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Transformation of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with linear plasmids containing 2μ sequences

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Linear plasmids were constructed by adding telomeres prepared from *Tetrahymena pyriformis* rDNA to a circular hybrid *Escherichia coli*-yeast vector and transforming *Saccharomyces cerevisiae*. The parental vector contained the entire 2μ yeast circle and the *LEU* gene from *S. cerevisiae*. Three transformed clones were shown to contain linear plasmids which were characterized by restriction analysis and shown to be rearranged versions of the desired linear plasmids. The plasmids obtained were imperfect palindromes: part of the parental vector was present in duplicated form, part as unique sequences and part was absent. The sequences that had been lost included a large portion of the 2μ circle. The telomeres were ~450 bp longer than those of *T. pyriformis*. DNA prepared from transformed *S. cerevisiae* clones was used to transform *Schizosaccharomyces pombe*. The transformed *S. pombe* clones contained linear plasmids identical in structure to their linear parents in *S. cerevisiae*. No structural re-arrangements or integration into *S. pombe* was observed. Little or no telomere growth had occurred after transfer from *S. cerevisiae* to *S. pombe*. A model is proposed to explain the genesis of the plasmids.

Key words: 2μ circle/plasmids/*Saccharomyces cerevisiae*/*Schizosaccharomyces pombe*/transformation

Introduction

Linear plasmids have emerged as indispensable tools for the study of telomere structure and function and may also prove to be generally useful in eukaryotic cells. With such plasmids it may, in fact, be possible to avoid some of the problems (such as plasmid instability, integration, and re-arrangements) that arise when circular plasmids are used in higher eukaryotic cells. To date, linear plasmids have been used almost exclusively in the yeast *Saccharomyces cerevisiae* (Szostak and Blackburn, 1982; Murray and Szostak, 1983; Dani and Zakian, 1983).

The ideal linear plasmid should be mitotically stable and should be able to replicate in the cells of many different species at a high copy number. The construction of such plasmids has been carried out essentially accordingly to the original protocol of Szostak and Blackburn (1982), which consists in the ligation of telomeres derived from *Tetrahymena thermophila* rDNA onto linearized yeast plasmids and subsequent transformation of *S. cerevisiae*. During replication in this organism, ~200 bp of the $C_{1\text{-}3}A$ telomeric sequences of *S. cerevisiae* telomeres are added to the ends of the *Tetrahymena* telomeres (Shampay *et al.*, 1984).

Acentric circular and linear plasmids are mitotically unstable in yeast. The stability of circular plasmids is greatly increased by the addition of sequences derived from the centromeric region of several *S. cerevisiae* chromosomes, called *CEN* sequences (Clarke and Carbon, 1980; Stinchcomb *et al.*, 1982). The increased stability engendered by *CEN* sequences is accompanied by a drastic drop in the copy number to a value of about one, which is a desirable feature in a mini-chromosome, but usually not in a plasmid. The mitotic stability of circular plasmids in yeast can also be increased by the addition of sequences derived from the stable endogenous DNA plasmid called the 2μ circle, which is contained in almost all strains of *S. cerevisiae* (Futcher and Cox, 1984). The replication of the plasmid is under the control of four loci: the origin of replication (*ORI*); two open reading frames, *REP1* and *REP2*, active in *trans*; and the *REP3* locus, close to *ORI* and active in *cis*. This control system allows the plasmid to replicate more than once per cell cycle when the copy number is low (Yayaram *et al.*, 1983). Another reading frame, *FLP*, is responsible for the high frequency site-specific recombination between two inverted repeats (*IRS*) which yields a mixture of two monomeric forms (A and B) of the plasmid and various multimeric forms (Broach *et al.*, 1982).

The development of techniques for high frequency transformation (Beach and Nurse, 1981) of the fission yeast *Schizosaccharomyces pombe* has made it possible to carry out gene cloning experiments in this simple eukaryote, as well as in *S. cerevisiae*. The experiments described here constitute an attempt to reach two distinct objectives: (1) to construct a stable, high copy number linear plasmid in *S. cerevisiae*; (2) to construct a linear shuttle vector able to replicate in both *S. cerevisiae* and *S. pombe*. To this end, *S. cerevisiae* was transformed with a linear *Escherichia coli*-yeast hybrid plasmid bearing *Tetrahymena* telomeres and containing the entire 2μ circle. The plasmids obtained were characterized and then used to transform *S. pombe*.

