Tetrahymena Mutants With Short Telomeres

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ABSTRACT

Telomere length is dynamic in many organisms. Genetic screens that identify mutants with altered telomere lengths are essential if we are to understand how telomere length is regulated *in vivo*. In *Tetrahymena thermophila*, telomeres become long at 30°, and growth rate slows. A slow-growing culture with long telomeres is often overgrown by a variant cell type with short telomeres and a rapid-doubling rate. Here we show that this variant cell type with short telomeres is in fact a mutant with a genetic defect in telomere length regulation. One of these telomere growth inhibited forever (tgi) mutants was heterozygous for a telomerase RNA mutation, and this mutant telomerase RNA caused telomere shortening when overexpressed in wild-type cells. Several other tgi mutants were also likely to be heterozygous at their mutant loci, since they reverted to wild type when selective pressure for short telomeres was removed. These results illustrate that telomere length can regulate growth rate in Tetrahymena and that this phenomenon can be exploited to identify genes involved in telomere length regulation.

TELOMERES are specialized chromatin domains at the ends of eukaryotic chromosomes. They have several known roles including protection of chromosomes from degradation and end-to-end fusion, ensuring complete replication of the chromosome terminus and positioning of chromosome ends in the nucleus (reviewed in Blackburn and Greider 1995). In most organisms, telomeric DNA is composed of simple tandem repeats in which one strand is G-rich and runs 5′ to 3′ toward the end of the chromosome, forming a single-stranded 3′ overhang (Klobutcher *et al.* 1981; Pluta *et al.* 1982; Henderson and Blackburn 1989; Makarov *et al.* 1997).

Conventional DNA polymerases cannot replicate a 3′ overhang, so in the absence of a repair mechanism, a chromosome is expected to get shorter with each round of DNA replication (Watson 1972; Lingner *et al.* 1995). Telomerase is an enzyme that compensates for telomere shortening by adding telomeric repeats to chromosome termini (Greider and Blackburn 1985, 1987). This ribonucleoprotein uses its RNA component as a template for telomere repeat addition (Greider and Blackburn 1989; Yu *et al.* 1990). Telomerase appears to be the primary enzyme responsible for telomere maintenance in organisms as diverse as ciliates, fungi, and vertebrates (Yu *et al.* 1990; Singer and Gottschling 1994; McEachern and Blackburn 1995; Bodnar *et al.* 1998; reviewed in Nugent and Lundblad 1998), although alternative pathways for telomere replication do exist (Lundblad and Blackburn 1993; Bryan *et al.* 1997).

In *Saccharomyces cerevisiae*, loss of *in vivo* telomerase activity results in ever-shortening telomeres and clonal cell senescence. There are five genes that can mutate to give this phenotype: TLC1, EST1, EST2, EST3, and EST4/CDC13 (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay *et al.* 1996; Nugent *et al.* 1996). Only two of these genes are necessary for telomerase activity *in vitro*: TLC1, the telomerase RNA component, and EST2/p123, the catalytic reverse transcriptase subunit of telomerase (Singer and Gottschling 1994; Lingner *et al.* 1997a,b). Est1 and Est4/Cdc13 proteins both bind single-stranded telomeric G-strand DNA, and these two proteins together with Est3 are necessary for *in vivo* function of the catalytic and RNA subunits of yeast telomerase (Lin and Zakian 1996; Virta-Pearlman *et al.* 1996). Biochemical approaches in ciliates have uncovered other proteins that copurify with telomerase—p43, p80 (and its mammalian homologs), and p95—and these proteins may also be essential for *in vivo* telomerase activity (Collins *et al.* 1995; Harrington *et al.* 1997; Lingner *et al.* 1997a; Nakayama *et al.* 1997).

Aside from proteins that are essential for *in vivo* telomere activity, a number of other factors are involved in regulating telomerase activity so that a cell's telomeres do not become too long or too short (reviewed in Shore 1997). Double-stranded telomere DNA-binding proteins like RAP1 (*S. cerevisiae* and *Kluyveromyces lactis*), TAZ1 (*Schizosaccharomyces pombe*) and TRF1 (human)

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are required for a cell to sense the amount of telomeric DNA at a chromosome end and mutations of such proteins result in altered telomere lengths (Longtine *et al.* 1989; Conrad *et al.* 1990; Krauskopf and Blackburn 1996; Cooper *et al.* 1997; Marcand *et al.* 1997; van Steensel and de Lange 1997). In *S. cerevisiae*, RAP1 interacts with two proteins (RIF1 and RIF2), which are also required for proper telomere length regulation (Hardy *et al.* 1992; Wotton and Shore 1997). Other *S. cerevisiae* genes known to cause changes in telomere length include the PIF1 DNA helicase, STN1 (an Est/Cdc13-interacting protein), and a novel gene TEL2 (Schulz and Zakian 1994; Runge and Zakian 1996; Grandin *et al.* 1997).

