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Nucleic Acids Research, 1993, Vol. 21, No. 3 497–504

Isolation of telomeric DNA from the filamentous fungus *Podospora anserina* and construction of a self-replicating linear plasmid showing high transformation frequency

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Received October 26, 1992; Revised and Accepted December 31, 1992

ABSTRACT

It has been previously shown that linear plasmids bearing *Tetrahymena* telomeric sequences are able to replicate autonomously in the filamentous fungus *Podospora anserina* (1). However, autonomous replication occurs in only 50–70% of the transformants, suggesting a defect in the recognition of the *Tetrahymena* telomeric template by the putative *P. anserina* telomerase so that only a fraction of entering DNA is stabilized into linear extrachromosomal molecules. We have cloned DNA sequences added to the *Tetrahymena* $(T_2G_4)_n$ ends of the linear plasmid. Nucleotide sequencing showed that these sequences are exclusively composed of T_2AG_3 repeat units. Hybridization experiments of *Bal*31 treated DNA showed that T_2AG_3 repeats are confined within 200 bp in chromosomal *P. anserina* telomeres. A new plasmid has been constructed so that after linearization, the terminal sequences contain T_2AG_3 repeats. This linear molecule transforms *P. anserina* with a high frequency (up to 1.75×10^4 transformants/μg), autonomous replication occurs in 100% of the transformants and the plasmid copy number is about 2–3 per nucleus. These results underscore the importance of the telomeric repeat nucleotide sequence for efficient recognition as functional telomeric DNA *in vivo* and provide the first step toward the development of an artificial chromosome cloning system for filamentous fungi.

INTRODUCTION

Telomeres are the specialized structures that form the termini of linear eukaryotic chromosomes. They are responsible for the complete replication of the extreme ends of chromosomal molecules and contribute to chromosome stability in protecting them from exonucleolytic degradation and end to end fusion events (2). Telomeric DNAs have been isolated from numerous evolutionary distant species ranging from yeast to human. Telomere function seems to be fully defined within a few hundred base pairs in ciliates or yeasts and thousands base pairs in vertebrates. The overall structure of all known eukaryotic telomeres consists of tandemly repeated short sequences of G- and C- rich complementary strands with an orientation specificity so that the G rich telomeric DNA runs 5′ to 3′ towards the terminus of a chromosome (3). Telomeric DNA sequences can be composed of precise repeats such as T_2G_4 in *Tetrahymena* (4) and T_4G_4 in *Oxytricha* (5). In other species such as *S.cerevisiae* (6), *S.pombe* (7) or *Dictyostelium* (8), the repeated units were found to be more heterogeneous. In ciliates and humans, at least, the 3′ end of the G rich strand is elongated by a ribonucleoprotein called telomerase (9, 10, 11, 12, 13). The conservation of the overall telomere structure among widely divergent eukaryotes is reflected in the observation that telomeric sequences from ciliates (6, 14), fission yeast (15) or humans (16, 17) can function as telomeres on linear plasmids in *S.cerevisiae*. However, heterologous telomeric DNAs exhibit a broad spectrum of activity in their ability to be recognized as functional telomeres *in vivo*. For instance, it has been reported (15) that after propagation in *S.cerevisiae*, YAC vectors are rarely replicated as linear minichromosomes when transferred into *S. pombe* cells. In roughly all the transformants, minichromosomes have undergone rearrangements such as circularization events often accompanied by deletions of internal sequences. In the same way, it has been reported that transformation of *S.pombe* cells with a linear plasmid bearing *Tetrahymena* telomeric sequences gives rise to only a very small fraction of transformants containing a plasmid with the correct structure (7). These observations suggest that neither *S.cerevisiae* nor *Tetrahymena* telomeric repeats are fully functional in *S.pombe*.

Similar features can be observed with the fate of linear plasmids bearing *Tetrahymena* telomeres in *P. anserina* : autonomous linear replication occurs in only 50–70% of the transformants, the remaining resulting from integration events into chromosomal DNA (1). As circular plasmids seem unable to replicate autonomously in *P. anserina* (18), transformation can only be achieved either by self replication of linear molecules or by integration in chromosomal DNA. The most likely explanation

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for the observed high number of integrative transformants is that *Tetrahymena* telomeric sequences are not perfectly recognized as telomeric templates in *P. anserina*. Therefore, a competition takes place between mechanisms involved in telomere recognition/elongation and those involved in recombination events giving rise to the final ratio of the two classes of transformants. This interpretation was supported by the observation that after propagation in *P. anserina*, the linear plasmid does not integrate anymore into chromosomal DNA when reintroduced in *P. anserina* protoplasts (18). Therefore, the addition of *P. anserina* telomere-like sequences onto *Tetrahymena* templates seems to fully inhibit recombination between the transforming plasmid and chromosomal DNA. Taken together, these observations have led us to investigate what could be the effect of homologous telomeric sequences on *P. anserina* linear vectors. We have cloned *P. anserina* telomeric repeats and shown that a sequence containing homologous telomeric repeats fully prevents integration of the transforming vector into chromosomal DNA.

MATERIALS AND METHODS

Strains

The characteristics of *P. anserina* were reviewed by Esser (19). The isolation and characterization of a *ura5-6* mutant deficient for OMPppase activity have been described elsewhere (20). The bacterial strain *Escherichia coli* DH5α (F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* (*ϕ80d lacZΔM15*); Bethesda Research Laboratories) was used for bacterial transformation and plasmid propagation. Bacterial plasmids used in this study were constructed by standard recombinant DNA techniques.

