Specific telomerase RNA residues distant from the template are essential for telomerase function

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The reverse transcriptase telomerase is a ribonucleoprotein complex that adds telomeric repeats to chromosome ends, using a sequence within its endogenous RNA component as a template. Although templating domains of telomerase RNA have been studied in detail, little is known about the roles of the remaining residues, particularly in yeast. We examined the functions of nontemplate telomerase residues in the telomerase RNA of budding yeast *Kluyveromyces lactis*. Although approximately half of the RNA residues were dispensable for function, four specific regions were essential for telomerase action in vivo. We analyzed the effects of mutating these regions on in vivo function, in vitro telomerase activity, and telomerase RNP assembly. Deletion of two regions resulted in synthesis of stable RNAs that appeared unable to assemble into a stable RNP. Mutating a region near the 5′ end of the RNA allowed RNP assembly but abolished enzymatic activity. Mutations in another specific small region of the RNA led to an inactive telomerase RNP with an altered RNA conformation.

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Ribonucleoprotein (RNP) complexes play central roles in essential cellular processes that include translation, rRNA, pre-mRNA and pre-tRNA processing, and telomere maintenance. In the ribosome and the spliceosome, large RNA molecules form a structural framework for the assembly of proteins and provide critical active site residues. The reverse transcriptase (RT) telomerase is also an RNP complex (for review, see Blackburn 1992, 1998), which uses a small part of an intrinsic RNA component as a template for telomeric DNA synthesis. Telomerase copies this template to synthesize new telomeric repeat units onto the termini of eukaryotic chromosomes. Telomeric DNA consists of a tandem array of repeat units whose length and number are often tightly controlled. The inability of conventional DNA polymerases to replicate the very ends of chromosomes would cause progressive loss of DNA sequences from the ends (Watson 1972; Olovnikov 1973). Telomerase action compensates for this loss and is thus required for long-term chromosomal maintenance.

Telomerase activity, purified partially from ciliate, human, and yeast extracts, has been characterized in detail in vitro (Morin 1989; Greider 1991; Lee and Blackburn 1993; Cohn and Blackburn 1995). In vitro enzyme action requires binding to a telomeric oligonucleotide primer and dNTP substrates, alignment of the primer 3′ end to the templating domain of telomerase RNA, and its extension by incorporation of nucleotide triphosphates complementary to the templating residues. Yeast telomerases typically synthesize only one repeat unit in vitro and stay bound to the product after completion of DNA synthesis (Prescott and Blackburn 1997a; Fulton and Blackburn 1998). The core catalytic components of the enzyme are telomerase RNA (*TER*) and a protein subunit containing conserved RT motifs (Weinrich et al. 1997; Beattie et al. 1998). Conserved aspartate residues in these motifs are required for catalysis (Counter et al. 1997; Harrington et al. 1997b; Lingner et al. 1997b; Weinrich et al. 1997). Additional proteins are associated with the core catalytic unit and may alter enzymatic properties or mediate the interaction of the enzyme with telomeres (Collins et al. 1995; Steiner et al. 1996; Harrington et al. 1997a; Nakayama et al. 1997; Gandhi and Collins 1998).

Telomerase RNA sizes vary widely among eukaryotes, from ~150 to 200 nucleotides in ciliates (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990) and ~400 to 450 nucleotides in mammals (Blasco et al. 1995; Feng et al. 1995) to 1200–1300 nucleotides in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* (Singer and Gottschling 1994; McEachern and Blackburn 1995). This size variability is accompanied by a lack of overall primary sequence conservation among each

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group of organisms, although core secondary structure is conserved among widely divergent ciliate RNAs (Romero and Blackburn 1991; Lingner et al. 1994; McCormick-Graham and Romero 1995). The templating domain has been the most well-studied portion of telomerase RNA. The consequences of telomerase RNA template mutations have been studied in vivo and in vitro in ciliates (for review, see Greider 1996 and references therein) and yeasts (Prescott and Blackburn 1997b) and examined minimally in mammals (Marusic et al. 1997). Most of these mutations were faithfully copied into telomeric DNA by the enzyme in vivo and in vitro. Strikingly, several of these mutations affected enzymatic properties such as processivity, fidelity, and proper termination of synthesis; in one case eliminating telomerase activity in vivo and in vitro (Gilley et al. 1995; Gilley and Blackburn 1996; Prescott and Blackburn 1997a,b). Hence, the template region of the RNA must contribute to active site functions. Less is known about the roles of the nontemplate regions of telomerase RNA, and in this regard the large size of the yeast telomerase RNAs is intriguing. An exciting possibility is that the RNA component of telomerase may contribute to the catalytic properties of the enzyme. In addition, telomerase RNA may provide a matrix for the assembly of the telomerase complex.

