Nonintegrative Transformation in the Filamentous Fungus *Podospora anserina*: Stabilization of a Linear Vector by the Chromosomal Ends of *Tetrahymena thermophila*

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The effect of the chromosomal ends of *Tetrahymena thermophila* on the stability of linear transforming molecules in the filamentous fungus *Podospora anserina* was tested. A derivative of an integrative vector for this fungus has been constructed, so that after linearization, the ends of the plasmid are the telomeric sequences of *T. thermophila*. After transformation, this linear molecule was maintained as an extrachromosomal plasmid with no integrated copies in about 50% of the transformants. Under selective conditions, there was approximately one linear molecule per 5 to 10 nuclei, and these extrachromosomal molecules were rapidly lost under nonselective conditions. The circular plasmid carrying an inverted repeat of *T. thermophila* telomeres could be linearized and processed in vivo.

Transformation of mycelial fungi has been described previously, and common features emerge from the results observed for different species: the transformation efficiency is low and the vectors are integrative (11, 25). In yeasts, integrative vectors were converted to autonomously replicating plasmids by the addition of sequences which act as replication origins. They are either autonomously replicating sequences (ARS) (10, 22) or the replication origin of the natural 2μm plasmid (2). These autonomously replicating plasmids exhibit a high transformation efficiency and instability when the transformed strains are grown under nonselective conditions. Attempts to obtain such vectors in mycelial fungi were made by the addition of chromosomal sequences into the integrative vectors (5). The absence of positive results may be due to the coenocytic structure of these organisms, which leads to the possibility of complementation between different nuclei of the hyphae. The nuclei which lose the transforming gene can be maintained by the presence of a few nuclei which have retained the plasmid. Genomic sequences of *Aspergillus nidulans* which promote autonomous replication of yeast plasmids have been cloned (21). When they were introduced into *A. nidulans*, they did not confer replicating activity on the plasmids (1). One of these sequences greatly enhances the transformation efficiency of *A. nidulans*, but the vector containing this *ans1* sequence remains integrative. In *Neurospora crassa*, a recombinant pUC8 plasmid carrying the *am* gene has been reported to be able to replicate autonomously (8). However, the transforming vector is rearranged and is present as high-molecular-weight concatemers; furthermore, some plasmid sequences are integrated into the chromosomal DNA.

For *Podospora anserina*, we have developed a system based on the transformation of an auxotrophic *ura5⁻* strain deficient for orotidylic acid pyrophosphorylase (OMPppase) by a recombinant vector (pPAura5-1) carrying the homologous *ura5* gene (3). This plasmid is integrative (19). We have tested the effect of the linearization of this vector on transformation. As it is known that broken DNA ends are highly

reactive in recombination, the linearized vector was stabilized by the addition of the ends of the rDNA plasmid of *Tetrahymena thermophila*. These sequences have been shown to be functional in *Saccharomyces cerevisiae* cells and to support the addition of yeast-specific telomeric sequences (20–23, 24). The resulting plasmids do not integrate and are maintained as linear molecules. We have constructed a derivative of pPAura5-1 which contains an inverted repeat of *T. thermophila* telomeres. This vector, pPATura2, was linearized, leaving the *T. thermophila* telomeres at each end, and the linear molecules were used to transform the *ura5* mutant. Among the *ura*⁺ transformants, a high proportion of mitotically unstable strains was obtained. In these strains, the transforming vector is maintained as extrachromosomal linear molecules which are present even under selective pressure at an average copy number lower than 1 per nucleus.

MATERIALS AND METHODS

Strains. The characteristics of *P. anserina* were reviewed by Esser (7). The isolation and characterization of a *ura5-6* mutant deficient for OMPppase have been described previously (19). The bacterial strain used for plasmid amplification was *Escherichia coli* BJ5183 (*endA sbcB recBC galK met strR thi-1 bioT hsdR*). Transformation of *E. coli* was performed as described by Hanahan (9).

