DESIGN AND CHARACTERIZATION OF A SYSTEM TO TEST SYNTHETIC DNA SEQUENCES FOR TELOMERE FUNCTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Specialized structures called telomeres are required for the stabilization and replication of eukaryotic chromosome ends, and of other linear DNA ends such as those of linear plasmids in the yeast *Saccharomyces cerevisiae*. In vitro DNA synthesis techniques were used to synthesize stretches of short, tandemly-repeating sequences similar to those known to be involved in telomere function in vivo. These sequences were cloned in a multiple cloning site in an *E. coli* circular plasmid, excised with restriction enzymes and used to construct perfect inverted repeats in *E. coli/S. cerevisiae* shuttle vectors.

To avoid the difficulties involved in the propagation of long inverted repeats in conventional *E. coli* cloning strains, the recBCsbcBrecF strain JC8111 was used for their construction and replication. An inverted 224 base pair repeat containing two copies of the yeast ARS1 region was faithfully propagated in this strain. However, most plasmids which contained inverted repeats of telomere-type tandemly repeating sequences underwent various degrees of rearrangement within the inverted repeat region.

Plasmids which yielded restriction fragments from the inverted repeat region of approximately the right mobility on agarose gels were used to transform *S. cerevisiae*. DNA was prepared from the transformed yeast clones and analyzed by agarose gel electrophoresis and Southern blotting to determine whether the transforming plasmids were replicating as circular or

linear forms. Resolution into stable linear plasmids was observed in several cases where an ARS1 region was immediately adjacent to the tandemly repeating sequences in both halves of the inverted repeat; it was not observed when the ARS1 regions were absent, nor when the telomere-type repeats were present in the opposite orientation to that found in vivo. However, interpretation of the results was hampered by the heterogeneity of the plasmid DNAs propagated in JC8111. For this reason it was concluded that this assay system would not be useful for an extensive study of telomere replication in yeast.

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LIST OF ABBREVIATIONS

ATP	riboadenosine 5'-triphosphate
bp	base pairs
DEAE	diethylaminoethyl
dpm	disintegrations per minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dATP	deoxyriboadenosine 5'-triphosphate
ddATP	dideoxyriboadenosine 5'-triphosphate
dCTP	deoxyribocytidine 5'-triphosphate
ddCTP	dideoxyribocytidine 5'-triphosphate
dGTP	deoxyriboguanosine 5'-triphosphate
ddGTP	dideoxyriboguanosine 5'-triphosphate
dTTP	deoxyribothymidine 5'-triphosphate
ddTTP	dideoxyribothymidine 5'-triphosphate
dNTP	deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
HCC	hexamine cobalt(III) chloride
hr	hour
kb	kilobase pairs
OD	optical density
RNA	ribonucleic acid

RNase	ribonuclease
rpm	revolutions per minute
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
vol	volume(s)
X-gal	5-bromo 4-chloro 3-indolylgalactoside

INTRODUCTION

The genetic material in eukaryotic nuclei is organized into linear structures called chromosomes, in contrast to the circular genomes of prokaryotes and eukaryotic organelles. Much evidence exists to suggest that each eukaryotic chromosome contains a single long DNA duplex molecule, whose two ends are located at the ends of the chromosome.[1]

McClintock[2] and other classical geneticists[3,4] have shown that chromosome ends possess unique properties that distinguish them from ends resulting from chromosome breakage. Whereas broken ends are highly unstable and recombinogenic, natural chromosome ends are non-recombinogenic and can stabilize the ends of broken chromosomes when transferred by duplicative transposition.[2-5]

At the level of the ends of the DNA molecule itself, another unique property is called for to allow their complete replication during the cell division cycle. All known DNA polymerases extend the DNA chain in the 5' to 3' direction, by adding mononucleotides to an RNA or DNA 3'-OH terminus.[6] At a linear DNA end, no 3' terminus exists to allow full replication of the 5'-ended strand, and cells and viruses with linear genomes must therefore have evolved mechanisms to prevent the gradual loss of terminal sequences during multiple rounds of replication.[5,7]

Consideration of these two unique properties of chromosome ends led to the concept of a "telomere" function encoded in the terminal DNA. The word is

derived from the Greek telos = end, by analogy with the function of chromosomal centromeres. Much interest has been focused on the questions of what DNA sequences can confer telomeric properties on a linear DNA end, whether a covalently-linked terminal protein may be required, and what genes and enzyme activities are involved in the regulation of telomere function.[5] The present study addresses the first of these problems.

The chief difficulties in studying chromosomal telomeres at the molecular level lie in their low concentration relative to total cellular DNA, and the lack of a functional assay for their isolation in conventional cloning vectors. One strategy for getting around this problem, employed by Button and Astell[8], is to use a chromosome walk from a cloned distal marker on a given chromosome to isolate progressively more telomere-proximal clones. A difficulty with this approach is that the telomeric fragment itself may well be unclonable by conventional techniques, if the end cannot be covalently fused to a conventional circular cloning vector.[8]

A second approach, originally employed by Szostak and Blackburn[9], has made use of the natural occurrence of identical, high copy number linear rDNA molecules in the macronucleus of the ciliated protozoan *Tetrahymena pyriformis*.[10] This in essence constitutes a ready-made pool of cloned "minichromosomes", in which telomeres are present at relatively high concentration.

An early study by Blackburn and Gall[10] provided convincing evidence of an unusual, repetitive sequence and structure present at the ends of the *Tetrahymena* rDNA minichromosomes. The inward-running (5'-ended)

DNA strand was composed of 20-70 repeats of the sequence 5'-CCCCAA-3', interspersed with occasional single nucleotide gaps. The complementary or 3'-ended strand contained the sequence 5'-TTGGGG-3' with a smaller number of nicks. The very end of the telomere was refractive to labeling by polynucleotide kinase and to the actions of several other enzymes which act on the ends of linear DNA fragments; it was hypothesized that it consisted of a hairpin loop linking the two strands, as suggested previously by Bateman[11].

Unfortunately, techniques for the stable transformation of *Tetrahymena*, which would allow functional analysis of the telomere DNA in a homologous system, have not yet been developed. However, the availability of fairly pure telomere DNA from the *Tetrahymena* rDNA pool made it possible to carry out a test of whether this DNA could also confer telomere activity on a linear plasmid in a quite different eukaryotic system: the yeast *Saccharomyces cerevisiae*. As reported by Szostak and Blackburn, the addition of two *Tetrahymena* telomeres to a cut (i.e. linearized) circular yeast plasmid in vitro, followed by reintroduction of the DNA into yeast , enabled the propagation of a stable linear plasmid in yeast cells. The astonishing implication of this result is that the mechanism of eukaryotic telomere function has been preserved throughout evolution since the divergence of the fungal and protozoan kingdoms.[5,9]

Subsequently, a number of experiments have shed light on those features of the telomeric DNA which are required for telomere function in eukaryotes. Most notably, stretches of a few hundred base pairs of a simple repeating sequence have been found at the very end of the DNA in a wide variety of

Table I. Canonical repeat sequences found at linear DNA termini in different organisms (5'-ended strand).

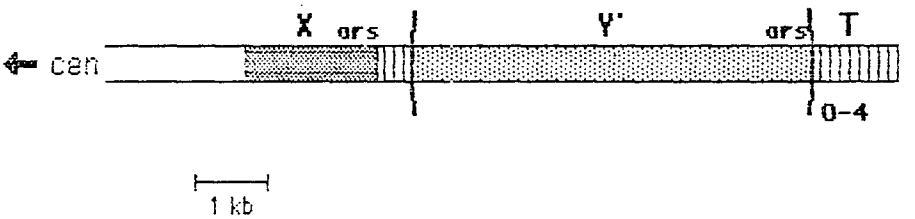
Organism	Basic repeat unit	Reference
Holotrichous ciliates		
Tetrahymena	CCCCAA	10,12
Glaucoma		12a
Paramecium		12b
Hypotrichous ciliates		
Oxytricha	CCCCAAAA	13
Stylonichia		14
Slime moulds		
Dictyostelium	$C_{1-8}T$	15
Physarum	$CCCTA_n$	16
Hemoflagellates		
Trypanosoma	CCCTAA	17
Fungi		
Saccharomyces	$C_{2-3}A(CA)_{1-3}$	18

organisms.[5,12-18] The canonical repeat sequence is characteristic of the individual species: Table I shows a list of those published to date. There are both tantalizing similarities and remarkable variations between the basic repeating units. The segregation of C's to the 5'-ended strand and G's to the 3'-ended strand is universally observed, and short repeating tracts of these nucleotides appear to be important, but beyond these, few rules can be discerned. It is not yet clear whether all of these sequences work in yeast, or whether only a subset of them is recognized by the yeast enzymes involved in telomere replication. It is also unclear whether these sequences have a direct effect on the secondary structure of the DNA, as has been observed with other unusual repetitive sequences[19-21], or whether the DNA sequence itself simply serves as a recognition signal for telomere-specific proteins[22-24].

A second important finding has been that probably all yeast telomeres are associated with adjacent ARS sequences, suspected to be chromosomal origins of replication.[8,18,25] These data have led to the hypothetical scheme of telomere organization shown in Figure 1, where an 'X' and a variable number of 'Y' type ARS regions are found immediately adjacent to the telomeric repetitive sequences. The *Tetrahymena* telomeres used in the original Szostak-Blackburn experiment also contained (fortuitously) a sequence which functions as an ARS in yeast.[9,26] This raises the possibility that close linkage to a replication origin is a requirement for telomere function. The 'X' and 'Y' regions which carry telomeric ARS activity in yeast are extensive enough that they could well contain other, as yet unidentified functions which are important for full telomere activity.[25,27,28] Few tests have yet been done of either of these possibilities.[5]

Figure 1. General arrangement of DNA sequences at chromosomal telomeres in *Saccharomyces cerevisiae*.

The diagram is based on those found in Chan and Tye[25], Dunn *et al.*[49] and Button and Astell[8]. Sequences are represented as follows: unique chromosomal DNA (▭); X ARS element (▩); Y' ARS element (▩); $C_{1-3}A$ tandem repeat regions (▥). The 'T' denotes the telomeric C1-3A repeats, 'ars' indicates the approximate location of the ARS function within each X and Y' element, and 'cen' indicates the direction of the centromere. The chief variable between different telomeres is the number of Y' elements each contains: this varies between none[8] and four[25]. The length of the T region also varies, even within a single telomere[8].



One further observation is relevant to the work described in this thesis. An interesting experiment by Szostak[29] demonstrated that the yeast telomere replication mechanism could recognize a circular analogue of a linear plasmid and resolve it into a stably propagating linear molecule. The circular version was constructed by ligating a cloned, exonuclease-digested *Tetrahymena* telomere to itself in vitro to produce a head-to head inverted repeat, and then ligating this into a circular yeast plasmid. Nine bp of non-telomere DNA at the centre of the inverted repeat, introduced during its construction, had no apparent effect on the efficiency of resolution.[29] This result gives tentative support to models of telomere replication which postulate formation of a telomeric inverted repeat during chromosome duplication[11,30,31], but other explanations cannot yet be ruled out.

The inverted repeat experiment provides an attractive system for assaying DNA sequences for telomere function in yeast. One methodological problem, however, is that long inverted repeats are generally not stably propagated in the common cloning host *Escherichia coli*.[32,33] It was for this reason that the original experiment used a ligation mix for yeast transformation, rather than a homogeneous population of molecules propagated by cloning in *E. coli*.[29] For an extensive series of experiments, however, it would be desirable to clone and characterize precisely the inverted repeats to be tested, before they were finally introduced into yeast and assayed for resolution and telomere function.

In 1984, Boissy and Astell reported[34] that a recBCrecF strain of *E. coli*, JC8111, was capable of replicating the long 5'-terminal palindrome of the Minute Virus of Mice genome, which had previously been shown to be

unstable in numerous common cloning strains[35]. This property is presumably related to the absence (or very low activity) of Endonucleases I and V and the recF endonuclease in this strain.[36,37]

This raised the possibility that JC8111 could be used to clone circular molecules carrying potential telomere-functional sequences as an inverted repeat. These clones could then be fully characterized by standard enzymatic and sequencing techniques, before introducing them into yeast and assaying for their replication as circular or linear molecules.

This thesis describes the design and testing of such an assay system, which could be used to test any desired repeat sequence for telomere activity. It is shown that the resolution of cloned circular molecules in yeast can be obtained, and the structures of some resolving and non-resolving plasmids are described. However, replication of the long inverted repeats by JC8111 is shown not to be accurate enough for the production of homogeneous DNA clones, rendering this assay system unsuitable for use in an extended study of telomere resolution in yeast.

MATERIALS AND METHODS

REAGENTS

Enzymes

Restriction enzymes were from Bethesda Research Laboratories (BRL), New England Biolabs or Pharmacia P-L Biochemicals. T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase and E. coli DNA polymerase I large (Klenow) fragment for end-labeling were from BRL. For DNA sequencing, the Klenow fragment from Promega Biotech was used.

Chemicals

All chemicals were of reagent or electrophoresis grade where applicable. Phenol was extracted with 1M Tris-Cl, pH 8, and stored under buffer at 4°C or -20°C in the presence of 0.1% hydroxyquinoline. Dimethylsulfoxide (DMSO) was stored in small aliquots at -20°C.

Radiochemicals

[α-^{32}P]-dATP, [α-^{32}P]-dCTP and [γ-^{32}P]-ATP, all with specific activity ~3000 Ci/mmole, were obtained from Amersham at 10mCi/ml and used within two weeks of purchase.

PLASMIDS

pBR322 [38] and pUC12 [39] were obtained from Caroline Astell and regrown in DNA maxipreps (see below) as necessary.