Telomeres are "normal" double-strand breaks, and some genes involved in double-strand break repair also have roles in telomere maintenance. The Ku heterodimer binds to DNA ends and functions in the repair of double-strand breaks by nonhomologous end joining (Gottlieb and Jackson 1993). Mutations in *S. cerevisiae* Ku70 and Ku80 homologs cause telomere shortening (Boulton and Jackson 1996; Porter *et al.* 1996) as do RAD50, MRE11, and XRS2, which are also involved in Ku-mediated double-strand break repair. Epistasis analysis suggests that RAD50 and MRE11 function in the telomerase pathway, whereas the Ku homologs are in a separate pathway required for telomere maintenance (Nugent *et al.* 1998). Other studies have shown that the DNA damage checkpoint mutants *tel1* (*S. cerevisiae*) and *rad1*, *rad3*, *rad17*, and *rad26* (*S. pombe*) have short telomeres (Greenwell *et al.* 1995; Dahlén *et al.* 1998). In sum, these findings suggest that telomeres are processed as a special kind of DNA damage.

In humans, telomeres of somatic cells shorten with age both *in vitro* and *in vivo* (Harley 1995). In addition, aberrant telomere shortening has been observed in aging-related genetic disorders such as Down syndrome, ataxia telangiectasia, and Hutchinson-Gilford progeria (Allsopp *et al.* 1992; Vaziri *et al.* 1993; Kruk *et al.* 1995; Pandita *et al.* 1995). It has been suggested that telomere shortening may regulate the aging process, perhaps by limiting a cell's proliferative capacity (Harley 1991). Human somatic cells that undergo telomere shortening lack *in vitro* telomerase activity as a consequence of repression of the human hTRT/hEST2 catalytic reverse transcriptase subunit of telomerase (Weinrich *et al.* 1997; Counter *et al.* 1998; Nakayama *et al.* 1998). Indeed, expression of hTRT/hEST2 in somatic cells in culture restores telomerase activity and telomere length and eliminates the *in vitro* replicative senescence normally observed in human somatic cells (Bodnar *et al.* 1998). Telomerase activation is observed in most immortalized cancers and is likely to play an important role in the immortalization process by ensuring maintenance of telomeres (Kim *et al.* 1994; Feng *et al.* 1995).

Although regulation of the telomerase catalytic subunit appears to be the primary mechanism of telomere length regulation in humans, a complete understanding of how telomere length is regulated will in part depend on genetic screens that identify mutants with altered telomere lengths. Here we show that a correlation between cell growth rate and telomere length can be used to select for telomere length regulation mutants in Tetrahymena.

MATERIALS AND METHODS

Nomenclature: The short telomere mutant phenotype is designated tgi (telomere growth inhibited forever). *tgi1* is the mutant strain described here. *tgi1-1* is the first mutant allele of the Tetrahymena telomerase RNA gene.

Cell growth and maintenance: For long-term storage 5-ml stock cultures were maintained at room temperature in 2% PPYS in a loosely capped stock tube. Every 3–4 weeks, 100 μl of each stock culture was transferred to a fresh 5-ml tube of 2% PPYS (2% proteose peptone, 0.2% yeast extract, and 10 μm $FeCl_3$). The telomeres of cells in stock cultures are relatively short, and this length serves as the baseline against which telomere growth is compared. For the tgi selection, Tetrahymena cultures were initiated by adding 100 μl of a room-temperature stock culture to a 250-ml flask containing 50 ml 2% PPYS. These cultures were maintained at mid- to late-log phase by gentle shaking and transferring about 200 μl of the culture to a fresh 250 ml flask every day. Cultures treated in this way were termed "continuous." Cultures that have undergone the tgi transition were identified by rapid cell growth and the appearance of short telomeres. The frequency of observation of spontaneous tgi mutants was greater if cells were transferred at late log cell densities of $3–4 \times 10^5$/ml due to the increased probability of transferring a rare mutant to the next flask.

