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Genetics

A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts

(*Candida/Kluyveromyces lactis*/telomeric repeat variants)

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ABSTRACT **Telomeric DNA sequences have generally been found to be remarkably conserved in evolution, typically consisting of repeated, very short sequence units containing clusters of G residues. Recently however the telomeric DNA of the asexual yeast *Candida albicans* was shown to consist of much longer repeat units. Here we report the identification of seven additional telomeric sequences from sexual and asexual budding yeast species. The telomeric repeat units from this group of relatively closely related species show more phylogenetic diversity in length (8–25 bp), sequence, and composition than has been seen previously throughout a wide phylogenetic range of other eukaryotes. We also show that certain strains of the asexual diploid species *Candida tropicalis* have two forms of telomeric repeats, which appear to differ by a single base pair. Despite their great diversity, the telomeric repeat units of *C. albicans*, *Saccharomyces cerevisiae*, and all of the species we have examined in this report share a conserved ≈6-bp motif of T and G residues resembling more typical telomeric sequences.**

Telomeres are the DNA and protein structures at the termini of eukaryotic chromosomes (1–3). They function to protect the chromosome ends from sequence loss that would otherwise gradually occur from the failure of DNA polymerases to completely replicate ends of DNA molecules (1–3). They also appear to function as a means by which cells can distinguish between chromosome ends and double-stranded DNA breaks, the latter of which can lead to blockage of the cell cycle and cell death (4, 5). The DNA sequences of telomeres have been found to be highly similar between even distantly related eukaryotes, typically consisting of tandem arrays of 5- to 8-bp repeating units characterized by clusters of G residues on the strand running 5′ to 3′ toward the chromosomal end (1–3). In ciliated protozoans, slime molds, and the budding yeast *Saccharomyces cerevisiae*, the terminal part of this G-rich strand exists as a single-stranded overhang *in vivo* (6–9). *In vitro*, the G-rich strand of telomeric sequences has been shown to be able to form unusual intramolecular and intermolecular structures stabilized by G·G base pairing (10–12). It has recently been shown *in vitro* that a subunit of an *Oxytricha* telomere end binding protein is capable of stimulating the appearance of unusual structures at salt levels well below those normally required for their formation (13). Whether any such structures involving non-Watson–Crick base pairing are involved in telomere function is unclear at present.

In certain ciliates and vertebrate cells it has been demonstrated that telomeric DNA repeats can be synthesized *de novo* onto DNA ends by a specialized ribonucleoprotein reverse transcriptase, telomerase (14–19). The telomerase enzymes of the ciliates *Tetrahymena thermophila* and *Euplotes crassus* contain identified RNA molecules, part of which serve as a template for the synthesis of the species-specific telomeric repeats (17, 20). Thus, mutations in the template region of the *T. thermophila* telomerase RNA lead to the production of terminal telomeric repeats that contain the corresponding mutation (21, 22). Such incorporation of sequence changes in telomeric repeats can in turn lead to severe effects on cellular and nuclear morphology and eventual death of the cell culture (21, 23).

Recently, the telomeres of the opportunistic pathogenic yeast *Candida albicans* were shown to consist of tandem copies of 23-bp repeats that lack any appreciable strand composition bias (24). This atypical telomeric repeat raised the questions of whether the *C. albicans* telomeres are synthesized by a telomerase and how widespread is the occurrence of such long telomeric repeats. Here we report that a number of other yeast species have telomeres composed of relatively long telomeric repeats and that while they are diverse in sequence, each contains a T+G motif reminiscent of more typical telomeric repeats.

MATERIALS AND METHODS

Strains. The yeast strains used in this study that have designations beginning with "B-" are National Institutes of Health strains generously provided by B. Wickes. The *Candida tropicalis* strain 1739-82 was provided by I. Polacheck (The Hebrew University–Hadassah Medical Center, Jerusalem). The *Candida glabrata* strain used in this work is American Type Culture Collection (ATCC) 34138.