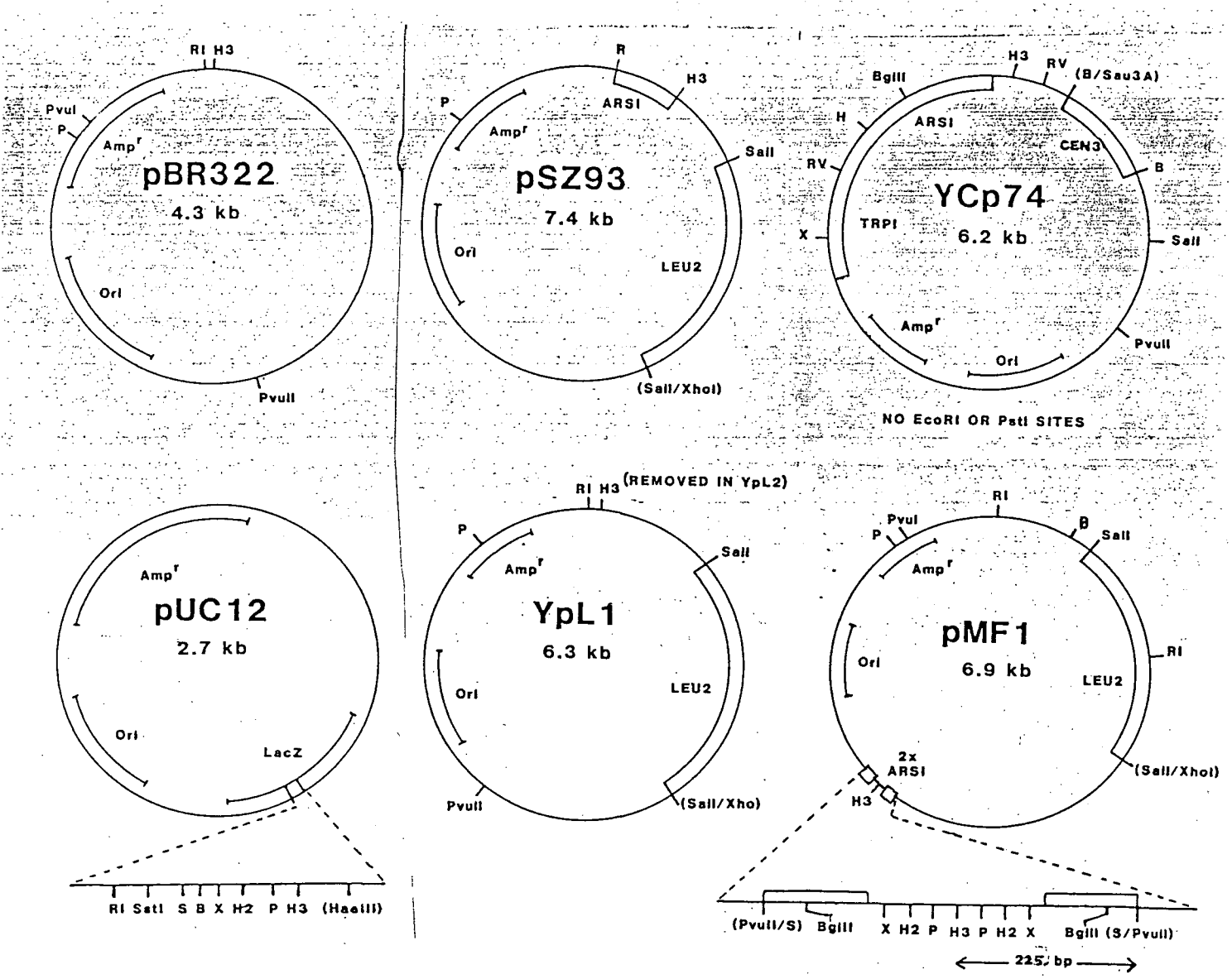
pSZ93 [40] was obtained from Linda Button. This plasmid contains almost all of pBR322 plus the yeast fragments ARS1 (in the EcoRI-HindIII region) and LEU2 (in the SalI site).

YRp74 and YCp74 were a gift of Johnny Ngsee (J. Ngsee and M. Smith, unpublished data). YRp74 is a derivative of YRp7 [41], modified to remove the EcoRI and PstI sites. The smaller PvuI-PvuII fragment from pBR322 was replaced by the equivalent fragment from pUC7, in which the PstI site in the ampR gene has been removed by mutagenesis. This leads to 46 bp from one end of the lacZ gene replacing the PvuII-HaeII region in pBR322. After insertion of the TRP1ARS1 EcoRI fragment in the EcoRI site (in the opposite orientation to that in YRp7) the remaining PstI site and the two EcoRI sites were removed by partial digestion, exonuclease digestion of the single-stranded tails, and religating (J. Ngsee, personal communication). YCp74 is identical to YRp74 except for the insertion of a 627 bp Sau3A fragment carrying the *S. cerevisiae* centromere from chromosome III, CEN3 [42], in the BamHI site. For unknown reasons, neither plasmid could be replicated in JM101 cells; they were routinely propagated in RR1.

The construction of all other plasmids used is described in the text. Restriction maps are summarized in Figure 2.

Figure 2. Restriction maps of plasmids used in this work.

Only the restriction sites relevant to these experiments are shown. All were predicted on the basis of DNA sequence analysis and verified by restriction enzyme digestion and gel electrophoresis. B=BamHI, H2=HincII, H3=HindIII, RI=EcoRI, RV=EcoRV, P=PstI, S=SmaI, X=XbaI. Amp^r, *E. coli* ampicillin resistance gene from pBR322; Ori, pBR322 origin of replication; LacZ, *E. coli* gene fragment complementary for the lacZΔM15 mutation; ARS1, CEN3, LEU2, TRP1, *S. cerevisiae* genes (standard abbreviations).



BACTERIAL STRAINS

Escherichia coli strains JM101, DH1, RR1 and JC8111 were obtained from glycerol cultures prepared by Marnie Thompson and maintained at -20°C. Cultures were regularly discarded and restarted from streaked cells.

YEAST STRAINS

Sacchyaromyces cerevisiae strain C1 [43] was obtained from Johnny Ngsee; it has a [α, lys2, trp1] genotype and was used with the YCp-derived plasmids.

S. cerevisiae strain A281 [44] was supplied by Linda Button; it is a derivative of A2, with the genotype [α, leu2-3,112, his3-11,15, can1, cir°].

BACTERIAL CULTURE

All bacterial strains were routinely grown in 2YT medium:

16g Bacto-tryptone
8g Bacto-yeast extract
5g NaCl
H_2O to 1 litre; pH adjusted to 7.2-7.4 with a few drops 5M NaOH.

Strains carrying plasmids were usually grown in the presence of 50 µg/ml ampicillin, added after autoclaving.

Culture plates contained identical media plus 1.5% Bacto-agar which was added before autoclaving.

All bacterial cultures were grown at 37°C.

YEAST CULTURE

Parent strains were grown in YEPD medium:

6g Bacto-yeast extract
12g Bacto-peptone
12g dextrose
H_2O to 600ml.

Strains carrying plasmids were grown in synthetic complete medium lacking one or more amino acids:

4g Bacto-yeast nitrogen base without amino acids
12g dextrose
20ml stock solution (see below)
H_2O to 600ml.

Stock solution: contained adenine sulfate, uracil, tryptophan, histidine, arginine, methionine (all 0.6 g/l), tyrosine, leucine, isoleucine, lysine (all 0.9 g/l), phenylalanine (1.5 g/l), glutamate (3 g/l), valine (4.5 g/l), and serine (11.25 g/l), minus either tryptophan or leucine for selection. 20 ml of

aspartate (3 g/l) and threonine (6 g/l), which need to be autoclaved separately, were sometimes added to the medium after autoclaving.

Culture plates contained identical media plus 2% Bacto-agar which was added before autoclaving.

All yeast cultures were grown at 30°C.

PREPARATION OF DNA

Bacterial plasmid minipreps

5 ml cultures were grown overnight in selective medium, and the alkaline lysis procedure was followed exactly as given in Molecular Cloning[45], p. 368, except that lysozyme was sometimes omitted. (In this alkaline lysis procedure, *E. coli* strains lyse without prior digestion with lysozyme [R.O. Shade, personal communication].) Usually the final wash with 70% ethanol was omitted, and instead the DNA was precipitated with 0.6 vol. isopropanol and 0.1 vol. 3M Na acetate following the RNase digestion, dried and finally resuspended in H_2O. This usually gave very clean results in restriction digests (1/5 of a prep per digest).

Bacterial plasmid maxipreps

Usually 500 ml cultures were grown overnight in selective medium with vigorous shaking, and the alkaline lysis procedure in Molecular Cloning[45], p. 90, followed with some modifications. Lysozyme was sometimes omitted.

The first high-speed centrifugation was carried out in a Beckman Ti45 rotor at 35,000 rpm for 30-60'. Supernatants were transferred to 150-ml Corex tubes, and the DNA recovered following isopropanol precipitation by centrifugation at 5000 rpm for 30'. Each pellet was dissolved in 10.0 ml H_2O. CsCl (10.0 g) was added, and the solution was transferred to a Beckman 7.5 cm quick-seal centrifuge tube. 150 µl ethidium bromide (10 mg/ml) was added to the tube before sealing and centrifugation in a Beckman Ti70.1 rotor for 18-24 hours at 60,000 rpm, 15°C.

To recover the DNA following its collection from the CsCl gradient, the sample was extracted several times with isoamyl alcohol and then dialysed against a large volume of TE for 2x1 hour. Na acetate (0.1 vol. of 3M solution) and 2.5 vol. ethanol were added to precipitate the DNA, and it was collected by centrifugation in an Eppendorf centrifuge for 10' at room temperature and dissolved in 50-500 µl H_2O. The yield of DNA was measured by diluting a small aliquot in 1 ml H_2O and measuring the absorbance at 260 nm; the sample was then diluted to 1 mg DNA/ml and stored at -20°C.

Carrier DNA.

Carrier DNA was used for yeast transformation and for increasing the yield when precipitating very low concentrations of DNA from ligation mixtures for bacterial transformation. A stock was prepared from 1 litre of JM101 cells according to the phenol-pH9 method[46], and stored at a concentration of 1.67 mg/ml following sonication.

Yeast DNA minipreps

The procedure given by Shirleen Roeder in the Cold Spring Harbour Manual on yeast genetics[47] was followed. This gave quite a low yield with selectively-grown cultures, so that the final preparation had to be isolated by centrifugation and resuspension of the pellet in TE. Generally 1/10 of a prep was used for one lane on a gel/Southern, or 1/30 for preps containing linear plasmids.

BACTERIAL TRANSFORMATION

Calcium chloride method

The procedure was followed as given in Molecular Cloning[45], except that the cells were generally used within 6 hours of preparation. 100-150 µl were spread on each selective plate following 40-60' incubation in 2YT. The top agar procedure was never used with ampicillin selection, since it gives rise to very diffuse colonies.

Hanahan method

Experiments on the effects of the Hanahan transformation protocol[48] on JC8111 are described in the text. The following method was finally adopted as giving the best results:

Cells were grown in 2YT containing 20mM $MgCl_2$. Good aeration greatly increases the growth rate and may improve transformation, hence the

volume of medium should not be greater than 1/10 volume of flask and shaking is carried out at 250 rpm. $MgCl_2$ makes cells easier to resuspend.

Cells should reach OD of 0.6-0.7 (550nm) in about 3 hr (for JC8111 - about 2 hr for other strains). Cells were chilled on ice and pelleted at 3000 rpm for 5', 4°C. The pellet was resuspended in one-third to one-half the original culture volume of transformation medium: 45mM $MnCl_2$, 10mM $CaCl_2$, 100mM RbCl, 3mM HCC (hexamine cobalt(III) chloride), 10% glycerol, filtered and stored at 4°C. Cells were incubated on ice for 15-30' and repelleted at 3000 rpm, 4°C. The pellet was resuspended in fresh transformation medium, 1/12.5 original culture volume. DMSO (7 µl for every 200 µl cells) was added and the cells were incubated on ice for 10'. A further, equal aliquot of DMSO was added and the cells were divided in 200 µl aliquots into chilled transformation tubes. The tubes were flash-frozen in a dry ice-ethanol bath and stored at -70°C.

For transformation, the cells were first thawed and DNA added as for the calcium chloride procedure.

YEAST TRANSFORMATION

The lithium acetate method was used as modified by Dunn *et al.*[49] Six transformations were carried out from each 100 ml yeast culture. Between 10 and 200 colonies were obtained from 1 µg transforming DNA.

ENZYME REACTION CONDITIONS

Restriction endonuclease digestion

Buffers for restriction endonuclease digestion were those suggested in Molecular Cloning[45], pp. 100-104. The table of salt conditions published in the New England Biolabs catalogue was also sometimes used as a guide as to the most suitable buffer for a double digest. Two to four times the theoretical amount of enzyme was used for most applications.

Ligations

Standard buffer conditions for ligation reactions were 25mM Tris-Cl, pH 7.5, 10mM $MgCl_2$, 1mM ATP and 1mM dithiothreitol. In several reactions (noted in the text) 1mM hexamine cobalt (III) chloride (HCC) was included, as this has been found to stimulate blunt-ended ligation by up to 10-fold[50].

DNA concentrations and other variables are given in the Results and Discussion where relevant.

Nick-translation

The nick-translation reaction for one Southern blot contained the following:

0.5 µg pBR322
5 µl 10x nick-translation buffer (250mM Tris-Cl, pH 7.5, 100mM $MgCl_2$, 1mM DTT)

5 µl 2mM $CaCl_2$

5 µl each 0.1mM dGTP, dCTP, dTTP

0.2 µl 0.1mM dATP

5 µl [α-^{32}P]-dATP

18 µl H_2O

0.5 µl DNase I, diluted 1:40,000 in nick-translation buffer

o.5 µl DNA polymerase holoenzyme

The reaction was incubated at 15°C for 2.5 hours, stopped by adding 5 µl 0.25M EDTA, heated at 70°C for 5' and placed on ice. Labeled probe DNA was separated from unreacted label on a 20cm x 1cm Ultrogel column and collected in 1-1.5 ml. About $5x10^7$ dpm were obtained as measured by Cerenkov counting.

Other enzyme reactions

T4 polynucleotide kinase and T4 DNA polymerase were used as described in Molecular Cloning[45], pp. 117-126. Klenow enzyme (DNA polymerase I large fragment) was used for end-labeling restriction fragments by adding enzyme (0.1-0.2 µl at 5 units/µl) and labeled dNTP (0.2-0.5 µl) directly to the restriction digest and incubating at room temperature for 10'.

DNA SEQUENCING

All sequencing was done on double-stranded DNA using a version of dideoxy sequencing[51]. Conditions were as given below.

Plasmid DNA (1 μg) was digested with a restriction enzyme in 10 μl buffer to linearize it. About 10 pmol of sequencing primer was added and the mixture was heated in a boiling water bath for 3-5' and then plunged into a water-ice or ethanol-dry ice bath to anneal. (Somewhat better results were obtained with the latter.) To the DNA mixture was added 1 μl [α-^{32}P]-dATP and 1 μl 12.5mM dATP and it was mixed thoroughly. 2 μl aliquots were then added to four tubes containing 2 μl of the sequencing mixes, as follow:

C: 5.5 μM dCTP, 250 μM ddCTP, 110 μM dGTP and dTTP;

T: 5.5 μM dTTP, 500 μM ddTTP, 110 μM dCTP and dGTP

A: 50 μM ddATP, 75 μM dCTP, dTTP and dGTP;

G: 5.5 μM dGTP, 300 μM ddGTP, 110 μM dCTP and dTTP.

