MOLECULAR AND CELLULAR BIOLOGY, June 1992, p. 2777–2783
0270-7306/92/062777-07$02.00/0

Vol. 12, No. 6

Isolation of Telomerelike Sequences from *Cryptococcus neoformans* and Their Use in High-Efficiency Transformation

JEFFREY C. EDMAN

Hormone Research Institute and Department of Laboratory Medicine, University of California, San Francisco, California 94143-0534

Received 7 October 1991/Accepted 24 March 1992

Development of a transformation system for the fungal human pathogen *Cryptococcus neoformans* is an important prerequisite for the identification of genes involved in virulence. It has previously been reported that low-efficiency transformation can be achieved by using the cloned *C. neoformans URA5* gene and *ura5* mutants. The introduction of linearized *URA5* vectors into *C. neoformans* resulted in unstable transformants which apparently harbored linear extrachromosomal DNA molecules. In this paper, the nature of these molecules is confirmed to be linear by exonuclease digestion. Recovery of the extrachromosomal DNA in *Escherichia coli* and sequence analysis demonstrates that repeats characteristic of telomeric DNA have been added to the ends of the introduced DNA. The recovered plasmids are capable of transforming at much higher efficiencies either in the supercoiled state (up to 200 transformants per μg) or the linear state (up to 90,000 transformants per μg).

Cryptococcus neoformans is a heterothallic basidiomycetous yeast that causes meningeal and systemic infections in AIDS patients and other immunocompromised patients (12). *C. neoformans* is a member of a genus comprising more than 20 species which share a broad range of morphological and biochemical characteristics, yet only *C. neoformans* is a frequent human pathogen. The nature of this virulence is thought to be due to three factors: the production of melanin, the presence of a polysaccharide capsule that inhibits phagocytosis, and the ability to grow at 37°C (5, 7–9). In order to readily dissect the roles that these factors play in the virulence of *C. neoformans*, a system for the routine genetic manipulation of the organism is necessary. A transformation system using the *C. neoformans URA5* gene has been reported elsewhere (3). This system utilized electroporation to introduce supercoiled and linear vectors containing the *URA5* gene into *C. neoformans ura5* mutants. It was shown that linearization of vectors resulted in 50- to 100-fold increases in transformation efficiencies (1 to 2 transformants per μg with supercoiled vectors versus 50 to 200 transformants per μg with linearized vectors). However, unlike other fungal systems, the increase in transformation efficiency was not the result of increased homologous recombination or integration of any sort. Rather, introduced DNA sequences were maintained (albeit unstably) as extrachromosomal DNA molecules with sizes and restriction maps consistent with those of linear DNA. In this paper, the linear nature of these molecules is confirmed. The linear vectors are modified by telomere addition, and the sequences of recovered telomeres are shown. Vectors containing telomere sequences show increased transformation efficiencies in the supercoiled state (up to 200 transformants per μg) and in the linear state (up to 90,000 transformants per μg).

MATERIALS AND METHODS

Construction of a new *C. neoformans URA5* vector and optimization of transformation. These studies began with the construction of a vector which contained a minimum of the *URA5* sequences necessary for transformation. A 2,000-bp *Bgl*II-*Bst*XI fragment from pURA5g2 (3) containing the *URA5* gene and flanking regions was blunt ended with T4 DNA polymerase and cloned into the *Eco*RV site of Bluescript SK$^-$. The resulting plasmid, pURA5x, is shown in Fig. 1.

A systematic optimization of electroporation parameters resulted in a protocol with a substantially increased efficiency compared with the one previously reported. *C. neoformans* JEC157 (α *ade2-27 ura5 lys1*) cells from a saturated overnight culture grown in YEPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) were diluted 1:50 in 50 ml of fresh YEPD. The culture was incubated for 5 to 6 h with vigorous aeration at 30°C (at an A_{600} of 0.7 to 1.0), collected by centrifugation, and resuspended in electroporation buffer (270 mM sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.0) containing 1 mM dithiothreitol. The cells were collected again and resuspended in 0.5 ml of electroporation buffer without dithiothreitol. Fifty microliters of the cell suspension was mixed with DNA (less than 5 μl in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), placed in the bottom of a 0.2-cm electroporation cuvette, and electroporated at 470 V and 25 μF (Bio-Rad GenePulser). These settings resulted in a time constant of 20 to 24 ms. After electroporation, the cells were plated immediately to SD (6.7 g of yeast nitrogen base per liter, 2% glucose) plates supplemented with 200 μg of lysine and adenine per ml. Using 100 ng of *Not*I-digested pURA5x, transformation efficiencies of 700 transformants per μg were routinely obtained.

