Volume 4 Number 5 1977 Nucleic Acids Research

Analysis of chromosomal integration and deletions of yeast plasmids

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Received 10 January 1977

ABSTRACT

Plasmid DNAs from six strains of Saccharomyces cerevisiae were compared. Three different plasmids were found, designated Scp 1, Scp 2 and Scp 3, with monomer lengths of 6.19, 6.06 and 5.97 kilobases as referenced to sequenced ØX174 DNA. DNA from each of the plasmids was inserted into a lambda vector DNA. Hybrid phage containing inserted DNA of the desired size were enriched by genetic selection and their DNAs analysed by rapid techniques. All three plasmids share the same organization, two unique sequences separated by two inverted repeats, and share basically the same DNA sequences. Scp 2 and Scp 3 differ from Scp 1 by missing a unique HpaI site and by having small overlapping deletions in the same region. The HpaI site in Scp 1 is, therefore, in a nonessential region and suitable for insertion of foreign DNA in the potential use of the yeast plasmid as a vector. Hybridization of labelled cloned plasmid DNA to restriction fragments of linear yeast DNA separated on agarose gels showed that the plasmid DNA was not stably integrated into the yeast chromosomal DNA.

INTRODUCTION

Certain strains of Saccharomyces cerevisiae (yeast) contain closed circular, double stranded DNA comprising about four percent of the total yeast DNA. It has been shown that this DNA has a monomeric circumference of approximately two microns and the same density as nuclear DNA[1,2,3]. There are 50 to 100 molecules per cell which are not located in the nucleus[4] or the mitochondria[5] but may be associated with a particulate fraction in the cytoplasm[6]. Bak, Christiansen and Christiansen[7] analysed the renaturation kinetics of yeast closed circular DNA and found a complexity of about six kilobases (kb) in agreement with its circumference. Petes and Williamson[8] showed that the circular DNA was self-replicating with one origin of replication as evidenced by observation of omega structures in the electron microscope. Thus, this DNA is a yeast plasmid. We refer to the most commonly observed type as Scp 1 (Saccharomyces cerevisiae plasmid) in preference to 2 micron or omicron DNA.

Recent studies of the structure of the yeast plasmids have been published by five groups of investigators[9,10,11,12,28]. Hollenberg et al.[9] analysed the structure of Scp 1 and found that there were two forms of the plasmid related by hypothetical recombination across a nontandem inverted sequence. They inserted these two forms of the plasmid, after cleavage with EcoRI endonuclease, into a bacterial plasmid vector and studied the clones obtained by electron microscopy and electrophoretic separation of various restriction fragments on agarose gels. This group and Guerineau, Grandchamp and Slonimski[10] proposed that the inverted repeat sequence might have the function of promoting integration into the chromosome as do certain repeated sequences in bacterial plasmids.

Our interest in the yeast plasmid arose because of its possible use as a self-replicating vector to allow transformation of yeast cells with DNA inserted into the plasmid DNA. No DNA transformation system has been developed for yeast cells, and they have no known DNA viruses. A related question is whether or not the yeast plasmid integrates into the nuclear or mitochondrial chromosomes and can carry an inserted fragment of DNA into the chromosome. Our first work on the structure of a yeast plasmid in preparation for an investigation of the above questions gave results which were similar to but quantitatively unlike those of C.P. Hollenberg et al.[9]. In the light of these results, we made precise measurements of various structural elements of the yeast plasmid as obtained from yeast cells and also from hybrids made by insertion of yeast plasmid DNA into a suitable strain of lambda bacteriophage. The yeast plasmid exists in the cell as monomers and multimeric forms[5], but we have only studied the monomeric form. In the course of these measurements two new species of yeast plasmids were discovered which have small alterations with respect to the more common type. Possible integration of the yeast plasmid was analysed. With certain reservations, we conclude that the plasmid does not stably integrate into the nuclear or mitochoncrial chromosomes.

MATERIALS AND METHODS

Preparation of yeast DNA. The procedure of Cryer, Eccleshall and Marmur[13] is used with the following modifications. The cells are grown to stationary phase in yeast extract (1%), peptone (2%) and dextrose (2%). Washed cells are incubated at room temperature in 0.5% β-mercaptoethanol for fifteen minutes. Washed, zymolase-prepared spheroplasts are lysed in sodium dodecyl sulfate plus proteinase K for five hours at 37° and 20 minutes at 60° (2 ml liquid per gram of cells), and the cell debris is spun

out at 25,000 G for twenty minutes. The supernatant is centrifuged through a 10 to 20% sucrose gradient onto a 1.8 g/ml cesium chloride shelf in a S W 25.2 rotor (24,000 RPM for 20 hours at 20°). The shelf solution is taken out after removing the upper layers (leaving behind a clear carbohydrate pellet) and adjusted to density 1.55 with solid cesium chloride and to 1 mg/ml in ethidium bromide. The linear DNA will often coprecipitate with anionic polysaccharide at this point and float to the top, where it is removed, leaving the closed circular DNA and most of the polysaccharide in solution which may be separated by centrifugation in the density gradient (S W 50.1, 30,000 RPM, 5°, 3-4 days). The precipitated linear DNA will redissolve after dilution with water, extraction with butanol and dialysis against 0.01 M tris·HCl, pH 7.5, 0.001 M EDTA (TE). It is then further purified by equilibrium centrifugation after dilution and adjustment to density 1.55 with CsCl and 0.5 mg/ml ethidium bromide. The yield is on the order of 0.5 mg DNA for 15 grams of cells.

Alkaline lysis of yeast cells for plasmid DNA purification. Spheroplasts are prepared and washed as above. They are thoroughly resuspended in 0.15 M sodium chloride, 0.1 M EDTA, pH 8.5 and lysed with stirring by making the solution 0.15 M in NaOH and 1% in sodium dodecyl sulfate (total volume fifty ml for 15 grams cell paste). The solution is stirred and pipetted vigorously to reduce viscosity. Then the pH is carefully brought up to pH 12.3, but not above, with NaOH, and kept there for twenty minutes. The lysate is neutralized after cooling on ice to a pH of about 8 with tris-HCl. It is made 1 M in potassium acetate and allowed to sit on ice for thirty minutes before sedimenting the potassium dodecyl sulfate-protein precipitate. 10 - 20 grams of well-washed and wet-ground nitrocellulose (Hercules RS 1/4 sec) is added to the supernatant and stirred for twenty minutes before being sedimented with the denatured linear DNA. The DNA in the supernatant is ethanol precipitated for an hour at -20°. The pellets are resuspended in TE buffer and made 1.55 g/ml with CsCl and 1 mg/ml ethidium bromide in a final volume of 25 ml. The closed circular DNA is separated from polysaccharides and some RNA by equilibrium centrifugation at 30,000 RPM, 5° for three days. The major central band is removed, extracted three times with isopropanol and dialysed. The resulting plasmid DNA is usually slightly contaminated with linear yeast DNA.