To each tube was then added 2 μl Klenow enzyme, diluted to 0.25 units/μl. The tubes were mixed by centrifuging them in an Eppendorf and incubated at 30°C for 15'. 2 μl 0.5mM dNTPs and 2 μl Klenow were added as a chase and left to react for another 15'. The reaction was stopped by adding 4 μl formamide dye stop mix to each tube.

The reaction mixtures were heated in a boiling water bath for a few minutes before loading them on a sequencing gel (about 3 μl/slot). Electrophoresis was carried out at 1400-1800 V for 1.5-3 hours, with metal plates used to distribute the heat evenly and reduce "smiling". The gel was dried down onto 3MM filter paper and autoradiographed on Kodak XAR-5 for 1-3 days.

GEL ELECTROPHORESIS

Polyacrylamide

8-20% polyacryamide gels were prepared as described in Molecular Cloning[45], pp. 174-177. Tris-borate buffer was routinely used for pouring and running all gels. Those gels used to isolate short DNA fragments or to separate end-labeled digestion products were run at 100-200 V for several hours or overnight. Those used to purify oligonucleotides or for DNA sequencing contained 7M urea and were run at 1000-1800 V for 1.5-3 hours. Gels for autoradiography were usually dried down onto 3MM filter paper before exposure.

Agarose

0.5-2% agarose gels were poured in preformed plastic trays of varying sizes, depending on the number of samples to be loaded. 0.5 µg/ml ethidium bromide was routinely added to the warm gel mix just before pouring. Tris-borate buffer was again used to pour and run all gels.

RECOVERY OF DNA FROM GELS

Electroelution

In most cases where DNA was to be recovered from polyacrylamide gels, electroelution into a dialysis membrane was used. Crushed gel was loaded into a short Pasteur pipette, a dialysis bag was fastened onto the end, and

the tube was used to form an electrical bridge between two buffer (20mM Tris-acetate, 1mM EDTA, pH8) reservoirs. Electroelution into the dialysis bag was carried out at 150 V for 2 hours, and DNA recovered from the bag by precipitation and centrifugation.

DEAE-paper and DEAE-membrane

This was the method of choice[52] for recovering DNA from agarose gels; it was also occasionally used for polyacrylamide gels. A slot was cut in the gel in front of the desired band and a small piece of DEAE-paper or DEAE-membrane, a little higher than the thickness of the gel and a little wider than the width of the band, was inserted. Electrophoresis was continued in the original direction until the band had run into the paper, which was then removed and rinsed with distilled H_2O. DNA was eluted from the paper by incubation in 1.5M NaCl in TE for 2 hours at 37°C and recovered by precipitation, redissolving in H_2O and reprecipitating to remove the salt before finally redissolving in H_2O or TE. DEAE-membrane (NA45, 0.45 µm, obtained from Schleicher and Schuell) proved superior to DEAE-paper, in that it was less prone to shred during the elution of the DNA.

SOUTHERN BLOTTING

The directions of the GeneScreen manufacturer[53] were followed, with some exceptions. The gel was incubated in 0.5 NaOH-1.5M NaCl for 30' at room temperature with gentle agitation to denature the DNA, and then neutralized in 0.5M Tris-Cl, pH 7.5-1.5M NaCl for 30'. It was then placed on a double thickness of filter paper dipped into a reservoir of 10x SSC, and covered with

the GeneScreen, six layers of filter paper cut to size, and a thick layer of paper towels. The transfer was allowed to continue until the gel reached a thickness of about 1 mm - no longer than 6 hours. The GeneScreen blot was then washed and dried as described.

Instead of the recommended dextran sulfate solution for prehybridization, the solution suggested in Molecular Cloning[45], p. 387 was used: 6xSSC, 0.5% SDS, 5x Denhardt's solution and 100 µg/ml denatured salmon sperm DNA. The filter was incubated for 2 hours at 65°C, and then probe was added in 1-1.5 ml buffer + 100 µg/ml salmon sperm DNA. Hybridization was allowed to take place at 65°C overnight (at least 12 hours). Washing and autoradiography were as described.

OLIGONUCLEOTIDE SYNTHESIS

Deoxyribooligonucleotides were synthesized using the phosphitetriester method as described[54], either by hand or in a Applied Biosystems 380A DNA Synthesizer. Several were supplied by Tom Atkinson (UBC) in their crude form. Purification was by electrophoresis in a 20% polyacrylamide gel containing 7M urea. The band of the appropriate mobility (usually the only one visible) was cut out, crushed and incubated overnight in 0.5M ammonium acetate/10mM magnesium acetate. The oligonucleotide was then isolated by passing the gel elution solution through a C_{18} SepPak cartridge, which under these ionic conditions retains the oligomer. The oligomer was then recovered in 1 ml 60% methanol and evaporated to dryness under vacuum.

RESULTS AND DISCUSSION

A. CONSTRUCTION OF POTENTIAL TELOMERIC RECOGNITION SEQUENCES

1. Constructing inserts for cloning.

The initial task of the project was to develop a procedure for producing long stretches of a simple canonical repeat sequence, in a form which could readily be used to construct inverted repeats in a suitable *E. coli*/yeast shuttle plasmid. The pUC plasmid cloning system[39] was chosen as a suitable means of introducing different restriction sites on either end of the repetitive "telomeric" sequence, since it readily allows characterization and sequencing of random clones.

The first sequence chosen as a target of the synthesis and cloning strategy was the C_4A_2/G_4T_2 canonical repeat from *Tetrahymena*.[10] At the time this work was carried out, this was the only sequence known definitely to be recognized in yeast in the Szostak telomere assay system.[29] This sequence would therefore serve as a useful control to ensure that Szostak's original result could be duplicated in the JC8111 system, before testing other sequences of unknown functional ability.

Two 12-mers were constructed using the phosphitetriester method of oligodeoxyribonucleotide synthesis (Materials and Methods). The sequences

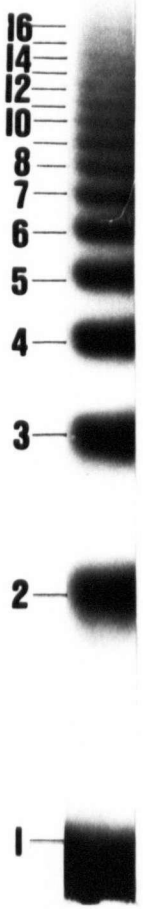
chosen were 5'-CCCCAACCCCAA-3' and 5'-GGGTTGGGGTTG-3'. When annealed, these oligomers should give rise to a mixture of two duplexes: one with 3-nucleotide 3' "sticky ends" and one wiith 3-nucleotide 5' "sticky ends". Each type of duplex can be ligated to itself to form long stretches of C_4A_2 and G_4T_2 repeats. The sticky ends ensure that the CA and GT sequences will be strictly segregated to the different strands, as is observed in the natural *Tetrahymena* telomere.

Following purification, isolation and kinasing of the two 12-mers, as described in Materials and Methods, 1 µg of the annealed duplex was incubated with T4 DNA ligase in a total volume of 10 µl for 2 hours at room temperature (see Materials and Methods for buffer conditions). A ligation done in parallel on linkers kinased with [γ-^{32}P]-ATP was used as a check on the effectiveness of the ligation reaction. As shown in Figure 3, up to 16 or more consecutive repeats of the basic 12-mer could be detected in the ligation mixture, corresponding to 30 repeats of the canonical C_4A_2 unit. This is well within the reported in vivo range of *Tetrahymena* telomere lengths, which contain 20-70 repeats of the basic unit[5]. Because one of the questions that we wished to address using this system was the effect of sequence length on the resolution reaction, it was decided to clone the entire mixture of ligated inserts and select a range of sizes for subsequent testing in yeast.

Since the sticky ends of the ligated duplexes did not correspond to any that could be produced by restriction enzyme digestion within the pUC multiple cloning site, it was necessary to clone them into a blunt ended restriction site. To 10 µl ligation reaction (stopped by adding EDTA to 5mM and heating

Figure 3. Ligation of oligomers to produce long stretches of telomere-type sequence.

A mixture of the two oligomers 5'-CCCCAACCCCAA-3' and 5'-GGGTTGGGGTTG-3' (1 µg each) was labeled with [γ-^{32}P]-ATP and polynucleotide kinase[45], and then incubated with DNA ligase for 10' at 0°C. 2.5 µl (0.25 µg DNA) of the reaction was fractionated on a 10% polyacrylamide gel. Autoradiography was carried out at -70°C for 8 hours. Numbers refer to lengths of the DNA fragments in each band, in multiples of the original 12 bp unit (equal to half the number of C_4A_2/G_4T_2 repeats each fragment contains).



for 15' at 70°C) were added 2 µl 10x T4 polymerase buffer (Materials and Methods), 1 µl 2mM dNTPs, 7 µl H_2O and 0.5 µl T4 DNA polymerase. The reaction was incubated for 30' at 37°C and then stopped by heating at 70°C for 15'. T4 DNA polymerase, in the presence of all four dNTPs, fills in 5' overhangs and chews back 3' overhangs, to generate a population of blunt-ended molecules.[45]

2. Cloning in pUC 12.

The HincII site in pUC 12 was chosen for insertion of the blunt-ended synthetic telomere sequences. A variety of approaches was used to try to clone the telomeric inserts into the pUC vector. These included straightforward ligation with a 10x molar excess of insert over vector (0.5 µg DNA in 30 µl volume); phosphatasing the cut vector with calf intestinal phosphatase before ligation; phenol extraction and ethanol precipitation of the vector and/or the inserts before ligation. In all cases where white colonies were isolated, following transformation of the ligation mix into JM101 and using the X-gal colour assay[39], subsequent analysis showed that inactivation of the β-galactosidase α-peptide was due to the loss of one or two base pairs before religation of the vector, giving a frame-shift mutation in the peptide coding sequence (data not shown).

The inserts were eventually cloned using a novel approach which was later applied to the construction of inverted repeats and to other cloning tasks involving blunt-ended ligation. HincII-cut pUC (0.4 µg) and 1 µg inserts were ligated in 20 µl ligation buffer with 2 units ligase for 30' at room temperature. This high DNA concentration and the high molar excess of

inserts over vector were designed to ensure that most vector molecules would be joined to at least one blunt-ended insert molecule. After 30', the reaction was diluted 10-fold to 200 µl ligation buffer, and a further aliquot of ligase was added and left to react overnight. The reaction was stopped before transformation by heating at 70°C for 15', 25 µl was added to 200 µl competent JM101 cells, and the transformation carried out as described in Materials and Methods.

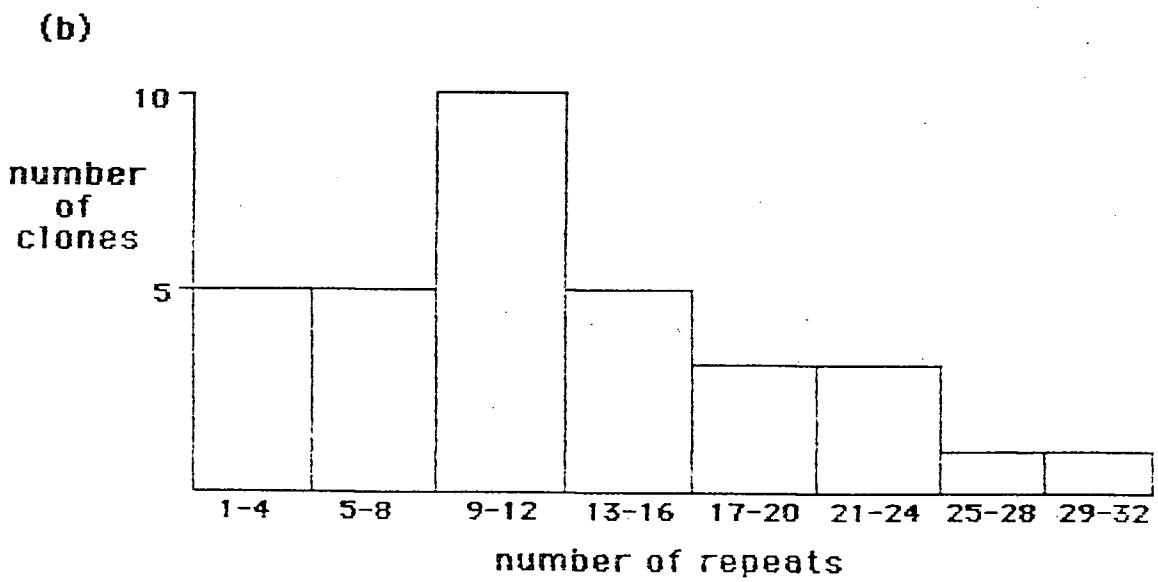
Approximately 250 colonies were obtained from four 25 µl transformations. Of these approximately 25% were white. (The high background of blue colonies was presumed to be due to the vector not being phosphatased before ligation; it was consistent with the number obtained from a control ligation in the absence of inserts.) White colonies were picked and grown in 5ml overnight cultures, and plasmid DNA minipreps prepared (Materials and Methods). Plasmid DNAs were digested with BamHI and PstI (which cut on either side of the HincII site; see Figure 2), labeled with [α-^{32}P]-dATP (0.25 µl) plus unlabeled dGTP and Klenow enzyme, and electrophoresed on a 10% polyacryamide gel with size markers (Figure 4a). Approximately 1/3 of the digests showed the presence of inserts of various sizes, ranging from ~20 to ~200 bp. The other white colonies were presumably due to frame-shift mutations at the HincII site as suggested above (see Figure 4a).

Figure 4b shows the size distribution of the 33 'A' clones characterized. Unexpectedly, it does not show the same pattern as the distribution of label in the original ligation mixture (Figure 3); in fact, no clones of the original 12 bp sequence (or 15 bp, with the overhangs filled in) were ever isolated. It may be that DNA fragments below a certain size were not good substrates

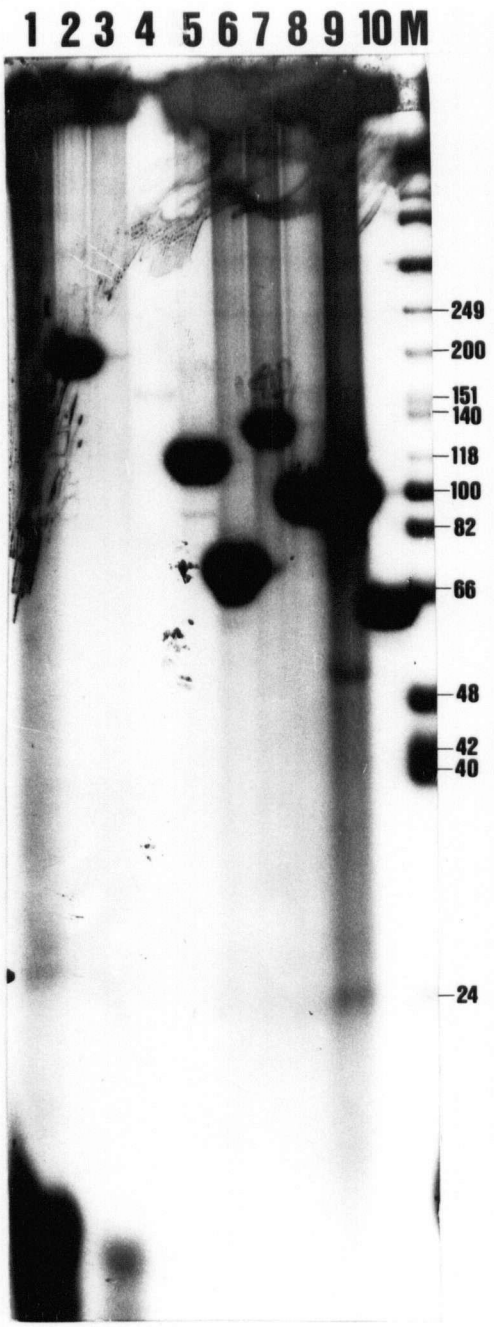
Figure 4. Cloning of telomere-type sequences constructed <u>in vitro</u> into pUC12.