Plasmids

The pPYACRC5 vector was contructed as follow: The 1.55 kbp *Eco*RI fragment carrying the *P. anserina ura5* gene (21) was blunt ended with the klenow fragment of DNA polymerase I and cloned into the filled-in *Sal*I site of pYAC4 (22). The resulting plasmid was named pPYAC4. The final construction, pPYACRC5, was created by replacing the *Bam*HI-*Sma*I fragment containing *TRP1*, *ARS1* and *CEN4* in pPYAC4 with the *BamHI-Sma*I fragment carrying *TRP1*, *ARS1*, *CEN4* and the cloning sites from pYAC-RC (23). The DNA fragment containing *Tetrahymena* telomeric repeats in pPYACRC5 was deleted by digestion with *Xho*I followed by religation to create the plasmid pPRC5. The pPATRC5 plasmid was constructed as follows. The plasmid pHUTEL-2-end (24) was digested with *Eco*RI and *Hae*III. The *Eco*RI-*Hae*III fragment of 0.5 kbp was cloned into the *Eco*RI-*Sma*I sites of pGEM7 creating the plasmid pEND2. The 0.5 kbp *Hind*III-*Xho*I fragment containing the human telomeric repeats (HTEL) was ligated to the 1.3 kbp *Sal*I fragment containing the kanamycin resistance gene (kan^r) from pUC4KN (a derivative of pUC4K (25, 26) in which *Not*I sites have been inserted into the polylinker flanking the gene). The ligated fragments were inserted in one step into the *Hind*III site of the plasmid p220.2 (27) to create p220.2TC. This plasmid contains a 'telomeric cassette' of two 0.5 kbp telomeric DNA fragments in opposing orientations flanking the kan^r gene which acts as a stuffer fragment and selectable marker. The entire 'telomeric cassette' has been removed by *Asu*II digestion and inserted into the *Cla*I site of pPRC5 to create pPATRC5.

Transformation of *P. anserina* and determination of the mitotic stability of the transformants

Protoplasts from the *ura5-6* strain were prepared and transformed as previously described (28, 29). The transformants selected on minimal medium were grown for 4 days on solid medium with uridine (100 mg/liter). From each transformant, small pieces of mycelium were picked at the thallus periphery and grown on minimal medium with or without uridine. The number of nuclei in each mycelial fragment was not known precisely but was higher than 1,000. If no regeneration of mycelium was observed on the minimal medium without uridine, it was concluded that the nuclei had lost the ura^+ transforming gene during growth on non-selective medium.

DNA isolation and Southern blotting

Genomic DNA of *P. anserina* was isolated from the mycelium. The mycelium was lyophilized, ground, and lysed in 100 mM Tris hydrochloride (pH 9.0) − 10 mM EDTA − 1% sarcosyl − 200 μg/ml proteinase K for 2 h at 65°C. After centrifugation (10,000×g, 10 min), the supernatant was extracted twice with phenol, once with chloroform, and nucleic acids were precipitated with ethanol. The DNA fiber was collected, washed several times with 70% ethanol, and dissolved in 10 mM Tris hydrochloride (pH 8.0) − 1 mM EDTA. Digestions with restriction enzymes were performed overnight under the conditions recommended by the supplier (Bethesda Research Laboratories). DNA samples were electrophoresed on agarose gels and transferred to nylon membranes (Hybond N+, Amersham). Filters were prehybridized at 65°C for 1 h in 5×SSC (1×SSC is 0.15 M NaCl plus 15 mM sodium citrate) − 40 mM Tris hydrochloride (pH 8.0) − 5×Denhardt's solution − 0.5% SDS − 100 μg of denatured salmon sperm DNA per ml. Double-strand DNA fragments were labeled with (α-^{32}P) dCTP (Amersham) using a Random Primed Labeling kit (Boehringer Mannheim). Hybridizations were carried out at 42°C for 16 h in 50% formamide − 5×SSC − 40 mM Tris hydrochloride (pH 8.0) − 5×Denhardt's solution − 0.5% SDS − 100 μg of denatured salmon sperm DNA per ml. The filters were then washed twice at 65°C in 0.1×SSC − 0.1% SDS for 30 min and subjected to autoradiography at −80°C with an intensifying screen. The synthetic probe $(T_2AG_3)_4$ was produced on an Applied Biosystem DNA synthesizer and labeled at the 5′ end with (γ^{32}P) ATP by using T4 polynucleotide kinase under the conditions recommended by the enzyme supplier (Bethesda Research Laboratories). Hybridizations were performed at 52°C for 16 h in 5×SSC − 40 mM Tris hydrochloride (pH 8.0) − 5×Denhardt's solution − 0.5% SDS − 100 μg of denatured salmon sperm DNA per ml. Filters were washed twice with 2×SSC − 0.1% SDS at 65°C and subjected to autoradiography.

*Bal*31 digestion

Genomic DNA from the *ura5-6* strain (480 μg) was mixed with 60 ng of *Hind*III digested λ DNA in 2.4 ml of 1×*Bal*31 buffer (20 mM Tris hydrochloride pH 7.2 − 600 mM NaCl − 12.5 mM $MgCl_2$ − 12.5 mM $CaCl_2$ − 1 mM EDTA) and equilibrated at 30°C for 10 min. After addition of 24 U of *Bal*31 (Boehringer Mannheim), 400 μl samples were removed at 0.5, 2, 7.5, 15, and 60 min. Reactions were terminated by adding EDTA to a final concentration of 50 mM and quick freezing. DNA samples were phenol extracted and precipitated with ethanol.