Blotting and Hybridization. DNA was blotted from agarose gels onto Nytran membranes (Schleicher & Schuell) according to procedures recommended by the manufacturer. Hybridizations were carried out according to the procedure of Church and Gilbert (25) in Na_2HPO_4 and 7% (wt/vol) SDS. Washes were carried out at the same temperature as the hybridization, in solutions containing 2% SDS and Na_2HPO_4, at the Na^+ concentrations given below. The hybridization probe used in Fig. 1 was a ^{32}P-labeled DNA fragment containing the telomeric repeats cloned from *C. albicans* strain WO-1 (24) prepared using a Multiprime kit (Amersham). The fragment was generated from PCR of a *C. albicans* telomeric clone and contained multiple tandem copies of *C. albicans* telomeric repeats and <10 bp on either end from the vector or from subtelomeric DNA. Hybridization was carried out at 55°C, with washes using 200 mM Na^+. For the *Kluyveromyces lactis* BAL-31 digest shown in Fig. 2, probing was done with a 5′ ^{32}P-end-labeled 25-base oligonucleotide identical in sequence to the *K. lactis* telomeric repeat shown in Fig. 3. Hybridization and washes were carried out at 49°C, with washes using 200 mM Na^+. For *Candida guillermondii*, probing was done with ^{32}P-labeled pCgui7, a pBluescript vector (Stratagene) carrying a 1.5- to 2-kb telo-

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Abbreviation: ATCC, American Type Culture Collection.

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meric clone from *C. guillermondii*. Hybridization and washes (in 200 mM Na^+) were carried out at 54°C. Sequences of the two oligonucleotides used in the hybridizations of Fig. 4 are ACGGATGTCACG (AC probe) and GTGTAAGGATG (AA probe) (the dimorphic base is underlined). Hybridization with the 5′ ^{32}P-end-labeled AC probe was at 47°C, and hybridization with the AA probe was at 24°C. Washes for both were in 2% SDS with 500 mM Na^+.

Cloning of Telomeres. Telomere-enriched libraries were constructed from genomic DNA by methods used previously for cloning *C. albicans* telomeres (24). Uncut yeast genomic DNA was ligated to a blunt-ended linearized plasmid vector, and then this ligated mix was digested with a restriction enzyme that cleaves both within the polylinker of the vector and within a few kilobases of at least some of the putative telomeric ends of the species in question. As with the previous cloning of *C. albicans* telomeres (24), no enzymatic pretreatment was done to produce blunt ends of the telomeres in the genomic DNA prior to the initial ligations. Plasmids were than recircularized with T4 DNA ligase and then transformed into *Escherichia coli* cells prior to screening for putative telomere clones by colony hybridization. The libraries from *Candida maltosa, Candida pseudotropicalis*, two strains of *C. tropicalis*, and *K. lactis* (ATCC 32143), all species whose genomic DNA showed multiple bands that cross-hybridized to the *C. albicans* telomeric repeat probe (see Fig. 1), were screened with this probe. A cloned *S. cerevisiae* telomere probe [repeat unit $TG_{2-3}(GT)_{1-6}$; refs. 26 and 27] was used to screen the telomere-enriched library from *C. glabrata*, whose genomic DNA cross-hybridized with this, but not with the *C. albicans*, telomeric probe (data not shown). *C. guillermondii* DNA did not appreciably cross-hybridize with either the *C. albicans* or the *S. cerevisiae* telomeric probes at the stringencies tested. The telomere-enriched library from this species was screened using total genomic *C. guillermondii* DNA as a probe. This procedure can be used to identify all clones containing repetitive DNA sequences, and we reasoned that telomeres should make up a significant fraction of the repetitive sequences found in telomere-enriched libraries. Typically, a few hundred *E. coli* transformants were obtained for each small library, and up to nine putative telomere clones were obtained from each. Nine repetitive DNA clones were obtained from *C. guillermondii*, three of which proved to be telomeric.

RESULTS

To determine the relationship of the complex telomeric repeat sequence of *C. albicans* to the more usual, simple telomeric sequences, genomic DNA from budding yeast species related to both *C. albicans* and *S. cerevisiae* (28) was analyzed by Southern blotting, using cloned *C. albicans* telomeric repeats as the hybridization probe. Under low-stringency hybridization conditions, we detected multiple cross-hybridizing bands in several species (Fig. 1). In some cases, the cross-hybridizing bands were diffuse in appearance, a characteristic feature of telomeric restriction fragments caused by different numbers of telomeric repeats in individual telomeres among a population of cells (2, 24). No hybridization of *C. albicans* telomeric repeats was detected to DNA from *S. cerevisiae*, a species with short, heterogeneous telomeric repeats of the sequence $TG_{2-3}(TG)_{1-6}$. Use of *S. cerevisiae* telomeric DNA as a hybridization probe to genomic DNA from a variety of yeast species produced a much greater degree of nonspecific hybridization and, with the exception of the species *C. glabrata*, generally did not reveal any obvious candidate telomeric fragments (data not shown).