(a) Plasmid DNAs were prepared from isolated transformed JM101 colonies and digested with BamHI and PstI, enzymes which cut on either side of the HincII insertion site in pUC12. The DNA fragments were end-labeled by incubation with DNA polymerase I Klenow fragment, dGTP and [α-^{32}P]-dATP, fractionated on 10% polyacrylamide, and autoradiographed at -70°C for 2.5 hours. A HinfI digest of øX174 DNA, labeled in a similar way, was used as a size marker. The figure shows a random sample of ten clones, seven of which (lanes 2,5-10) clearly contain inserts with a size range of 65-200 base pairs, or approximately 10-30 repeats of the basic 6-mer unit. The faint contaminating bands seen in several lanes were ignored as insignificant when these experiments were first carried out; however, in light of later results (see below), these bands may in fact have resulted from deleted or recombined telomere-type inserts.

(b) A total of 33 insert-containing clones were identified and approximately sized using the above approach. The size distribution of the insert DNAs shows a distinct maximum at 9-12 repeats of the basic 6-mer.



(a)



for T4 DNA polymerase, and were thereby excluded from the blunt-ending reaction.

3. Sequencing the pUC12 clones.

Since the cloned inserts contained no known restriction enzyme sites, the only reliable way to characterize them was by sequencing. This is in principle readily accomplished in the pUC system, since appropriate universal primers from each side of the multiple cloning site are commercially available for use in double-strand enzymatic sequencing.

A number of attempts were made to sequence the inserts directly from the plasmid miniprep DNA, using the protocol given in Materials and Methods for double-strand sequencing. These efforts proved almost totally unsuccessful, with numerous artifact bands present in the sequencing gel, no matter what attempts were made to clean up the preparation by RNase digestion and precipitation of the DNA.

Eventually the strategy was adopted of characterizing the inserts initially on the basis of size, as described above, picking a suitable range of sizes and growing up these plasmids in an overnight 500ml culture. The DNA from these large preparations was then purified on a CsCl gradient as described in Materials and Methods. Using this approach, readable sequences were obtained for 10 different clones of the C_4A_2/G_4T_2 repeating sequence, covering a length range of 21 to over 190 bp of the canonical repeat. Sequences for four of these clones which were used in later experiments are given in Figure 5a.

Figure 5. Sequences of telomere-type inserts cloned in pUC12.

Inserts are identified by the sequence of their basic repeat unit and the number of consecutive nucleotides of that repeating sequence that they contain. All sequences were obtained using double-strand dideoxy sequencing from the pUC/M13 universal forward primer. For each insert, the complete sequence between the flanking PstI and XbaI sites is shown, in the 5' to 3' direction.

(a) 'A' sequence: $(CCCCAA)_n \bullet (GGGGTT)_n$.

A21: CTGCAGGTGGGTT$(GGGGTT)_2$GGGGGACTCTAGA

A37: CTGCAGGTCGTT$(GGGGTT)_5$GGGGGACTCTAGA

A69: CTGCAGGNN$(CCCCAA)_{11}$CCCGACTCTAGA

A190: CTGCAGGTCGGGTT$(GGGGTT)_{30}$... The resolution of the gel was not sufficient to read all the way through to the Xba site, but 31 complete 6-mer repeats could be seen.

(b) 'B' sequence: $(CA)_n \bullet (GT)_n$.

B23: CTGCAGGTC$(CA)_{11}$CGACTCTAGA

B49: CTGCAGGTC$(CA)_{24}$CGACTCTAGA

B95: CTGCAGGTC$(CA)_{47}$CGACTCTAGA

At this point a naming system was adopted for the sequenced plasmids. The C_4A_2/G_4T_2 repeat unit was designated sequence A, and a plasmid carrying an insert of 37 bp of this canonical repeat sequence will be referred to as pA37, while the insert itself is known simply as A37.

The entire synthesis, cloning and sequencing strategy was subsequently applied to a second canonical repeat sequence, the simple CA/GT repeat. The two oligos synthesized for this were 5'-$(CA)_6$-3' and 5'-$(GT)_6$-3'. On annealing, these would result in a duplex with one-nucleotide sticky ends. The cloning protocol described above gave rise to approximately 70 white colonies out of a total of 250 from a single transformation. From twelve plasmid minipreps, six plasmid DNAs were obtained which contained inserts ranging from ~20 to ~115 bp, as judged by gel sizing (data not shown). Three clones were purified on CsCl and sequenced (Figure 5b).

Since these experiments were done, improved procedures for sequencing plasmid miniprep DNAs have been reported. These methods include additional DNA purification steps such as the use of NACS52 columns (R.O. Shade, personal communication) or Sepharose CL-4B columns (R. Lansman, personal communication.)

B. TRANSFORMATION OF JC8111.

Having obtained suitable clones of potential telomere recognition sequences in pUC12, the next step was to construct inverted repeats from them in a suitable *E. coli*/yeast shuttle vector. As discussed in the Introduction, most strains of *E. coli* do not faithfully replicate long inverted repeats when they

are introduced in plasmid DNA. For this reason Szostak's original experiment was performed using a ligation mixture, rather than attempting to clone and characterize a plasmid of the desired structure.[29]

It was hoped to circumvent this difficulty through the use of the *E. coli* strain JC8111 as a cloning vehicle, as outlined in the Introduction. The one major drawback to the use of this strain was its exceptionally low transformation efficiency. In the original study by Boissy and Astell[34], an efficiency of $4x10^3$ transformants/μg DNA was reported for uncut plasmid DNA, some 500-fold lower than the efficiency obtained with DH1 cells.

During the work described in this thesis, JC8111 rarely gave efficiencies above $5x10^2$ transformants/μg DNA using the calcium chloride transformation procedure (Materials and Methods). Since cutting and ligating DNA can reduce transformation efficiencies by 100-fold or more, this efficiency is too low to allow routine isolation of recombinant molecules.

It was therefore decided to try applying the Hanahan protocol[48] for very high efficiency transformation of *E. coli*. This technique had been reported to give up to 10^8 transformants/μg DNA with DH1 and other strains, an increase of about 100-fold over the calcium chloride technique. A series of experiments was done to test the effect of different ingredients in the transformation mix. As shown in Table II, the version of the Hanahan protocol which involves snap-freezing the cells in 10% glycerol was found to give transformation efficiencies as high as $2x10^5$ transformants/μg DNA, an increase of 400-fold over the calcium chloride technique.

Table II. Transformation efficiency of JC8111 cells made competent in different media.

Cells were grown and treated as described by Hanahan[48]. Composition of the transformation medium was as given. Addition of 10mM K-MES, pH 6.1 to any of the media resulted in a 2-10 fold decrease in transformation efficiency (data not shown). Cells were assayed immediately after preparation in all experiments.

Transformation medium	Transformation efficiency (colonies/μg pBR322)
50mM $CaCl_2$	500
45mM $MnCl_2$ 10mM $CaCl_2$	800
45mM $MnCl_2$ 10mM $CaCl_2$ 75mM DTT	5000
45mM $MnCl_2$ 10mM $CaCl_2$ 100mM RbCl 7% DMSO 75mM DTT	12,000
45mM $MnCl_2$ 10mM $CaCl_2$ 100mM RbCl 3mM HCC 7% DMSO 75mM DTT	20,000
45mM $MnCl_2$ 10mM $CaCl_2$ 100mM RbCl 3mM HCC 7% DMSO 10% glycerol + snap-freezing	50,000-200,000

The effects of some other variables in the transformation procedure were tested, including the effects of growth time, pH of the transformation medium, repeated freezing and thawing of the cells and time of incubation before plating. These results are summarized below.

Growth time. There was little variation in transformation efficiency among cells prepared from cultures over an OD range at 550nm of 0.4 to 0.9 - not more than 2-3 fold in any experiment. The best results were obtained from cells which grew to OD 0.6-0.7 within 3 hours.

Effect of buffer. It was found that inclusion of the 10mM K-MES, pH 6.1 called for in the original Hanahan protocol - or 10mM K-MES over a range of other pHs - reduced transformation efficiencies from 2- to 10-fold. In subsequent transformations buffer was routinely omitted from the transformation medium (see Materials and Methods for complete protocol). Similarly the presence of Tris in the transforming DNA sample (for instance in ligation buffer) has been observed to reduce transformation efficiencies in some experiments. DNA was therefore usually precipitated with ethanol and resuspended in distilled H_2O before mixing it with JC8111 cells for a transformation.

Cell treatment. Repeated freezing and thawing of the cells had no effect on transformation efficiencies, and neither did incubation in growth medium for times longer than one hour prior to plating. (Because JC8111 grows noticeably more slowly than standard *E. coli* strains, it was a possibility that the low recovery of transformants was due to incomplete induction of ampicillin resistance at the time of plating. However, the failure to obtain

more transformants from longer transformation times suggests that this is not the case. The observation that, regardless of whether the $CaCl_2$ or the Hanahan technique is used, JC8111 remains about 1000 times less efficient at transformation than DH1, further suggests that this difference is an inherent property of the strain, independent of the transformation conditions. Although it is tempting to speculate that the low transformation efficiency is related to the recBCsbcBrecF genotype of this strain, there is no direct evidence that this is the case).

Using the protocol given in Materials and Methods, transformation efficiencies for JC8111 of $5x10^4$ transformants/μg plasmid DNA were routinely obtained. Despite several attempts, the 4-fold increase over this seen in some experiments could not reliably be reproduced. (Subsequently, it has been discovered that if the heat shock step during transformatiion is done at 37°C rather than 42°C, the efficiency of transformation is increased by at least two-fold (R.O. Shade, personal communication).)

C. REPLICATION OF YCp PLASMIDS

1. Construction and characterization in *E. coli*

The first sets of experiments involving construction of inverted repeats in an *E. coli*/yeast shuttle plasmid were done using the centromeric plasmid YCp74 (a gift of Johnny Ngsee). YCp74 carries a 1.5 kb EcoRI fragment containing the TRP1 and ARS1 sequences from yeast and a 627 bp Sau3A fragment containing CEN3, cloned into the EcoRI and BamHI sites,

respectively, of pBR322. (See Materials and Methods and Figure 2 for details on YCp74 construction.)

The rationale behind using a centromeric yeast plasmid for the resolution experiments was that it might allow more sensitive detection of small differences in resolution efficiency than would an acentric plasmid. Acentric circular yeast plasmids undergo a large increase in mitotic stability (approximately 5-fold) when they are linearized by telomere addition[55]; one consequence is that even a poorly resolving plasmid could largely take over a selectively-grown yeast culture, making it very difficult to measure the original frequency of resolution. Centromeric circular plasmids, by contrast, are much less stable when linearized[56], so that plasmids which had a less than 100% chance of resolution in one replication cycle might be propagated in their circular form efficiently enough to be detectable even after many generations of cell division.

Attempts to construct identical inverted repeats were carried out in YRp74, a plasmid identical to YCp74 but lacking the 627 bp CEN3 insert. However, no suitable clones were ever obtained. This was probably due simply to chance, since the frequency of isolation of appropriate recombinant clones in JC8111 was very low (see below).

Two of the 'A' sequence clones were used to construct inverted repeats in the PvuII site of YCp74. Fifty micrograms of each of pA21 and pA190 were digested with either PstI and SmaI or XbaI and HaeIII, and the digests electrophoresed on a 1.5mm thick polyacrylamide gel, overnight. The gel was stained with 0.5 µg/ml ethidium bromide and the bands containing the

telomeric inserts were cut out. DNA was isolated from the bands by electroelution, as described in Materials and Methods, and dissolved in a final volume of 25 µl TE.

PvuII-cut YCp74 (0.5 µg) and 5 µl of the insert preparation were ligated together in 20 µl ligation buffer for 30' at room temperature. The mixture was then diluted to 200 µl ligation buffer and PvuII added to prevent recircularization of the original plasmid. Following overnight ligation, the mixture was heated at 65°C for 15'; NaCl was added to 50mM, and PvuII was used for a final 1 hour digestion at 37°C. 25 µl aliquots were then used directly to transform competent JC8111 cells ($2x10^5$ transformants/µg pBR322).