Growth of lambda bacteriophage and preparation of DNA. Bacteriophage are cultured and DNA prepared as described previously[14]. Rapid preparations of lambda DNA are made directly from well cleared

plate stocks. This technique utilizes the large quantity of unpackaged DNA in lambda lysates. We use purified agarose in the plates instead of agar because unpurified agar greatly inhibits restriction enzymes. The plate is overlayed in the cold with 5 ml of cold λ diluent (10 mM tris-HCl, pH 7.5, 10 mM $MgSO_4$) overnight. To 4 ml of this is added 0.4 ml 0.5 M EDTA (pH 8.5), 0.2 ml 2 M tris base and 0.2 ml 10% sodium dodecyl sulfate (SDS). After mixing, 10 microliters of diethyloxydiformate is added with shaking and the tubes are put at 65° for 30 minutes uncapped in a hood. They are cooled on ice, and one ml of 5 M potassium acetate is added. After one hour on ice, they are spun at 25,000 G for ten minutes, and the nucleic acids in the supernatant are precipitated with ethanol (11 ml). After one hour at -20°, the precipitate is redissolved in 0.4 ml TE buffer, finally adding tris-HCl, pH 7.5 to 0.1 M. Five microliters is usually an appropriate amount for gel electrophoresis. The whole procedure may be scaled down by a factor of ten for large numbers of preparations to be done in small capped tubes. The major contaminants are E. coli DNA and RNA. The latter can be degraded by treatment with ten micrograms per ml of pancreatic RNase for 15 minutes at 25° before adding the EDTA and SDS.

Alkaline lysis of E. coli for purification of plasmid vector DNA. This procedure is carried out similarly to the method of Sharp et al.[15]. Lysozyme is used to prepare spheroplasts from washed stationary phase cells (cells from a liter in 50 ml 10% sucrose, 50 mM NaCl, 50 mM tris-HCl, 5 mM EDTA, 1 mg/ml lysozyme, pH 8.0 at 25° for 15 minutes). The cells are lysed with 2 ml of 4 M NaOH and 25 ml 2% SDS, 0.15 M EDTA pH 8.5 which clears the solution. The rest of the procedure is identical to that followed with the yeast cells above. The yield of PMB9 DNA from HB101 is about one milligram per liter of saturated cells without chloramphenicol.

Preparation and characterization of bacterial plasmid hybrids. Viable molecular hybrids are prepared and transfected as previously described[16] with the following modifications specific to plasmid vectors. The plasmid vector used, PMB9, is a tetracycline resistance plasmid with length about 5.5 kb derived from ColE1 (provided by H.W. Boyer). It has one EcoRI site in a nonessential region. All plasmid DNAs were purified as described above. For the hybrid discussed in the text, 30 μg/ml Scp 2 EcoRI-A fragment (derived by cleavage of a lambda-Scp 2 hybrid) and 20 μg/ml of EcoRI cleaved PMB9 DNA were mixed and incubated with DNA ligase for 18 hours. The DNA was then used to transform calcium treated HB101 ($recA^-rK^-mK^-$). After preincubation in broth at 37° for 20 min,

cells were spread on plates with tetracycline (15 μg/ml). One nanogram of the EcoRI cleaved and DNA ligase treated PMB9 DNA gave 40% of the transformation efficiency obtained with untreated PMB9 DNA. Twelve of the potentially hybrid colonies were grown to saturation in 40 ml cultures and their DNA's prepared quickly by a crude lysate method of A. Rambach. The washed cells were converted to spheroplasts by incubation in 15% sucrose, 100 mM EDTA, 50 mM tris-HCl, pH 8, 1mg/ml lysozyme, at 0° for 15 minutes and lysed at 0° for 10 minutes by making the solution 0.05% in Triton X 100 (total volume of 4 ml). The solution is centrifuged at 25,000 G for 30 minutes at 2°. 10 microliters of diethyloxydiformate is added to the top two ml of supernatant and heated to 65° for 15 min. The mixture is centrifuged after cooling on ice and the supernatant is ethanol precipitated. The pellets are resuspended in 0.5 ml TE. Ten microliters is cleaved with EcoRI endonuclease in the appropriate buffer for examination by agarose gel electrophoresis. Six of the twelve colonies contained PMB9 with the Scp 2 EcoRI-A fragment inserted.

Length determinations of restriction fragments. Fragments longer than 1 kb were coelectrophoresed on 0.4% to 0.7% agarose gels[14] with EcoRI, HindIII and EcoRI-HindIII fragments of λCI857 DNA. Restriction endonucleases were purchased from New England Biolab except EcoRI which was purified as described previously[14] and HindIII and BamI which were kindly supplied by S. Goff. The lengths of λ DNA and various restriction fragments were recalibrated (P. Philippsen and R. Davis, manuscript in preparation) using the available sequence information for ØX174 DNA (F. Sanger, personal communication). From these data, the size of lambda DNA is taken as 49.0 kb. Fragments smaller than 1 kb were coelectrophoresed on 1.3% or 1.5% agarose gels and fragments smaller than 0.15 kb on 6% polyacrylamide gels with HindII fragments of ØX 174 DNA.

RESULTS

Restriction fragment analysis of plasmid DNA. Yeast DNA isolated as described in the methods section by sodium dodecyl sulfate and proteinase K lysis of spheroplasts and centrifugation through a sucrose velocity gradient contains linear and closed circular DNA. Cleavage with HindIII or EcoRI restriction nuclease gives a characteristic spectrum of bright bands from repeated DNA superimposed on many faint bands after agarose gel electrophoresis (Fig. 1, c and f). Further purification of the DNA on cesium chloride - ethidium bromide density gradients shows that five of

the repeated HindIII fragments and four of the repeated EcoRI fragments are derived from the closed circular DNA region of the density gradient (Fig. 1, d and g). Linear DNA from the density gradient gives only the known repeated fragments from ribosomal DNA[17] as shown for the HindIII fragments in Figure 1b. Thus, it is possible to separate the major repeated DNA bands seen with HindIII or EcoRI endonuclease digestions into those which originate from linear DNA (ribosomal DNA) and those from closed circular DNA (plasmid DNA). The one exception is the plasmid EcoRI-C fragment which is coincident on agarose gel electrophoresis with the second largest EcoRI fragment of ribosomal DNA. However, these fragments are not coincident on polyacrylamide gels.