Transformations: Microinjection of C3*rmm1* cells from 30° log phase cultures was performed essentially as described (Tondrovi and Yao 1986), using a Picospritzer II (General Value) and 1-mm glass micropipettes (World Precision Instruments, Inc.) pulled with a David Kopf Instruments model 700C, except that groups of cells were removed to drops of 2% PPYS immediately after injection. Injectees were isolated as single cells; placed into microtiter wells; grown in 200 μl 2% PPYS, and 250 μg ml^{-1} penicillin, 250 μg ml^{-1} streptomycin at 30° for 3 days; and selected with 100 μg ml^{-1} paromomycin (ICN). Transformants took 9–16 days to appear. The 50-ml 30° log phase cultures of these transformants were grown under selective conditions (100 or 200 μg ml^{-1} paromomycin) for 1 wk, and then growth was continued either in the absence or in the presence of paromomycin selection (these growth conditions produced identical phenotypes). During the first few days of growth in 50-ml cultures, some transformants were cryopreserved (modified from Flacks 1979). For cryopreservation, cells were starved overnight in 10 mm Tris (pH 7.4) at 30°, incubated in 8% DMSO for 30 min at room temperature, frozen in 200 μl aliquots at −80° for 4 hr, and then immersed in liquid nitrogen. For recovery, cells were thawed quickly at 37°, added to a 37° 2% PPYS 50-ml flask, and allowed to recover at 30° overnight without shaking. Cultures were selected with paromomycin as above.

Oligonucleotides, PCR, and sequencing: DNA oligonucleotides were gel purified on denaturing polyacrylamide gels as described (Henderson *et al.* 1987). Telomerase RNA gene PCR was carried out using sense primer 9 (5′ATACCCGCT TAATTCATTCAGA, 3′ end is at +22 nt in the telomerase RNA gene); antisense primer 73 (5′GTAGAACTTTTAATAG GATCAATGTCTCATAAATA, 3′ end is 25 nt downstream of

telomerase RNA gene); 0.2 mm magnesium chloride; 30 ng genomic template DNA; and 40 cycles of 95° for 1 min, 42° for 2 min, and 74° for 3 min. For sequencing, mutant and wild-type *tgi1* PCR products (Figure 2A, line 8) were excised, eluted in 10 mm Tris, 1 mm EDTA (pH 8.0), precipitated, and sequenced as recommended (Promega fmol DNA sequencing system). To determine the level of mutant telomerase RNA gen in *tgi1*, primer 9 and 5′ ^{32}P-labeled primer 10 (5′AAAAA TAAGACATCCATTGATAAATAGTGTATCAAATG, 3′ end is at +122 nt in the telomerase RNA gene) were used for PCR, separated on a 6.5% gel polyacrylamide gel, exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), and quantitated.

Subcloning: A 550 bp-*Dde*I telomerase RNA gene fragment of pCG1 (Greider and Blackburn 1989) as subcloned with *Kpn* linkers into the multiple-cloning site of pBluescript KSII+ (Stratagene, La Jolla, CA) to produce pSA1-wt. *tgi1* 9/73 PCR products containing the *tgi1* mutation were subcloned into the pSA1-wt telomerase RNA gene to produce pSA2-*tgi1-1*. pSA1-wt or pSA2-*tgi1-1* telomerase RNA genes were subcloned into the *Kpn*I site of the pRD4-1 polylinker to produce pSA3-*tgi1-1* and pSA4-wt. Plasmid DNA for microinjection was made using a Midiprep kit (QIAGEN, Chatsworth, CA). Cycle sequencing of these plasmid preps provided the expected telomerase RNA gene sequences.

DNA and RNA preparation: To prepare Tetrahymena genomic DNA, 100 μl of a cell pellet was mixed with 200 μl NDS (2% SDS, 0.5 m EDTA, 0.01 m Tris · HCl, pH 9.5) and 100 μl 2 mg/ml pronase, incubated for at least 12 hr at 55°, stored at −20°, mixed with 300 μl H_2O, extracted at least two times with phenol/chloroform (1:1), precipitated with 1 ml cold 95% ethanol, washed once with 70% ethanol, resuspended in 100 μl H_2O, reprecipitated with 7 μl 3 m NaOAc and 250 μl 95% ethanol, washed three times with 70% ethanol, and resuspended in 30–50 μl H_2O. Genomic RNA was prepared as described (Avilion *et al.* 1992).

Southerns: For all Southern blots, *Pst*I-digested genomic DNA was separated on 1% agarose gels, blotted to a nylon membrane (Magnagraph) as recommended. The probe pTre1 was labeled with digoxygenin (Boehringer Mannheim Genius kit, Indianapolis). pTre1 contains a portion of the rDNA gene immediately adjacent to the telomere and is useful for identifying telomeric restriction fragments (Larson *et al.* 1987). Blots were developed using Lumi-Phos 530 (Boehringer Mannheim) as recommended.