Preparation of *C. neoformans* DNA and Southern analysis. Five-milliliter cultures of transformants were grown to saturation in SD plus adenine and lysine at 30°C. Cells were collected at approximately 1,500 rpm in an IEC clinical centrifuge. The pelleted cells were resuspended in 1 ml of a solution containing 1 M sorbitol, 1% β-mercaptoethanol, and 1 mg of Mureinase (U.S. Biochemicals) per ml and transferred to a 1.5-ml microcentrifuge tube. The suspension was incubated at 37°C for 1 h and collected at 3,000 rpm in a microcentrifuge (IBI model IMV-15). The pelleted spheroplasts were lysed by the addition of 500 μl of 50 mM EDTA–1% sodium dodecyl sulfate–100 μg of RNase A per ml. The samples were then incubated for 30 min at 65°C with

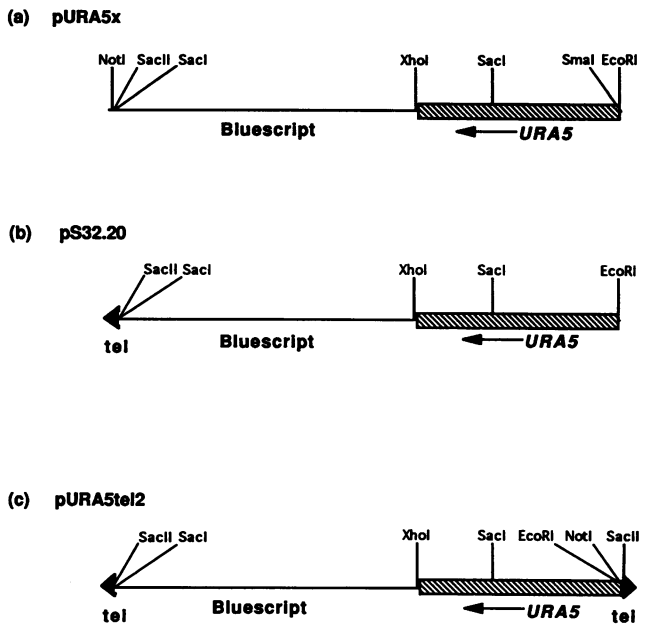

FIG. 1. Maps of *Pst*I-linearized plasmids described in this study. (a) pURA5x; the *Bgl*II-*Bst*XI fragment of pURA5g2 was treated with T4 DNA polymerase and inserted into the *Eco*RV site of Bluescript SK$^-$. (b) pS32.20, one of the recovered plasmids described in the text as containing one telomere. (c) pURA5tel2, a plasmid containing two telomeres constructed as described in Material and Methods. In order to stabilize the inverted repeats of telomeres, pURA5tel2 also contains the 1.3-kb *Pst*I fragment of pUC4K encoding the kanamycin resistance gene of Tn*903* (13).

occassional mixing. Two hundred microliters of 5 M potassium acetate was added, and the mixture was inverted several times. After incubation on ice for 30 min, the samples were spun at 15,000 rpm in a microcentrifuge. The supernatant was transferred to a new tube, and 500 μl of isopropanol was added. DNA was spooled from this mixture on a capillary tube and transferred to another microcentrifuge tube containing 400 μl of TE (10 mM Tris-HCl, pH 8.0–1 mM EDTA). The DNA was heated to 65°C for 30 min and precipitated with 800 μl of 90% ethanol–0.7 M ammonium acetate. After collection by centrifugation, the DNA pellet was resuspended in 50 μl of TE. This procedure yields 10 to 25 μg of DNA. Southern blots were prepared on nylon membranes after electrophoresis in 0.8% agarose gels and hybridized to digoxigenin-labeled probes (Genius, Boehringer-Mannheim). Blots were washed according to the manufacturer's instruction and visualized by using the chemiluminescent substrate AMPPD (Tropix).

DNA sequencing. All DNA sequencing was performed on double-stranded templates of restriction fragment subclones on an ABI model 470 DNA sequencer.

Exonuclease treatment of transformant DNA. Before agarose gel electrophoresis was performed, samples of DNA from transformants and of DNA from untransformed cells plus added supercoiled or *Not*I-digested pURA5x were treated with 80 U of T7 gene 6 exonuclease (U.S. Biochemicals) for 30 min at 37°C in 50 mM Tris-HCl, pH 8.1–5 mM $MgCl_2$–20 mM KCl–5 mM 2-mercaptoethanol. The same samples incubated in buffer alone served as controls.

Construction of a vector containing two telomeres. In order to create a stable inverted repeat of telomere sequences, pS32.20 (see below) was digested with *Sac*I and *Pst*I. Two *Sac*I sites are present in pS32.20, one derived from the polylinker of Bluescript and one in the *URA5* gene. The single *Pst*I site is directly adjacent to the added telomere sequence (see Fig. 4). After phenol-chloroform extraction and ethanol precipitation, the *Pst*I fragment of pUC4K containing the Tn*903* kanamycin resistance gene was added (13). The mixture was then ligated with T4 DNA ligase and used to transform *E. coli* JM101. Ampicillin- and kanamycin-resistant transformants were selected. The resulting plasmid contained the kanamycin resistance gene flanked by inverted repeats of the telomere sequences. This plasmid was digested with *Sac*II, which releases the inverted repeat and the kanamycin resistance gene as a single fragment. This fragment was then inserted into the *Sac*II site of a derivative of pURA5x from which the *Pst*I site in the polylinker had been removed by treatment with T4 DNA polymerase. Ampicillin- and kanamycin-resistant transformants were isolated. The resulting plasmid, pURA5tel2 (Fig. 1), was confirmed to be correct by restriction mapping and sequencing of telomere regions.