Transformation of *P. anserina* protoplasts. Protoplasts were prepared from the *ura5-6* mutant by the protocol previously described (3) with the following modifications. Cell walls were digested in 25 mM phosphate (pH 5.8)–0.8 M sorbitol buffer by using Glucanex (80 mg/g of mycelium) as enzyme source. Glucanex is a commercially available preparation of β-D-glucanases (Novo-Ferments S.A., Basel, Switzerland). Protoplasts were separated from mycelial debris by filtration, collected by centrifugation, and suspended in STC buffer (0.1 M Tris hydrochloride [pH 7.5], 0.01 M CaCl₂, 0.8 M sorbitol) at a concentration of 2×10^9 cells per ml. DNA (10 to 20 μg in 25 μl of H₂O) was mixed with 100 μl of prepared protoplasts for 30 min at room temperature, and then 1.25 ml of 60% polyethylene glycol 4000, 0.01 M Tris hydrochloride (pH 7.5), and 0.01 M CaCl₂ was added.

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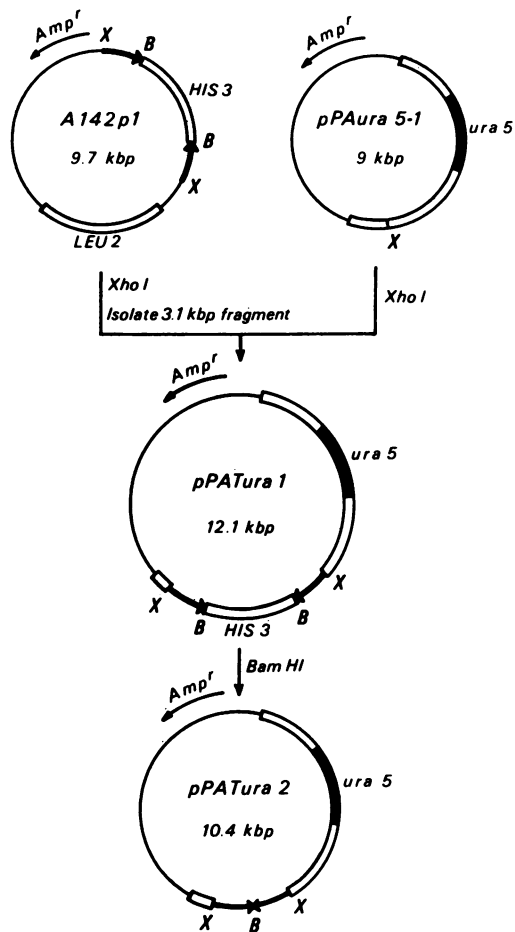FIG. 1. Construction of the vectors. *LEU2* and *HIS3* are yeast genes. The 0.7-kb *XhoI-BamHI* fragments are the chromosomal ends of *T. thermophila*. Plasmid pPAura5-1 has been described previously (3). Only the *BamHI* and *XhoI* sites used for the construction are shown.

After 20 min at room temperature, protoplasts were pelleted and suspended in 1 ml of STC buffer. Transformed protoplasts were mixed at 40°C in melted selective medium containing 0.8 M sucrose and 2% agar and overlaid on the same medium.

Determination of mitotic stability of the transformants. The transformants were grown for 4 days on solid medium supplemented with uridine (100 mg/liter), after which time the diameters of the mycelia were about 8 cm. From each thallus, eight small pieces of medium (1 mm³) containing mycelial fragments were picked at the colony periphery and deposited on minimal medium. 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, it was deduced that the nuclei had lost the *ura*⁺ transforming gene during growth on the nonselective medium.