Experiments with ciliate telomerase RNAs in vivo have revealed the importance of nontemplate residues (Bhattacharyya and Blackburn 1997). When the nontemplate portion of the *Tetrahymena* telomerase RNA is replaced with the corresponding region from another ciliate species, *Glaucoma*, and the chimeric RNA is expressed in *Tetrahymena*, it assembles into an only partially functional enzyme with an aberrant cleavage activity. In vitro studies by reconstitution of enzyme activity from in vitro synthesized telomerase RNA and partially purified cell extracts have shown that mutations in some regions outside the template domain of *Tetrahymena* telomerase RNA abolish or significantly reduce reconstituted activity (Autexier and Greider 1998). Similarly a 30-nucleotide nontemplate region of the human telomerase RNA is necessary for reconstitution of enzyme activity (Autexier et al. 1996). However, these studies did not distinguish whether these sequences are required for enzyme activity itself or for telomerase RNP assembly.

Here we report a detailed examination of the role of nontemplate residues in the telomerase RNA (*TER1*) of the budding yeast *K. lactis*. Each of the 12 *K. lactis* telomeres contains an average of 15 tandem 25-bp repeats, with the range being 10–20 repeats. *TER1* RNA contains a 30-nucleotide template region that is complementary to 1⅕ telomeric repeat units (McEachern and Blackburn 1995), and an in vitro telomerase activity in *K. lactis* has been described recently (Fulton and Blackburn 1998). We report that although approximately half of the *TER1* RNA sequences are dispensable, several small sequences are critical for enzyme function in vivo and in vitro. Two small regions are essential for activity, although mutations in them still allow telomerase RNP assembly. Interestingly, mutating one of these regions abolishes detectable telomerase activity both in vivo and in vitro and significantly changes the conformation of the mutant telomerase RNA as well as the telomerase RNP complex. This is the first demonstration that in yeast telomerase RNAs, residues far from the template play essential roles in the telomerase RNP and its activity both in vitro and in vivo.

Results

A BclI *template mutation provides a marker for assaying in vivo functions of* TER1 *RNA*

To investigate the roles of telomerase RNA sequences outside the template, we created a series of *TER1* deletion or substitution mutants and assayed their function in vivo. Function was assessed in a *K. lactis* strain deleted for the entire *TER1* gene (strain *7BΔTER1*). As described previously for a *TER1* allele containing a 300-bp deletion including the templating domain (McEachern and Blackburn 1995), telomeric repeats in strain *7BΔTER1* were progressively lost from the chromosome ends (Fig. 1A, lanes 5,6). Upon further passaging, the cells grew very poorly, forming small senescent colonies with extremely rough edges and critically short telomeric fragments. The occasional healthy, smooth survivor colonies contained cells that had regenerated long tracts of telomeric DNA by an alternative *RAD52*-dependent recombination pathway (McEachern and Blackburn 1996; Fig. 1A, lanes 7,8). As described previously, such survivor colonies with elongated telomeric DNA continue to be prone to telomeric shortening and additional episodes of growth senescence (McEachern and Blackburn 1996). When the *7BΔTER1* strain was transformed with a centromeric plasmid expressing the wild-type *TER1* gene (pTERWT), wild-type length telomeres were regained. These telomeres were stably maintained after numerous serial passages (Fig. 1A, lanes 9,10), each passage representing ~25 cell doublings.