Telomere-enriched libraries were constructed (see *Materials and Methods*) from genomic DNA from seven budding

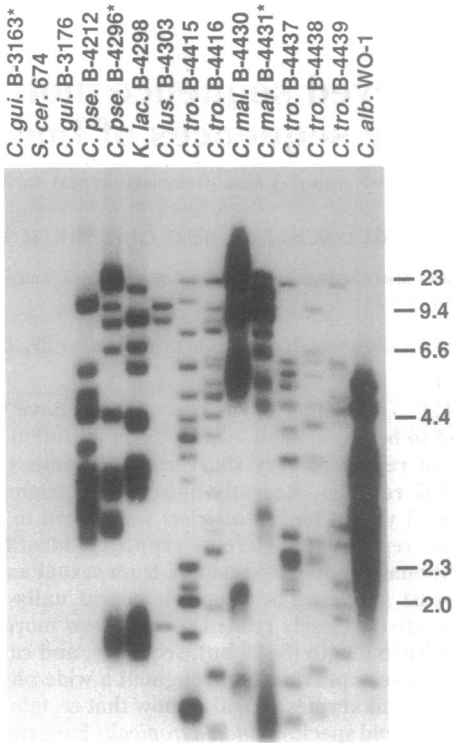

FIG. 1. Hybridization of *C. albicans* telomeric repeats to genomic DNAs of various yeast species. Genomic DNAs of eight yeast species were digested with *Eco*RI, electrophoresed in a 0.8% agarose gel, blotted, and probed with a ^{32}P-labeled telomeric fragment from *C. albicans* WO-1. DNA size markers, measured in kilobase pairs, are shown at the right. The species and strains used here are indicated above each lane: *C. gui., C. guillermondii; S. cer., S. cerevisiae; C. pse., C. pseudotropicalis; K. lac., K. lactis; C. lus., Candida lusitaniae; C. tro., C. tropicalis; C. mal., C. maltosa*; and *C. alb., C. albicans*. Asterisks indicate strains from which telomeres were cloned.

yeast species and strains. Putative telomeric clones from these species were identified by their ability to hybridize to known yeast telomeric repeats [either the 23-bp *C. albicans* repeat or the $TG_{2-3}(GT)_{1-6}$ repeat of *S. cerevisiae* (used for *C. glabrata*)] or by screening for end-linked repetitive DNA sequences without the use of a specific probe (used for *C. guillermondii*). Sequencing putative telomere fragment inserts from the different yeast species identified clones that contained tandem repeats with unit lengths of 8–25 bp, as discussed below. With a single exception (discussed below), the repeats showed no sequence variations within a species. In every case the repeat array extended to the very end of the insert, directly abutting vector sequences, as has been found previously for cloned telomeres. The repeat-containing clone from each species hybridized back to the same pattern of restriction fragments observed originally with the *C. albicans* or the *S. cerevisiae* probe used for library screening. Most of the hybridizing bands were preferentially sensitive to BAL-31 nuclease (Fig. 2 and data not shown), indicating that the bulk of the repeat sequences are present at the ends of chromosomes. The lengths of the tracts of repeats cloned from the different yeast species were typically between 250 and 600 bp, although those from the two *C. tropicalis* strains were only 130–175 bp. That this species has particularly short telomeres was also supported by their very rapid loss during BAL-31 digestion and by their relatively weak hybridization, even with species-specific telomere probes (data not shown).

Fig. 3 shows an alignment of these newly discovered telomeric repeat unit sequences together with those of *C. albicans* and *S. cerevisiae*. Two striking features are appar-