RESULTS

***C. neoformans* transformants are mostly unstable and contain extrachromosomal DNA.** Consistent with the previous study performed with pURA5g2, the majority of transformants with *Not*I-digested pURA5x were unstable (0 to 5% of cells retained uracil prototrophy after 15 to 20 generations on nonselective medium). As with the low-efficiency protocol, 80 to 90% of transformants were unstable. The unstable transformants harbor extrachromosomal DNA molecules that contain the *URA5* sequences. Southern analysis of undigested DNA samples from 10 *Not*I-digested pURA5x transformants demonstrates the spectrum of transformants obtained (Fig. 2a). Of the 10 transformants, 5 (lanes 5 through 8 and 10) contained extrachromosomal DNA that hybridized to a pURA5x probe and that migrated slightly more slowly than *Not*I-digested pURA5x added to untransformed DNA (lane VEC). One transformant contained extrachromosomal DNA that was smaller than the input DNA (lane 1). Two transformants contained extrachromosomal DNAs roughly twice the size of *Not*I-digested pURA5x (lanes 2 and 3). The remaining transformants did not contain detectable extrachromosomal *URA5* sequences and presumably are the result of integration events (lanes 4 and 9).

Extrachromosomal DNA in unstable transformants is linear. The nature of the extrachromosomal DNA was investigated by restriction analysis and exonuclease sensitivity. As shown above, when *Not*I-digested pURA5x (4,893 bp) is used for transformation, extrachromosomal DNA that migrates slightly more slowly than cloned *Not*I-digested pURA5x is present in some transformants (Fig. 2a; lanes 5 through 8 and 10). If the extrachromosomal DNA in these transformants were retained as unrearranged linear molecules, then digestion with *Xho*I would be expected to yield fragments of 2,900 and 2,000 bp. As can be seen in Fig. 2b, lanes 5 through 8 and 10, such products are obtained from the transformants harboring extrachromosomal DNAs of roughly the same size as *Not*I-digested pURA5x. (The one transformant with a smaller plasmid [the same as in Fig. 2a, lane 1] is shown in Fig. 2b, lane 1 and demonstrates that this plasmid had undergone a deletion of the Bluescript portion of pURA5x.) The *Xho*I digestion products migrate slightly more slowly than native pURA5x digested with *Not*I and *Xho*I (on the order of 100 bp). A *Sma*I digestion (with expected products of 4,866 and 27 bp) was also performed,