Reverse transcription: Reverse transcription was in 10 μl reactions. A total of 0.1 pmol of gel-purified 5′ ^{32}P-labeled oligo-10 was incubated with 1 μl genomic RNA (5–10 μg), 0.87× telomerase buffer (1× = 5 mm Tris-Acetate, pH 8.5, 5 mm potassium acetate, 5 mm 2-mercaptoethanol, 1 mm spermidine, 1 mm $MgCl_2$), 10 mm $MgCl_2$, 1 mm dNTPs, 2.5 U RNasin (Promega, Madison, WI), and 1.25 units avian myeloblastosis virus reverse transcriptase (Promega) at room temperature for 10 min, then at 50° for 1 hr. Reactions were precipitated with 20 ng glycogen (Boehringer Mannheim), 15 μl 2.5 M ammonium acetate, and 135 μl 95% ethanol at −70° for 10 min, spun in a microfuge for 10 min, washed once with 70% ethanol, dried in a speed vac, resuspended in 3 μl loading buffer (90% formamide, 5% sucrose, 0.01% xylene cyanol, and 0.01% bromophenol blue), boiled 4 min, and separated on a prerun 6%, 7 m urea, 0.6× TBE-sequencing gel at 1000–1400 V. Gels were dried and exposed to X-ray film (Fuji).

RESULTS

Telomere length is temperature-sensitive: Previous studies showed that *T. thermophila* cells grown as stationary stock cultures at room temperature have short telomeres which elongate in log phase cultures at 30° (Larson *et al.* 1987). In our efforts to understand this phenomenon we tested whether telomere length might be sensitive to growth temperature rather than growth phase, and, indeed, telomeres of log phase cultures remained short at room temperature and elongated when shifted to 30° (Figure 1A). Thus, telomere length is temperature-sensitive in rapidly dividing Tetrahymena cells. This temperature-sensitive phenotype could be conferred by a single gene or by a general metabolic effect. Telomeres of another protozoan, the African trypanosome, elongate during log phase growth in mice (Bernards *et al.* 1983), and our data suggest that this phenotype might be a function of temperature rather than log phase growth.

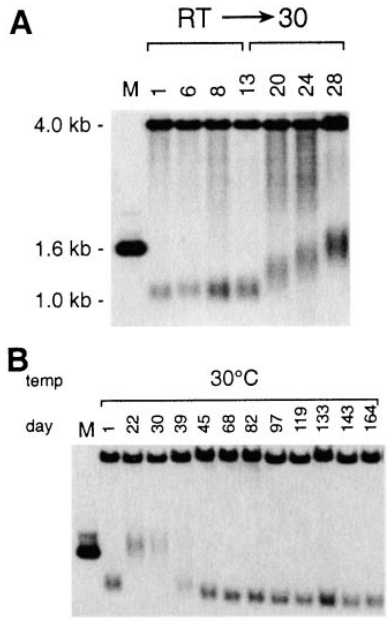

Figure 1.—Tetrahymena telomere length dynamics. (A) Telomere length in Tetrahymena is temperature-sensitive. A Tetrahymena culture (strain SB255) was grown at room temperature in log phase for 2 wk (lanes marked 1, 6, 8, and 13, corresponding to days in culture) and then at 30° for 2 wk (lanes 13, 20, 24, and 28). After shifting to the higher temperature the ~1.2-kb telomere restriction fragment increased in length significantly. (B) tgi mutations cause telomere shortening during rapid cell growth at 30°. A continuous log phase culture of Tetrahymena cells was grown at 30°. Telomeres elongate over time as in panel A, but then telomeres undergo relatively rapid shortening, presumably due to a spontaneous mutation, and remain short thereafter. This is how macronuclear tgi mutations are generated. All panels show Southern blots probed with pTre1, a plasmid containing the 3′ end of the Tetrahymena rDNA gene, which detects the telomeric restriction fragment (1.0–1.6 kb) of the rDNA chromosome, and a 4-kb band, which is an internal nontelomeric rDNA restriction fragment (Larson *et al.* 1987).

Identification of Tetrahymena mutants with short telomeres: After one month of log phase growth at 30°, a Tetrahymena culture not only has long telomeres but