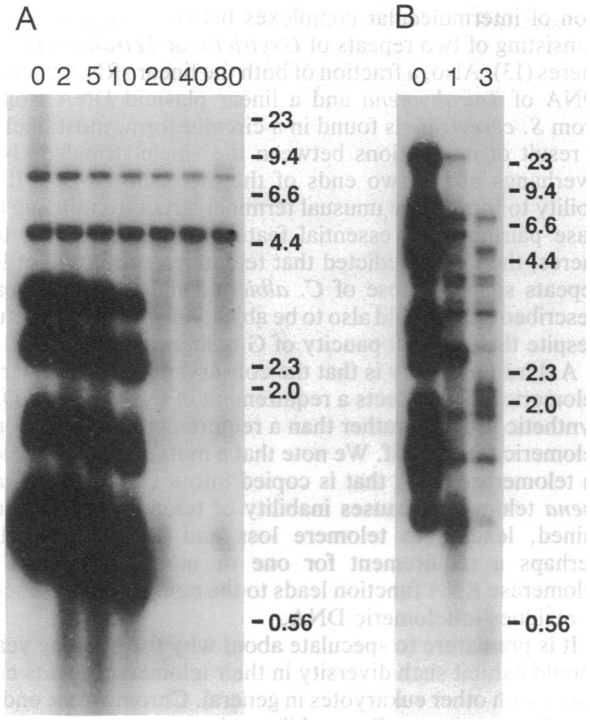

FIG. 2. BAL-31 nuclease sensitivity of genomic copies of the tandem repeats in *K. lactis* ATCC 32143 (*A*) and *C. guillermondii* B-3163 (*B*). Uncut yeast genomic DNAs were incubated with BAL-31 nuclease for increasing periods of time (given in minutes above each lane), digested with *Eco*RI, electrophoresed in a 0.8% agarose gel, and blotted onto a Nytran membrane. Hybridizations were carried out using telomeric probes specific for each species. For *C. guillermondii*, most hybridizing bands were lost by the 1-min time point, and approximately three other bands were shortened but not lost at 3 min. These latter bands presumably are homologous to the particular subtelomeric sequences present in pCgui3. DNA size markers (in kilobase pairs) are indicated at the right of each panel.

ent. First, there is considerable variety with respect to both telomeric repeat unit lengths and sequence complexities, especially when compared to the telomeric repeats known from other eukaryotes. Second, despite these considerable variations between species, all of these yeast species, including *S. cerevisiae*, share an ≈6-bp motif (boxed) containing only T and G residues that resembles typical telomeric sequences.

The sequence relationships among the telomeric repeats in Fig. 3 are generally consistent with the phylogenetic rela-

Species	Telomeric Repeat
C. alb.	A C G G A T G T C T A A C T T C T T G G T G T
C. tro. 4414	A C/A G G A T G T C A C G A T C A T T G G T G T
C. tro. 4443	A A G G A T G T C A C G A T C A T T G G T G T
C. mal.	A C G G A T G C A G A C T C G C T T G G T G T
C. gui.	A C T G G T G T
C. pse.	A C G G A T T T G A T T A G T T A T G T G G T G T
K. lac.	A C G G A T T T G A T T A G G T A T G T G G T G T
C. gla.	C T G G G T G C T G T G G G G T
S. cer.	T $(G)_{2-3}$ $(T\ G)_{1-6}$
	5' To End→ 3'

FIG. 3. Sequences of telomeric DNA repeats from several budding yeast species. The orientation with respect to the chromosome end is indicated. The box indicates the positions of base pairs that appear to be most conserved among the various telomeric repeats. The species shown here are *C. albicans* (*C. alb.*), *C. tropicalis* (*C. tro.*), *C. maltosa* (*C. mal.*), *C. guillermondii* (*C. gui.*), *C. pseudotropicalis* (*C. pse.*), *K. lactis* (*K. lac.*), *C. glabrata* (*C. gla.*), and *S. cerevisiae* (*S. cer.*). Alternate alignments of the G-rich *C. glabrata* repeat are possible.

tionships of these yeasts deduced from comparison of ribosomal RNA sequences (28). As would be expected, the telomeric repeats of the two strains both considered to be *C. tropicalis* have nearly identical sequences. Likewise, the 25-bp telomeric repeats of the closely related *K. lactis* and *C. pseudotropicalis* differ at only one position. The telomeric repeat sequences from *C. albicans*, *C. maltosa*, *C. pseudotropicalis*, *C. tropicalis*, and *K. lactis* are 23–25 bp in length, with differences largely or entirely confined to the central part of the repeat. The 16-bp repeat unit from *C. glabrata*, the species in this study that may be most closely related to *S. cerevisiae* (28), is very G-rich, which probably contributes to its cross-hybridization to the heterogeneous and shorter *S. cerevisiae* telomeric repeats. All these budding yeast sequences, including the most common form of the irregular *S. cerevisiae* repeats, have a perfect or near match to the boxed 6-bp T+G sequence.