Cloning and sequencing of *P. anserina* telomeric repeats

The cloning procedure of Wang and Zakian (30) was followed. Total DNA prepared from a transformant containing the pPYACRC5 linear plasmid was digested with *Cla*I and extracted with phenol-chloroform. The digested DNA (5 μg) was treated with T4 DNA polymerase (final concentration, 67 mU/ml) at 15°C for 15 min in the presence of a high concentration of the four deoxynucleotides (final concentration of each deoxynucleotide, 100 μM), extracted with phenol-chloroform, precipitated with ethanol, and self-ligated with T4 ligase at a low DNA concentration (10 ng/μl) to circularize plasmid DNA. The ligation products were concentrated by ethanol precipitation and used to transform *E.coli* DH5α competent cells (Bethesda Research Laboratories) to ampicillin resistance. Sequencing was performed on double strand plasmid DNA by Sanger's method (31) using the synthetic primer shown in Fig. 1.

RESULTS

Cloning of *P. anserina* telomeric DNA

In order to obtain a YAC library of *P. anserina* chromosomal DNA we have constructed a modified YAC vector by the addition of the *P. anserina ura5* gene (21) to the pYACRC vector (23). The resulting plasmid, pPYACRC5 (Fig. 1), carries all sequences from pYACRC and is able to transform yeast AB1380 spheroplasts as other YAC vectors. Transformation of the *P. anserina ura5-6* strain either with the previously described pPATura1 linear vector (1) or with *BamH*I cut pPYACRC5 gives rise to similar results: transformation frequency is in the range of 200−1000 transformants/μg of DNA, 50−70% of the transformants are highly unstable through vegetative growth and the linear plasmid is transmitted with a very low frequency through meiosis. This suggests that the yeast sequences *ARS1* and *CEN4* are not functional in *P. anserina*. Fig. 2 shows molecular analysis of unstable *P. anserina* transformants carrying the pPYACRC5 linear plasmid. Total DNA was cut with *Pst*I, run on an agarose gel and probed with radiolabeled pBR322. This probe detect weakly the 1.8 kbp fragment which contains only 30% of pBR322 sequences. However, the hybridization pattern is in good agreement with the *Pst*I restriction map of the linear plasmid shown in Fig. 1B. But the comparison of restriction patterns between plasmid DNA extracted from *E.coli* (Fig. 2, lane 2 and 6) and plasmid DNA in *P. anserina* transformants (Fig. 2, lanes 3 to 5) reveals that the linear plasmid has been modified during propagation in *P. anserina*. Internal *Pst*I fragments detected by the probe have exactly the same mobility as in the control lane whereas terminal fragments migrate slightly slower. As it has been previously shown in *S.cerevisiae* (6) and *S.pombe* (7) one might expect that *Tetrahymena* telomeric sequences have been healed *in vivo* by addition of host specific telomeric sequences. It can be estimated from this experiment that at least 100 to 150 bp of DNA have been added to the ends of the linear plasmid. This is in agrement with the 200 bp increase of telomeric ends we previously reported for the linear vector (1).

To isolate these sequences we used the enzyme T_4 DNA polymerase which has been shown to enable the isolation of telomeres with virtually no loss of telomeric DNA (30). Genomic DNA from an unstable transformant was digested with *Cla*I which cuts only once in the plasmid (Fig. 1), treated with T_4 DNA polymerase and self ligated to circularize plasmid DNA.

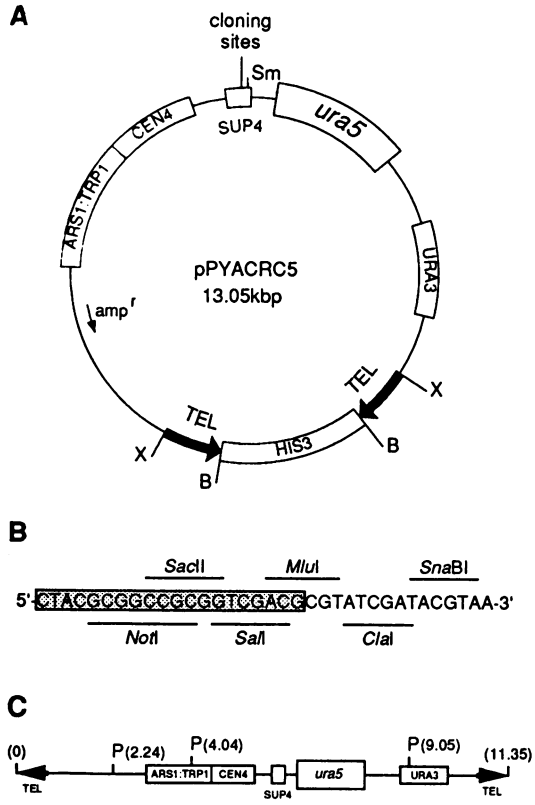

Figure 1. (A) Map of the pPYACRC5 vector. *SUP4*, *TRP1*, *HIS3* are yeast genes. *ARS1* and *CEN4* are sequences from yeast's chromosome IV: *ARS1* is an autonomous replication sequence while *CEN4* provides centromere function. The TEL sequences are derived from the termini of *Tetrahymena* macronuclear rDNA molecules. The *ura5* gene is from *P. anserina*. pBR322 derived sequences are shown as thin lines. (B) nucleotide sequence of the pPYACRC5 polylinker. The shaded box corresponds to the nucleotide sequence of the primer used for sequencing. (C) *Pst*I restriction map of *Bam*HI linearized pPYACRC5. The sizes (kbp) are indicated from the left end of the linear molecule. Restrictions sites are: B, *Bam*HI; P, *Pst*I; Sm, *Sma*I; X, *Xho*I.