Yeast plasmid DNA was isolated from total DNA as the lower band in cesium chloride - ethidium bromide density gradients or isolated from yeast cells by alkaline lysis as described above. The latter method is preferable because of better overall yields and higher purities with regard to contaminating nuclear and mitochondrial DNAs. Plasmid DNA isolated from six strains was analysed by cleavage with HindIII and EcoRI endonucleases. Three different patterns were observed. The corresponding plasmids were designated Scp 1, Scp 2 and Scp 3. Scp 1 was found in two a mating type and one α mating type haploid his 3 strains and one α mating type heterotroph (S288c) obtained from G. Fink. The restriction patterns of Scp 1 are shown in Figure 1, d and g. The restriction patterns of Scp 2, a plasmid isolated from a MAL GAL a type haploid (D585-11C) are shown in Figure 1, e and h. Two HindIII and two EcoRI fragments are deleted by about 0.1 kb as compared to Scp 1. The same two HindIII fragments are deleted even more (0.2 kb) in Scp 3, a plasmid isolated from a haploid strain disomic in chromosome six originating with R.K. Mortimer. Cleavage of Scp 3 with EcoRI yields only one large fragment of about 6 kb. Size determinations of these restriction fragments are compiled in Table I. Electrophoresis on 8% polyacrylamide gels failed to show any small HindIII or EcoRI fragments from plasmid DNA. Other restriction endonucleases tested were BamI, SalI, SstI, PstI and HpaI. The first three enzymes did not cleave the plasmid, the latter two did. PstI endonuclease converts closed circular Scp 1, Scp 2 and Scp 3 DNA into about 6.2, 6.1 and 6.0 kb long linear DNAs, respectively. It shortens the HindIII fragments A and C from all plasmids by 0.31 kb, and the EcoRI fragments B and D of Scp 1 and Scp 2 by 0.23 kb. HpaI endonuclease cleaves Scp 1 DNA once and shortens the Scp 1 EcoRI fragments B and D by 0.59 kb. Scp 2 and Scp 3

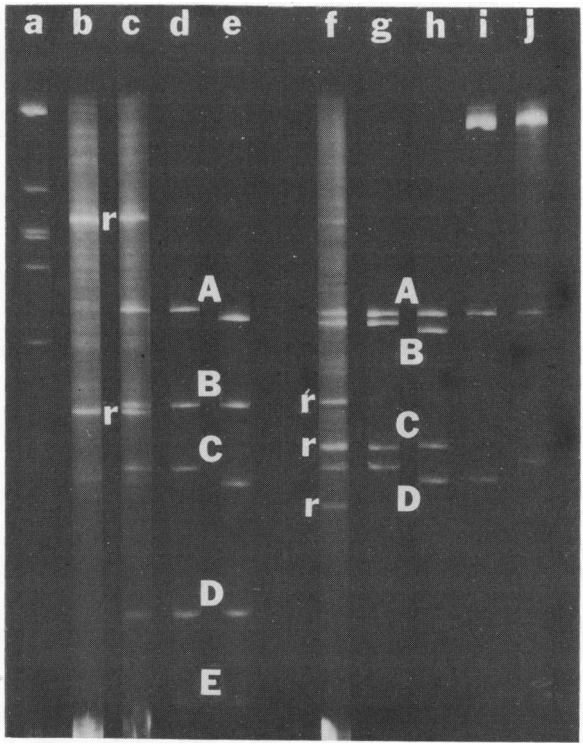


Figure 1. Restriction spectra of whole yeast and plasmid DNAs. Agarose gel (0.7%) electrophoresis of HindIII endonuclease digests of (b) purified linear S288c DNA - 1.5 μg, (c) unfractionated S288c DNA - 1.5 μg, (d) Scp 1 DNA - 0.4 μg and (e) Scp 2 DNA - 0.4 μg and EcoRI endonuclease digests of (a) lambda DNA as a standard - 0.4 μg, (f) unfractionated S288c DNA - 1.5 μg, (g) Scp 1 DNA - 0.4 μg, (h) Scp 2 DNA - 0.4 μg, (i) purified λgt2 - Scp 2 DNA - 0.6 μg and (j) λgt2 - Scp 1 DNA from quick isolation procedure - 0.6 μg. Small r's denote ribosomal DNA bands and capital letters plasmid DNA bands.

DNA are not cleaved by HpaI endonuclease. A unique cleavage site for PstI and HpaI endonuclease in yeast plasmids has also been found by Livingston and Klein[12] and Beggs et al.,[11] respectively.

Determination of the monomer lengths of Scp 1, Scp 2 and Scp 3. The published data on the monomer length of plasmid DNA vary between 1.88 and 2.2 microns[3,5]. Because the micron is not an absolute standard for DNA length measurement with the electron microscope, it was not possible to decide whether the yeast plasmids differed in size among the various strains. Therefore, we measured in the electron microscope the lengths of yeast plasmid DNA from two strains with ϕX174 double stranded DNA as a standard (donated by S. Eisenberg and C. Hutchison). ϕX174 DNA

consists of 5375 ± 10 nucleotides according to sequence data (F. Sanger, personal communication). The monomer circles from Scp 1 DNA consist of 6181 ± 64 nucleotide pairs and from Scp 2 of 6089 ± 54 nucleotide pairs (35 measurements each).

The sum of all the lengths of the fragments from each plasmid's restriction pattern as given in Table 1 is double the measured monomeric length. This is in agreement with the recent demonstrations by other laboratories [9, 11, 12, 28] that yeast plasmids exist in two forms. The size of the plasmids as determined by gel electrophoresis is, therefore, half the sum of the restriction fragment lengths--6.19 kb for Scp 1, 6.06 kb for Scp 2 and 5.97 kb for Scp 3. These values are in good agreement with those obtained by electron microscopic measurements and will be used for the final structural map (Fig. 2).

Cloning of yeast plasmid DNA. In order to allow study of specific cloned plasmid segments, viable molecular hybrids were made with various EcoRI endonuclease cleaved plasmid DNAs and λgt2 DNA. λgt2 is a derivative of λgt-λEcoRI-C [18] with the EcoRI site between the λ EcoRI-C fragment and the right arm of λgt removed by mutation. λgt2 DNA has one central

Table 1. Sizes (nucleotide pairs) of EcoRI and HindIII plasmid fragments.