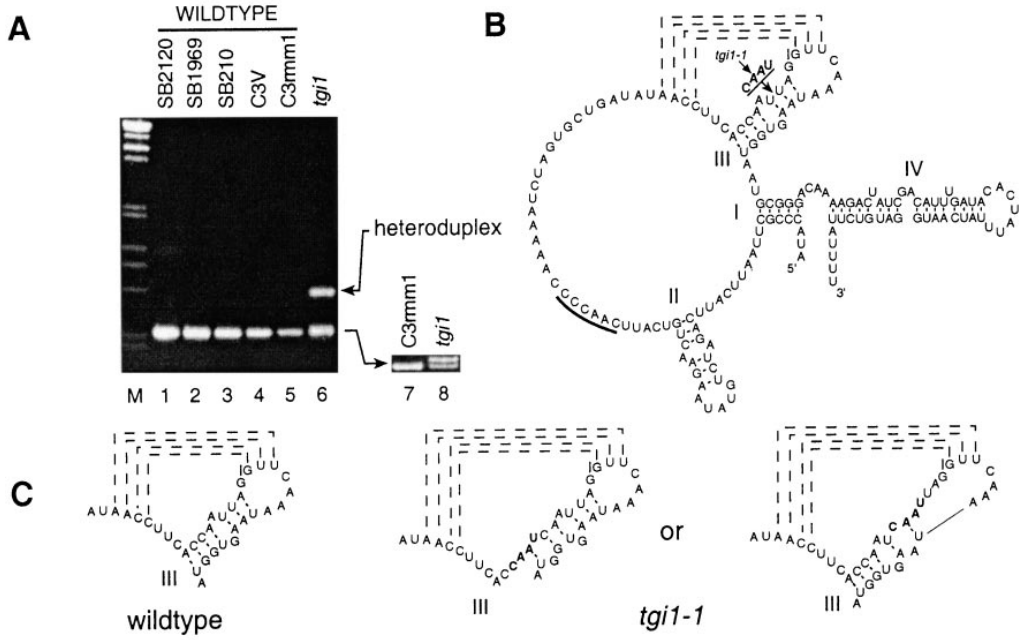

Figure 2.—*tgi1* has a telomerase RNA mutation. (A) Telomerase RNA gene PCR amplification products from several wild type Tetrahymena strains (lanes 1–5 and lane 7) and from the *tgi1* mutant (lanes 6 and 8). Lanes 1–6 were separated on a 5% polyacrylamide gel. The top band appearing in the *tgi1* lane results from formation of DNA heteroduplexes between PCR products from two alleles of the *tgi1* gene coexisting in the same PCR reaction. Mutant and wild type homoduplex telomerase RNA genes of *tgi1* appeared as a single band in lane 6 but, when digested with *Taq*I and displayed on a 7.5% PAG, the 4-bp difference in the mutant and wild-type telomerase RNA gene restriction fragments could be resolved (lanes 7 and 8). (B) Predicted secondary structure of the *Tetrahymena thermophila* telomerase RNA component (Romero and Blackburn 1991). The four base pair insertion of *tgi1-1* (shown in bold) is located in stem III. (C) The *tgi1-1* insertion results in a direct repeat that could slip in two directions to alter pseudoknot structure.

also has a slower doubling rate (Larson *et al.* 1987). Under these conditions, the culture is often overgrown by variant cells with short telomeres and a rapid-doubling rate (Figure 1B) (Larson *et al.* 1987). Because this short telomere/rapid growth rate phenotype is retained for hundreds of generations of log phase growth at 30°, this phenotype is designated tgi. Larson *et al.* (1987) suggested that a tgi phenotype could be the result of a spontaneous mutation. Here we confirm this possibility by showing that *tgi1* has a telomerase RNA mutation that causes telomere shortening at 30°.

***tgi1* has a telomerase RNA mutation:** Tetrahymena cells have two nuclei, a transcriptionally quiescent diploid-germline micronucleus and a polyploid (45C to 60C) somatic macronucleus that is transcriptionally active. We isolated several independent tgi mutants from vegetative (nonmating) cultures grown at 30°. Because the mutations were macronuclear (not in the germline), they could not be readily analyzed by standard genetic methods. Therefore, we took a candidate gene approach to the characterization of these tgi mutants. Since the Tetrahymena telomerase RNA gene is known to be involved in telomere length regulation (Greider and Blackburn 1989; Yu *et al.* 1990), we examined it for alterations in several tgi strains. PCR analysis of the telomerase RNA gene from *tgi1* revealed a heteroduplex product, which indicated that a mutation was present (Figure 2A, lane 6). The wild-type-sized PCR band could be resolved on a high-resolution gel as a doublet (Figure 2A, lane 8) whose products were sequenced directly and shown to be the wild-type telomerase RNA gene and a mutant allele of the telomerase RNA gene (*tgi1-1*), which had a 4-bp insertion located in stem III of the proposed telomerase RNA secondary structure (Figure 2B; Romero and Blackburn 1991). The mutant and wild-type telomerase RNA PCR products were present in *tgi1* at an apparent ratio of 60% mutant/40% wild type (Figure 2A, lane 8). The loop in stem-loop III forms a pseudoknot that has been conserved over a large evolutionary distance (Figure 2B), suggesting that it is critical for normal telomerase function (Bhattacharyya and Blackburn 1994; McCormick-Graham and Romero 1995). The *tgi1-1* insertion produced a small tandem duplication in stem III's primary sequence. Although this mutation does not preclude stem formation, it is likely to perturb the structure of the pseudoknot (Figure 2C). Preliminary analysis revealed that high levels of the *tgi1-1* mutant telomerase RNA had no gross effect on telomerase processivity of crude S100 extracts *in vitro*, suggesting that the *tgi1-1* mutant telomerase is catalytically active (S. Ahmed and E. Henderson, unpublished data).