Transformation of highly competent *E.coli* cells with the ligation mixture gave rise to one Ap^R clone. Restriction analysis of the rescued plasmid revealed that it had the expected structure. The fragment containing putative *P. anserina* telomeric DNA was sequenced by using the synthetic primer shown in Fig. 1B. Close to the primer, the sequence starts with a 25 units track of the repeat T_2AG_3, followed by the *Tetrahymena* T_2G_4 telomeric sequence. Thus, the *in vivo* processing of *Tetrahymena* telomeric templates consists of the addition of a homogeneous track of the repeated sequence T_2AG_3. Restriction analysis of the rescued plasmid showed that the *Xho*I-*Sal*I fragment containing telomeric repeats is 800 bp in length. Assuming that the *Xho*I-*Bam*HI fragment containing *Tetrahymena* telomeric repeats is 700 bp in pPYACRC5, about 50 bp of *Tetrahymena* DNA have been removed by the healing process.

T_2AG_3 repeats are the component of chromosomal *P. anserina* telomeres

To demonstrate that chromosomal *P. anserina* telomeres are also composed of T_2AG_3 repeats we set up hybridization experiments using the synthetic probe $(T_2AG_3)_4$. Genomic DNA from the

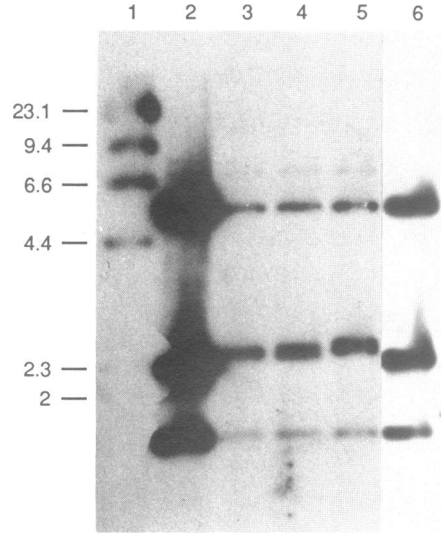

Figure 2. Southern blot analysis of the linear plasmid after propagation in *P. anserina* transformants. DNA samples were electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, and probed with a mixture of ^{32}P-labeled pBR322 DNA (2.5 ng/ml; 10^9 cpm/μg) and ^{32}P-labeled λ DNA (0.5 ng/ml; 10^8 cpm/μg). Lane 1, *Hind*III digested λ DNA (0.2 μg); lane 2 and 6, *Bam*HI-*Pst*I digested pPYACRC5 DNA (0.5 ng), (lane 6 corresponds to a 10 times shorter exposure to autoradiography); lanes 3 to 5, *Pst*I digested DNA (1 μg) from three unstable pPYACRC5 transformants. Numbers to the left refer to DNA size standards (kilobases).

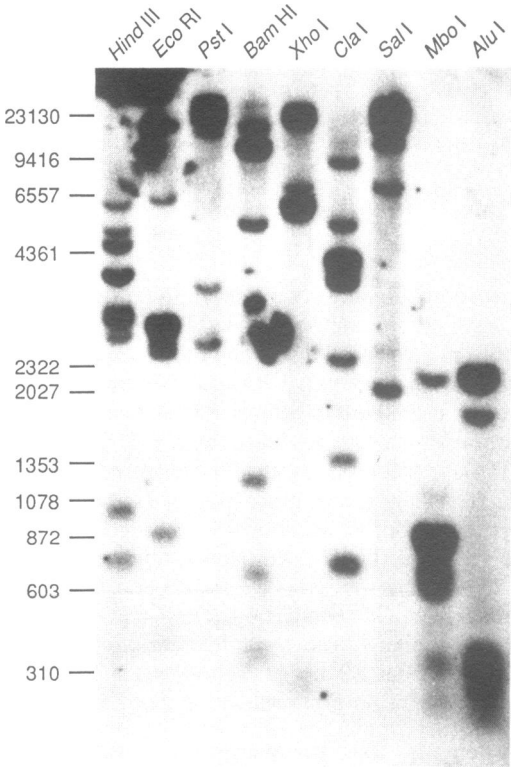

Figure 3. Southern blot analysis of *P. anserina* genomic DNA with the telomeric probe $(T_2AG_3)_4$. Genomic DNA (10 μg) from the *ura5-6* strain was digested with different restriction enzymes, electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, and probed with ^{32}P-labeled $(T_2AG_3)_4$. Numbers to the left refer to DNA size standards (base pairs).