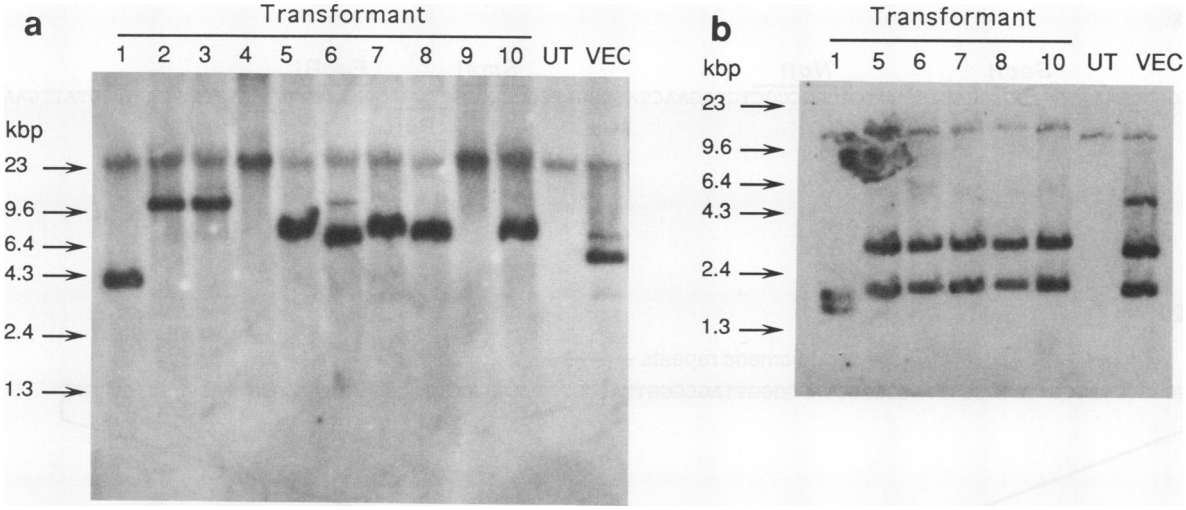

FIG. 2. Southern analysis of transformants. (a) Undigested DNA samples were run on an 0.8% agarose gel. Lanes: 1 through 10, DNA samples from 10 independent transformants; UT, untransformed DNA; VEC, untransformed DNA plus 10 pg of *Not*I-digested pURA5x. (b) DNA samples were digested with *Xho*I and separated on an 0.8% agarose gel. Lanes: 1, 5, 6, 7, 8, and 10, six of the transformants from panel a harboring extrachromosomal DNA; UT, DNA from untransformed cells; VEC, 10 pg of *Not*I-digested pURA5x added to untransformed DNA prior to *Xho*I digestion. Nylon membrane transfers were hybridized to digoxigenin-labelled pURA5x. Molecular size standards (in kilobase pairs) are indicated on the left.

and a single fragment of approximately 5,000 bp was seen (not shown). These data suggest that the ends of the molecules are being modified and that substantial alterations to the plasmid are not occurring in those transformants with extrachromosomal DNA molecules that are approximately the same size as *Not*I-digested pURA5x.

If the extrachromosomal DNA in *C. neoformans* transformants is linear, then it should be sensitive to exonucleases. Figure 3 shows that these sequences are sensitive to T7 gene 6 exonuclease. DNA from a single transformant (transformant 10; same DNA preparation as Fig. 2a, lane 10) was treated with exonuclease and was degraded. Supercoiled pURA5x added to untransformed *C. neoformans* DNA is resistant, and *Not*I-digested pURA5x is sensitive. Similar results were obtained with *Bal* 31 nuclease (not shown).

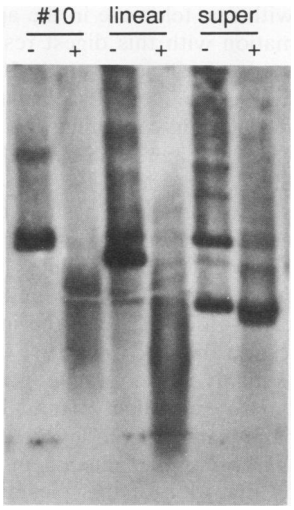

FIG. 3. Exonuclease sensitivity of extrachromosomal DNA in transformants. DNA from a single transformant (transformant 10 from Fig. 2a) harboring extrachromosomal DNA of approximately the same size as *Not*I-digested pURA5x was treated with T7 gene 6 exonuclease as described in Materials and Methods. *Not*I-digested pURA5x and supercoiled pURA5x served as positive and negative controls, respectively. Pluses indicate that enzyme was added, and minuses indicate buffer-only controls. Nylon membrane transfers were hybridized to digoxigenin-labelled pURA5x.

Recovery of extrachromosomal DNA into *Escherichia coli*. Attempts to recover plasmids in their native state from *C. neoformans* have been unsuccessful. This is undoubtedly due to the linear nature of the DNA. Repair of staggered ends with Klenow or T4 DNA polymerase followed by ligation could potentially allow the recovery of these molecules in a circular form. However, if telomeres were added to the ends, this would result in the formation of an inverted repeat, which is known to be unstable in *E. coli* (2). Using this protocol, no transformants that had restriction maps similar to that of pURA5x were obtained. In order to remove the possible inverted repeat, DNA from transformants was digested with either *Sma*I or *Xho*I. *Sma*I would remove a small fragment containing one telomere and leave the remainder of the plasmid intact. *Xho*I would remove the entire *URA5* gene and leave a telomere attached to Bluescript. After repair with T4 DNA polymerase and ligation, *E. coli* transformants were obtained for those extrachromosomal DNAs whose sizes were consistent with that of linear pURA5x (1 to 10 *E. coli* transformants per μg of *C. neoformans* DNA).

Sequence of the recovered DNA. Seven plasmids (five from the *Xho*I digestion and two from the *Sma*I digestion) were sequenced around the putative telomere addition site, and all demonstrated the addition of simple repeats characteristic of telomeric DNA. The number of repeats was between 7 and 12. The repeat was AGGGGGTT in most cases, but there was some variability in the number of G residues (of 65 total repeats from the seven recovered plasmids, 3 were AGGGGTT and 2 were AGGGTT). Because of the enzymatic treatment necessary for the circularization of the plasmids, there could be a larger number of repeats present in the plasmid when it is in *C. neoformans*. Data from other