Results

Construction of the plasmid vector

Starting material for the construction was plasmid CV20, which contains the entire 2μ circle in the B form and the *LEU2* gene from *S. cerevisiae* cloned into pBR322. To be able in the future to use our plasmids in yeast strains prototrophic for leucine, we introduced the *neo* gene into this vector (the *neo* gene codes for resistance to the antibiotic G418 in eukaryotes and to kanamicin in prokaryotes). CV20, which has three *Cla*I sites, was digested partially with *Cla*I and totally with *Bam*HI. The vector was then ligated to the 1400-bp *Cla*I-*Bam*HI fragment prepared from plasmid pBRneo and containing the *neo* gene. Transformation of *E. coli* strain SF8 with the ligation mixture and selection for kanamycin-resistant colonies yielded the recombinant plasmid designated CVneo. A linear version of the restriction map for this plasmid, which is ~13.9 kb in length is given in Figure 3a.

Construction of linear plasmids in S. cerevisiae

Telomeres for the construction of linear plasmids were obtained from the macronuclear rDNA of *T. pyriformis*. This molecule

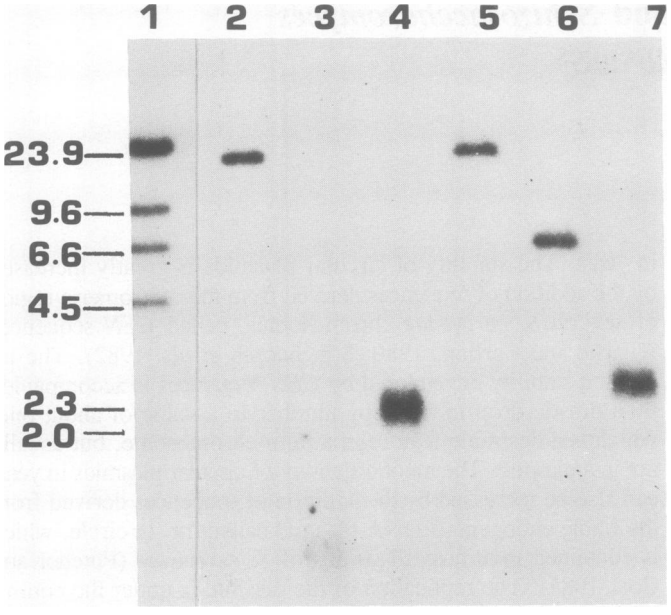

Fig. 1. Hybridization of DNA extracts of pScT6 to a ^{32}P-labelled C_4A_2 probe. **Lane 1:** *Hind*III-digested λ DNA hybridized to a ^{32}P-labelled λ DNA probe; **lane 2:** undigested pScT6; **lanes 3–7:** pScT6 digested with *Bam*HI, *Eco*RI, *Hind*III (not digested), *Pst*I and *Kpn*I. In each digest only one fragment homologous to C_4A_2 is revealed by autoradiography.

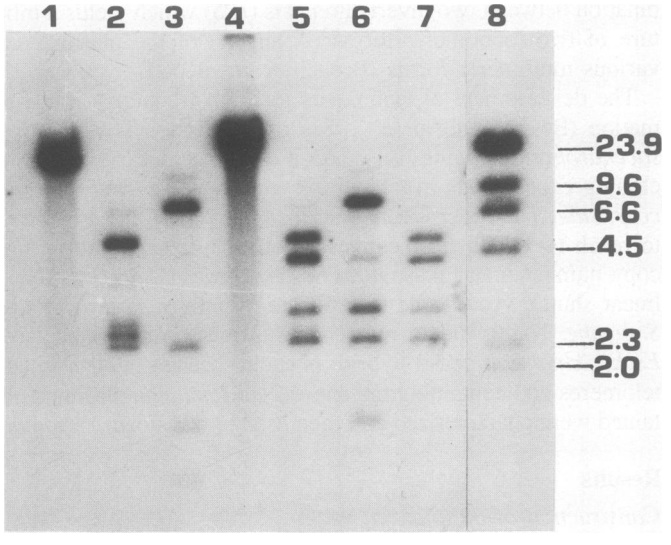

Fig. 2. Hybridization of DNA extracts of pScT6 and CVneo to a ^{32}P-labelled CVneo probe. **Lane 1:** undigested pScT6; **lanes 2** and **3:** pScT6 digested with *Eco*RI and *Hind*III; **lane 4;** undigested CVneo; **lanes 5** and **6:** CVneo digested with *Eco*RI and *Hind*III; **lane 7:** purified CVneo DNA digested with *Eco*RI; **lane 8:** *Hind*III-digested λ DNA hybridized to a ^{32}P-labelled λ DNA probe.