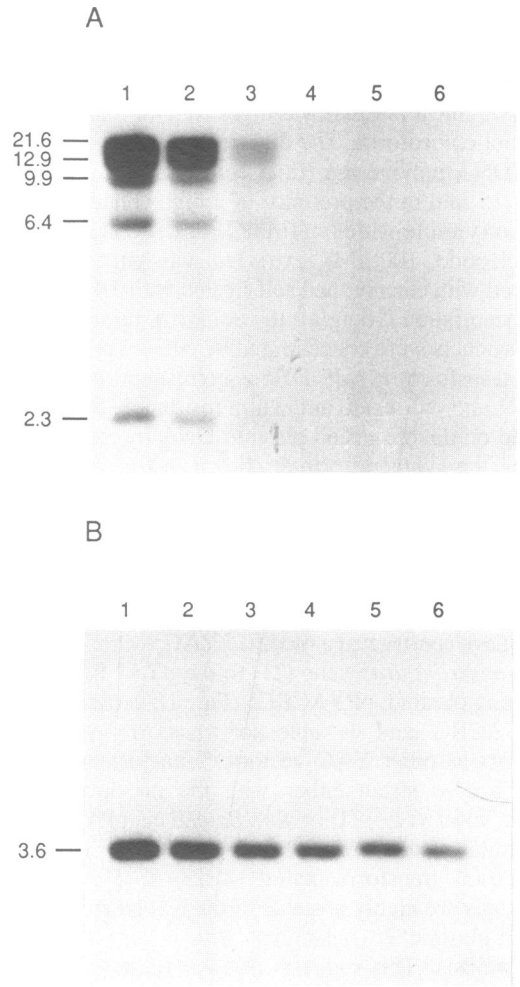

Figure 4. $(T_2AG_3)_4$ hybridization of *Bal*31-treated *P. anserina* DNA. Genomic DNA from the *ura5-6* strain was treated with *Bal*31 nuclease for 0 (lane 1), 0.5 (lane 2), 2 (lane 3), 7.5 (lane 4), 15 (lane 5), or 60 min (lane 6) and subsequently digested with *Sal*I. **(A)** DNA samples (10 μg) were electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled $(T_2AG_3)_4$. **(B)** The membrane shown in (A) was reprobed with the ^{32}P-labeled 1.55 kbp *Eco*RI fragment containing the *ura5* gene. Size (kilobases) and position of detected fragments are indicated to the left of each panel.

ura5-6 strain was digested with different restriction enzymes, run on an agarose gel and probed with the radiolabeled oligonucleotide $(T_2AG_3)_4$ (Fig. 3). Several genomic DNA fragments hybridize with the probe at high stringency, demonstrating that some chromosomal DNA fragments do contain T_2AG_3 sequences. In the *Hind*III digested DNA, eight bands can be discerned with some bands being doublets or triplets. It can be estimated that at least twelve DNA fragments are detected by the probe. Assuming that *P. anserina* genome contains seven chromosomes (32, 33), this result is in good agreement with the expected number of telomeres. The considerable variation in the intensities of the fragments detected may also traduce some variation in the number of telomeric repeats present at each chromosome end. This can be observed particularly for some of the small fragments which give a very weak signal suggesting they contain very few repeats. In addition, the bands detected are rather broad and heterogenous especially for the smallest fragments from *Mbo*I or *Alu*I digests. This may

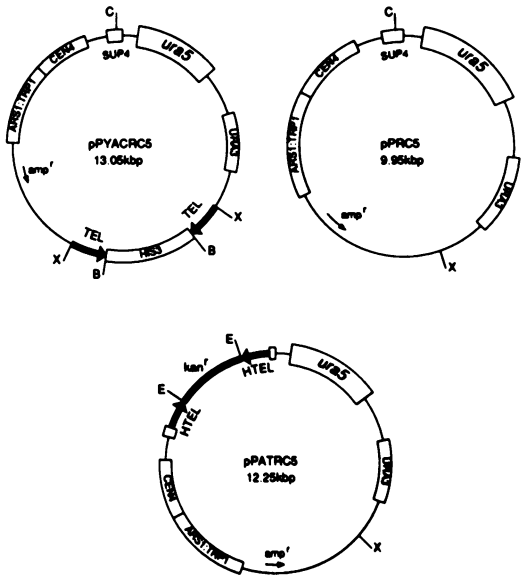

Figure 5. Maps of the linear vectors with different ends. The symbols used are as in Fig.1. In pYACRC5, the ends are *Tetrahymena* telomeres. In pPRC5, the telomeres have been deleted. pPATRC5 contains human telomeres (HTEL) with T_2AG_3 telomeric repeats, separated by the kanamycin resistance gene (kan^r). Restriction sites are: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; X, *Xho*I.

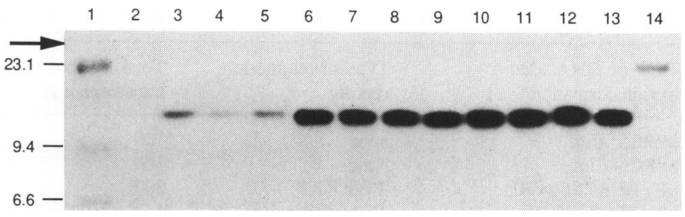

Figure 6. Southern blot analysis of DNA from *P. anserina* transformants. Total DNA (1 μg) was electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, and hybridized with a mixture of ^{32}P-labeled pBR322 DNA (2.5 ng/ml; 10^9 cpm/μg) and ^{32}P-labeled λ DNA (0.5 ng/ml; 10^8 cpm/μg). Lane 1 and 14, *Hind*III digested λ DNA (0.2 μg); lane 2, DNA from the *ura5-6* strain; lanes 3 to 5, DNA from three unstable pPYACRC5 transformants; lanes 6 to 13, DNA from eight pPATRC5 transformants. Numbers to the left refer to DNA size standards (kilobases). The position of the ethidium bromide stained genomic DNA is indicated by the arrow.

reflect some heterogeneity in size as generally observed for DNA fragments ending with telomeres (34). Most of the fragments detected by the $(T_2AG_3)_4$ oligonucleotide are very small in the *Mbo*I and *Alu*I digested DNAs suggesting that the telomeric repeats can be as short as 300 bp or even shorter.