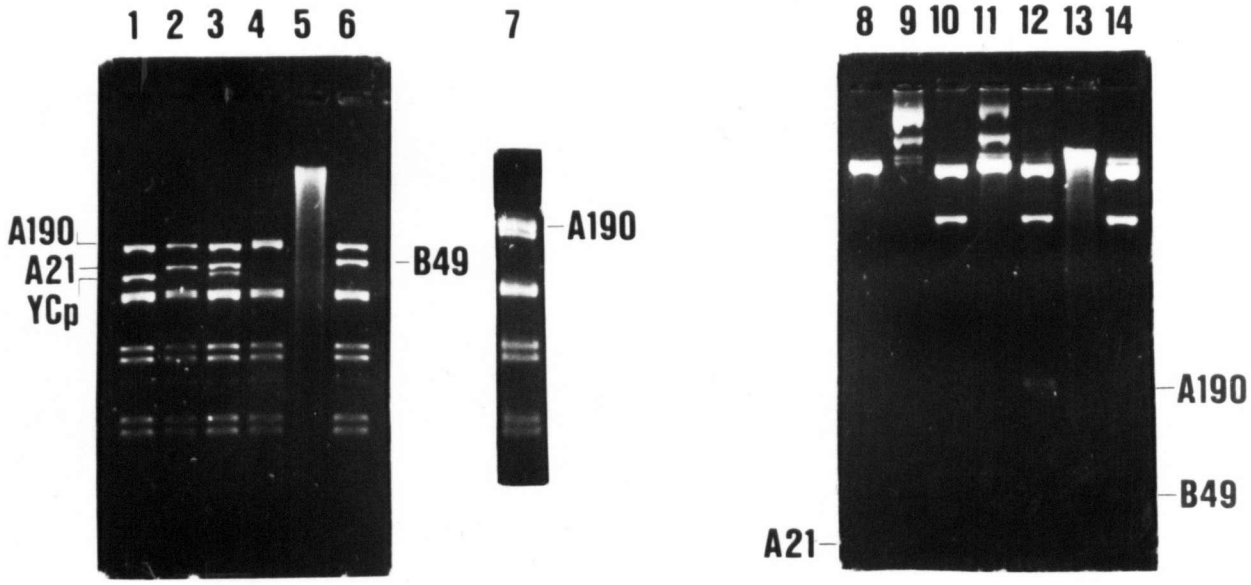
Five colonies from each transformation were grown up and plasmid DNA minipreps prepared. TaqI digestion was used as an initial screen for recombinant clones. Clones which carried an insert of approximately the expected size, and appeared otherwise intact when the Taq digest was electrophoresed and visualized on an agarose gel, were further characterized by a HindIII/BamHI digest. It proved necessary to repeat the ligation and transformation procedure in order to isolate appropriate clones of YCpA21XH and YCpA190XH. (The XH or PS designation refers to the enzymes used to isolate the insert - either XbaI/HaeIII or PstI/SmaI - and therefore to the orientation of the sequences relative to the centre of the inverted repeat. Refer to Figures 2 and 5 for further details.)

Eventually one clone of each of the A21PS, A21XH, A190PS and A190XH inverted repeats in YCp74 was isolated, grown in a large scale DNA prep and

Figure 6. Restriction enzyme analysis of inverted repeats constructed in YCp74.

Plasmid DNAs purified on a CsCl gradient were digested with the appropriate restriction enzyme and fractionated on a 1.2% agarose gel. Lanes 1-7, TaqI digests; lanes 8,10,12,14, BamHI; lanes 9,11,13, PstI. Plasmids were YCp74, lanes 1,8,9; YCpA21PS, lanes 2,10; YCpA21XH, lanes 3,11; YCpA190PS, lanes 4,12; YCpA190XH, lanes 5,7 (miniprep),13; YCpB49PS, lanes 6,14.

The A21PS, A190PS and B49PS plasmids all appear to contain a perfect, stable inverted repeat of the right length (size marker data from other experiments, not shown), while the A21XH plasmid is clearly unstable. (This may explain why no band corresponding to the excised inverted repeat is visible in the PstI digest, lane 11.) The A190XH clone behaved somewhat inexplicably. In two separate plasmid preps purified on CsCl the recovered DNA appeared to be degraded (lanes 5,13). However, on subcloning back into JC8111 and isolation in a plasmid miniprep, a normal plasmid carrying an apparently stable inverted repeat was observed (lane 7 and data not shown).



purified on a CsCl gradient. The same procedure was carried out to prepare a clone of YCpB49PS. Results of restriction enzyme digestion of these five clones are shown in Figure 6, with the parent plasmid YCp74 shown for comparison. The BamHI and PstI digests were chosen because these enzymes cut at either end of the inverted repeats, in the PS and XH orientations respectively (see Figure 2). Liberation of an appropriately sized fragment by these enzymes is thus convincing evidence that the plasmid contains the desired sequence.

Neither of the two XH clones passed this test. Interestingly, the YCpA21XH clone was unstable in JC8111, although the remaining four clones appeared to be propagated faithfully. (Inconsistent results were obtained with YCpA190XH - see Figure 6.) The unstable YCpA21XH clone was twice subcloned by transformation into JC8111 and isolation of single colonies; each time no transformants carrying a stable full-length insert were isolated (Figure 7).

As a final step towards characterization of these clones prior to their transformation into yeast, double-strand enzymatic sequencing was carried out on four of them. To avoid the necessity for subcloning the inserts into a sequencing vector, a 15 nucleotide-long primer was synthesized (automated method, see Materials and Methods), which was predicted from a computer search to hybridize uniquely to one side of the PvuII site in YCp74. (The primer, 5'-CGCTTCACGACCACG-3', consisted of nucleotides 2034-2048 of the pBR322 sequence in Molecular Cloning[45].) Figure 8 shows the data obtained by carrying out enzymatic sequencing using the protocol in Materials and Methods and the PvuII primer on these four clones.

Figure 7. Plasmid YCpA21XH remains unstable when subcloned in JC8111.

Plasmid YCpA21XH (lane 1 and Figure 5, lane 3) was transformed into JC8111, six isolated colonies were picked, plasmid minipreps prepared, and TaqI digests fractionated on a 1% agarose gel (lanes 2-7). The plasmid carrying the most stable insert (lane 3) was used to transform JC8111 for a second time, and the same procedure carried out on another 7 colonies (lanes 8-14). Each time no transformants were recovered which carried a stable, non-deleted form of the A21 insert; in fact, the majority had the insert entirely or nearly entirely deleted.

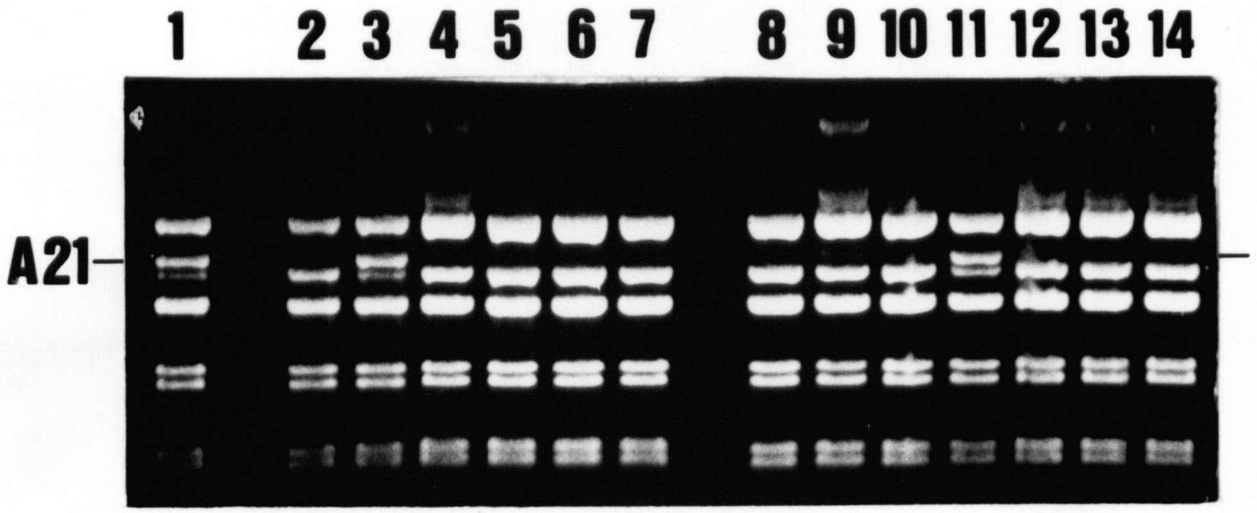


Figure 8. Double-strand dideoxy sequencing of inverted repeats cloned in YCp74.

Dideoxy sequencing was carried out on CsCl-purified plasmid DNAs containing inverted repeats of the A21, A190 or B49 sequences, using a synthetic oligonucleotide primer which hybridized to vector sequences on one side of the insert (Materials and Methods). The arrows mark the beginning of each insert sequence. In only one case, YCpA21PS, was a clear sequence obtained, showing the predicted sequence except for a missing G-C base pair at the PvuII/SmaI junction. The deleting clone of YCpA21XH gave readable sequence for 10-20 base pairs within the inverted repeat, which agreed with the sequence predicted from the pUC multiple cloning site. Beyond this region the sequence became unreadable, presumably reflecting the clone's heterogeneity. YCpA190PS and YCpB49PS both gave strong stop bands a few base pairs within the inverted repeat, as expected for long inverted repeats sequenced at 30°C. CsCl-purified clones of YCpA190XH were unsuitable for sequencing purposes (see Figure 6).

YCpA21PS
C T A G
centre of inverted repeat (PstI site)
YCpA21XH
C T A G
YCpA19OPS
C T A G
stop
YCpB49PS
C T A G
stop

The A21PS clone appears to carry a perfect inverted repeat of the expected sequence, with the exception of a missing base pair at the PvuII/SmaI junction. (Not all the sequence of the second half of the repeat can be read from the gel, but what can be seen is consistent with the above interpretation.) The deletogenic A21XH clone contains 6 unambiguous base pairs of the expected sequence (immediately following the PvuII/HaeIII junction), and from then on artifact bands become stronger and more numerous, presumably reflecting the heterogeneous composition of the DNA preparation.

Sequencing of the B49PS and A190PS clones ran into the expected problem with sequencing a long perfect inverted repeat[51]: namely, that self-association of the sequenced strand of DNA acts as a strong stop signal for the Klenow polymerase, generating a sharp band across all four lanes of the gel and preventing a readable sequence from appearing beyond that point. While the existence of this stop band is a good indication that the DNA does contain a long inverted repeat, it is of course no guarantee that the sequence of the repeat is the desired one. In this case, however, this seemed so probable as not to require further analysis before the plasmids were tested in yeast.

2. Replication in yeast.

The five clones in YCp74 described above were used to transform yeast strain C1 to tryptophan auxotrophy using the lithium acetate method (Materials and Methods). Yeast DNA minipreps were prepared from isolated colonies according to the procedure in Materials and Methods. DNA was

digested with restriction enzymes, separated on 0.7% agarose, blotted to GeneScreen and hybridized with nick-translated pBR322 as described.

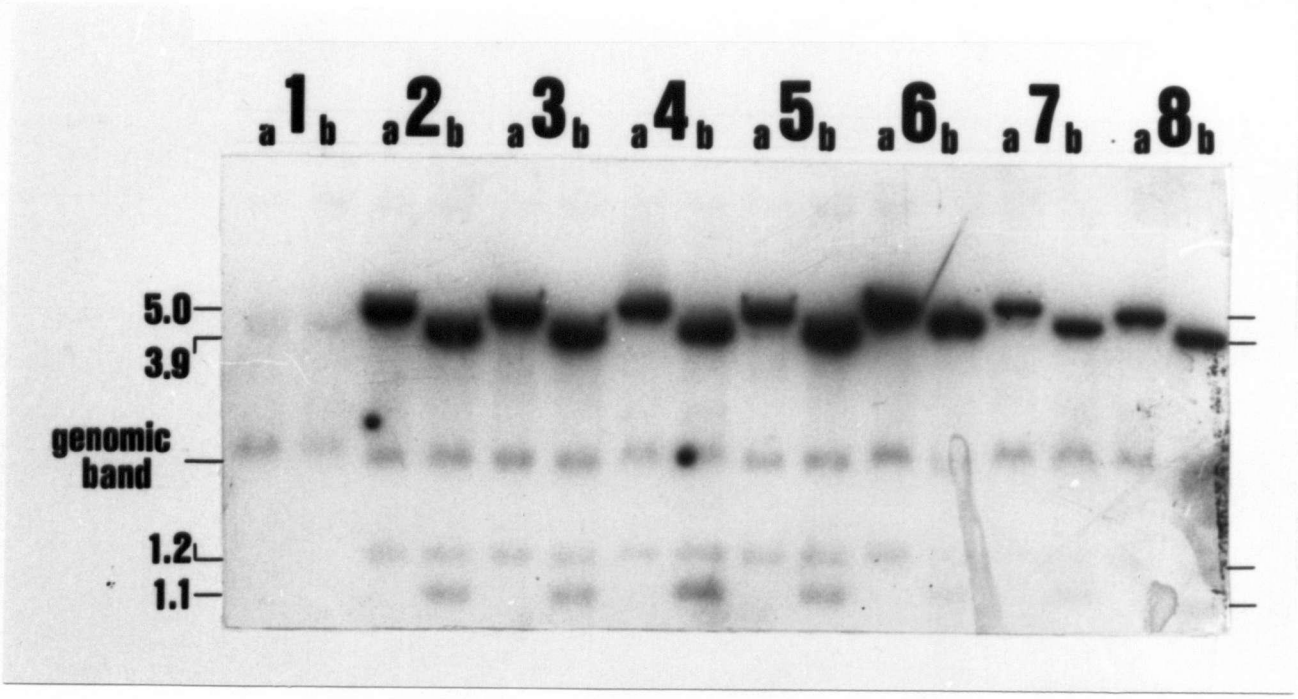
Figure 9 shows a typical autoradiograph of DNA from yeast cells transformed with four of the five clones. As can be seen, each of the eight transformants gave restriction patterns consistent with the original circular plasmid being replicated intact, without resolution. These experiments were repeated on a total of a further four A21XH, three A190XH and five B49PS yeast clones, these being the plasmids which carried an inverted repeat in the appropriate orientation for resolution. In all cases similar results were obtained (data not shown).

There are two possible explanations for the failure to observe resolution in the YCp plasmids, assuming that the cloned inverted repeats do in fact contain the intended sequences. One is that the presence of the CEN sequence, or some other sequence present on the plasmids, in some way interferes with the recognition or resolution of the inverted repeats. On the basis of the data presented here, this cannot be ruled out.

Another possibility, however, is that in order for the telomeric inverted repeats to be recognized, they must be closely linked to an ARS sequence. All telomeres so far characterized as functional in yeast appear to carry an ARS activity within a few hundred base pairs of the terminal sequences.[8,18,25] In order to test this possibility, a new cloning vector, pMF1, was constructed as described in the following section.

Figure 9. YCp74-derived plasmids with telomere-type inverted repeats replicate as circles in yeast.

DNA was prepared from 50-ml yeast cultures, digested with restriction enzymes, fractionated on an agarose gel, blotted to GeneScreen and hybridized with nick-translated pBR322. Lane 1, host strain C1 DNA; 2,3, two clones of C1 transformed with YCpA21PS; 4,5, C1/YCpA21XH; 6,7, C1/YCpA190PS; 8, C1/YCpA190XH. (a) EcoRV digest; (b) EcoRV/SalI double digest. Numbers refer to size of DNA in band (in kilobase pairs). Restriction patterns are those predicted from a circular map of the plasmids (Figure 2).



D. REPLICATION OF pMF1 PLASMIDS

1. Construction of pMF1.

The first object of this new approach was to obtain an ARS sequence which could readily be inserted next to the cloned telomere sequences in pUC12. The method chosen was to clone an ARS separately in the pUC12 BamHI site. This would leave suitable restriction sites for joining it to the cloned 'A' and 'B' sequences, and inserting it into a pBR322-based shuttle plasmid.