has a restriction map very similar to that of *T. thermophila* rDNA and contains two *Bam*HI restriction sites at a distance of ~750 bp from each end (Niles and Jain, 1981). rDNA purified as described in Materials and methods was digested with *Bam*HI and telomeric fragments were electroeluted from a preparative agarose gel. These *Bam*HI fragments were ligated to CVneo linearized with *Bam*HI, and the ligation mixture was used to transform *S. cerevisiae* strain YT3247. 22 Leu$^+$ clones were obtained, 19 of which contained circular plasmids. In fact they produced multiple bands on blots prepared with α ^{32}P-labelled CVneo probe and they transformed *E. coli* to kanamicin resistance. For the remaining three clones only one plasmid DNA band was revealed by hybridization of the blotted filters with the same probe. One of the three plasmids, pScT4, migrated on agarose gels as a linear molecule of 18 kb and the two others, pScT5 and pScT6, migrated as linear molecules of 19.5 kb. The length of these plasmids was greater than expected. In fact a 750-bp telomeric fragment should have been added to each end of the linearized 13.9-kb CVneo plasmid vector, giving rise to a 15.4-kb molecule. Furthermore, ~200 bp of the repeats characteristic of *S. cerevisiae* telomeres should have been added *in vivo* to each extremity giving rise to a molecule of ~16 kb.

To confirm that the plasmids were linear and to explain the excessive size of the molecules we determined the physical map of one of the plasmids, pScT6, by restriction and hybridization analyses.

Structure of plasmid pScT6

pScT6 DNA extracts digested with several enzymes were separated on agarose gels and the blotted filters were hybridized with ^{32}P-labelled probes. The hybridization bands revealed by autoradiography were compared with the corresponding bands obtained by digestion of CVneo DNA.

To measure the length of the plasmid telomeres we used a ^{32}P-labelled C_4A_2 probe prepared from *T. thermophila* rDNA on the assumption that *T. thermophila* and *T. pyriformis* rDNAs possess the same telomeric sequences. With this probe the *Bam*HI digest revealed one smeared band (see lane 3 of Figure 1) with an average size of ~1200 bp. The smearing is a distinctive feature of small telomeric fragments and is caused by the characteristic size heterogeneity of telomeres (Blackburn and Gall, 1978). The size of the fragment demonstrated that ~450 bp of telomeric sequences had been added by *S. cerevisiae*. Clearly the size of the plasmid could not be explained in terms of telomeric growth. With the same probe only one hybridization band was seen with all the other enzymes used, instead of the expected two bands, one for each end of the plasmid. The sizes of the fragments obtained were: 2450 bp for the *Eco*RI digest, 7500 bp for *Hind*III and 2750 bp for *Kpn*I. These results demonstrate that the distal sites of the three enzymes used are situated symmetrically on the molecule. Hybridization of the *Eco*RI and *Hind*III digests with the ^{32}P-labelled CVneo probe, as shown in Figure 2, revealed all the fragments present in the plasmid including the terminal fragments described above. The internal fragments were then characterized by hybridization of the same digests with a pBRneo probe and a 2μ probe prepared as described in Materials and methods. This made it possible to establish that the *neo* gene was not present on the plasmid and that two fragments were present which share homology with the same 1686-bp 2μ fragment containing *ORI*. All the results described above made it possible to assemble the physical map of pScT6 displayed in Figure 3b. The plasmid is an imperfect palindrome from which all of the *neo* gene sequences and part of the 2μ sequences are missing. The unique central portion consists of 4590 bp of 2μ sequences flanked on both sides by 7450 bp of identical sequences terminating with the *LEU2* gene and the telomere. The physical map of pScT6 is shown in Figure 3b.

The copy number of pScT6 was nine copies per plasmid-bearing cell, as determined by the hybridization experiments described in Materials and methods. The copy number was also determined for pScT4 and pScT5 and was found to be 13 and 10 respectively.

