approximately 1 μg DNA with 1·0 unit of S1 nuclease in 50 μl of 30 mM NaCl, 1 mM $ZnCl_2$, 35 mM sodium acetage, pH 4·75, for 2 min at 37°C. All regions of DNA which were altered by converting protruding ends to blunt ends were characterised by DNA sequence analysis according to the method of Maxam and Gilbert (1977).

RESULTS AND DISCUSSION

Construction of the autonomously replicating plasmids YEp351 and YEp352

The yeast/*E. coli* shuttle vector YEp351 was constructed by ligation of the entire pUC18 sequence to a fragment of DNA containing both the 2 μ circle sequences necessary for replication and the wild type yeast *LEU2* gene. pUC18 was linearised at the unique *NdeI* site and made blunt-ended by filling in the protruding 5' ends in the presence of Klenow fragment. The *LEU2* gene and the replication origin of the yeast 2 μ circle were obtained as a single 2·95 kb *BamHI-XbaI* fragment from the plasmid pC4B (Broach, 1983). This fragment was also made blunt-ended by treatment with Klenow fragment. The two fragments were ligated and used to transform *E. coli* RR1. Clones harboring plasmids with the β-lactamase gene were selected on ampicillin medium and the presence of the *LEU2* gene was verified by replica plating the transformants onto minimal medium supplemented for all the requirements of RR1 except leucine. Several ampicillin-resistant and leucine-prototrophic transformants were confirmed by restriction mapping to have plasmids containing both fragments used in the ligation (Figure 1). This plasmid designated YEp351 has all the unique sites of the multiple cloning region of pUC18, except for *EcoRI* and *KpnI*, each of which also occurs once in the *LEU2* sequence (Andreadis *et al.*, 1984).

A similar approach was used to obtain the shuttle vector YEp352 with the yeast *URA3* gene as a selectable marker. This construction entailed ligation in a single reaction of three separate fragments of DNA: pUC18 linearised at the *NdeI* site, a 1·4 kb *RsaI* fragment of the yeast 2 μ circle containing the sequences necessary for autonomous replication (Broach, 1981), and the wild type *URA3* gene isolated as a 1·1 kb *HindIII-SmaI* fragment.

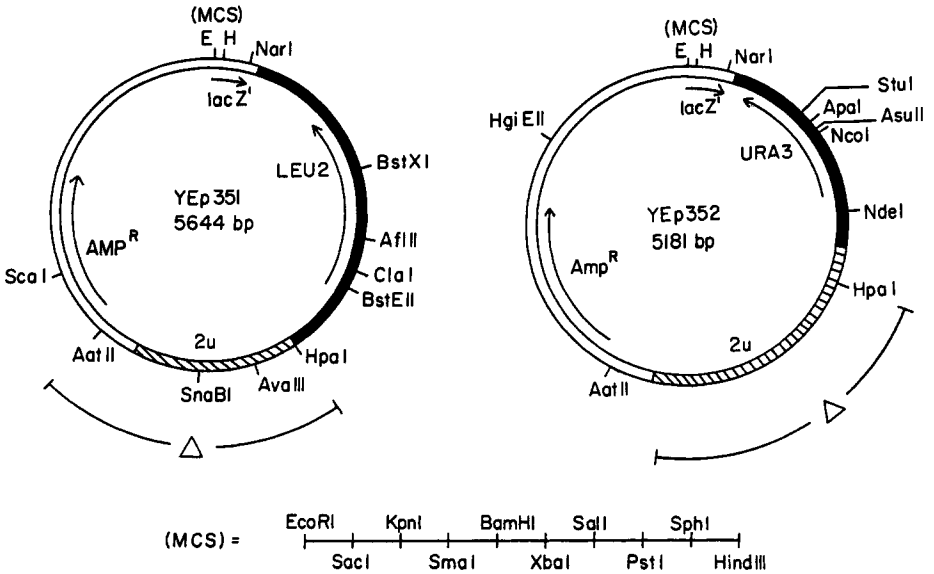

Figure 1. Restriction maps of YEp351 and YEp352. Only unique restriction sites are shown. (MCS) represents the multiple cloning region of pUC18 (Yanisch-Perron *et al.*, 1985) including *EcoRI* (E), *SstI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI*, and *HindIII* (H). Nucleotide number 1 in each vector is the 'A' of the initiation codon of *lacZ'*. The solid areas represent yeast genomic sequences, the open areas represent PUC18 sequences, and the cross-hatched areas represent yeast 2 μ circle sequences. In YEp351 the yeast genomic sequence includes nucleotides 286–2256, the 2 μ circle sequence includes necleotides 2257–3241, and the pUC18 sequence includes nucleotides 3442–285. In YEp352 the yeast genomic sequence includes nucleotides 286–1383, the 2 μ circle sequence includes nucleotides 1384–2768, and the pUC18 sequence includes nucleotides 2769–285. The sequences marked Δ have been deleted from YEp351 and YEp352 to construct YIp351 and YIp352, respectively. The sequence deleted from YEp351 includes nucleotides 2259–3492 and the sequence deleted from YEp352 includes nucleotides 1611–2489.