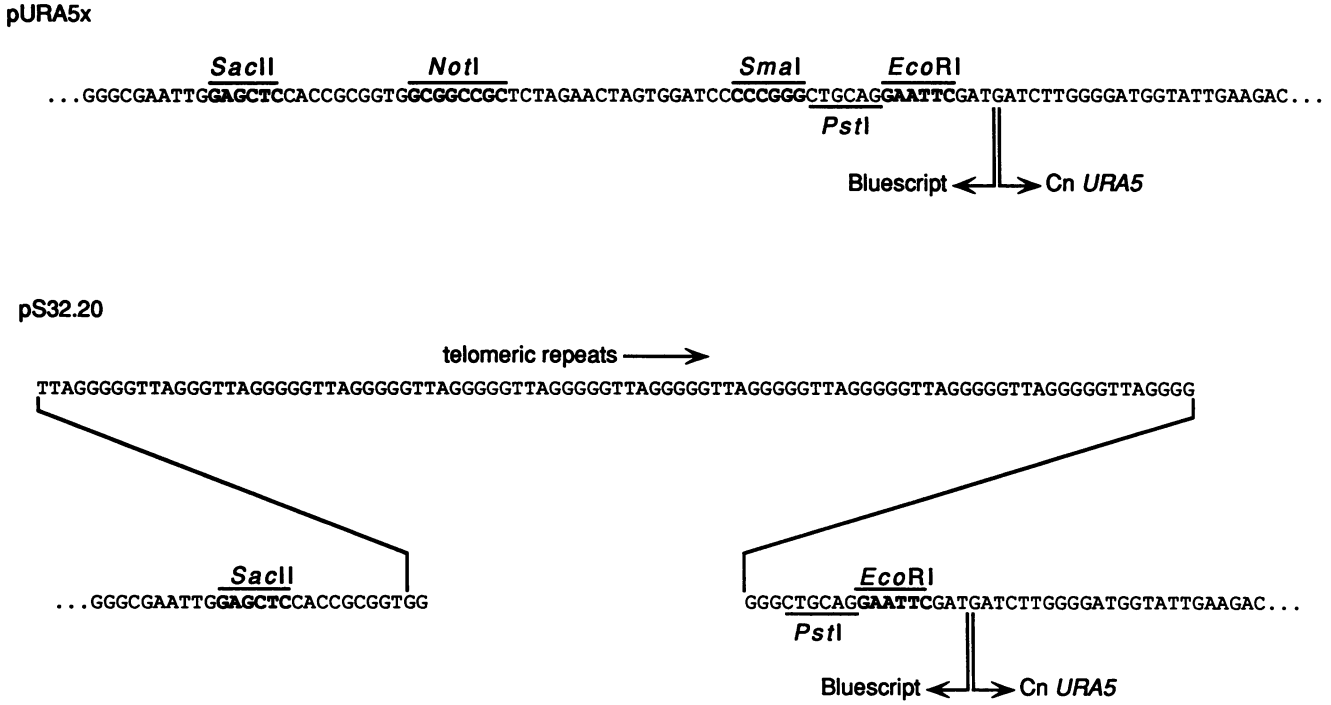

FIG. 4. Sequences of the multiple cloning site region of pURA5x and of recovered plasmid pS32.20. The telomeric repeats present in recovered plasmid pS32.20 are shown.

telomeres suggest that a 12- to 16-nucleotide 3′ overhang is present on the G-rich strand (1). Using this as a guide, I conclude that one or two repeats are probably lost in the recovery procedure. Ten to twelve repeats would represent 80 to 96 bp. This would be consistent with the subtle size increases demonstrated by Southern analysis of the plasmids in *C. neoformans*. The sequence of pS32.20 (from the *Sma*I digestion) is shown in Fig. 4.

Telomeric DNA enhances transformation efficiencies for supercoiled and linearized plasmids. It has been previously demonstrated that transformation of *C. neoformans* with supercoiled DNA results in low transformation efficiencies (1 to 10 per μg). Linearization of pURA5x with *Pst*I results in a 10- to 15-fold increase in efficiency (Table 1). The recovered plasmid pS32.20 was tested for its transformation efficiency. The addition of telomeric DNA enhanced transformation efficiency for supercoiled DNA 14-fold (218 transformants per μg). Digestion of pS32.20 with *Pst*I results in a linear molecule with one telomere in the appropriate orientation. Transformation with this digest resulted in an even greater enhancement of efficiency to 49,100 transformants per μg (a 202-fold increase over *Pst*I-digested pURA5x).

TABLE 1. Transformation efficiencies of plasmids

Plasmid	Transformation efficiency[a] (*transformants*/μg)		
	Single experiment	Avg of 4 expts (range)	Fold increase relative to pURA5x[b]
Undigested			
pURA5x	10	15 (0–30)	
pS32.20	190	218 (70–450)	14
pURA5tel2	260	193 (30–360)	13
*Pst*I digested			
pURA5x	200	243 (80–490)	
pS32.20	51,000	49,100 (47,200–51,000)	202
pURA5tel2	74,800	59,500 (33,700–90,200)	244

[a] The standard transformation contained 100 ng of DNA. DNA concentrations were determined by fluorescence. Supercoiled plasmids were isolated by CsCl gradient centrifugation. After restriction enzyme digestion, plasmids were extracted with phenol-chloroform, ethanol precipitated, and resuspended in TE. DNA concentration was then determined.

[b] Comparisons are only within the undigested and *Pst*I-digested groups.