DNA preparation and analysis. Plasmid construction is shown in Fig. 1 and was done by general molecular methods (12). Genomic DNA of *P. anserina* was isolated from the mycelium. The mycelium was lyophilized, ground, lysed in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–2% sarcosyl at 60°C for 2 h, and then treated with proteinase K (100 μg/ml) for 2 h at 37°C. After centrifugation (10,000 × *g*,

10 min), nucleic acids were precipitated from the supernatant with 15% polyethylene glycol 6000–0.75 M NaCl for 1 h on ice. The mixture was centrifuged, and the pellet was dissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. The DNA was then purified by CsCl-ethidium bromide density gradient centrifugation. The DNA preparations from the wild type and various transformants (about 4 μg) were treated overnight with restriction enzymes. They were separated on agarose gels and transferred to nitrocellulose filters. Filters were prehybridized at 65°C for 3 h in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0])–1× Denhardt solution (0.2 g of Ficoll [Pharmacia Fine Chemicals] per liter, 0.2 g of bovine serum albumin per liter, 0.2 g of polyvinylpyrrolidone per liter). Hybridizations with probes were carried out at 45°C for 16 h in 50% formamide–50 mM Tris hydrochloride (pH 7.4)–4× SSC–200 μg of salmon sperm DNA per ml. The filters were then washed at 65°C in 1× SSC–0.2% sodium dodecyl sulfate, dried, and subjected to autoradiography at −80°C with Kodak X-Omat S film and an intensifying screen.

RESULTS

Construction of derivatives of pPAura5-1 containing rDNA *T. thermophila* telomeres. The ends of the linear rDNA plasmid of *T. thermophila* have been shown to function as stable chromosomal ends in *S. cerevisiae* (24). To test the effect of these sequences on vector stability in *P. anserina*, derivatives of pPAura5-1 containing these telomeric sequences have been constructed (Fig. 1). The 3.1-kilobase (kb) *XhoI* fragment of the plasmid A142p1 (a gift of J. W. Szostak) contains an inverted repeat of *T. thermophila* telomeres separated by the yeast *HIS3* gene. This *XhoI* fragment was excised and purified by agarose gel electrophoresis. It was ligated with the *XhoI*-linearized pPAura5-1. The ligated DNA was used to transform *E. coli* BJ5183. The Amp⁺ clones were probed with the nick-translated *XhoI* fragment of A142p1. Several positive clones were obtained, and one of them was retained (pPATura1). Its structure is shown in Fig. 1. A derivative, pPATura2, was obtained by digestion of pPATura1 with *BamHI* and recircularization. The ligated DNA was used to transform *E. coli* BJ5183. Plasmid DNA was extracted from Amp⁺ transformants and digested with restriction enzymes to identify the plasmid in which the yeast *HIS3* gene had been excised. The juxtaposed inverted repeat leads to some rearrangements of the plasmid in the bacteria. However, it is possible to prepare this plasmid from bacterial clones which have been controlled for the absence of rearranged molecules.

Transformation of the *ura5-6* mutant of *P. anserina* by linearized pPATura2. We observed previously (19) that the prototrophs obtained from the transformation of the *ura5-6* mutant by the circular pPAura5-1 vector are highly stable even under nonselective conditions. This high stability results from the integration of the transforming vector in the chromosomal DNA. To test the effect of *T. thermophila* telomeres, plasmid pPATura2 either in its circular form or after linearization at the *BamHI* site was used to transform the *ura5-6* mutant. pPATura2 linearized at the *XhoI* sites was used as control (a linear vector without telomeres). The mitotic stability of the transformants was determined as described in Materials and Methods. All the *ura*⁺ strains obtained from the transformation by *XhoI*-linearized pPATura2 were found to be stable (Table 1). By contrast, some of the *ura*⁺ transformants obtained with *BamHI*-linearized pPATura2 were unstable and lost the *ura*⁺ pheno-

type when grown on the nonselective medium. The frequency of the unstable strains, estimated from three independent transformations, was about 50%. Unstable strains were also obtained after transformation with the circular pPATura2, but at a significantly lower rate. Owing to the coenocytic structure of mycelium and the absence of uninucleated cells, the kinetics of the loss of the $ura5^+$ gene was difficult to determine. However if small pieces of mycelium were picked at different times during the growth, we found that after 24 h of growth, when the diameter of the mycelium was about 2 cm, 50% of the mycelium fragments had lost the ura^+ transforming gene in the absence of selective pressure. After 48 h, no mycelial fragment picked at the edge of the thallus could regenerate a mycelium on minimal medium.