In order to demonstrate that T_2AG_3 sequences have a telomeric location, we showed that the DNA fragments detected by the probe are lost after brief digestion of intact DNA with the *Bal*31 exonuclease (Fig.4). After 7.5 min of *Bal*31 treatment, all the DNA fragments homologous to the $(T_2AG_3)_4$ probe are completely deleted whereas the same blot probed with the *ura5* gene shows that internal chromosomal sequences remain undigested even after 60 min of *Bal*31 treatment. As *Hind*III digested λ DNA was added to *P. anserina* genomic DNA before *Bal*31 treatment, the kinetic of exonuclease activity could be easily assessed by probing the same blot with λ DNA (data not shown). About 200 bp were removed after 7.5 min of digestion, suggesting that T_2AG_3 repeats are confined within the terminal 200 bp of *P. anserina* chromosomes. Because *Bal*31 can remove telomeric repeats very quickly, this value may be largely underestimated, but it is in agreement with the small size observed above for some of the telomeric fragments.

Theses results demonstrate that *P. anserina* telomeres are composed of short tracks of T_2AG_3 repeats and therefore, confirm the expected telomeric nature of DNA sequences added to the termini of linear plasmids after replication in *P. anserina*.

Effect of telomeric sequences containing T_2AG_3 repeats on autonomous replication of a linear plasmid in *P. anserina*

In order to assess the effect of T_2AG_3 telomeric repeats, we constructed the plasmids shown in Fig. 5. The three plasmids carry the *ura5* selective marker but differ by their termini after linearization with the appropriate restriction enzyme. In pPYACRC5 *Bam*HI digested DNA, the termini are composed of *Tetrahymena* telomeric sequences whereas there are no telomeres in *Xho*I or *Cla*I linearized pPRC5. As human telomeres have been shown to be also composed of T_2AG_3 repeats (16, 17), we used human telomeric sequences (HTEL) to construct the plasmid pPATRC5. The human telomeric DNA has been previously isolated by complementation of a deficient yeast artificial chromosome (16) and has been shown to induce the formation of functional telomeres when reintroduced in mammalian cells (35). From the *Eco*RI sites in pPATRC5, the telomeric sequence corresponding to an *Eco*RI-*Hae*III DNA fragment issued from pHUTEL-2-end (24) starts with 12 bp of yeast telomeric repeats, followed by about 500 bp of T_2AG_3 repeats and a few related variants such as $GTAG_3$ and $TCAG_3$.

As shown in table 1, either *Cla*I or *Xho*I linearized pPRC5 DNA transforms the *P. anserina ura5-6* strain with a low frequency and the *ura*$^+$ phenotype is stable through vegetative growth. Transformation occurs by integration of the plasmid in chromosomal DNA as we reported previously for linear plasmids without telomeric ends (1, 20). Transformation frequency shows a 3 fold increase when protoplasts are transformed with the *Bam*HI linearized pPYACRC5 plasmid, bearing *Tetrahymena* telomeric repeats. About 70% of the transformants are unstable and loose the *ura*$^+$ phenotype upon growing on non selective medium whereas the remaining 30% are stable through vegetative growth. Transformation frequency of the linear plasmid bearing T_2AG_3 repeats (*Eco*RI linearized pPATRC5) shows nearly a 60 fold increase over the transformation frequency observed with *Bam*HI cut pPYACRC5 and reaches 1.75×10^4 transformants/μg DNA. All the pPATRC5 transformants tested were unstable when grown on non selective medium but unlike pPYACRC5 transformants, the loss of the *ura*$^+$ phenotype was not complete. After 4–5 days culture upon non selective conditions, pieces of mycelium picked at the edge of the thalli were able to regenerate a mycelium with a lag time of 48h after transfer on minimal medium without uridine. The delay observed for growth recovery on selective medium indicates that some nuclei still contained the *ura*$^+$ gene after propagation on non selective medium. This could arise either by stabilization events such as integration in chromosomal DNA or by persistance of a few nuclei containing the linear replicating plasmid. To examine these two possibilities, transformants were subcultured several times on minimal medium supplemented with uridine until loss of the *ura*$^+$ phenotype. All the transformants lost the *ura*$^+$

Table 1. Transformation frequency of the linear plasmid bearing human telomeric DNA and mitotic stability of the transformants

Type of DNA used in transformation	Type of telomeric repeat	Total number of transformants[a]	Number of transformants tested	% of viable transformants[c]	% of unstable transformants[d]
pPRC5 *XhoI*	none	120	20	100	0
pPRC5 *ClaI*	none	100	20	100	0
pPYACRC5*BamHI*	TTGGGG	307[b]	48	91	66
pPATRC5 *EcoRI*	TTAGGG	1.75×10^{4}[b]	100	100	100

[a] Protoplasts were prepared from the *ura5-6* mutant. Equal number of protoplasts (5×10^{7}) were transformed with 1 μg of plasmid DNA preparation.
[b] Calculated from two independant transformation experiments.
[c] Transformants which were able to grow after transfer from transformation plates to minimal medium.
[d] Transformants were grown on minimal medium supplemented with uridine (0.1 mg/ml). After 4–5 days, four small pieces of mycelium were picked at the edge of the thalli and grown on minimal medium with or without uridine to determine the ura^{+} or ura^{-} phenotypes