Transformation of S. pombe

Minipreparations of DNA from *S. cerevisiae* strains harbouring plasmids pScT4, pScT5, pScT6 and CVneo were used to

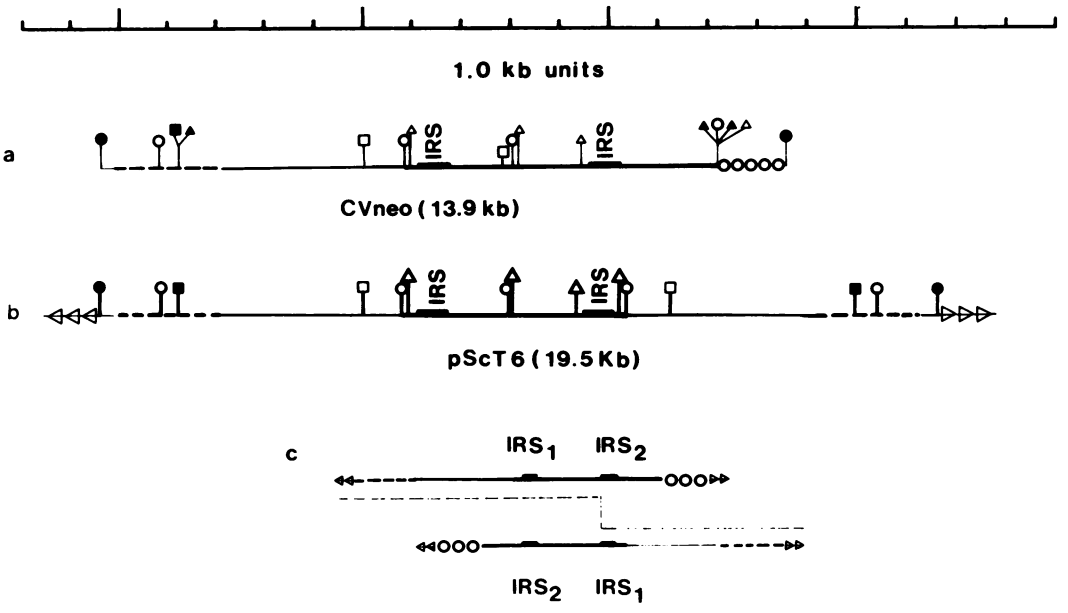

Fig. 3. (a): restriction map of the circular recombinant plasmid CVneo (13.9 kb) shown in linear form, as if cut at the *Bam*HI site. (b): restriction map of the linear plasmid pScT6 (19.5 kb). The two plasmids are aligned at the *Bam*HI site. (c): schematic representation of a hypothetical recombination event between two linear molecules consisting of the entire CVneo vector and telomeres. Symbols: thin line (———) = pBR322; thick line (▬) = 2μ DNA; circles (°°°°) = *neo* gene; dashed line (----) = *LEU2* gene; triangles (△△△) = telomeres; IRS_1 and IRS_2 indicate the inverted repeats of the 2μ plasmid. The cleavage sites for the restriction enzymes are indicated as follows: *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, and *Pst*I.

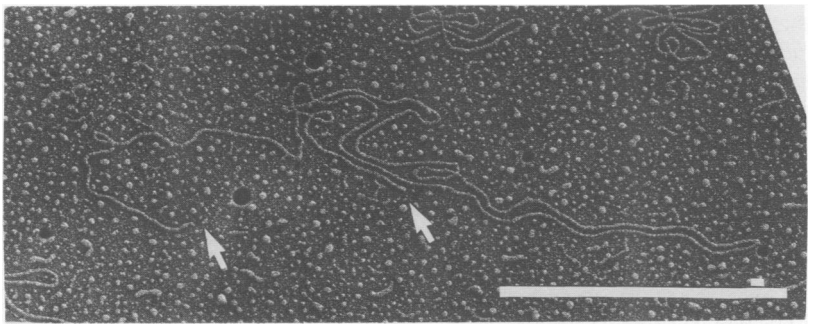

Fig. 4. Electron micrograph of plasmid pSpT6, Arrows indicate the ends of the linear molecule. Bar = 0.5 μm.

transform *S. pombe leu 1.32*. For each transformation, samples containing ~50 ng of plasmid DNA and ~200 ng of high mol. wt. chromosomal DNA were used. The same efficiency of transformation was obtained with linear and circular plasmids, and in all cases a number of Leu^+ colonies grew on minimal plates after 5–6 days of incubation at 30°C. The efficiency of transformation was ~300 transformants/μg DNA and one transformant per 5 x 10^6 viable regenerated protoplasts. Mini-preparations of DNA were obtained from several *S. pombe* Leu^+ clones and samples were analysed by agarose gel electrophoresis for the presence of linear plasmids. Ethidium bromide stained gels showed that all Leu^+ clones obtained by transformation with linear plasmids gave a single plasmid DNA band and that the putative linear plasmids were present at high copy numbers. Three plasmids (pSpT4, pSpT5 and pSpT6) were selected for further study. Several Leu^+ clones obtained by transformation with CVneo were also analysed. We did not observe any integrative transformation either with the linear or with the circular plasmids, and all plasmids rescued from *S. pombe* were of the same size and structure as the original plasmids in *S. cerevisiae*.