Fragment Designation	Scp 1 Size	Scp 2 Size	Scp 2 Δ to Scp 1	Scp 3 Size	Scp 3 Δ to Scp 1
EcoRI-A	4000 ± 25	4000 ± 25	0	6000 ± 60	--
B	3830 ± 35	3700 ± 45	130	--	--
C	2350 ± 15	2350 ± 15	0	--	--
D	2190 ± 10	2060 ± 15	130	--	--
HindIII-A	4000 ± 20	3880 ± 20	120	3780 ± 20	220
B	2730 ± 10	2730 ± 10	0	2730 ± 10	0
C	2180 ± 20	2060 ± 25	120	1960 ± 20	220
D*	1280 ± 15	1280 ± 15	0	1280 ± 15	0
E	910 ± 10	910 ± 10	0	910 ± 10	0

Measurements were made on agarose gels by coelectrophoresis with marker molecules (see Methods). The mean of several measurements is given and the interval corresponds to the maximal deviation observed. This deviation primarily reflects the relative proximity of a marker band.

*Fragment HindIII-D is present in double molar amounts in all three restriction patterns.

EcoRI site in a nonessential region and has a sixteen percent deletion with respect to wild type lambda DNA. It can grow unlike other λgt vectors[18] without DNA inserted at its EcoRI site. Bacteriophage hybrids with long DNA inserts may be selected by infecting E. coli pel^- (penetration of λ) strains [19,20]. Lambda phages with DNA sizes much smaller than normal grow very poorly on these strains. The efficiency of growth is inversely related to the size of the deletion in the lambda DNA. Therefore, λ hybrid phages can only grow efficiently on a pel^- strain if the size of the vector DNA plus the inserted DNA is about wild type size. Some characteristic data for this genetic size selection on the pel^- strain GL1[19], using two different λgt vectors, are given in Table 2. Other size selection ranges can be obtained by using longer or shorter λ vectors.

As shown in Table 2, the use of λgt2 lends itself to the selection of hybrids containing a single six kb monomeric plasmid. Plasmid DNA of each of the three types was partially cleaved with EcoRI endonuclease and mixed at 40 μg/ml with an equal concentration of completely cleaved λgt2 DNA. The EcoRI termini were covalently joined as described previously[16]. After calcium transfection into SF8[16], the pools of about 1000 plaques obtained were grown on a strain (2574) obtained from W. Arber which is similar to GL1 but is rK^- mK^- like SF8. In the case of Scp 1 hybrids, twelve plaques were selected. DNA was prepared from the isolated clones by the rapid procedure described in the methods section and characterized after cleavage with restriction nucleases. The rapid DNA preparation technique allows the processing of many isolates in a few hours. The DNA (40 - 100 μg per plate) is sufficiently pure to allow observation of the molecules in the electron microscope or cleavage by most restriction endonucleases (see Figure 1j).

Six of the twelve lambda-Scp 1 hybrid DNAs examined had just two EcoRI plasmid DNA fragments inserted. Four of the six had fragments A and D (e.g., Figure 1j) and two had B and C showing directly the existence of two plasmid forms. The natural order of the fragments was proven by analysis of heteroduplex molecules in the electron microscope [21] (see, for example, the micrograph of Table 4d). Similar results were obtained with λgt2 - Scp 2 hybrid plaques. Figure 1i shows an example of a clone containing the EcoRI fragments A and D from Scp 2; note the smaller D fragment as compared to Scp 1. λgt2 - Scp 3 hybrid phages contain 6 kb long EcoRI fragments (Table 1). The assignment of each of the HindIII fragments to either one of the two plasmid forms was made by cleavage of

Table 2. Growth of λgt-hybrid phages on the pel⁻ strain GL1.

Vector System	Insert Size	Hybrid Size	Relative Growth
λgt1	0	73% λ	0
(left arm 21.8 kb	1 - 12 kb	75 - 95% λ	$<10^{-2}$
right arm 13.9 kb)	12 - 15 kb	95 - 101% λ	1
λgt2	0	84%	10^{-4}
(left arm 21.8 kb	0 - 6 kb	84 - 95% λ	$<10^{-2}$
right arm 19.5 kb)	6 - 9 kb	95 - 100% λ	1

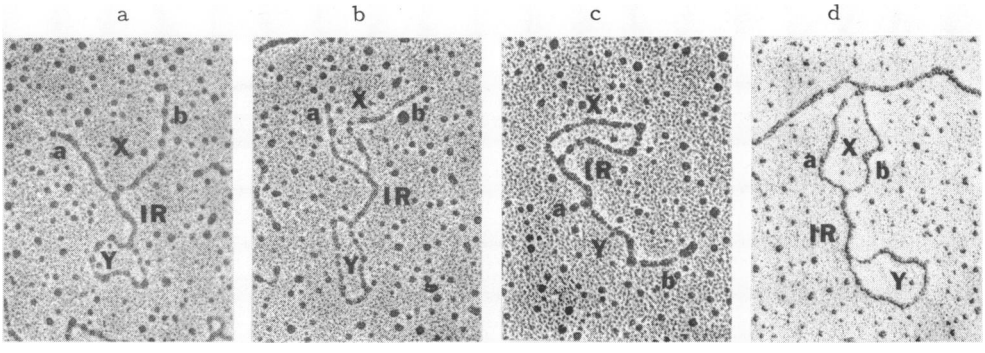
The actual efficiency of plating of normal sized lambda on GL1 compared to C600 is 10 to 20 percent. Bacteriophage with small deletions grow with somewhat lower efficiencies and smaller plaque sizes. The actual efficiencies vary with growth conditions. The upper size limit of the hybrids obtained also depends on the growth media. Magnesium ions at 0.01 molar are required to stabilize oversize virions; however, this interferes with the growth of even full size lambda on GL1.

various λgt2-hybrid DNAs with HindIII endonuclease alone and together with EcoRI endonuclease. The fragments Hind III-B, C, D and EcoRI-A, D belong to one form, designated XY; the fragments HindIII-A, D, E and EcoRI-B, C belong to a second form, designated XY'. The two forms of Scp 3 were identified by their HindIII fragments because the two forms each give identically sized EcoRI fragments. A detailed analysis is given below.