Since ARS1 was already available in the YRp74 and YCp74 plasmids, it was chosen as the target sequence. YRp74 was digested with HindIII, and approximately 1 µg of the 867 bp fragment carrying ARS1 was isolated from an agarose gel using the DEAE membrane technique (Materials and Methods). This fragment was then redigested with RsaI and HinfI, and the DNA electrophoresed on an 8% polyacrylamide gel. The 189 bp band predicted to carry the whole of the ARS function was visualized by ethidium staining and reisolated from the gel on DEAE membrane. Half of the recovered DNA was mixed with 200ng BamHI-cut pUC12 in 10 µl T4 DNA polymerase buffer containing all four dNTPs. The mixture was incubated with T4 DNA polymerase for 30' at 37°C to blunt-end all the DNA molecules. The reaction was then stopped by heating at 70°C for 15'. Ligase was added in a total volume of 20 µl ligation buffer and the mixture incubated for 30' at room temperature; it was then diluted to 100 µl ligation buffer to promote recircularization and incubated overnight.

The ligation mixture was used to transform JM101 to ampicillin resistance. Several hundred colonies were recovered, nearly all of them white in the X-gal colour assay. Plasmid minipreps were prepared from 48 clones. One of these carried an insert of about 200 bp between the EcoRI and XbaI sites; the others presumably had had their BamHI sites filled in and religated. The recombinant clone was grown in a 500ml culture, and 1.34 mg plasmid DNA was recovered from a CsCl gradient. Sequencing of the insert using both forward and reverse universal primers gave unambiguous sequence for the entire cloned region, and showed that the plasmid, pUCR1, had precisely the sequence predicted on the basis of the cloning strategy (Figure 10).

In an initial attempt to test the effect of the ARS sequence on resolution of the inverted repeats, it was inserted next to the A69 and B95 sequences as an EcoRI-XbaI fragment, replacing the original EcoRI-XbaI region from the pUC multiple cloning site. The ARS-A69 and ARS-B95 dimers were then excised from the recombinant plasmids as PstI-SmaI fragments, isolated from an agarose gel on DEAE membrane, and ligated to PvuII-cut YRp74 or YCp74 as for the previous YCp constructions. The ligation mixtures were used to transform JC8111 to ampicillin resistance. Eleven colonies were recovered from the four ligations. Inspection of a TaqI digest of the plasmid DNAs suggested that all the clones either were the same as the parent plasmid, or had undergone massive deletion events which left little more intact than the ampicillin resistance gene and the pBR322 origin of replication (Figure 11).

It was concluded that the introduction of an inverted repeat of a sequence (ARS1) already present elsewhere on the same plasmid probably stimulated

Figure 10. Sequence of the ARS1-containing insert in pUCR1.

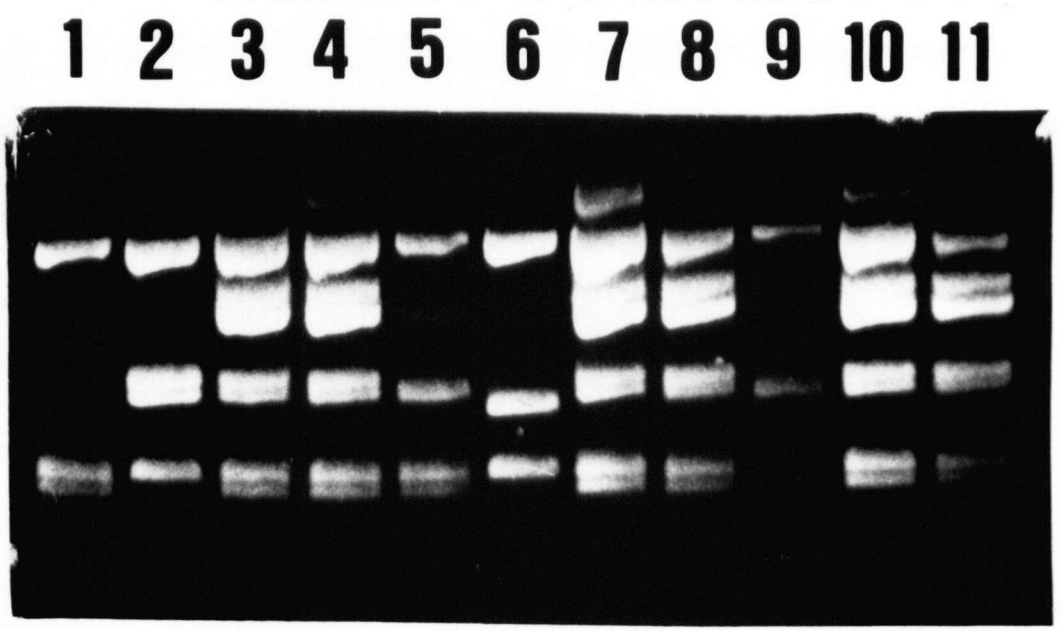
Dideoxy sequencing was carried out on pUCR1 from the universal forward and reverse primers which hybridize to either side of the pUC multiple cloning site. Enough sequence could be read from each direction to overlap in the centre of the insert and give unambiguous data for the full 189 bp.

The sequence agrees with that published for the TRP1ARS1 fragment in YRp7 [41], except that four base pairs have been removed from the centre of the former PstI site (as expected from the construction of YCp74: see Materials and Methods). pUC sequence in italics, ARS consensus sequence in boldface. The flanking SmaI and XbaI sites and the internal BglII site are underlined.

5'-*CCCGGGGATC* ACAATCAATC AAAAAGCCAA ATGATTTAGC ATTATCTTTA CATCTTGTTA TTTTACAGA**T** **TTTATGTTTA** GATCTTTTAT GCTTGCTTTT CAAAAGGCCG GCAAGTGCAC AAACAATACT TAAATAAATA CTACTCAGTA ATAACCTATT TCTTAGCATT TTTGACGAAA TTTGCTATTT TGTTAGAGT*G* *ATCCTCTAGA*-3'

Figure 11. Rearranged plasmid clones obtained from ligation of ARS1-containing inverted repeats into YCp74 and YRp74.

100ng YCp74 or YRp74 cut with PvuII was ligated with 100-200ng PstI-SmaI fragment containing ARS1 inserted next to the A69 or B95 telomere-type sequences, in a total volume of 40 µl ligation buffer. The ligation mix was used to transform JC8111 cells to ampicillin resistance. Plasmid miniprep DNA was prepared from the 11 colonies recovered, digested with TaqI and fractionated on a1% agarose gel. Lanes 1,2, A69R1/YRp74 ligation; lanes 3,4, A69R1/YCp74; lanes 5,6, B95R1/YRp74; lanes 7-11, B95R1/YCp74. Lanes 3,4,7,8,10,11 seem to be derived from recircularized vector, while lanes 1,2,5,6,9 correspond to drastically deleted forms of the original plasmid.



recombination between the copies of the sequence, even in JC8111. It was therefore decided to construct a new shuttle vector which carried no other copies of ARS1 (or any other ARS or CEN sequence), and which could be used routinely for the insertion and testing of different potential telomeric sequences.

Plasmid YpL1 is pBR322 containing a 2.2 kb SalI-XhoI fragment carrying the LEU2 gene. It was constructed by fusing the LEU2-carrying PstI-HindIII fragment from pSZ93 (Materials and Methods) with the complementary PstI-HindIII fragment from pBR322. Plasmid YpL2 was constructed by cutting YpL1 at the single HindIII site, filling in the ends with Klenow polymerase and dNTPs and religating, thus removing the HindIII site. Both plasmids were grown in 500ml cultures and purified on CsCl gradients.

pMF1 was constructed by ligating the SmaI-HindIII ARS1 fragment from pUCR1 as an inverted repeat in the PvuII site of YpL2. The SmaI-HindIII fragment was isolated from 5 µg pUCR1 by the DEAE membrane technique, and half was incubated with 250 ng PvuII-cut YpL2 in 10 µl ligation buffer containing 1mM HCC (see Materials and Methods) and 1 unit ligase for 30' at room temperature. The reaction was then diluted to 100 µl ligation buffer (without HCC) to promote recircularization, and ligated overnight; the DNA was then phenol-extracted, precipitated, dissolved in H_2O, redigested with PvuII, reprecipitated and taken up in 10 µl H_2O. This was used to transform competent JC8111 cells ($\sim 3x10^5$ colonies/µg pBR322) to ampicillin resistance. A total of 25 colonies was obtained.

Plasmid miniprep DNA was prepared and cut with BglII and BamHI, and the digests were fractionated on an agarose gel. Nine out of 24 clones carried BglII sites in the appropriate region. One of these clones was picked and grown up in a 500ml culture, and 70 µg plasmid DNA isolated from a CsCl gradient. This clone was named pMF1.

Figure 12 shows the results of restriction enzyme digestion of pMF1 by enzymes predicted to cut within the inverted ARS repeat. All of them give rise to fragments of the appropriate size, including HindIII which should cut exactly within the centre of the inverted repeat. It is notable that even after prolonged overdigestion of the pMF1 clone, a small fraction of the DNA remains uncut by these enzymes. This is probably due to a low frequency of deletion of the entire inverted repeat during pMF1 propagation in JC8111 (see below).

Since pMF1 contained the longest inverted repeat so far constructed in JC8111 (2 x 228 bp), it was tested for its ability to replicate intact in other *E. coli* strains. 250 ng pMF1 was used to transform JC8111, JM101, DH1 and RR1 cells prepared according to the Hanahan protocol. The competence of the latter three strains was aboout 100 times that of JC8111, as tested with pBR322. Whereas over 80 colonies were recovered from a single JC8111 plate, only four JM101, two DH1 and 44 RR1 colonies were recovered. Plasmid DNA was isolated from all the JM101 and DH1 colonies and six RR1 colonies, digested with RsaI and electrophoresed on an agarose gel (Figure 13). As can be seen, all twelve clones had undergone major deletions. Eleven of the twelve had clearly lost the band containing the inverted repeat; the remaining one, RR#1 (Figure 13, lane 9), had been drastically

Figure 12. The inverted repeat in pMF1 can be cut by the predicted restriction enzymes.

pMF1 (lanes 1-6) and YpL2 (lanes 7-12) DNAs were digested with restriction enzymes and fractionated on a 1.2% agarose gel. Lanes 1,7, HindIII/BamHI; lanes 2,8, XbaI/BamHI; lanes 3,9, SalI; lanes 4,10, PstI; lanes 5,11, BglII/BamHI; lanes 6,12, RsaI. Although the XbaI, PstI and BglII digests all liberate short inverted repeats from the centre of the ARS1 inverted repeat in pMF1, only the 304 bp fragment in the BglII digest (arrow) ran slowly enough to be visible on the gel.

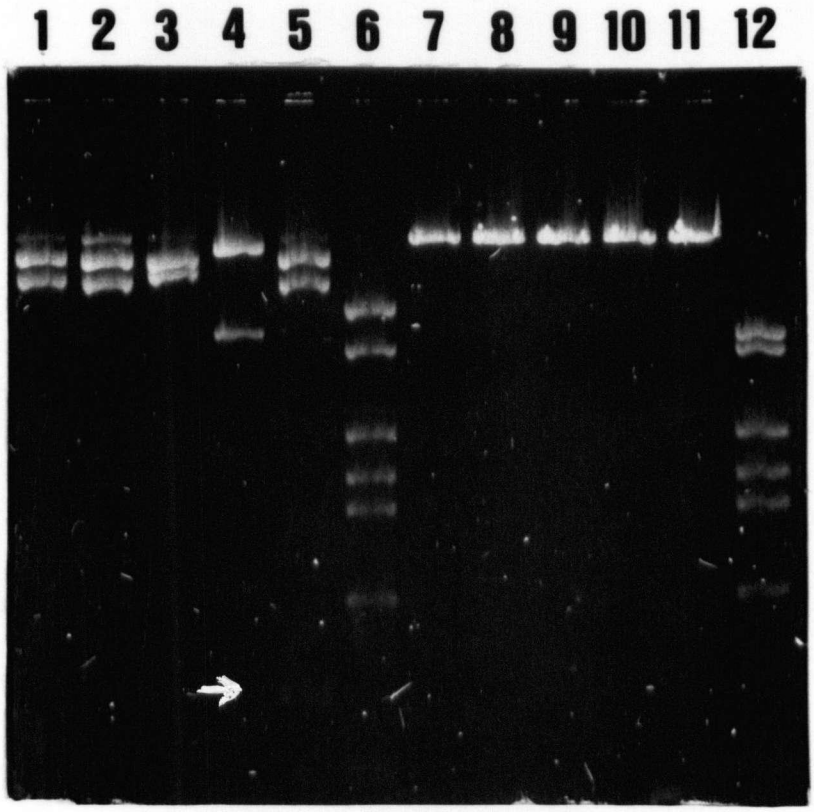
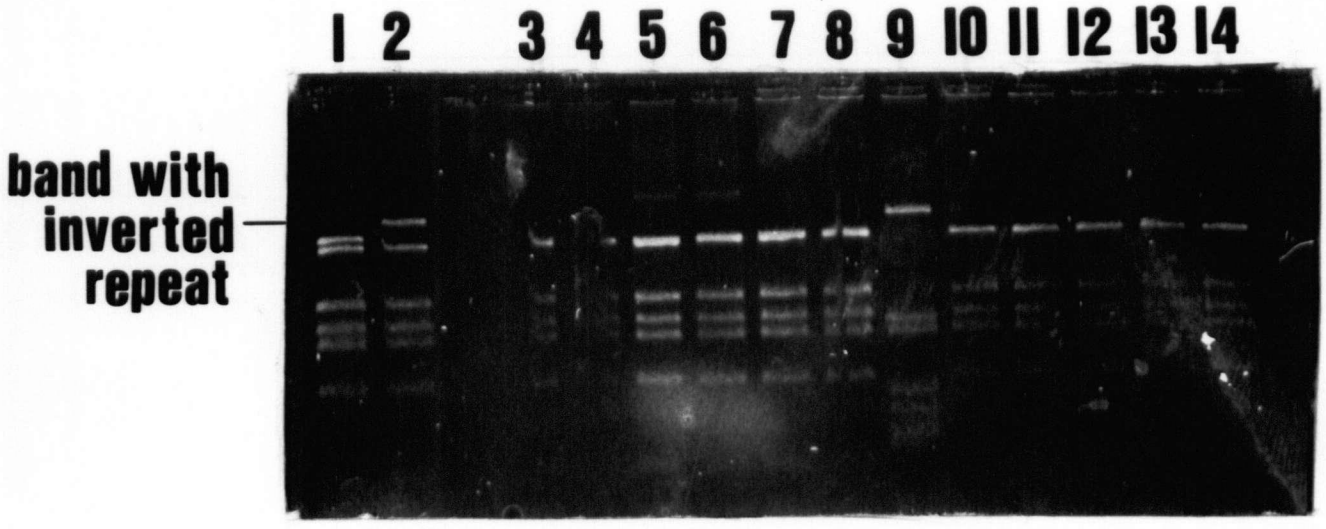


Figure 13. The 224 base pair inverted repeat in pMF1 is wholly deleted in DH1, JM101 and RR1 cells.