The instability which might reflect the presence of a nonintegrated vector was confirmed by genetic analysis. Different transformants were crossed with the $ura5$-6 strain, and the progeny were tested for prototrophy. All the stable transformants give rise to ura^+ spores in a Mendelian ratio, as described previously (19). By contrast, the frequency of ura^+ strains is very low (under 10^{-2}) in the progeny of the crosses between the unstable transformants and the $ura5$-6 strain. Furthermore, we observed that the ura^+ genotype was not transmitted to the progeny when the unstable transformants were used as male strains. When they were used as female strains, most of the perithecia did not contain ura^+ spores. Only very few perithecia did contain some ura^+ spores, and even in these cases, a non-Mendelian ratio was observed between ura^+ and ura^- spores in the asci. The ura^+ strains obtained from these crosses exhibited the same instability as the primary transformants. These results provide evidence that the transforming $ura5^+$ gene is not integrated into chromosomal DNA in the unstable strains.

Molecular analysis of the unstable transformants. The presence of the $ura5^+$ gene on extrachromosomal molecules in the unstable transformants was examined by analysis of the DNA of transformants. First, total undigested DNA was run on agarose gels and probed with a nick-translated DNA fragment corresponding to the $ura5$ gene (3). When DNA from stable ura^+ transformants was probed, the $ura5$ fragment hybridized only with chromosomal DNA (Fig. 2). However, with the DNA of unstable transformants, an additional hybridization band was observed. Such a result was obtained with unstable strains obtained from the transformations with either circular or linearized pPATura2. This band also hybridizes with the chromosomal ends of *T. thermophila* (data not shown) and with pBR322 (Fig. 3). When the DNA was probed with pBR322, no trace of hybridization was observed at the level of the genomic DNA. This means that in the unstable strains, no pBR322
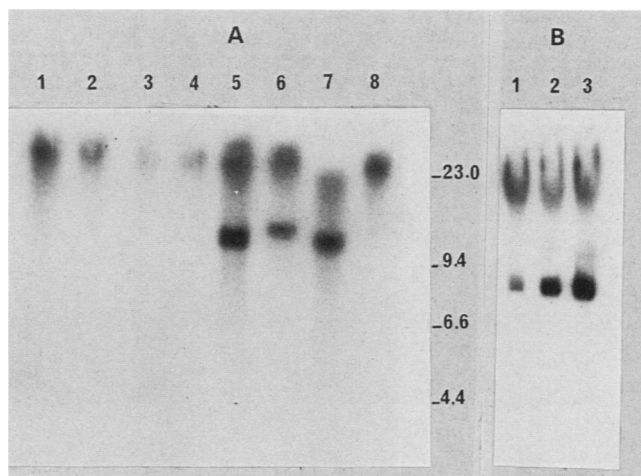

FIG. 2. Southern blot hybridization with the $ura5$ gene of total DNA from the transformants. (A) Total DNA (about 4 μg) from stable transformants (lanes 1 to 4), unstable transformants (lanes 5 to 7), and the $ura5$-6 mutant (lane 8) were run on a 0.5% agarose gel, transferred to nitrocellulose, and probed with the nick-translated fragment carrying the $ura5$ gene. On the right are indicated the positions of size markers (in kilobase pairs). The position of the additional band in lane 6 is higher than in lanes 5 and 7, owing to the presence of contaminating ethidium bromide in this DNA preparation. (B) Total DNA was extracted from an unstable transformant which was grown on minimal medium (lane 3) or on minimal medium supplemented with uridine for 12 h (lane 2) or 24 h (lane 1). DNA was run on a 0.3% agarose gel, transferred to nitrocellulose, and probed with the $ura5$ gene.

TABLE 1. Mitotic stability of the transformants

Type of pPATura2 used	No. of transformants tested[a]	% of unstable transformants[b]
Linearized at *XhoI* sites	63	0
Circular	52	15
Linearized at the *BamHI* site	85	53

[a] Protoplasts were prepared from the $ura5$-6 mutant. Equal numbers of protoplasts (2×10^8) were transformed with 10 μg of plasmid DNA preparations. Transformants were selected on minimal medium.