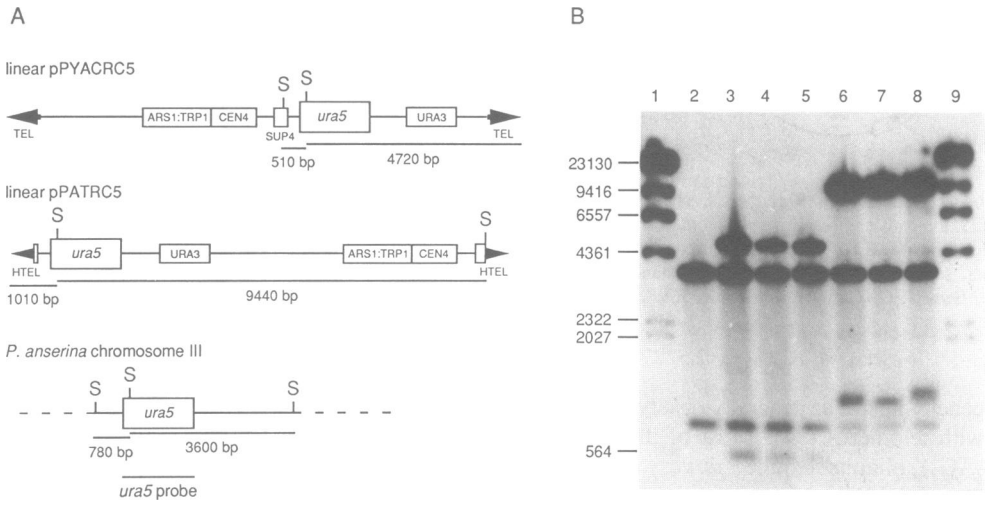

Figure 7. Determination of the plasmid copy number in pPYARC5 and pPATCR5 transformants. **(A)** Location of *SalI* sites (S) in the linear plasmids and in the DNA region encompassing the chromosomal *ura5* gene. Thin lines represent the expected size of the DNA fragments detected by the *ura5* probe. The symbols used in the drawing are as in Fig. 1 and 5. **(B)** Southern blot analysis of DNA from pPYACRC5 and pPATRC5 transformants. Total DNA (1 μg) was digested with *SalI*, electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, and probed with a mixture of the ^{32}P-labeled 1.55 kbp *EcoRI* fragment carrying the *ura5* gene (2.5 ng/ml; 10^{9} cpm/μg) and ^{32}P-labeled λ DNA (0.5 ng/ml; 10^{8} cpm/μg). Lanes 1 and 9, *HindIII* digested λ DNA (0.2 μg); lane 2, DNA from the *ura5* recipient strain; lanes 3 to 5, DNA from three unstable pPYACRC5 transformants; lanes 6 to 8, DNA from three pPATRC5 transformants. The autoradiogram was exposed for 72 h. Relative intensities were determined by using a scanning densitometer on X-ray films exposed for 2, 16 and 48 h. Numbers to the left refer to DNA size standards (kilobases).

phenotype after 2 or 3 passages on non selective medium suggesting that the observed increased stability of the transformants does not result from integration events.

To determine whether autonomous linear replication occured in pPATRC5 transformants, the presence of pBR322 sequences on extrachromosomal molecules was examined by analysis of DNA extracted from transformed strains. Total undigested DNA was run on an agarose gel and probed with radiolabeled pBR322 (Fig. 6). A single band of the correct size is detected for each transformant and no hybridization is observed with chromosomal DNA. This means that the linear plasmid replicates autonomously without integration of pBR322 sequences into chromosomal DNA. The comparison of band intensities between DNAs from pPYACRC5 and pPATRC5 transformants clearly indicates a higher plasmid copy number in pPATRC5 transformants.

Using the single copy chromosomal *ura5* gene as a reference, we determined the amount of plasmid DNA in pPYACRC5 and pPATRC5 transformants. Total DNA was cut with *SalI*, run on an agarose gel and probed with the 1.55 kbp *EcoRI* fragment carrying the *ura5* gene (21). Fig. 7A shows *SalI* restriction maps of the linear plasmids and the location of *SalI* sites encompassing the chromosomal *ura5* gene. As the size of *SalI* DNA fragments detected by the probe is different between chromosomal and plasmid DNA, the comparison of bands intensity in each lane gives an accurate measurement of the plasmid copy number in each transformant. From the quantitation of the autoradiogram shown in Fig. 7B we calculated an average plasmid copy number of 0.4 and 2.7 per nucleus for pPYACRC5 and pPATRC5 transformants respectively. This result provides evidence that the replacement of *Tetrahymena* telomeric templates by human telomeric repeats leads to a seven fold increase in plasmid copy number and may explain the increased mitotic stability of pPATRC5 transformants.

In order to know if the plasmid copy number has an effect on the meiotic stability of the linear plasmid, pPATRC5 and pPYACRC5 transformants were crossed with the *ura5-6* strain. With pPYACRC5 transformants, the frequency of ura^{+} strains in the progeny was very low (below 10^{-2}), as previously reported for linear plasmids bearing *Tetrahymena* telomeric repeats in *P. anserina* (1). The frequency of transmission of the

linear pPATRC5 plasmid through meiosis was in the same order of magnitude, indicating that the increased plasmid copy number in pPATRC5 transformants does not improve the frequency of transmission of the plasmid through meiosis.