Analysis of selfannealed Scp 1, Scp 2 and Scp 3 DNA. The three plasmids were cleaved once by an appropriate restriction enzyme, denatured, renatured for a short time and examined in the electron microscope. Self-annealed molecules were seen forming one single-stranded loop, one short double-stranded region and two single-stranded ends (Table 3a, b, c). The double-stranded region corresponds to non-tandem inverted repeats and is 0.62 kilobase in Scp 1, 2 and 3. The single strands in each molecule correspond to the two regions of unique sequences (X and Y) between the inverted repeats. The shorter unique region, Y, has a size of 2.24 kb in all three cases. (The small deviation in Scp 3 is not taken as significant.) The EcoRI cleavage site in Scp 3 is located in the Y-region close to one of the inverted repeats (Table 3c). The larger unique region, X, differs in length among the plasmids. This region contains the unique PstI site of all three plasmids as demonstrated for Scp 1 and Scp 2 in Table 3a and b, respectively.

The locations of EcoRI sites were determined by analysis of heteroduplexes between various λgt-plasmid hybrid DNAs and λgt2 DNA. An example is given in the electron micrograph and data of Table 3d, which characterizes a heteroduplex between λgt2 DNA and a λgt2 hybrid containing Scp 2 DNA opened at the EcoRI site in the X region. Other λgt2 hybrids with Scp 1, Scp 2 and Scp 3 DNAs inserted at the EcoRI site in the Y region were seen with the position of that EcoRI site close to one of the inverted repeats.

Table 3. Sizes (Nucleotide pairs) of unique and repeated plasmid sequences.



Scp 1 cleaved with PstI		Scp 2 cleaved with PstI		Scp 3 cleaved with EcoRI		Heteroduplex λgt2 - Scp 2/λgt2	
50 molecules		25 molecules		25 molecules		35 molecules	
IR	624 ± 20	IR	621 ± 16	IR	622 ± 18	IR	622 ± 18
X	2695 ± 112	X	2575 ± 98	X	2530 ± 98	X	2580 ± 60
Y	2238 ± 98	Y	2243 ± 84	Y	2205 ± 90	Y	2240 ± 78
X_a	1000 ± 72	X_a	900 ± 46	Y_a	255 ± 30	X_a	1142 ± 46
X_b	1695 ± 102	X_b	1675 ± 104	Y_b	1950 ± 80	X_b	1438 ± 54

The size of the inverted repeats (IR) was determined using double stranded ϕX174 DNA as a standard. The sum of the unique sequences (total plasmid size minus two times the inverted repeat size) was then proportionally divided among the single stranded parts of the molecules. The intervals given are twofold the standard error.

Structural map of yeast plasmids. The unique PstI site present in all three plasmids investigated was taken as the origin of the structural map. The coordinates of the other positions were determined from the following experimental data: 1) total length of the plasmids, 2) lengths of the restriction fragments, 3) restriction fragment analysis of cloned plasmid DNA showing two forms of plasmids, 4) length measurements of self-annealed single stranded plasmid DNA. The resulting structural map for the two forms, XY and XY', is presented in Figure 2 and the assignment of the