Construction of a plasmid containing two telomeres. Since pS32.20 had only one telomere and since that telomere was present on the Bluescript side of the *Pst*I-linearized molecule, it would seem reasonable to assume that the presence of two telomeres on the vector might further enhance transformation efficiencies. Such a vector (pURA5tel2) was constructed as described in Materials and Methods and is shown in Fig. 1c. In order to retain the inverted repeat of telomere sequences in this vector, a *Pst*I fragment containing the Tn*903* kanamycin resistance gene was inserted between the telomeric sequences. This allows positive selection for the maintenance of the inverted repeat by propagating *E. coli* harboring the plasmid in the presence of kanamycin. Upon digestion of this plasmid with *Pst*I, the fragment containing the kanamycin resistance gene is released, exposing both telomeres in the appropriate orientation. Transformation of *C. neoformans* with *Pst*I-digested pURA5tel2 results in a transformation efficiency of 59,500 transformants per μg (Table 1). This slight increase in efficiency over pS32.20 is probably not significant. However, two individual transformations with pURA5tel2 resulted in transformation efficiencies approaching 100,000 transfor-

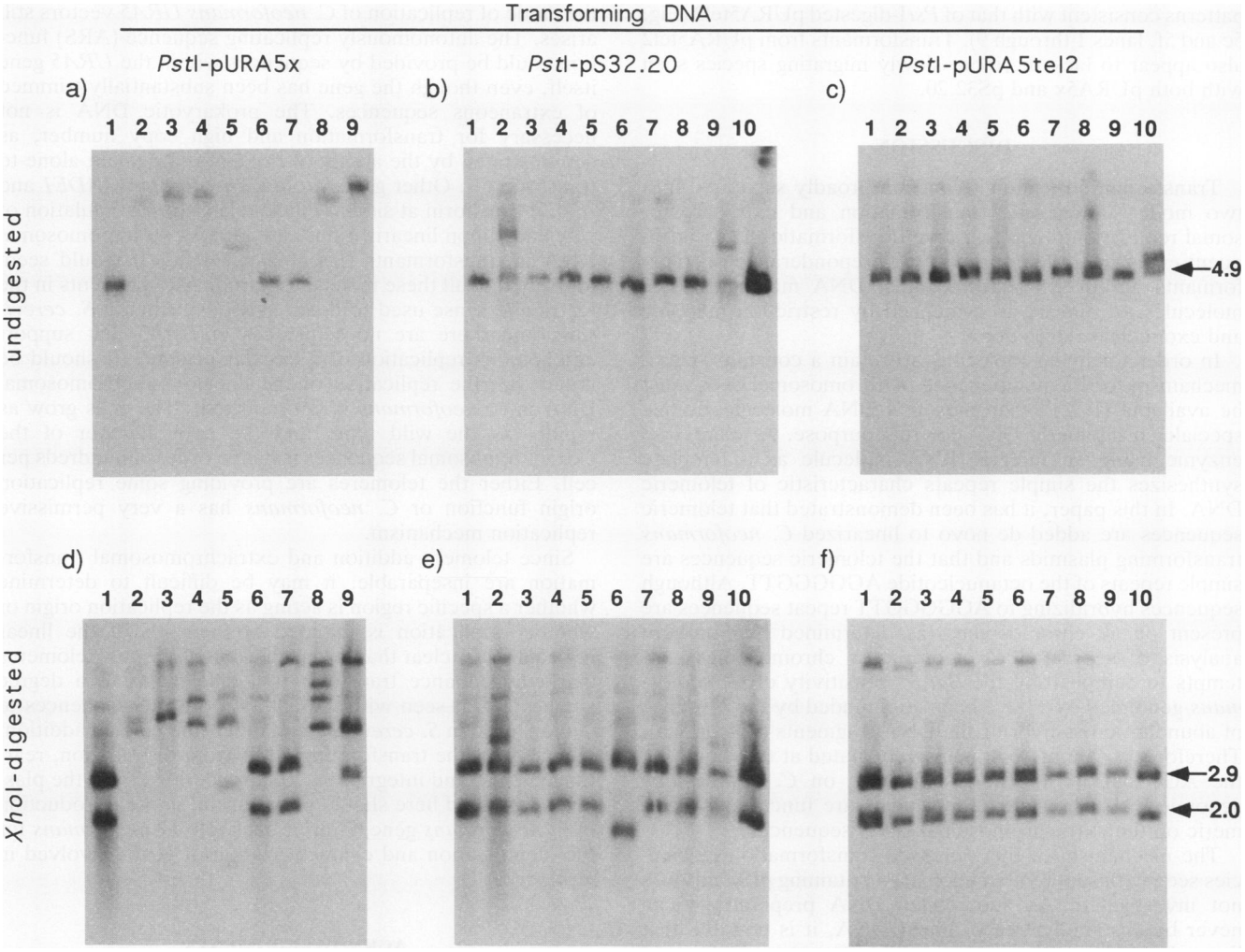

FIG. 5. Southern blots of undigested (a through c) and *Xho*I-digested (d through f) DNA from transformants. (a) lanes: 1 through 9, undigested DNA from pURA5x transformants. (b) Lanes: 1 through 9, undigested DNA from pS32.20 transformants; 10, 10 pg of *Not*I-digested pURA5x added to untransformed DNA. (c) Lanes: 1 through 9, undigested DNA from pURA5tel2 transformants; 10, 10 pg of *Not*I-digested pURA5x added to untransformed DNA. (d through f) Lanes contain same samples as in panels a through c digested with *Xho*I prior to electrophoresis. Nylon membrane transfers were hybridized to digoxigenin-labelled pURA5x.