[b] The transformants were grown on minimal medium supplemented with 100 μg of uridine per ml. After 4 days, 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.

sequences have been integrated into the chromosomal DNA. The extrachromosomal molecules is slightly larger than the linearized pPATura2. This extrachromosomal band may be a linear plasmid, since other bands which would correspond to supercoiled and relaxed species or to multimers were not observed. The linear structure of this plasmid was confirmed by digestion of the DNA with *PstI*, a restriction enzyme that cleaves the transforming plasmid only once in the Amp^r gene. Southern blots of the digested DNA were probed with nick-translated pBR322 DNA (Fig. 3). Two bands were observed, the expected result if the plasmid was linear in the transformants.

To test whether there is a correlation between the instability of the transformants and the presence of this band, DNA was prepared from an unstable strain which was grown in a minimal medium for 24 h or for 12 and 24 h in a medium supplemented with uridine. DNA was extracted, run on an agarose gel, and probed with the $ura5$ gene (Fig. 2). The presence of uridine in the growth medium results in a marked decrease of the intensity of hybridization at the level of the extrachromosomal band. This result can be correlated with the instability of the strain on nonselective medium and suggests that the $ura5^+$ transforming gene is located only on the linear extrachromosomal molecules.

The DNA of two unstable strains was digested with *XhoI* and probed with the $ura5$ gene and the 0.7-kb *XhoI* fragment of pPATura2 corresponding to *T. thermophila* chromosomal ends. DNAs from the $ura5$-6 mutant and from a stable transformant were used as controls (Fig. 4). The DNA fragment containing *T. thermophila* telomeres hybridized with several *XhoI* fragments ranging from 9 to 2 kb in all the strains. These fragments may correspond to *P. anserina* chromosomal ends but possibly also to some internal se-
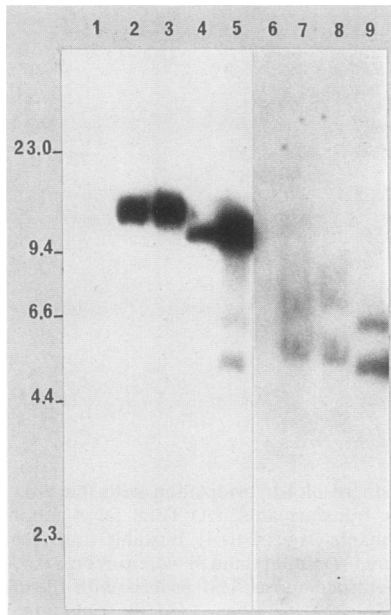
FIG. 3. Southern blot hybridization with pBR322 of the DNA from unstable strains. DNA was prepared from the *ura5-6* mutant (lanes 1 and 6) or from two unstable strains (lanes 2, 3, 7, and 8). Either total DNA (lanes 1 to 3) or DNA digested with *PstI* (lanes 6 to 8) was run on a 0.3% agarose gel. Southern blots were probed with nick-translated pBR322 DNA. Controls: lane 4, 0.05 ng of pPATura2 digested with *Bam*HI; lane 5, a mixture of 0.5 ng of pPATura2 digested with *Bam*HI and 0.01 ng of pPATura2 digested with *Bam*HI and *PstI*; lane 9, 0.05 ng of pPATura2 digested with *Bam*HI and *PstI*. The positions of size markers are shown on the left.

quences, as reported for *S. cerevisiae* (20). For the unstable strains, the additional bands correspond to the ends of the linear extrachromosomal molecule. These fragments are about 0.9 kb, which is larger than the fragments (0.7 kb) in pPATura2.

When the DNA was probed with the *ura5* gene, a band of 4.1 kb common to all the strains was revealed. It corresponds to the chromosomal endogenous fragment carrying the *ura5* gene. For the stable transformant (Fig. 4, lane 6), an additional band with the same intensity corresponds to the integrated copy of the transforming gene. For the two unstable strains (lanes 7 and 8), the additional band has the size of the central *Xho*I fragment of the pPATura2. However, the intensity of the hybridization with this fragment is much weaker than with the endogenous *ura5* locus. This difference suggests that the linear molecules would be present at a copy number below 1 per nucleus.