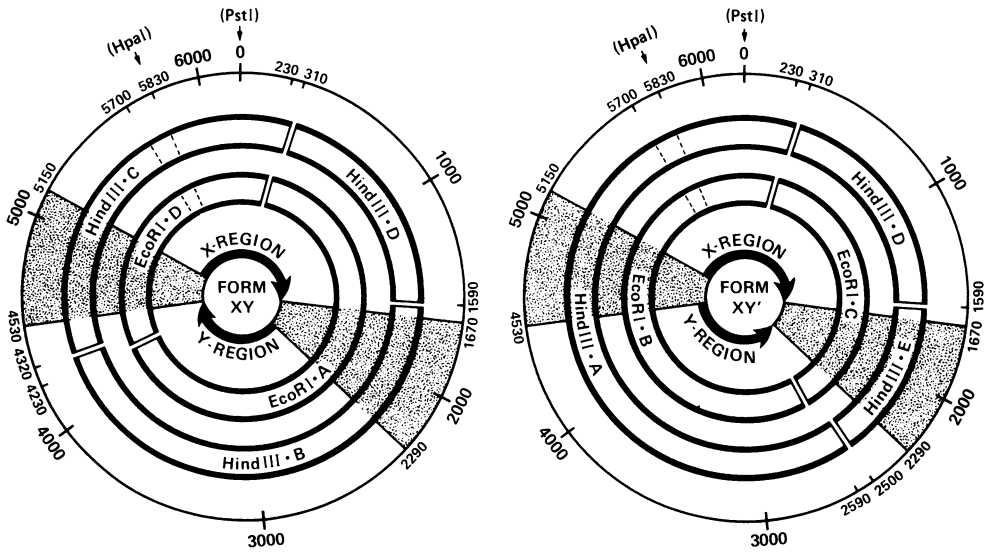


Figure 2. Structural map of the yeast plasmids Scp 1, Scp 2 and Scp 3. The concentric circles represent for each plasmid form the sequence organization (inner part), the order of restriction fragments (middle part) and the map coordinates (outer part). The shaded areas mark the inverted repeats. The deletion in Scp 2 is shown by the dashed lines. Scp 3 has a deletion in the same region. Identical map positions in form XY and XY': The map coordinates start at the unique PstI site present in the X region of Scp 1, Scp 2 and Scp 3 proceeding in a circle up to 6, 190 nucleotide pairs, the length of the most abundant plasmid Scp 1. The direction was set arbitrarily so that the EcoRI site present in the X-regions maps at postion 230 (see Results and Table 3). The HindIII site 310 nucleotide pairs from the PstI site in all plasmids (see Results) maps next to the EcoRI site. The corresponding EcoRI - HindIII fragment of 80 nucleotide pairs was identified in digests of Scp 1 and Scp 2 DNA and not of Scp 3 DNA because the EcoRI site at 230 is missing in Scp 3. One inverted repeat starts 1680 ±100 nucleotide pairs distant from the PstI site and 1440 ± 50 nucleotide pairs distant from the EcoRI site (Table 3). The latter value was taken because of its relatively small standard error. The 620 nucleotide pairs long inverted repeat maps therefore between 1670 and 2290. The Y-region is 2240 ± 80 nucleotide pairs long in all plasmids (Table 3) and maps therefore between 2290 and 4530. The other inverted repeat maps accordingly between 4530 and 5150. The last part of the map between 5150 and 6190 contains the HpaI site present only in Scp 1 at map position 5830 and the deletions found in Scp 2 and Scp 3 (see Results below). Restriction sites in form XY: (Fragments EcoRI-A, D and HindIII-B, C, D) The EcoRI-D fragments maps from 4230 to 230 because it contains the PstI site and the deletions (see Results and Table 1). The EcoRI-A fragments lies in the remaining map region from 230 and 4230. The HindIII-C fragment maps from 4320 to 310 because it also contains the PstI site and the deletions. This leaves the region from 310 to 4320 for the HindIII fragments B and D. A decision between the two possible locations of the two fragments was made using the EcoRI site at 4230. The HindIII-B fragment is cleaved by EcoRI endonuclease. A corresponding EcoRI - HindIII fragment of about 100 nucleotide pairs was observed in digests of Scp 1, Scp 2 and Scp 3 DNA. The HindIII-D fragment maps, therefore, from 310 to 1590, and the HindIII-B fragment from 1590 to 4320. Restriction sites in form XY' (Fragments EcoRI-B, C and HindIII-A, D, E): The EcoRI-B fragment maps from 2590 to 230, because it contains the PstI site and the deletions (see Results above and Table 1). The EcoRI-C fragment lies in the remaining map region from 230 to 2590. The HindIII-A fragment maps from 2500 to 310, because it also contains the PstI site and the deletions. This leaves for the HindIII-D and E fragments only the remaining map region from 310 to 2500. A decision between the two possibilities was made on the strength of the proven sequence homology between the two forms of the plasmids (see Results below). The HindIII-D fragment maps, therefore, as in form XY from 310 to 1590, leaving for the HindIII-E fragment, the map region from 1590 to 2500.

coordinates is described in the figure legend.

The structural maps of Scp 1, Scp 2 and Scp 3 show no differences except for the small deletions in Scp 2 and Scp 3, the missing HpaI site in Scp 2 and Scp 3 and a missing EcoRI site in Scp 3. The question remains to be answered whether or not the three plasmids in fact have basically the same DNA sequences. Heteroduplexes between different λgt-plasmid hybrid DNAs were investigated in the electron microscope. No major sequence heterogeneities besides the known small deletions (see below) were seen among any of the regions of Scp 1, Scp 2 and Scp 3. Moreover, heteroduplexes between cloned DNA from the forms XY and XY' of all three plasmids showed that the X and Y regions of the two forms have the same DNA sequences but the Y regions are inverted with respect to each other. This extends the results obtained by Hollenberg, et al.[9] and Guerineau, et al.[10] with yeast plasmids isolated from strains H1 and DR19, respectively.

Location of the deletions in Scp 2 and Scp 3. Both forms of the plasmid Scp 2 and Scp 3 are deleted by 0.13 kb and 0.22 kb, respectively, as compared to Scp 1 (Table 1). From measurements in the electron microscope (Table 3) and from mapping of restriction fragments (Figure 2), it is already clear that these deletions are located somewhere between map positions 5150 and 6190. A more precise mapping was made possible by analyzing heteroduplex molecules formed between corresponding cloned restriction fragments from Scp 1 and Scp 2 or Scp 3.

λgt hybrid DNA containing the 3.70 kb EcoRI-B fragment from Scp 2 and λgt hybrid DNA containing the 3.83 kb EcoRI-B fragment from Scp 1 together with another 2.78 kb yeast fragment were cleaved with EcoRI endonuclease, mixed, denatured and renatured. An aliquot was examined in the electron microscope (Figure 3). Some of the 3.7 kb duplex molecules carried a very small deletion loop, with one end point at 0.16 ± 0.01 fractional length of the Scp 2 EcoRI-B fragment. This result was confirmed and the absence of other smaller deletions demonstrated by treating another aliquot of the renatured molecules with S1 nuclease from Aspergillas oryzae (Miles) which should cleave across any deletion loops[22].

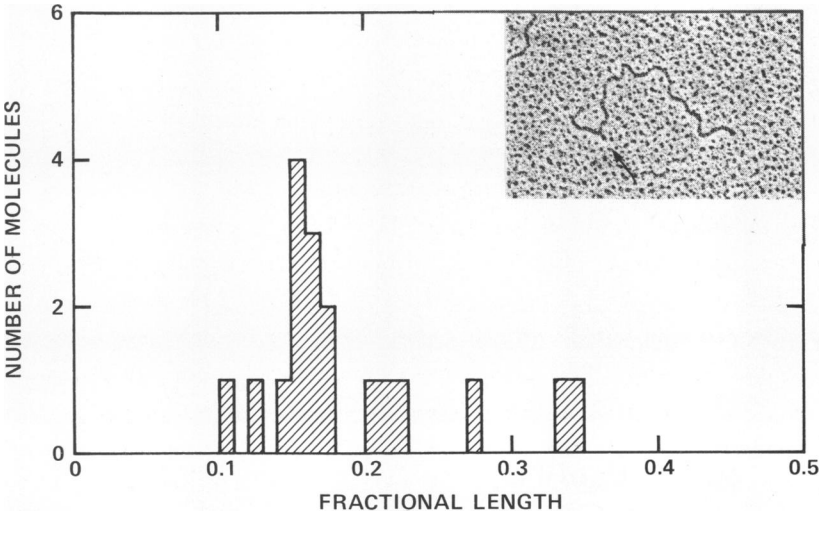


Figure 3. Electron microscopic investigation of the deletion in Scp 2. Eighteen out of 150 renatured Scp 1 and Scp 2 EcoRI-B molecules showed a small knob (example shown). The distance from these knobs to the nearest end is plotted as the fractional length of the Scp 2 EcoRI-B fragment.

Figure 4 shows the band patterns on agarose gels before and after S1 treatment. The cleavage with S1 nuclease clearly produced two new fragments, with sizes of 0.84 and of about 0.16 fractional length of the Scp 2 EcoRI-B fragment (Figure 4c). These measurements map the deletion between positions 5700 and 5830 in the structural map of Figure 2.