A low-mobility heteroduplex was observed in our PCR analysis of *tgi1* because wild-type and *tgi1-1* PCR products annealed to form a bent heteroduplex which migrated slowly in the gel (Figure 2A, lane 6). Telomerase RNA gene PCR analysis of seven other tgi mutants revealed no aberrantly migrating PCR bands, so we sequenced each of these PCR products directly (from −110 nt upstream of the telomerase RNA gene to 24 nt downstream of the gene), and their sequences were all exactly the same as wild type with no hint of a heterozygous

mutation being present (data not shown). Therefore, *tgi1* was the only mutant harboring an allele of the telomerase RNA gene, and the seven other tgi mutants presumably have defects at different loci.

The *tgi1-1* telomerase RNA causes telomere shortening: To determine whether the *tgi1-1* telomerase RNA mutation was responsible for the tgi phenotype, we introduced the mutant gene into wild-type cells by microinjection. To accomplish this, the *tgi1-1* telomerase RNA gene was cloned into pRD4-1, a Tetrahymena vector, which contains a high-copy number origin of replication and carries a ribosomal DNA (rDNA) gene that confers resistance to paromomycin (Yu and Blackburn 1989). pRD4-1 has been previously used to overexpress telomerase RNA genes in Tetrahymena cells, resulting in transformed cells with mutant phenotypes that were dominant to the endogenous wild-type telomerase RNA gene (Yu *et al.* 1990; Gilley *et al.* 1995). For the experiments described here, both wild-type and *tgi1-1* mutant telomerase RNA genes were subcloned into the 3′ nontranscribed spacer (3′NTS) of pRD4-1 to produce pSA3-*tgi1* and pSA4-wt. Since the *tgi1* mutant was isolated from a C3*rmm1* Tetrahymena strain (Larson *et al.* 1986), mutant or wild-type plasmids were injected into C3*rmm1* cells for isogenic phenotype analysis. Several sets of mutant and wild-type transformants were obtained and grown in 30° log phase cultures for several months. Some transformants were cryopreserved immediately after transformation and revived later for growth analysis. DNA, RNA, and protein extracts were made from these cultures in order to monitor the effects of the introduced telomerase RNA genes on telomere metabolism. A reverse transcription assay for telomerase RNA from five independent pSA3-*tgi1-1* transformants demonstrated that the injected telomerase RNA genes were expressed at high levels for several weeks (Figure 3, A and B; data not shown). However, the exogenous telomerase RNA genes were gradually lost from the pRD4-1 rDNA vector due to recombination with the endogenous rDNA chromosome (Figure 3), as has been observed with the 3′ NTS of the pRD4-1 vector alone (Yu *et al.* 1990).

Of six mutant transformants, five acquired sustained short-telomere phenotypes when the *tgi1-1* mutant telomerase RNA was present, and these phenotypes reverted to wild type once the *tgi1-1* telomerase RNA was lost (Figure 3A–C). In contrast to the sustained short telomere phenotypes of transformants expressing the *tgi1-1* telomerase RNA, all four wild-type telomerase RNA transformants exhibited only slight telomere shortening during a single time point of the growth period (Figure 3D). These data indicate that the *tgi1-1* telomerase RNA causes telomere shortening *in vivo*, and it is therefore likely to be responsible for the short telomere phenotype of the *tgi1* mutant.

tgi1-1 transformants were under two selective pressures. The first pressure selects for cells with shorter