Plasmid minipreps were prepared from DH1, JM101 and RR1 cells transformed to ampicillin resistance with pMF1, digested with RsaI and fractionated on 1% agarose. YpL2 and pMF1 DNAs were included for comparison. Lane 1, YpL2; 2, pMF1 (in JC8111); 3,4, pMF1 in DH1; 5-8, pMF1 in JM101; 9-14, pMF1 in RR1. The inverted-repeat-containing band has clearly been deleted in all clones from all three strains, except for lane 9 where the plasmid has been drastically rearranged.



rearranged and it seemed likely that its inverted repeat was likewise no longer intact.

The similar appearance of the deleted clones in all three strains suggests a common mechanism for removal of the inverted repeat. Based on the number of colonies recovered, this process occurs more efficiently in RR1 (a recA+ strain). It may therefore involve the product of the recA gene.

2. Construction of pMF1 derivatives.

pMF1 was constructed so as to furnish a convenient cloning vector for the potential telomere sequences cloned in the pUC12 HincII site. The cloned sequences are flanked by unique XbaI and HindIII sites from the pUC12 multiple cloning site, and these sites are also unique to the centre of the inverted ARS repeat in pMF1 (see Figure 2). pMF1 therefore offered an easy means of cloning telomeric sequences between the ARS repeats, in either orientation.

In order to construct the pMF1 derivatives, 1 µg pMF1 was overdigested 5-10 fold with HindIII or XbaI, phenol extracted, precipitated in ethanol and redissolved in H_2O. 5 µg of plasmids pA21, pA37, pA69, pB23, pB49 or pB95 was digested with HindIII and XbaI, and the smaller DNA fragment isolated from an agarose gel using the DEAE membrane technique. Half of this isolated fragment was mixed with 250 ng cut pMF1 DNA, ligated in 10 µl ligation buffer + HCC for 30' at room temperature, and then diluted to 100 µl ligation buffer (without HCC) and ligated overnight. The ligation was stopped with a phenol extraction, carrier DNA (2 µg) was added, and the DNA was

precipitated with 2.5 vol. ethanol, taken up in 20 µl H_2O, and 2.5 µl used to transform JC8111 to ampicillin resistance. 10-100 colonies/transformation were obtained in fairly high competence cells ($5x10^4$ colonies/µg pBR322).

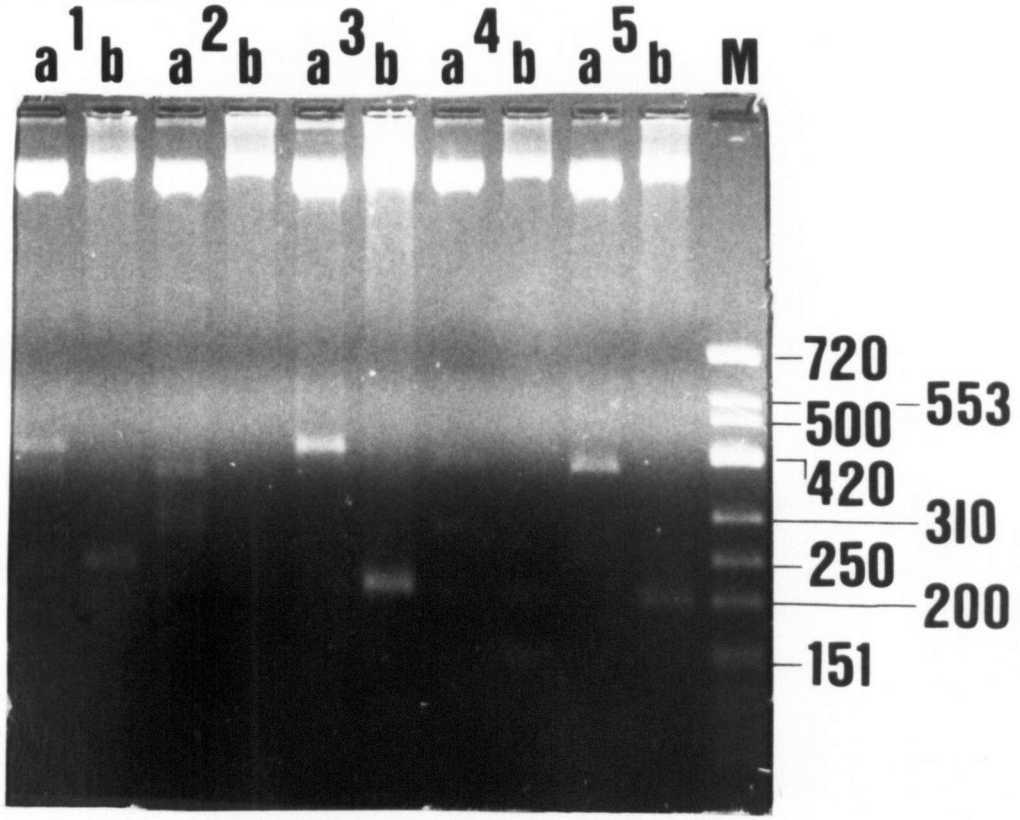
To characterize clones carrying the appropriate inverted repeat inserts, plasmid minipreps were prepared from 5ml overnight cultures, digested with RsaI or BglII, and electrophoresed on an agarose gel. Clones carrying inserts of the right size were grown in 500ml cultures and plasmid DNA purified on a CsCl gradient. Figure 14 shows the results of BglII digestion on these purified DNAs. A portion of each digest was heated in boiling water and chilled on ice before loading into a gel slot: this clearly identified some faint bands visible in these and other digests as being snapback (half-length) forms of the excised inverted repeats (Figure 14). Although in at least two of the clones - pMF1A37+ (lane 4) and pMF1B23+ (lane 2) - some deletion bands were also clearly present, it was considered worthwhile to transform these DNAs into yeast and assay for their replication as circular or linear plasmids.

3. Replication in yeast.

Transformation of yeast strain A281 to leucine auxotrophy was carried out as described in Materials and Methods, using 1 µg of each of the various plasmids. While the number of auxotrophic colonies recovered varied between experiments, no consistent difference in efficiencies between different plasmid preparations could be discerned which might be related to their replication properties in yeast. Colonies were picked and grown overnight in 40-50ml selective cultures (lacking leucine). At this point

Figure 14. Sizing of the inverted repeats in the pMF1 derivatives by BglII digestion.

Plasmid DNAs purified on CsCl were digested with BglII and fractionated on 1.2% agarose. Lane 1, pMF1B49+; 2, pMF1B23+; 3, pMF1A69+; 4, pMF1A37+; 5, pMF1A21+. (a) Digest loaded on gel as normal; (b) digest heated in boiling water for 3' and quick-chilled on ice before loading. Marker lane contains øX174 DNA cut with HinfI; numbers refer to fragment lengths in base pairs. The (b) lanes demonstrate 'snap-back' of the excised inverted repeats, resulting in half-length molecules which can also be detected as faint bands under normal loading conditions. These are visible in lane 1a, and much more prominent in many other experiments (data not shown).



distinct differences in replication efficiency could be observed: some clones gave a thick, cloudy suspension of yeast cells after overnight culture, similar to a culture of A281 in non-selective medium; while others, including those transformed with the parent plasmid pMF1, gave translucent suspensions which clearly contained far fewer yeast cells, and which did not increase in yield even after culture for a further 12 hours.

Yeast DNA minipreps were performed as described in Materials and Methods, and the resulting uncut DNA was electrophoresed on a 0.7% agarose gel in the presence of ethidium bromide (0.5 µg/ml) and visualized under UV light. Figure 15 shows typical results from two sets of transformations. Figure 15a compares two sets of pMF1A69+ yeast transformants (from two different *E. coli* clones) with two sets of pMF1A69- transformants. (The + and - designate plasmids which carry a telomeric inverted repeat in the appropriate or inappropriate orientation for resolution, respectively. A set of transformants from a '+' plasmid called pMF1A69X2 is also included; this clone did not resolve in yeast, and was subsequently shown to contain a contaminating DNA insert from the pA69 plasmid prep (data not shown).) All of the + transformants yielded much more DNA than did the - transformants, and a faint band is visible in these samples which migrates more slowly than a linearized pMF1 band on the same gel (Figure 15a, lane 1), yet faster than the bulk of the chromosomal DNA.

Figure 15b shows sets of three transformants from each of the A21+, A37+, B23+ and B49+ transformations. These show considerable variability in DNA yield, but linear plasmid bands are visible in B23#2 (lane 11) and all of the B49 clones (lanes 14-16). No such band is visible in the A21#2 clone (lane

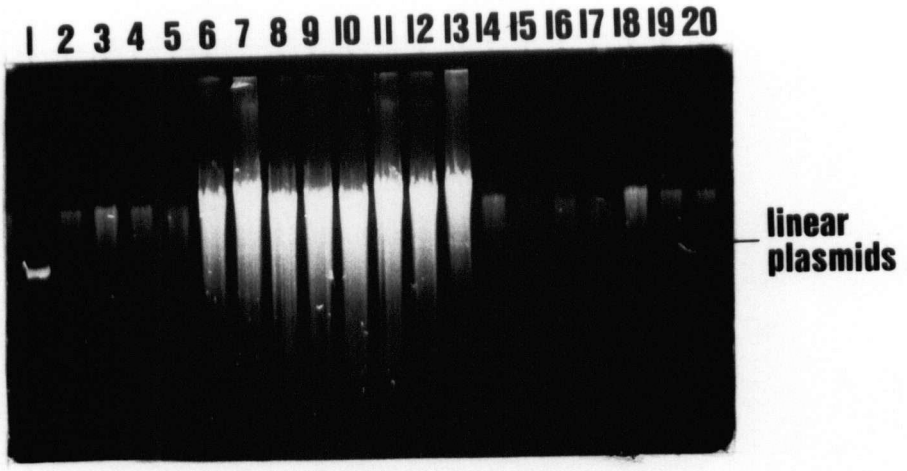
Figure 15. Detection of linear plasmids in uncut DNA from yeast cells transformed with pMF1 derivatives.

DNA was prepared from 50ml selectively-grown yeast cultures and electrophoresed on 0.7% agarose in the presence of 0.5 μg/ml ethidium bromide. One-fifth of a DNA miniprep was loaded in each lane: differences in fluorescent intensity are due to differences in plasmid stability and hence in DNA yield.

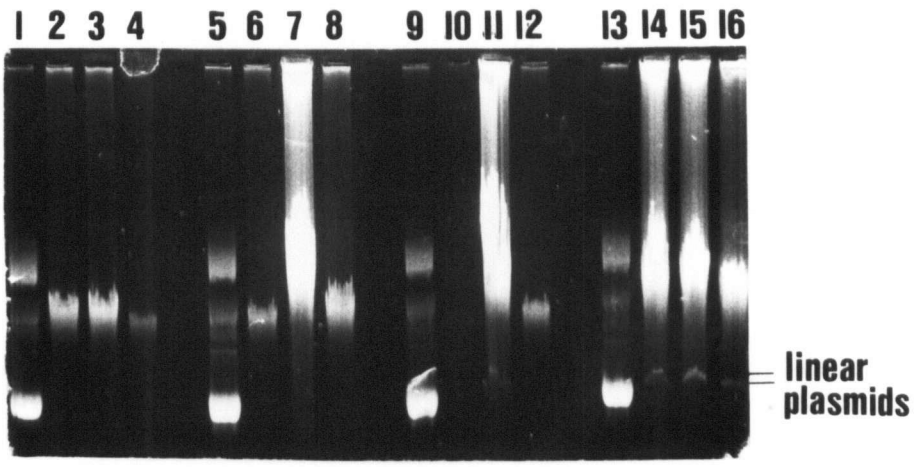
(a) Lane 1, pMF1 cut with HindIII; 2-5, DNA from A281 cells transformed with pMF1A69X2; 6-9, A281/pMF1A69+1; 10-13, A281/pMF1A69+2; 14-16, A281/pMF1A69-1; 17-19, A281/pMF1A69-2; 20, A281/pMF1. A faint linear plasmid band can be seen in lanes 6-13.

(b) Lane 1, uncut pMF1A37; 2-4, A281/pMF1A37+; 5, uncut pMF1A21+; 6-8, A281/pMF1B23+; 9, uncut pMF1B23+; 10-12, A281/pMF1B23+; 13, uncut pMF1B49+; 14-16, A281/pMF1B49+. Linear plasmid bands can be seen in lanes 11, 14-16, but not in lane 7; the stability of the latter transformant may be due to chromosomal integration of the LEU2 gene.

(a)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
linear plasmids

(b)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
linear plasmids

7), and subsequent data reinforce the interpretation that this clone does not carry a linear plasmid. The increased stability of the leucine auxotrophy marker may result from an integration event (data not shown).