The mapping of the deletion in Scp 3 was more difficult, because Scp 3 has only one EcoRI site at map position 2590 (Figure 2). The Scp 1 EcoRI-B fragment (map positions 2590 - 230) was renatured with linear Scp 3 DNA opened at position 2590 and in another experiment with the long Scp 3 EcoRI-HindIII fragment ranging from map position 2590 to 310. The source for these plasmid fragments was again DNA from λgt-plasmid hybrids cleaved with EcoRI endonuclease alone or together with HindIII endonuclease. The λDNA fragments were not removed. Eight molecules of the right length were identified in the electron microscope carrying a small deletion loop starting 3100 ± 200 nucleotide pairs distant from the EcoRI site at map position 2590. This locates one deletion in Scp 3 between map positions

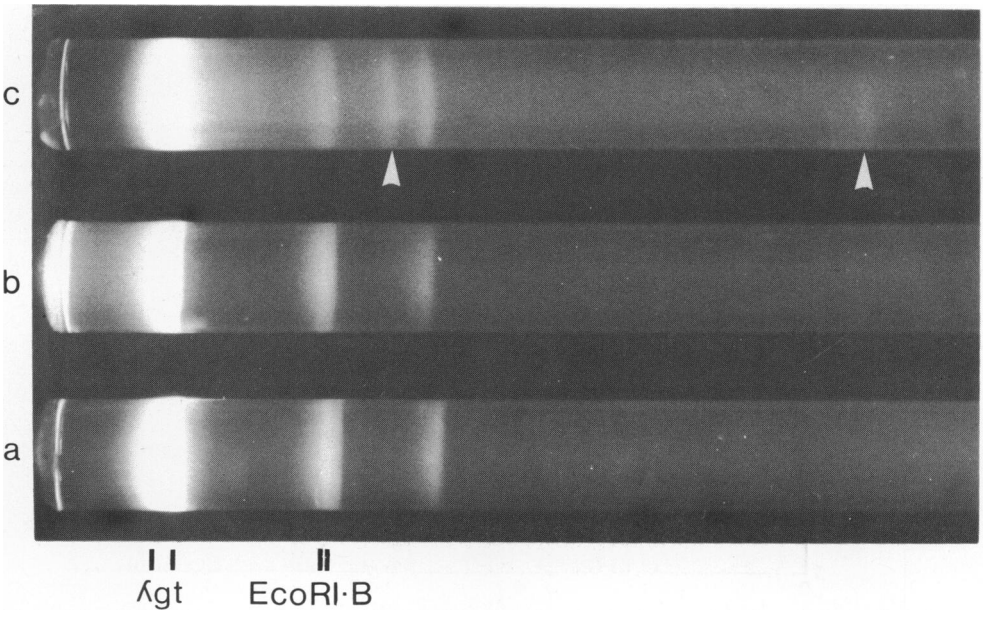


Figure 4. Mapping of the deletion in Scp 2 with S1 nuclease. The products of the following reaction steps (see text) were separated by electrophoresis on 1.3% agarose gels: (a) cleavage with EcoRI endonuclease, (b) denatured and renatured and (c) cleaved with S1 nuclease. The arrows denote the S1 cleavage products. The sizes were determined using the coelectrophoresing fragments of known length and an adjacent standard gel of λ HindIII fragments (not shown).

5500 and 5900, but does not exclude the existence of other small deletions. The fact that the HpaI site present in Scp 1 at map position 5830 is missing in Scp 2 and Scp 3 suggests that the deletions of Scp 2 and Scp 3 overlap (Figure 2).

Analysis of integration of the plasmid DNA. If plasmid DNA sequences are integrated into chromosomal DNA, they must be present in high molecular weight linear yeast DNA. This can be examined by searching for fusion fragments, restriction fragments containing both plasmid DNA and chromosomal DNA. There would be a set of two fusion fragments for each plasmid integrated at a different site. The technique used to identify these fragments was to hybridize with a labelled probe across a gel of separated restriction fragments from yeast DNA depleted of closed circular DNA. The probe used was a hybrid between the vector PMB9 and the plasmid fragment EcoRI-A (see Methods), which was labelled by nick translation [23] with DNA polymerase I (donated by M. Goldberg) and all four α^{32}P-labelled deoxynucleotide triphosphates (New England Nuclear, specific activity greater than 100 Curies per millimole). Yeast DNA was cleaved with various restriction enzymes and fractionated on 0.7% agarose gels. The gels were photographed with a long wavelength UV source to note band positions, and the DNA was denatured, neutralized, and eluted onto nitrocellulose strips in situ [24] to examine sequences in bands down to less than 1.5 kb. About 10^6 disintegrations per minute of probe was hybridized to each strip by the method described by Denhardt, [25] and finally, the washed strips were autoradiographed to locate plasmid sequences.

In order to ascertain the sensitivity of this assay, the gels were loaded with one nanogram of either Scp 1 or Scp 2 DNA cut with EcoRI endonuclease and mixed with lambda DNA as a carrier. All four EcoRI yeast plasmid fragments were seen as dark bands on the autoradiographs after hybridization with the EcoRI-A plasmid probe. It should be noted that the EcoRI-A fragment has extensive sequence homology with all the other EcoRI fragments (see Figure 2). If one assumes that the DNA content of yeast is about 10^4 kb [26], then a six kilobase unique fragment would make up 1.2 nanograms out of the 2 micrograms of total yeast DNA used in a gel. The above control experiment indicates that fragments the size of plasmid EcoRI fragments may be seen at unique or even less than unique representation in the genome. Large DNA does not elute from the gels as completely as smaller DNA [24], but control experiments with unique levels of uncleaved λgt2 - Scp 2 DNA gave clear signals with the same probe.

The results of hybridizations using various whole yeast DNAs are given in Figure 5. Strips a, b and c are made with DNA from D585-11C (single colony isolate), freed of closed circular DNA and cleaved with EcoRI, HindIII, and BamI endonucleases. Strip a shows hybridization to only the four EcoRI plasmid fragments due to open circular plasmid DNA in the original yeast DNA preparation. In strip b, hybridization is seen only at the positions at plasmid HindIII fragments A, B and C; the smallest two HindIII fragments have run off the gel in this experiment. The two EcoRI and HindIII plasmid fragments which gave hybridization signals have only the inverted repeat region homologous with EcoRI fragment A. In strip c, the only bands seen are those representing monomer and dimer open circular plasmid DNA near the top of the gel, as expected, since the Scp 2 plasmid DNA is not cleaved by BamI endonuclease. Strip e is similar to strip a but the probe and yeast DNA were from different preparations. In this case a faint band (arrow) is seen at the position of linear plasmid DNA, presumably due to some partial EcoRI endonuclease cleavage.