mants per μg (the highest transformation efficiency observed with pS32.20 was 51,000 transformants per μg).

Southern analysis of transformants obtained with telomere-containing plasmids. As noted above, transformation with plasmids lacking telomeres results in a substantial fraction of transformants containing integrated *URA5* sequences. Twelve transformants derived from *Pst*I-digested pURA5x, pS32.20, and pURA5tel2 were analyzed by Southern analysis. Of 12 *Pst*I-digested pURA5x transformants, Southern analysis of undigested DNA indicated that 8 harbored extrachromosomal *URA5* sequences. Nine of these transformants are shown in Fig. 5a and d. The remainder contained either integrated forms (Fig. 5a; lanes 2 through 4, 8, and 9) or high-molecular-weight multimers of the plasmid (such multimers cannot be distinguished from integrated sequences with the gel system used). *Xho*I digestion of the transformants indicated that six contained extrachromosomal sequences that were consistent with simple addition of telomere sequences to the introduced plasmid (hybridizing fragments of 2,900 and 2,000 bp). The others contained more-complex additions or rearrangements. In addition, most of the transformants contained low but detectable amounts of higher-molecular-weight extrachromosomal DNAs. These may represent the presence of a small quantity of multimeric species and were not studied further.

When pS32.20 or pURA5tel2 transformants were analyzed, all transformants (12 of each) contained extrachromosomal sequences, as indicated by the presence of low-molecular-weight *URA5* sequences in the undigested Southern blot (Fig. 5b). *Xho*I digestions revealed that in the pS32.20 transformants, 11 had retained the structure of *Pst*I-digested pS32.20. Two of these transformants also contained extrachromosomal DNA molecules that were roughly twice the size of *Pst*I-digested pS32.20 (Fig. 5b, lanes 1 and 9). One transformant had suffered a small deletion of the *URA5* portion of the plasmid (Fig. 5e, lane 6). This deletion has occurred on the end that does not contain a telomere (Fig. 1b). All of the pURA5tel2 transformants had undigested and *Xho*I digestion

patterns consistent with that of *Pst*I-digested pURA5tel2 (Fig. 5c and 5f, lanes 1 through 9). Transformants from pURA5tel2 also appear to lack the more slowly migrating species seen with both pURA5x and pS32.20.

DISCUSSION

Transformation in fungi has been broadly separated into two modes: integrative transformation and extrachromosomal replication (4). Integrative transformation is an infrequent event in *C. neoformans*; the preponderance of transformants harbor extrachromosomal DNA molecules. The molecules are linear, as evidenced by restriction mapping and exonuclease digestion.

In order for linear molecules to retain a constant size, a mechanism for the maintenance of chromosomal ends must be available (1, 14). Chromosomal DNA molecules utilize specialized telomeric DNA for this purpose. A telomerase enzyme using an integral RNA molecule as a template synthesizes the simple repeats characteristic of telomeric DNA. In this paper, it has been demonstrated that telomeric sequences are added de novo to linearized *C. neoformans* transforming plasmids and that the telomeric sequences are simple repeats of the octanucleotide AGGGGGTT. Although sequences hybridizing to AGGGGGTT repeat sequences are present on all chromosomes (as determined by Southern analysis of separated *C. neoformans* chromosomes), attempts to demonstrate the *Bal* 31 sensitivity of *C. neoformans* genomic DNA have been confounded by the presence of abundant cross-hybridizing DNA fragments (not shown). Therefore, it cannot be conclusively stated at this time that the AGGGGGTT repeat is telomeric on *C. neoformans* genomic DNA; however, the repeats are functionally telomeric on the extrachromosomal DNA sequences.

The mechanism of the increased transformation efficiencies seen with supercoiled telomere-containing plasmids was not investigated. As supercoiled DNA preparations can never be absolutely free of linear DNA, it is possible that linear DNA in such preparations is the only transforming species. If this is true, then it is not surprising that the supercoiled telomere-containing plasmids transform at higher efficiencies. Even with random linearization, the 3,000-fold increase in transformation efficiency demonstrated by the *Pst*I-linearized telomere-containing plasmids would be adequate to explain the increased efficiency seen with telomere-containing supercoils.