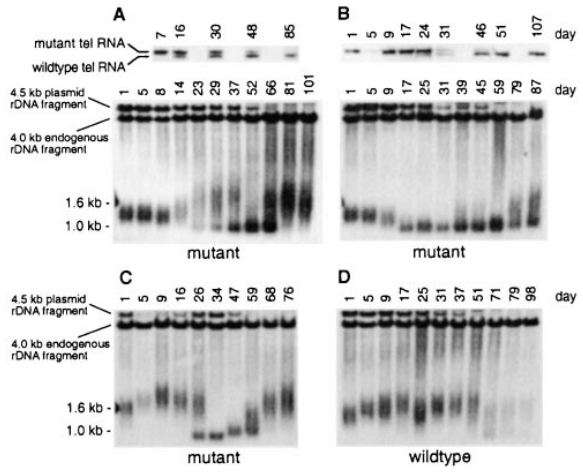

Figure 3.—Overexpression of mutant and wild-type telomerase RNA genes demonstrates that the *tgi1* mutation can cause telomere shortening *in vivo*. The top panels in A and B show reverse transcription products of the telomerase RNA to indicate relative levels of mutant and wild-type telomerase RNA during transformant growth. The lower panels in A–D show telomere length dynamics of three mutant transformants (A–C) and one wild-type transformant (D). The transformant in panel C was revived from cryopreservation, which resulted in an initially low-copy number of the mutant gene. Transformation was accomplished using the rDNA vector pRD4-1. Telomere shortening, a tgi phenocopy, correlated with the presence of the mutant gene but was relieved when the mutant gene was lost. This was not observed in the wild-type control. A restriction enzyme polymorphism between the plasmid-borne (4.5-kb) and endogenous (4.0-kb) rDNA allows the copy number of the transformed telomerase RNA genes to be monitored.

telomeres that replicate quickly; this phenotype depends on the presence of the *tgi1-1* transgene. A second pressure selects against cells that have a transgene integrated in the 3′ NTS of the rDNA locus (in this case, the *tgi1-1* transgene). The culture in Figure 3A, days 23–52, had two populations of telomeres, which probably represent two populations of cells—a population of cells with long telomeres that had completely lost the *tgi1-1* gene from the rDNA locus and a population of cells with short telomeres that was losing the *tgi1-1* transgene. The cells with short telomeres took over the culture, suggesting that complete loss of the *tgi1-1* transgene is not as advantageous as telomere shortening. However, the *tgi1-1* transgene was quickly and completely lost from a transformant culture once the culture's telomeres all became short (Figure 3, A–C). There was no selection for the *tgi1-1* transgene once a culture's telomeres all became short because it takes several weeks for the culture's telomeres to elongate again and cause the cell cycle to slow.

One complication in analysis of transformant phenotypes in log phase Tetrahymena cultures at 30° is that spontaneous tgi mutations can arise, as was observed for the wild-type transformant in Figure 3D (days 71–98) and for the sixth mutant transformant, which became

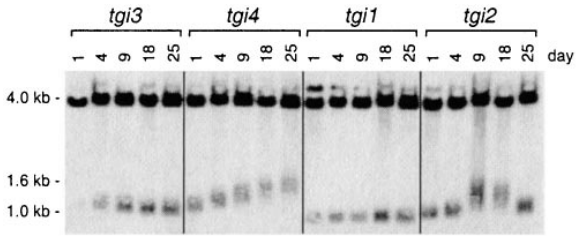

Figure 4.—Some *tgi* mutants revert under nonselective growth conditions. Four *tgi* strains were stored as room temperature stock cultures for a year. Under these conditions cells replicate rarely, only during transfer every 3–4 weeks. Two of the four mutant stock cultures (*tgi2* and *tgi4*) regained their ability to lengthen telomeres at 30°, supporting a model for comaintenance of *tgi* alleles under selective pressure but assortment to homozygosity when the pressure is removed.

tgi in the midst of phenotypic analysis (data not shown). Several other mutant transfomants became tgi when their telomeres elongated after losing the injected *tgi1-1* telomerase RNA gene from the rDNA locus (data not shown). This second round of telomere shortening might have depended on recombination of the *tgi1-1* transgene with the endogenous locus such that the *tgi1-1* allele was present at a low level in a large population of cells with lengthening telomeres. In this situation, a cell harboring the *tgi1-1* allele at the endogenous locus might have a selective advantage given the short telomere/fast cell cycle phenotype conferred by *tgi1-1*. However, no trace of the *tgil-1* telomerase RNA gene was detected by PCR in these new tgi mutants (data not shown), so novel spontaneous tgi mutations are likely to be responsible.

Other tgi mutants are also probably heterozygous: A Tetrahymena macronucleus contains 45–60 copies of each chromosome, and these chromosomes assort stochastically due to amitotic macronuclear division. Therefore, a Tetrahymena culture usually becomes homozygous for any macronuclear allele within several hundred generations (Larson *et al.* 1986; Orias and Bradshaw 1992). Any tgi mutation that arises must struggle for survival among 45–60 wild-type copies of its gene. Therefore, tgi mutations are dominant and provide a growth advantage to cells that carry them. It is reasonable to expect that our tgi mutants would eventually become homozygous for their mutations because they were grown under selective pressure for short telomeres for hundreds of generations after their short telomere phenotypes appeared. To test this possibility, four tgi mutants were stored under nonselective conditions (in stock tube cultures at room temperature) for at least a year, then their telomere phenotypes were examined at 30°. Two of the mutants, *tgi2* and *tgi4*, reverted to wild type under nonselective conditions and exhibited telomere growth at 30° (Figure 4), suggesting that they were heterozygous at the mutant locus and that removal of selective pressure for the mutant allele led to its loss. Although *tgi2*'s telomeres initially grew, they quickly became short again, so its mutation may have been present at a low-copy number in the stock tube culture (Figure 4).