In the cloned telomere from *C. tropicalis* strain B-4414, we found two telomeric repeat sequences that differed at one position in the repeat, as shown in Fig. 3: six of the approximately seven repeat units in the B-4414 telomere were homogeneous (and will be termed the AC repeat), but the remaining repeat (henceforth termed the AA repeat) was identical to the homogeneous telomeric repeats cloned from strain *C. tropicalis* B-4443 (Fig. 3).

To determine the distribution of the AA and AC variant repeats among the telomeres and strains of *C. tropicalis*, genomic DNA from several *C. tropicalis* strains, including B-4414 and B-4443, and a control *C. albicans* strain was probed with oligonucleotide probes specific for either the AA or the AC repeat (Fig. 4). Only strains B-4414 and 1739-82, and to some extent the *C. albicans* telomeres, hybridized with the AC repeat-specific oligonucleotide probe (Fig. 4). However, genomic DNA from all of the *C. tropicalis* strains tested, including B-4443, but not from *C. albicans*, hybridized well with the oligonucleotide specific for AA repeats (Fig. 4 and data not shown). These results clearly indicate that both B-4414 and 1739-82 contain at least two forms of telomeric repeats, which most likely are variably interspersed in different telomeres, because signal ratios with the two probes differed between individual telomeric fragments in both panels of Fig. 4.

DISCUSSION

The recent demonstration that the telomeres of *C. albicans* are composed of complex 23-bp repeats (24) raised the possibility that the mechanisms involved in the function or maintenance of *C. albicans* telomeres might differ markedly from more typical eukaryotic telomeres, which are composed of 5- to 8-bp repeats and are synthesized by telomerase. The results presented here demonstrate that, like *C. albicans*, several other yeast species contain telomeres composed of relatively long repeat units. The telomeres that we have identified include repeat lengths of 8, 16, 23, and 25 bp. Although the variation in size and sequence of these different telomeric repeats is large, each contains a highly similar ≈6-bp sequence composed of T and G residues that is reminiscent of more typical telomeres, including the short heterogeneous repeats of *S. cerevisiae*. Although embedded in an otherwise highly variable telomeric sequence, the conserved T+G motif is in the same orientation as the G-rich strand of other eukaryotes' telomeres.

Superficially, the long telomeric repeat units of *C. albicans* and other yeasts are similar to the diverse 31- to 53-bp repeats that have been identified at the ends of the linear mitochondrial DNA of six *Tetrahymena* species (29). However, these mitochondrial terminal repeats lack any identifiable conserved sequence motif. In some *Tetrahymena* species the telomeric repeats at the left and right sides of the mitochon-

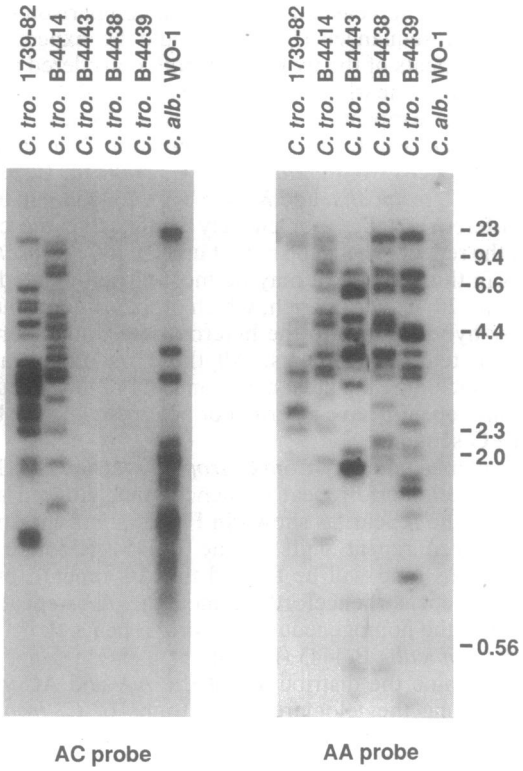

FIG. 4. Two types of telomeric repeats are present in certain *C. tropicalis* (*C. tro.*) strains. Approximately equal amounts of genomic DNAs from five representative strains of *C. tropicalis*, and from *C. albicans* (*C. alb.*) WO-1 were digested with *Cla* I, electrophoresed in an 0.8% agarose gel, blotted, and probed with oligonucleotides specific to either the AC form of *C. tropicalis* telomeric repeat (*Left*) or the AA form of repeat (*Right*). The specificity of the AA probe is indicated by its failure to hybridize with the *C. albicans* telomeres, despite only one base mismatch and the fact that the *C. albicans* cells used here have much longer telomeres (and therefore many more telomeric repeats per telomere) than do *C. tropicalis* strains. The shortness of the *C. tropicalis* telomeres may explain why they appear to be particularly homogeneous in size, as indicated by the relative sharpness of individual telomeric bands (see also Fig. 1).

drial DNA are markedly different (29). The telomere length distribution also differs between the two ends (30). These two observations are more compatible with the proposal that they are maintained by recombination (29, 30) and not telomerase, since telomerase activity appears to act distributively among all the nuclear telomeres in a given cell (31).