Several other results confirm this low number of copies. Protoplasts were prepared from a stable and an unstable transformant grown under selective conditions on minimal medium. Equal numbers of protoplasts were added to minimal medium with or without uridine. The same number of protoplasts of the stable strain regenerated on both media. However, only half the number of protoplasts obtained from the unstable strain could regenerate on the minimal medium compared with medium supplemented with uridine. This result confirms that many of the nuclei do not contain the *ura*+ gene. As there are an average of three nuclei in the protoplasts, one can estimate that only about 1 of 5 to 10 nuclei would contain the plasmid. The specific activity of OMPppase in crude extracts of different strains has been measured. For stable transformants containing one inte-

grated copy of the vector, the specific activity of OMPppase is identical to that of the wild type (B. Turcq, unpublished results). In unstable transformants, this activity is only 20 to 25% of that of the wild-type strain.

DISCUSSION

In *S. cerevisiae*, transformation by linear plasmid DNA molecules with free ends leads to the integration of the molecules into the chromosomal DNA even if the plasmid contains a replication origin (15, 16). For integrative plasmids, the linearization leads to a marked increase of the efficiency of transformation and targeting effect, which have been explained by the reactivity of free ends in recombination. However, no marked effect on transformation was observed in filamentous fungi when vectors are linearized. Furthermore, analysis of the integration site did not reveal a targeting effect; most of the integrations of linear fragments occurred outside of the homologous locus (17).

It has been shown that in *S. cerevisiae*, linear molecules can be stabilized if their ends are chromosomal ends. The used chromosomal ends were isolated from linear plasmids carrying rDNA genes which are present in the macronuclei of the ciliated protozoans *T. thermophila* or *Oxytricha* sp. (18, 24). In vivo, such chromosomal ends are processed and converted into yeast telomeres by the addition of the characteristic C1A3 repeat (20). The resulting linear plasmids are no more reactive in recombination.

We have tested the effects of the ends of *T. thermophila* linear plasmids on the transformation of the filamentous fungus *P. anserina*. pPAura5-1 is an integrative vector which contains the *ura5* gene of *P. anserina* (3). It can be used to
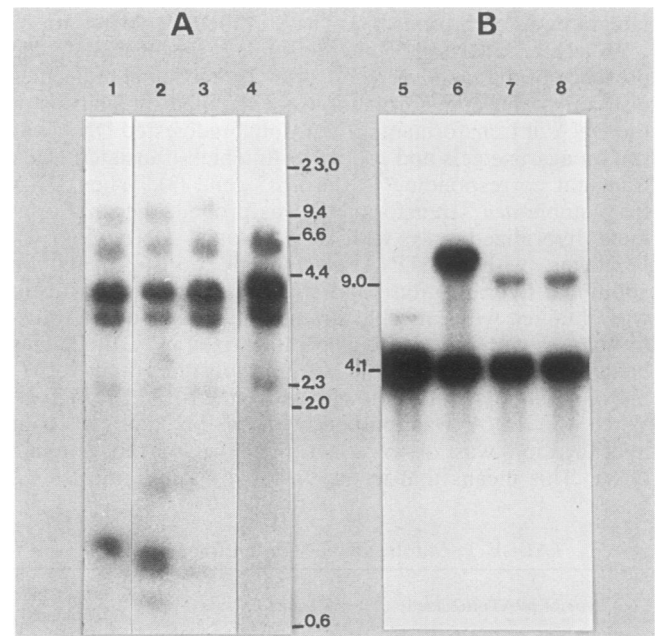