The three pSpT plasmids were hybridized with the C_4A_2 ^{32}P-labelled probe: pSpT4 gave a single DNA band of 18 kb and pSpT5 and pSpT6 each gave a single DNA band of 19.5 kb, confirming that the plasmids were linear and of the same size as the original plasmids. The digestion of pSpT6 with *Bam*HI, *Eco*RI, *Hind*III and *Kpn*I and hybridization with the C_4A_2 probe revealed the presence of only one telomeric band as already observed with the *S. cerevisiae* transformants. The sizes of the fragments obtained were very similar to those of pScT6 and in particular the *Bam*HI fragment did not appear to differ in size from the corresponding pScT6 fragment by more than 100 bp, if at all. Furthermore hybridization of pSpT6 undigested DNA with the 2μ DNA probe showed that the 2μ fragment was still present in the plasmid.

The copy number of the plasmid was shown to be at least 85 by means of the densitometric procedure given in Materials and methods. This high copy number made it possible to prepare pSpT6 DNA by electro-elution from agarose gels. The DNA was subsequently prepared for electron microscopy according to Davis *et al.* (1971) with 25% formamide. Examination under the Philips EM400 electron microscope, operating at 80 kV, showed that the DNA consists of linear molecules measuring ~19.5 kb; one such molecule is displayed in Figure 4.

Discussion

We have constructed linear plasmids by adding telomeric ends

of *T. pyriformis* to a linearized vector and shown that the telomeric fragments of the plasmids hybridze to a C_4A_2 probe. Thus, *T. pyriformis* possesses the same telomeric sequence as *T. thermophila*. Our initial objective was the construction of a linear plasmid in *S. cerevisiae* containing the entire 2μ circle. We obtained three very similar linear plasmids, but all three contain only part of the 2μ circle. The plasmids have the structure of imperfect palindromes, which suggests that they are the products of recombination events. The simplest hypothesis that we can propose to explain the physical map of these linear plasmids is that two linear molecules containing the entire plasmid vector have recombined as shown in Figure 3c to give a plasmid of the pScT type. This and other more complex hypotheses have two features in common: (i) only molecules containing the intact *LEU2* gene can survive under our selection conditions; (ii) the recombinant plasmids must have a selective advantage over the parental molecules. In all three linear plasmids recombination occurred at, or very close to, the inverted repeats of the 2μ circle; the inverted repeats are therefore preferred sites for mitotic recombination even in the absence of FLP function.

Our second objective, the construction of a linear shuttle vector able to operate in both *S. cerevisiae* and *S. pombe*, was successful. Linear plasmids can replicate in *S. pombe* and telomeres that function in *S. cerevisiae* also function in *S. pombe*, with apparently little or no modification. The C_4A_2 sequences contained in the pScT plasmids are still present and the length of the telomeres is very similar in both series of plasmids. We do not know whether specific telomeric sequences are added to the plasmids by *S. pombe*; if the addition is extensive, it must occur at the expense of other sequences. Although the two yeasts are not closely related it is not impossible that they possess and use the same $C_{1\text{-}3}A$ repeats in their telomeres. The requirements for efficient extrachromosomal replication of linear plasmids containing 2μ sequences appear to be similar in the two yeast hosts since introduction of the plasmids constructed in *S. cerevisiae* into *S. pombe* does not result in structural rearrangements or integration into the genome.

A feature of the pSpT plasmids that has already proved useful in simplifying the preparation of significant quantities of plasmid DNA from *S. pombe* is their high copy number in this yeast species. This phenomenon could be due to a relatively inefficient complementation of the *S. pombe LEU1* function by the *S. cerevisiae LEU2* gene, since the two genes, which both encode the same enzyme (β-isopropylmalate dehydrogenase), do not exhibit sequence homology (Beach *et al.*, 1982).