DISCUSSION

Telomeric DNA and structure are similar among otherwise widely divergent eukaryotes. Telomere repeats from the ciliate *Tetrahymena* can seed telomere formation on linear plasmids in *S.cerevisiae* (36), *S.pombe* (7), and *P. anserina* (1). The telomere terminal transferase activity in *Tetrahymena* cell extracts recognizes any G-rich telomere strand as a primer (9, 10). Such a process could explain how the putative yeasts and *P. anserina* telomerases recognize heterologous telomeric repeats. However, linear plasmids bearing *Tetrahymena* telomeric sequences transform *P. anserina* with a low frequency and autonomous replication occurs in only a fraction of the transformants. This observation suggests that the primary defect is in heterologous telomeric DNA recognition by the putative *P. anserina* telomerase. We have shown here that the healing of *Tetrahymena* telomeric templates results in the addition of an homogeneous track of T_2AG_3 repeats. Hybridization experiments demonstrated that T_2AG_3 repeats are the component of chromosomal telomeres and are confined within the terminal 200 bp of each *P. anserina* chromosome. As there is only a single base change between telomeric repeats from *Tetrahymena* and *P. anserina*, this could explain why *Tetrahymena* telomeric DNA can seed telomere formation in *P. anserina*. Alternatively, this single base difference may be deleterious for efficient recognition and/or elongation by the putative *P. anserina* telomerase. To test the effect of homologous telomeric sequences, we constructed the plasmid pPATRC5 in which *Tetrahymena* repeats from pPYACRC5 have been replaced by a human telomeric DNA fragment containing T_2AG_3 repeats. The change in telomeric templates results in a 60 fold increase in transformation frequency, autonomous replication occurs in 100% of the transformants and the plasmid copy number is shifted from 0.4 to 2.7 per nucleus. The most likely explanation for these results is that homologous T_2AG_3 repeats are more efficiently recognized as telomeric DNA than *Tetrahymena* repeats. However, the shift in plasmid copy number remains an intriguing result. After propagation in the transformants, it is likely that both pPYACRC5 and pPATRC5 plasmids have acquired functional *P. anserina* telomeres so that the main difference between the two linear plasmids concerns internal sequences immediately adjacent to the termini. It is to notice that we could never demonstrate that the linear plasmid with *Tetrahymena* telomeres was bearing a specific sequence functioning as a replication origin in *P. anserina* and the replication is thought to initiate at non specific sequences. Therefore, the seven fold increase in plasmid copy number could be mediated by a replication origin lying on human derived telomeric sequences. It has been shown that the X and Y elements in *S.cerevisiae* subtelomere domains can function as autonomous replicating sequences in yeast cells (36). However, the human telomeric DNA fragment used in this study corresponds to the terminal repeats of pHUTEL-2end (24) and does not contain any subtelomeric sequence. If no particular sequences are responsible for the increased plasmid copy number in pPATRC5 transformants, this suggests that *Tetrahymena* sequences have a deleterious effect on the replication or on the maintenance of the pPYACRC5 plasmid. Thus, the seven fold increase in plasmid copy number would be the consequence of the removal of *Tetrahymena* sequences rather than the addition of a putative replication origin. This seems to be true since previous attempts to isolate chromosomal elements conferring a high plasmid copy number had failed : if some DNA sequences can increase transformation frequency, none have been found to have an effect on the plasmid copy number (18). The 700 bp *Tetrahymena* telomeric fragment contains approximately 400 bp of unique AT-rich DNA followed by 300 bp of T_2G_4 repeats (37). It is not clear whether the low copy number of the pPYACRC5 plasmid could be due to the presence of the T_2G_4 track or to internal sequences. Further experiments are needed to confirm the unexpected effect of *Tetrahymena* sequences on the maintenance of linear plasmids in *P. anserina*.

In *Fusarium oxysporum*, linear self replicating plasmids are produced by the *in vivo* rearrangement of normally integrative circular vectors. The termini of the linear plasmids are composed of T_2AG_3 repeats and the plasmid copy number is approximately 10 per haploid genome (38). As telomere formation seems to be sufficient to allow autonomous replication in *F. oxysporum* and *P. anserina*, the existence of replication origins as specific DNA sequences remains to be investigated.

The replacement of *Tetrahymena* telomeric template by human telomeric sequences results in a net increase on the mitotic stability of the linear plasmid but does not improve its transmission through meiosis. Therefore, the observed increase in mitotic stability can simply be explained as a consequence of a more efficient replication of the linear plasmid rather than an improved fidelity of transmission through mitosis. However, it has been recently demonstrated that human telomeres are tightly associated with the nuclear matrix and it seems likely that the T_2AG_3 repeats mediate the anchorage of the telomere (39). As telomeric repeats are identical between *P. anserina* and human, the same attachment may occur in *P. anserina* . Thus, a single base difference in the sequence of the telomeric repeat may destabilise the anchorage to the nuclear matrix and explain the difference in stability between the two linear plasmids. It is not presently known if the attachment to the nuclear matrix is mediated by a nucleoprotein complex forming at the telomere but it is demonstrated that some proteins bind specifically to the telomere in ciliates (40, 41) and in yeast (42, 43). In this last case, binding of the protein RAP1 to the telomeric end has been shown to have a direct effect on telomere lengh and on chromosome stability. If such proteins exist in *P. anserina*, a single nucleotide change in the sequence of the telomeric repeat of the linear plasmid may also affect the stability of the molecule.

In *S.cerevisiae* and *S.pombe*, centromeric DNAs have been shown to allow artificial minichromosomes to be segregated properly through nuclear divisions (44, 15). Experiments are now in progress to use the plasmid pPATRC5 as a cloning vector to isolate a functional *P. anserina* centromere.

ACKNOWLEDGEMENTS

We thank Joel Bégueret and Michel Perrot for helpful discussions and critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique and the Université de Bordeaux II. J.P.J. was supported by a grant from the Ministère de la Recherche et de la Technologie.

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