The conservation of a T+G motif in the yeast nuclear telomeric repeats that are otherwise very dissimilar in repeat unit length and sequence suggests that these repeats are likely to share some common feature in their synthesis or function. A possible function of this T+G motif might involve proteins that bind to telomeres. One or more of the three classes of telomere-interacting proteins that have been identified—telomerase (18), proteins that bind to the single-stranded overhang at the telomere terminus (32, 33), and proteins that bind to double-stranded telomeric repeats (34)—may be highly conserved in function, thus constraining telomeric repeat sequences.

An alternative explanation for the conservation of the T+G motif may be the formation of unusual base-paired DNA structures involving G·G pairing. Numerous studies have shown that the G-rich strand of typical telomeric sequences is capable of forming unusual structures, including multistranded forms, *in vitro* (ref. 10; reviewed in ref. 12). There is no conclusive evidence that any of these structures are formed *in vivo*; however, *in vitro*, the β-subunit of the *Oxytricha* telomere end binding protein catalyzes the formation of intermolecular complexes between oligonucleotides consisting of two repeats of *Oxytricha* or *Tetrahymena* telomeres (13). Also, a fraction of both the linear rRNA-encoding DNA of *Tetrahymena* and a linear plasmid DNA isolated from *S. cerevisiae* is found in a circular form, most likely as a result of interactions between the single-stranded G-rich overhangs of the two ends of the molecule (9, 35). If the ability to form some unusual terminal structure utilizing G·G base pairing is an essential feature of chromosomal telomeres, then it is predicted that telomeres composed of long repeats such as those of *C. albicans* and the other yeasts described here should also to be able to form such a structure, despite their overall paucity of G residues.

A third possibility is that the conservation of G clusters in telomeric DNA reflects a requirement of the telomeric DNA synthetic process, rather than a requirement of a function of telomeric DNA itself. We note that a mutation of a C residue in telomerase RNA that is copied into a G by the *Tetrahymena* telomerase causes inability of telomeres to be maintained, leading to telomere loss and senescence (21)—perhaps a requirement for one or more C residues for telomerase RNA function leads to the conserved presence of G residues in telomeric DNA.

It is premature to speculate about why the budding yeasts should exhibit such diversity in their telomeric repeats compared with other eukaryotes in general. Chromosome ends of the dipteran insect *Drosophila melanogaster* appear to be maintained by periodic additions of retrotransposons of complex sequence to chromosomal termini (36, 37), further suggesting that the more common, very simple G-rich telomeric repeats, while widely found, are not invariably necessary for telomeric DNA function.

A model of telomere maintenance by telomerase predicts that there will be a telomerase RNA gene that contains a sequence identical to that of a telomeric repeat, which is used as a template in the synthesis of new telomeric repeats. A corollary prediction of the telomerase model is that a species could exhibit two forms of telomeric repeats if it carries two copies of a telomerase RNA gene that differ in the sequence of the template for telomere synthesis, just as has been found experimentally *in vivo* in *Tetrahymena* when two different telomerase RNAs are present (22). As described here, certain strains of the diploid species *C. tropicalis* do contain alternate forms of telomeric repeats that appear to differ by a single base substitution. While not excluding alternate mechanisms for telomere maintenance, this observation appears most consistent with the possibility that *C. tropicalis* encodes two variant telomerase RNA genes.

For must eukaryotes, telomeric repeats are too short to be useful for identifying telomerase RNA genes by nucleic acid hybridization; however, the long telomeric repeats we have found in species such as *C. albicans* and *K. lactis* make it possible to probe directly for telomerase RNA gene candidates. BAL-31 nuclease digestion experiments have demonstrated the existence of such telomerase RNA gene candidates in *K. lactis* (see Fig. 2) and *C. albicans* and *C. glabrata* (M.J.M., unpublished results), consistent with the possibility that telomerase is involved in telomere synthesis in these species.

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