Materials and methods

Strains and plasmids

S. cerevisiae YT3247 (*leu* 2-3, *leu* 2-112, *his* 3-11, *his* 3-15, [cir^0]) was kindly provided by A. Hinnen. *S. pombe* (*leu* 1.32 h$^-$) was a gift from P. Nurse. The *E. coli* strain used was SF8 (*rec* BC, *hsd* R$^-$, *hsd M*$^+$) (*Struhl et al.*, 1976). The amicronucleate *T. pyriformis* strain GLC6 was furnished by H. Lipps. The plasmids used in this work were: CV20 (Broach and Hicks, 1980), pBRneo (Southern and Berg, 1982) and pGY39 (Kiss *et al.*, 1981).

Media

S. cerevisiae was grown on YEPD/2 (0.5% yeast extract, 1% peptone, 2% glucose) and on the minimal medium MC (0.67% yeast nitrogen base without amino acids, 2% glucose). *S. pombe* was grown on YD (1% yeast extract, 2% glucose) and on the minimal medium MP (0.67% yeast nitrogen base without amino acids, 0.5% KH_2PO_4, 200 μg/ml L-asparagine, 2% glucose). MC and MP were supplemented with 80 μg/ml leucine when necessary. 2% agar was added to all media for plates. *E. coli* was grown on LB (0.3% yeast extract, 1% tryptone, 0.5% NaCl), supplemented with 1.5% agar for plates. *T. pyriformis* was grown on YEPD/2 at 23°C under aeration.

Transformation conditions

S. cerevisiae was transformed according to Beggs (1978). *S. pombe* was transformed according to Beach and Nurse (1981) except that a 3-fold higher protoplast concentration was used. Transformation of *E. coli* was carried out by the method described by Cohen *et al.* (1972).

Preparation of probes and hybridization conditions

DNA probes were ^{32}P-labelled by nick-translation as described by Maniatis *et al.* (1982). The 847-bp *Hin*dIII fragment, containing the *T. thermophila* C_4A_2 sequences, was obtained from plasmid pGY39. The 1686-bp 2μ DNA fragment, containing *ORI*, was obtained by digesting plasmid CV20 with *Eco*RI and *Hpa*I. From the same plasmid the 2150-bp *LEU2* fragment was obtained by digestion with *Ava*I and *Sal*I. In all cases, the desired fragments were electro-eluted from agarose gels.

Transfer of DNA from agarose gels to nitrocellulose filters was carried out as described by Southern (1975). Hybridization reactions (Maniatis *et al.*, 1982) were performed at 68°C with the exception of those in which the C_4A_2 probe was used, which were carried out at 60°C. Kodak X-AR-5 film was used for autoradiography and intensifying screens (Dupont Cronex Lightning-plus) were used at −70°C whenever necessary.

Extraction of DNA

Circular plasmid DNA was prepared from *E. coli* cultures by the method of Bolivar and Backman (1979) and purified on CsCl ethidium bromide gradients. Minipreparations of yeast DNA from cells grown to late exponential phase in MC or MP medium were carried out according to Struhl *et al.* (1979). To determine the copy number in *S. cerevisiae*, DNA was extracted according to the procedure of Cryer *et al.* (1975). *T. pyriformis* rDNA was prepared according to Wild and Gall (1979) from exponential cultures and electro-eluted from agarose gels.

Determination of copy number

The copy number of the pScT plasmids was determined by hybridization of DNA extracts digested with *Bam*HI with a ^{32}P-labelled *LEU2* probe. Sections of the filters corresponding to the two bands containing the chromosomal and plasmid *LEU2* genes were cut out and the radioactivity counted in a scintillation counter. The ratio of chromosomal to plasmid counts was halved, as two copies of the *LEU2* gene are present on the plasmid.

Technical problems prevented the above procedure from being used in *S. pombe*. The copy number of pSpT6 was determined by quantitative analysis of DNA extracts run on agarose gels using as a reference known amounts of marker DNA run on the same gels. The gels stained with ethidium bromide and photographed under appropriate conditions were scanned with a Joyce densitometer. By this method a lower limit to copy number is determined.

In all cases the copy number of the plasmids was corrected for the fraction of plasmid-bearing cells.

Enzymes and chemicals

Restriction endonucleases (New England Biolabs), alkaline phosphatase (Boehringer), DNaseI (Miles), DNA polymerase (New England Biolabs), and T4 ligase (New England Biolabs) were used according to the suppliers' specifications. [α^{32}P]ATP, specific activity 3200 Ci/mmol, was purchased from New England Nuclear. All other chemicals were of the highest purity form.

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