FIG. 4. Southern blot hybridization of *Xho*I-digested DNA. DNAs from the *ura5-6* mutant (lanes 3 and 5), two unstable strains (lanes 1, 2, 7, and 8), and a stable transformant (lanes 4 and 6) were digested with *Xho*I, run on 0.8% agarose gel, transferred to nitrocellulose, and probed with the nick-translated 0.7-kb *Xho*I-*Xho*I fragment carrying the chromosomal ends of *T. thermophila* (A) or with the *ura5* gene (B). On the right of panel A are shown the positions of size markers. On the left of panel B is shown the position of the central *Xho*I fragment of pPATura2 and the position of the *Xho*I chromosomal fragment carrying the *ura5* gene.

transform $ura5^-$ strains of this species. We have constructed a derivative of this vector which contains two chromosomal ends of *T. thermophila* as an inverted repeat. The resulting vector pPATura2 can be linearized to give a molecule whose ends are the telomeric sequences of *T. thermophila*. This linearized vector was used to transform the *ura5-6* mutant. About 50% of the transformants exhibit high mitotic instability when grown on nonselective medium. These unstable strains contain linear molecules which carry the ura^+ transforming gene. Such linear molecules are also observed in unstable strains obtained from transformation with the circular pPATura2. Then, like in yeasts (23), the inverted repeat of *T. thermophila* telomeres can be resolved in vivo, generating a linear plasmid. The organization of these molecules is the same as that of the transforming vector. However, these molecules are slightly larger than the linearized pPATura2. These results can be interpreted by reference to the results observed in yeasts. pPATura2 should be processed in vivo so that the ends are no more reactive. It is likely that processing consists in the addition of *P. anserina* telomeric sequences, creating functional telomeres. This is now under investigation by rescue of the linear plasmids which are present in the unstable strains.

Transformation with linear pPATura2 also gives stable strains in which the ura^+ transforming gene is integrated into the chromosomal DNA. Such stable strains were also observed in yeasts but with a lower frequency (24). Preliminary analysis has shown that in most cases, these strains contain chromosomally integrated pBR322 sequences but have lost *T. thermophila*-derived ends.

The linear plasmid is present in unstable strains at an average number of copies of about 1 per 5 to 10 nuclei. This low copy number of the ura^+ gene is sufficient to provide the growth of the mycelium and to maintain ura^- nuclei by complementation. Several hypotheses can be proposed to explain the presence of a majority of ura^- nuclei. It is known that protoplasts contain several nuclei. If only one of them is transformed by the linearized plasmid, the protoplast would give rise to a ura^+ mycelium which would contain a mixture of ura^+ and ura^- nuclei. This ratio would be maintained during growth, with no selection of one class of nuclei over the other. This does not seem to be true, because ura^+ spores which were obtained in the progeny of the crosses between the unstable transformants and the ura^- mutant gave mycelia in which the ratio of ura^+ and ura^- nuclei is also highly unbalanced. Since the spores contain only one nucleus, the ura^+ gene is lost during growth. Under selective conditions, the ura^+ nuclei will be maintained at a ratio sufficient to provide growth of the mycelium.

An alternative would be that the linearized plasmid is inefficiently replicated or unequally transmitted to daughter nuclei. As the plasmid is maintained in the strains under a selective pressure, is is replicated. It is known that in *S. cerevisiae*, ARS are located near the telomeric sequences (6). We do not know the nucleotide sequence of the *Xho*I fragment containing the *T. thermophila* telomeres, but an ARS could be present on this fragment. However, this hypothetical ARS would not be very efficient, and the replication rate of the linear molecules would be low compared with that of chromosomal DNA. If, in yeasts, specific sequences are necessary for the replication of transforming plasmids, in *Xenopus* egg nuclei, such sequences would not be required (13), and the existence of elements equivalent to yeast ARS remains to be demonstrated in eucaryotic organisms other than yeasts (26).

In *S. cerevisiae*, in the absence of a centromere, the plasmids have a strong tendency to be retained in the mother nuclei during mitosis (14). It is likely that pPATura2 does not contain a centromeric sequence, so that its mitotic segregation would be affected. Cloning *P. anserina* genomic sequences that stabilize the linearized pPATura2 may provide a way to isolate elements which are involved in the stability of chromosomes. Such sequences have been characterized in *S. cerevisiae* (4) but not yet in other eucaryotic organisms.

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