The latter fragment was isolated from the 1·2 kb *HindIII* fragment containing *URA3* (Rose *et al.*, 1984) cloned into pUC18. Both the pUC18 and the *URA3* fragments were made blunt-ended with Klenow polymerase prior to the ligation. Following transformation of *E. coli*, clones were selected for ampicillin resistance and uracil independence. Surviving clones which also contained the 2 μ circle sequence were identified by colony hybridisation using the 1·4 kb *RsaI* fragment of this episome as a probe. Several clones were confirmed by restriction mapping to have plasmids containing all three fragments used in the ligation.

One such plasmid was further modified by removal of the second *XbaI* and *PstI* sites present in the 2 μ circle and in the *URA3* segments of the vector, respectively. The plasmid was first partially digested with *XbaI* and the linear plasmid consisting of a mixture cut either in the muliple cloning region of pUC18 or in the 2 μ circle region was isolated. The linear plasmid was treated with Klenow fragment and religated. After transformation of *E. coli* RR1, ampicillin-resistant clones were screened for plasmids containing the *XbaI* site in the pUC18 multiple cloning region but lacking the site in the 2 μ circle region. This plasmid was used to eliminate the *PstI* site in the 5' non-coding region of the *URA3* gene by an analogous procedure. In this case the 3' protruding ends of the *PstI* sites were removed by digestion with S1 nuclease. The resultant vector YEp352 is unique for all six base pair recognition sites of the pUC18 multiple cloning region (Figure 1).

To derive the complete nucleotide sequences of YEp351 and YEp352 all the regions at the ligated junctions and at the destroyed *PstI* and *XbaI* sites were sequenced. In every case the sequences were consistent with what was expected based on the known sequences of the DNA fragments used for the constructions. The complete nucleotide

sequences of YEp351 and YEp352 are available upon request.

Properties of YEp351 and YEp352

In addition to having a greater diversity of unique restriction sites for directed ligation of DNA fragments, the presence in YEp351 and YEp352 of the lacZ′ sequence of the pUC vectors allows detection of *E. coli* clones containing plasmids with inserts in the multiple cloning site of the lacZ′ region (Viera and Messing, 1982). This has been verified for both shuttle vectors by transformation of the host strain *E. coli* TB1 and plating in the presence of X-gal. Clones containing either native vector exhibit the characteristic blue colour indicative of functional β-galactosidase, while plasmids with inserts in the multiple cloning region give rise to colourless colonies.

YEp351 and YEp352 transform yeast with the high efficiency seen with other shuttle vectors containing the 2 μ circle replication origin such as YEp13 and YEp24. Like other episomal yeast plasmids, YEp351 and YEp352 segregate during vegetative growth in yeast, a useful property for ascertaining that the expression of a new phenotype is caused by the presence of the plasmid. After growth for 30–40 generations in non-selective medium, approximately 90 per cent of the cells show retention of the plasmid.

Construction of the integrating plasmids YIp351 and YIp352

The introduction of genes into yeast can be achieved either on autonomously replicating plasmids or on plasmids capable of integrating into chromosomal DNA (Hinnen *et al.*, 1978). Integration is required for stable transformation when the plasmid lacks a yeast origin of replication (Struhl *et al.*, 1979). The transformation efficiency of integrating plasmids has been shown to be greatly enhanced when they are introduced as linear fragments whose free ends have homologous sequences in chromosomal DNA (Orr-Weaver *et al.*, 1981).

The two autonomously replicating plasmids YEp351 and YEp352 were converted to integrating plasmids by removal of 2 μ circle sequences essential for their replication in yeast. In the case of YEp351 all 2 μ circle sequences were removed by digestion with *HpaI* and *AatII*. The linear vector lacking the yeast replication origin was purified and recircularised via unimolecular ligation of the non-homologous free ends. Similarly, the yeast replication origin was deleted from YEp352 by cleavage at the *SspI* and *HpaI* sites located within the 2 μ circle sequence. In this case some 2 μ circle sequence remains in the religated vector, however, replicative function has been lost since the *SspI* site lies within the defined sequence known to function as the 2 μ circle origin of replication (Broach, 1981). The resulting integrative plasmids YIp351 and YIp352 contain *LEU2* and *URA3*, respectively, as selectable markers and retain the pUC18 multiple cloning region (Figure 1).

YIp351 and YIp352 were used to transform yeast both as circular plasmids and as linear fragments cleaved at unique restriction sites within *LEU2* or *URA3*, respectively. The linearised plasmids gave rise to approximately 1000 transformants per μg DNA. Every transformant tested for retention of the selectable marker during growth in non-selective medium showed greater than 99 per cent stability, indicating integration of *LEU2* and *URA3* into chromosomal DNA. Transformation with the circular plasmids yielded a small number of prototrophic transformants over a high background of small colonies which did not grow when subsequently patched onto selective media. The small colonies may have arisen on the transformation plates from cells which acquired a plasmid that did not integrate into chromosomal DNA, enabling division for a limited number of generations.

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