The de novo addition of telomerelike sequences has been observed for the ascomycete *Fusarium oxysporum* (11). The *F. oxysporum* telomere sequences also increased transformation frequencies. Unlike *C. neoformans*, the linear extrachromosomal DNA in *F. oxysporum* has undergone partial duplication of the transforming DNA. The fate of DNA introduced into *C. neoformans* appears to be most similar to that of DNA introduced into *Paramecium tetraurelia* (6). In *P. tetraurelia*, microinjection of supercoiled DNA into the macronucleus results in apparent random linearization and the addition of telomerelike sequences to the ends of the DNA. Linear DNA is directly modified by the addition of telomeric repeats. In addition, the molecules replicate with great efficiency. The observation that virtually any DNA fragment appears to undergo the same fate led the authors to suggest that, like *Xenopus* oocytes (10), *P. tetraurelia* contains a permissive replication system.

Unlike *Xenopus* oocytes and *P. tetraurelia*, *C. neoformans* does not have a distinct life cycle requiring rapid replication of DNA molecules. Therefore, the question of the mode of replication of *C. neoformans URA5* vectors still arises. The autonomously replicating sequence (ARS) function could be provided by sequences within the *URA5* gene itself, even though the gene has been substantially trimmed of extraneous sequences. The prokaryotic DNA is not necessary for transformation and high copy number, as demonstrated by the ability of the *URA5* fragment alone to transform (3). Other genes from *C. neoformans* (*ADE1* and *ADE2*) transform at similar efficiencies, show stimulation of efficiency upon linearization, and harbor extrachromosomal DNA in transformants (unpublished data). It would seem unlikely that all these molecules harbor ARS elements in the restrictive sense used to define ARS elements in *S. cerevisiae*, and there are no sequences in *URA5* that support autonomous replication of a circular plasmid. It should be noted that the replication of the linear extrachromosomal DNA in *C. neoformans* is very efficient. The cells grow as rapidly as the wild type, and the copy number of the extrachromosomal sequences is on the order of hundreds per cell. Either the telomeres are providing some replication origin function or *C. neoformans* has a very permissive replication mechanism.

Since telomere addition and extrachromosomal transformation are inseparable, it may be difficult to determine whether a specific region is acting as the replication origin or whether replication is initiated at the ends of the linear molecule. It is clear that at least in *C. neoformans*, telomeres markedly enhance transformation efficiencies to a degree similar to that seen with the addition of ARS sequences to vectors used in *S. cerevisiae* and other fungi (4). In addition, they protect the transforming DNA from degradation, rearrangements, and integration. Vectors derived from the plasmids described here should prove useful in the introduction of *C. neoformans* gene libraries back into *C. neoformans* for the identification and characterization of genes involved in virulence.

ACKNOWLEDGMENTS

I wish to acknowledge the continuous help and advice of K. J. Kwon-Chung.

This work was supported by National Institutes of Health grant AI29312.

REFERENCES

1. **Blackburn, E. H.** 1990. Telomeres: structure and synthesis. J. Biol. Chem. **265**:5919–5921.
2. **Collins, J.** 1981. Instability of palindromic DNA in *E. coli*. Cold Spring Harbor Symp. Quant. Biol. **45**:409.
3. **Edman, J. C., and K. J. Kwon-Chung.** 1990. Isolation of the *URA5* gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. Mol. Cell. Biol. **10**:4538–4544.
4. **Fincham, J. R. S.** 1989. Transformation in fungi. Microbiol. Rev. **53**:148–170.
5. **Fromtling, R. A., H. J. Shadomy, and E. S. Jacobson.** 1982. Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans*. Mycopathologica **79**:23–29.
6. **Gilley, D., J. R. Preer, K. J. Aufderheide, and B. Polisky.** 1988. Autonomous replication and addition of telomerelike sequences to DNA microinjected into *Paramecium tetraurelia* macronuclei. Mol. Cell. Biol. **8**:4765–4772.
7. **Kwon-Chung, K. J., I. Polachek, and T. J. Popkin.** 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. **150**:1414–1421.
8. **Kwon-Chung, K. J., and J. C. Rhodes.** 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus*

neoformans. Infect. Immun. **51:**218–233.

9. **Littman, M. L., and E. Tsubura.** 1959. Effect of degree of encapsulation upon virulence of *Cryptococcus neoformans*. Proc. Soc. Exp. Biol. Med. **101:**773–777.
10. **Mechali, M., and S. Kearsey.** 1984. Lack of specific sequence requirement for DNA replication in *Xenopus* eggs compared with high specificity in yeast. Cell **38:**55–64.
11. **Powell, W. A., and H. C. Kistler.** 1990. In vivo rearrangement of foreign DNA by *Fusarium oxysporum* produces linear self-replicating plasmids. J. Bacteriol. **172:**3163–3171.
12. **Rippon, J. W.** 1988. Medical mycology, 3rd ed. The W. B. Saunders Co., Philadelphia.
13. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
14. **Zakian, V. A.** 1989. Structure and function of telomeres. Annu. Rev. Genet. **23:**579–604.