DISCUSSION

The abundance of chromosomes in Tetrahymena has made it a pioneering system for biochemical and molecular analysis of telomeres. Here we show that mutants with defects in telomere length regulation can be identified in Tetrahymena. This *in vivo* approach will complement ongoing studies that rely on site-directed mutagenesis of Tetrahymena telomerase components *in vitro* (Yu *et al.* 1990; Autexier and Greider 1994; Gilley *et al.* 1995).

The *tgi1-1* mutation: In a previous Tetrahymena study, overexpression of three telomerase RNA template mutations resulted in lethal phenotypes, and the only cells that survived to grow at normal doubling rates were ones that had completely lost their mutant telomerase RNA gene (Yu *et al.* 1990). In contrast, transformants expressing high levels of the *tgi1-1* mutant telomerase RNA grew at healthy doubling rates of 3–4 hr (data not shown). Such mild effects on cell viability were expected because tgi mutants grow vigorously and must maintain a basal level of telomerase-mediated telomere repair. Indeed, wild-type cells expressing the *tgi1-1* mutant telomerase RNA experienced gradual telomere shortening over a three-week period. The *tgi1-1* mutation is likely to perturb the stem III pseudoknot of the telomerase RNA (Figure 2C). It has recently been shown that telomerase RNA mutations affecting stem III and the pseudoknot have little effect on telomerase activity *in vitro*, suggesting a potential role for this conserved pseudoknot *in vivo* (Autexier and Greider 1998).

Telomere length and the cell cycle: tgi mutants had only 0.2% less genomic DNA to replicate than their siblings with long telomeres, yet their doubling rates were about 10% faster (data not shown). Thus, it is unlikely that having less genomic DNA to replicate resulted in a faster doubling rate. How, then, might telomere length influence Tetrahymena's growth rate? A number of studies suggest that telomeres are monitored by cell cycle checkpoint mechanisms. Loss of a chromosomal telomere in *S. cerevisiae* has been shown to cause RAD9-dependent cell cycle arrest (Sandell and Zakian 1993). In addition, mutations in either of two *S. cerevisiae* genes STN-1 or EST4/CDC-13 cause temperature-dependent telomere elongation and damage, which activates the RAD9-dependent G2/M checkpoint and results in slow growth (Grandin *et al.* 1997). Since Tetrahymena also has a similar temperature-sensitive slow-growth phenotype that correlates with long telomeres (Figure 1, Larson *et al.* 1987), perhaps a temperature-sensitive Tetrahymena homologue of either STN-1 or CDC-13 is involved. Finally, we note that the human

ataxia telangiectasia mutated gene and its yeast homologue TEL1 both affect telomere length and cell cycle checkpoints that monitor DNA damage, as do a number of *S. pombe* checkpoint mutants (Friedberg *et al.* 1995; Greenwell *et al.* 1995; Morrow *et al.* 1995; Dahlén *et al.* 1998). Similar telomere cell-cycle interactions may be responsible for slow doubling rates of Tetrahymena cells with long telomeres.

tgi, a molecular balancing act? Each Tetrahymena macronucleus is polyploid (45C–60C), so a spontaneous tgi mutation must be dominant to have a selective advantage. Although tgi mutations must be dominant, *tgi1* was a macronuclear heterozygote for its telomerase RNA mutations, and both *tgi2* and *tgi4* reverted under nonselective growth conditions. These results suggest that tgi mutants are commonly macronuclear heterozygotes at their mutant loci (Figures 2 and 4). Because a Tetrahymena macronucleus normally assorts to homozygosity at all loci, we propose that a mixture of wild-type and mutant tgi alleles confers a selective advantage for many tgi mutants. These semi-dominant tgi mutations could result from either a lethal loss-of-function mutation that requires some basal level of wild-type gene activity or from a gain-of-function mutation that requires an interaction between mutant and wild-type gene products. Tetrahymen's polyploid macronucleus is the ideal environment for balancing mutant and wild-type gene ratios and may allow for selection of mutants whose effects vary from subtle to extreme. This characteristic should facilitate cloning as complementation vectors become available in Tetrahymena (Gaertig *et al.* 1994).

A drawback of the system presented here is that the mutations are macronuclear. This means that they cannot be subjected to conventional genetic analyses, and they may be lost upon storage under nonselective conditions (Figure 4). The recently realized ability to generate germline tgi mutations should facilitate mapping of tgi genes (E. Hamilton and E. Orias, personal communication). By combining both forward and reverse genetic approaches, we hope to fully exploit the tgi phenotype and unveil a broad spectrum of informative mutations in the telomere length regulation machinery.

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