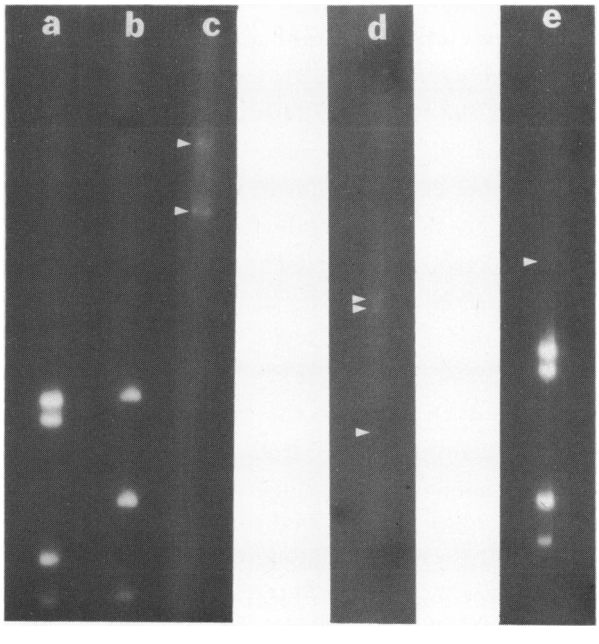


Figure 5. Examination of plasmid sequences in linear yeast DNA. Autoradiographs of ^{32}P-labelled plasmid probe hybridized to electrophoretically separated restriction fragments of yeast DNA (2 μg) eluted after denaturation onto a nitrocellulose strip. D585-11C DNA cleaved with (a), (e) EcoRI, (b) HindIII and (c) BamI. (d) A 364a X H 79-20.3 α DNA cleaved with EcoRI. Arrows indicate faint bands not seen after reproduction.

The preparations of D585-11C DNA used have an amount of open circular DNA in them which was never quantitated but is about twice the unique level. Two preparations of yeast DNA were obtained from B. Hall which were prepared under different conditions from strains having Scp 1 plasmid DNA. These DNAs were used in experiments like the above, but only very faint bands at the plasmid fragment positions were seen indicating that these preparations contained less than a unique amount of open circular plasmid sequences. No other bands were seen. An example is shown in gel d for DNA cleaved with EcoRI.

These results strongly support the conclusion that under the conditions of growth for the strains used in this study there is no stable integration of the yeast plasmid. No hybridization was seen to hypothetical fusion fragments of a different size than plasmid restriction fragments. Examination of the restriction map of the yeast plasmid (Figure 2) shows that there is no integration site on the plasmid which would not give one new EcoRI or HindIII restriction fragment partially homologous to the plasmid EcoRI-A fragment and also larger than 1.5 kilobases. This eliminates the necessity of identifying small fusion fragments from an integrated plasmid. It leaves only the small probability that the fusion fragments produced with three different restriction enzymes are coincident with the plasmid restriction fragments. A reservation concerning the stability of the integration exists since a plasmid rapidly changing its site of integration among a fairly large set of different sites would not be seen in these experiments. It would also not be seen if it was only integrated a small percentage of the time. Under the purification conditions used, the D585-11C DNA has a normal amount of mitochondrial DNA based on the ability to isolate this DNA (unpublished results). Therefore, the above conclusion also covers the mitochondrial genome.

DISCUSSION

Scp 2 and Scp 3 contain the first deletions characterized in a eukaryotic plasmid DNA. The yeast strains carrying these plasmids presumably originated as different isolates from nature. It is of interest to note that the major structures of the three plasmids, most particularly the repeated sequences, remain constant. Furthermore, the positions of the deletions are not adjacent to the repeated sequences indicating that they are not related to any hypothetical recombination event between those regions.

We have shown that the deletion of Scp 2 and the deletion and missing

EcoRI site of Scp 3 are present in both the XY and the XY' forms of the plasmids and that the two forms are homologous except for an inversion event. This adds additional evidence toward the existence of some interconversion between the two forms in vivo. We and others [9, 11] have found no evidence of any such interconversion when the cloned monomeric plasmid of either form is propagated in E. coli for many generations using either viral or plasmid vectors. If a plasmid was discovered carrying a sequence marker of some type for the Y region, then a haploid yeast strain carrying this plasmid and one carrying either Scp 2 or Scp 3 could be mated and the plasmids of the diploid examined for mixed plasmid forms. Such an experiment could be valuable in elucidating the role of the nontandem inverted repeat sequences.

Our work casts doubt on the suggestion [9, 11] that the nontandem inverted repeat sequences might function like those in certain bacterial plasmids to enable integration of the circular DNA into the chromosome. Earlier work[4] showed that the level of closed circular DNA in isolated nuclei was at about the same level as contaminating mitochondrial DNA. We found no evidence that plasmid DNA sequences were covalently coupled to other DNA sequences. Such a result not only precludes stable integration of the plasmid but also denies the existence of chromosomal sequences homologous to the plasmid EcoRI-A fragment which could allow integration of the plasmid by recombination as proposed for the nontandem inverted repeat units. Other work has shown that such sequences would have been seen even if they were as short as 100 bases. It is, therefore, suggested that the plasmid is entirely cytoplasmic.

Yeast cells are a desirable system for the possible cloning of eukaryotic DNA. They are easily grown, allow an advanced level of genetic manipulation and are more likely to transcribe and translate eukaryotic genes than E. coli. So far, however, they have not been transformed with externally applied DNA. The plasmid DNA is the only known small DNA replicon in yeast and as such is a possible vector. A first problem in the use of such a vector is determining a position where DNA may be inserted without rendering the resultant hybrid inviable. We have shown that the HpaI site in Scp 1 is apparently such a site since this region is not necessary for replication as concluded from its deletion in Scp 2 and Scp 3. The plasmid could be opened at this site and DNA inserted after addition of polyadenylate and polythymidylate tails, respectively[27]. Small DNA fragments carrying restriction sites capable of generating cohesive ends for

cloning of like restriction fragments could be added at the HpaI site with this method. Assuming that such a hybrid DNA molecule could be put into the yeast cell and established in the cytoplasm, the question remains whether productive transcription and translation of genes on the plasmid occurs in the cytoplasm.

ACKNOWLEDGEMENTS

We thank C.P. Hollenberg and P.W.J. Rigby for useful information related to the plasmid and DNA hybridization, respectively, and appreciate Sue Crissman for preparation of this paper. This work was supported in part by Public Health Service Grant GM 21891 from the National Institutes of General Medical Sciences. P.P. was supported by a fellowship from Studienforderung Cusanuswerk, and J.R.C. was a postdoctoral fellow on a Tumor Biology Training Grant from the National Institutes of Health.

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