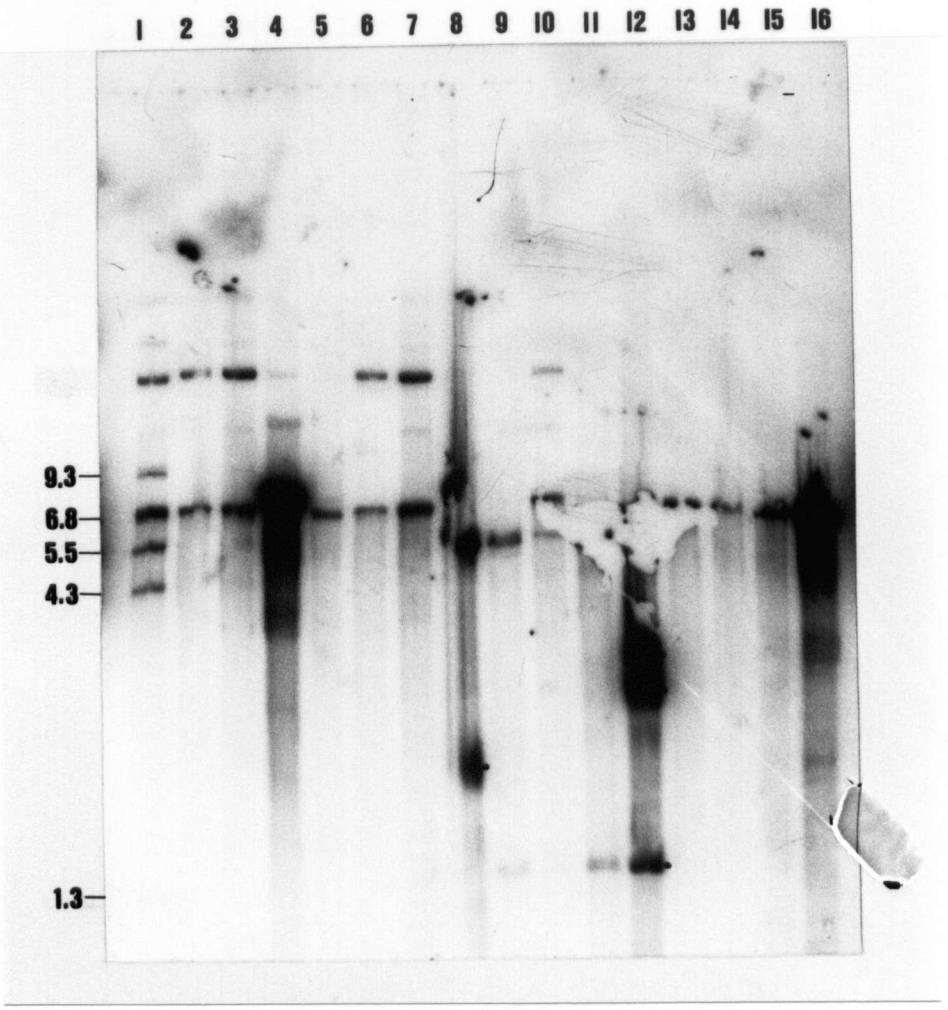
The other cases of improved transmittance of the leucine auxotrophy phenotype are consistent with resolution of the transforming circular plasmid into a linear plasmid. Szostak has already observed that linear plasmids replicate more stably than their circular analogues, provided that they don't carry a CEN marker.[55,56] To confirm that the high-DNA-yield clones did in fact contain linear plasmids, and that these were derived from the introduced circular pMF1 derivative via the predicted resolution event, the DNA was further analyzed by digestion with restriction enzymes, electrophoresis on an agarose gel, blotting to GeneScreen and probing with nick-translated pBR322 as described in Materials and Methods.

Figure 16 shows the results of a detailed comparison of yeast DNAs from clones transformed with pMF1, pMF1A69- and pMF1A69+. As expected from previous studies on linear yeast plasmids[5], the linearized pMF1A69+ plasmid seems to have had extra DNA added at its ends: approximately 100 bp at one end and 400 bp at the other, although the fuzziness of the bands (caused by overloading or by the inherent variability of telomere ends) makes this only a rough estimate. Other linear yeast plasmids were not analyzed in such detail, but all the data obtained suggest that the uncut DNA assay is a reliable marker for plasmid resolution.

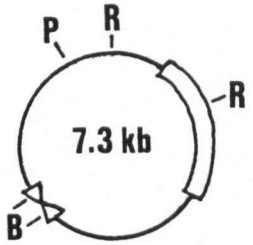
Figure 16. Southern blotting of DNA from a pMF1A69+ yeast transformant confirms the presence of a linear pMF1-derived plasmid.

For each lane, 1/10 of a yeast DNA miniprep was digested with the appropriate restriction enzyme and loaded in a total volume of 50 µl. Digests were fractionated on a 0.7% agarose gel, blotted to GeneScreen and probed with nick-translated pBR322. Lanes 1,5,9,13, A281 transformed with pMF1; 2,6,10,14, A281/pMF1A69-1; 3,7,11,15, A281/pMF1A69X2; 4,8,12,16, A281/pMF1A69+2. Lanes 1-4, uncut DNA; 5-8, PvuI digests; 9-12, EcoRI digests; 13-16, BglII digests. (Lower molecular weight bands in lane 1 are due to contamination by marker DNA fragments from a neighbouring lane.) PvuI and EcoRI cut at one and two sites respectively in the original plasmids; BglII cuts within the ARS sequence in the inverted repeats.

The interpretation of the observed digest patterns is shown below the photo: plasmid pMF1A69+2 has resolved at the expected site and 100-400 bp of telomeric DNA has been added to each end of the resulting linear plasmid. P=PvuI site, R=EcoRI sites, B=BglII sites. The open rectangle denotes the LEU2 gene; the open triangles denote the inverted repeat sequences (ARS1 plus the A69 insert); the filled triangles represent yeast telomeric sequences added to the linearized plasmid. Lengths given for the different restriction fragments were measured from a plot of mobility versus log molecular weight of the size standards in lane 1.

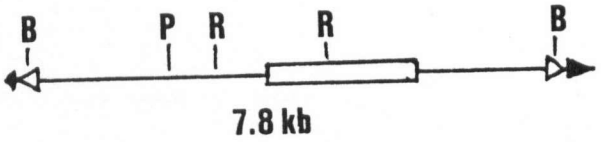


pMF1A69 −



PvuI → 7.3 kb
EcoRI → 1.5, 5.8 kb

pMF1A69 +



PvuI → 2.1, 5.7 kb
EcoRI → 1.5, 2.8, 3.5 kb

E. ACCURACY OF REPLICATION IN JC8111

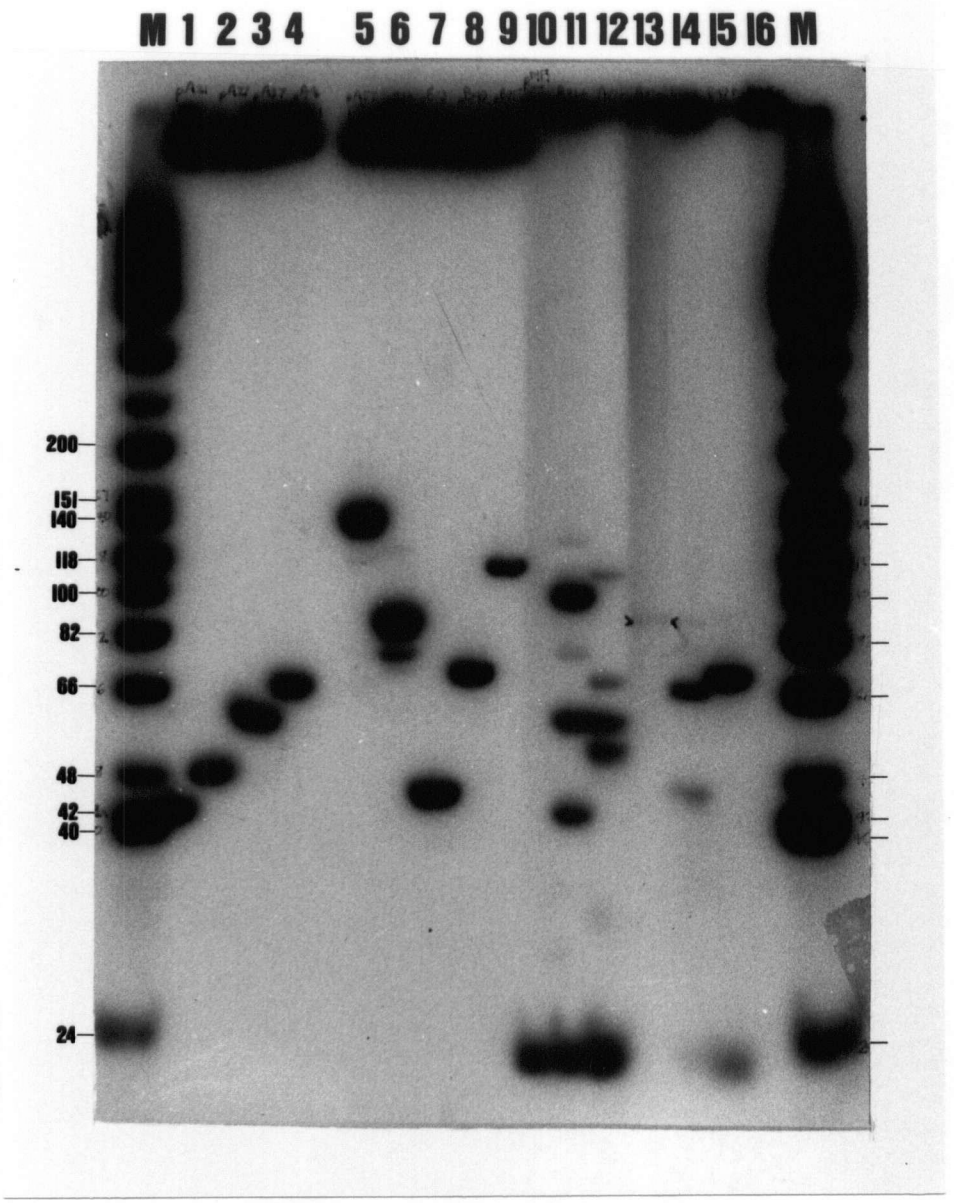
Analysis of the linearized plasmids and their replication in yeast was not pursued further, because of a discovery that cast severe doubts on the usefulness of this system as a means of assaying sequences for telomere function.

As a preliminary step to chemical sequencing of the pMF1 derivatives which had been used to transform yeast, plasmid DNA was digested with XbaI and HindIII, end-labeled with [α-^{32}P]-dATP and Klenow polymerase, and electrophoresed on a 10% polyacrylamide gel for several hours. The gel was dried down onto 3MM filter paper and autoradiographed for 6 hours (Figure 17). Unexpectedly, only two out of the eight pMF1 derivatives tested yielded single insert bands of the right mobility (lanes 13,15). All of the rest gave at least two bands, and some (e.g. pMF1A21+, lane 11) gave several bands, none of which had the appropriate mobility compared to the standards. Furthermore, at least one of the 'A' sequence clones in pUC12 was heterogeneous (lane 6), although this had not been detected in the original screening procedure.

It was also noted that several clones gave bands which co-migrated with the 24 bp band from pMF1, implying that a fraction of the plasmids in the clone had undergone an exact deletion of the inserted telomeric sequences. Taken together, these results indicate that the inverted repeats which carried stretches of short, CA/GT type tandem repeats were quite unstable in JC8111 - much more so than the long but non-repetitive inverted repeats in pMF1 or the 5'-terminal of the Minute Virus of Mice genome[34]. As noted

Figure 17. Telomere-type inverted repeats are generally not faithfully replicated in JC8111.

Plasmid DNAs purified on CsCl were digested with XbaI and HindIII to liberate the cloned 'A' and 'B' inserts from the pA and pB plasmids and the pMF1 derivatives. The digests were labeled with [α-^{32}P]-dATP and Klenow enzyme, and fractionated on 10% polyacrylamide. Marker lanes, øX174 DNA digested with HinfI and labeled in a similar fashion. The gel autoradiograph was deliberately overexposed so as to reveal any contaminating DNAs. Lane 1, pA21; 2, pA27; 3, pA37; 4, pA46; 5, pA57; 6, pA69; 7, pB23; 8, pB49; 9, pB95; 10, pMF1; 11, pMF1A21+; 12, pMF1A37+; 13, pMF1A69+2; 14, pMF1B23+; 15, pMF1B49+; 16, pMF1B95+. Note that pMF1A69+ (lane 13) and pMF1B49+ (lane 15) are the only plasmids containing a telomere-type inverted repeat which show a single insert band, of the appropriate mobility (compare lanes 6, 8), on this gel. In fact, the pMF1A69 clone is too under-labeled to interpret this result with any confidence.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
200
151
140
118
100
82
66
48
42
40
24

previously, there is also the possibility that pMF1 itself undergoes complete deletion of its hairpin at low frequency, accounting for the small fraction of uncut DNA observed in some prolonged restriction digests (Figure 12 and data not shown).

The original goal of the project was to develop a simple, reliable means of assaying different canonical repeat sequences for their ability to function as telomeres in yeast. For this it was felt that the DNA preparations used to transform yeast should be homogeneous and susceptible to complete sequencing if necessary. The data presented here suggest that JC8111 is incapable of replicating DNAs which can be resolved in yeast with sufficient accuracy to meet these criteria.

CONCLUSIONS

The results from the work described above bear on two different topics: telomere resolution in *Saccharomyces cerevisiae*, and the replication of inverted repeats in *Escherichia coli*.

Although the results in yeast cannot be rigorously interpreted because of the non-heterogeneous nature of the transforming DNA, a few conclusions can be drawn. First, a wholly artificial telomere-like inverted repeat, containing a non-telomeric ARS (ARS1) and CA-type sequences synthesized in vitro, was recognized and resolved in *S. cerevisiae* A281 cells. This enables one to conclude with a high degree of confidence that no other, as yet uncharacterized, elements are required for the functional definition of a yeast telomere, at least on a linear plasmid. (It does not rule out the possibility that other elements might be involved in the functioning of naturally occurring yeast telomeres.)

Are both the ARS function and the CA-type sequences necessary for an inverted repeat to be recognized and resolved by the yeast telomere replication system? The inverted ARS repeat in pMF1 is not resolved on its own, implying that CA sequences are absolutely required for resolution to take place. The data on the function of CA sequences in the absence of a linked ARS are more ambiguous, since these experiments were carried out in the YCp74/yeast strain C1 system. As noted previously, the failure to observe resolution in the YCp74-derived plasmids could be due to the

cloning of inappropriate sequences, a *cis* effect from another plasmid sequence, or the lack of an ARS function tightly linked to each telomere. The last explanation is the most attractive, but neither of the others can be ruled out on the basis of the data presented here.

The effect of CA sequence length on the resolution reaction could not be properly addressed in this assay system because of misreplication of the CA sequences in JC8111. The two plasmids with the most stable inverted repeats, pMF1A69+ and pMF1B49+, both showed 100% resolution in yeast, suggesting that the lower length limit for telomere resolution is even shorter than these insert lengths.

In the work on construction and replication of inverted repeats in JC8111, several results stand out. pMF1, which carries an inverted 224 base pair repeat, is almost stable in this strain while wholly unstable in three common cloning strains (recA+ or recA-). This result confirms and strengthens the conclusions of Boissy and Astell[34] on the unique replication properties of JC8111.

Nonetheless, those plasmids which carried long inverted repeats of short tandem repeats were generally unstable in JC8111. The results with a given plasmid were quite unpredictable. Whereas YCpA190PS and pMF1A69+ appeared to be fairly stable, some plasmids carrying much shorter inverted repeats (e.g. YCpA21XH or pMF1B23+) were never isolated in a stable form. The data from Figure 17 and unpublished observations further indicate that previously stable plasmids can become unstable during propagation in

JC8111, although the reverse - a stable subclone arising from an unstable clone - was never observed (see e.g. Figure 7).

Overall, the data on the use of JC8111 as a cloning host point to the lack of real understanding that exists over *E. coli* recombination systems. It seems clear that any sequence cloned in this strain should be very carefully characterized before and after cloning to guard against the possibility of subtle deletion or rearrangement, prior to any further experiments being carried out.

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