To differentiate between telomeric repeats added by telomerase containing the mutant RNAs and the preexisting wild-type repeats, we marked the template of the *TER1* RNA with a mutation: a single base change in the template (A458G, *TER1–BclI* allele), resulting in the creation of a *Bcl*I restriction site (M. McEachern and E.H. Blackburn, unpubl.). Telomeric repeats synthesized by *TER1–BclI* incorporate the single base change without otherwise altering the telomeric repeat sequence (D. Hager and M. McEachern, pers. comm.). Unlike previously described mutations in the *TER1* template region (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996), the *TER1–BclI* mutation is phenotypically silent and does not detectably affect telomere function and telomere length regulation. The *7BΔTER1* cells transformed with a centromeric plasmid expressing the *TER1–BclI* gene (pTERBclI) regained stably maintained wild-type length telomeres, with no change in telomere length and no apparent colony or cellular phenotype observed even after multiple serial cell passages (Fig. 1A, lane 11; data not shown). The *Bcl*I mutation was incor-

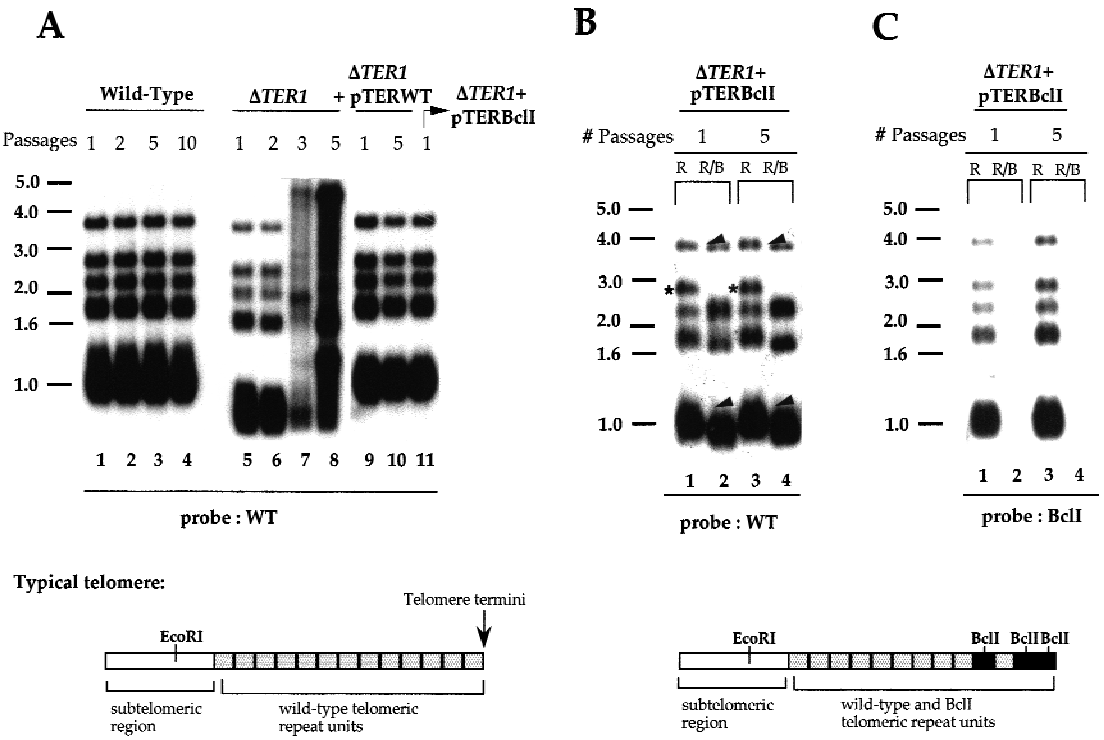

Figure 1. Deletion of *TER1* results in telomere shortening, which is complemented by *TER1–BclI*. Southern blots of yeast genomic DNA showing telomeric profiles from wild-type strain 7B520, Δ*TER1*, or derivatives thereof. DNAs are digested with *Eco*RI (*A*) or in pairs of digestions with *Eco*RI (R) and *Eco*RI plus *Bcl*I (R/B) (*B,C*) and probed with radiolabeled oligonucleotide probes for telomeric repeats as indicated. The wild-type (WT) probe (oligonucleotide Klac 1–25) hybridizes to wild-type and mutant repeats. The *Bcl*I probe (oligonucleotide KTelBcl) hybridizes only to *Bcl*I-marked repeats. The same filter was probed first with the *Bcl*I probe (*C*) and subsequently with the wild-type probe (*B*). DNA markers in kilobases are indicated to the *left* of each panel. All 12 telomeres are visualized. The band around 1 kb contains seven telomeres and the band just above 1.6 kb contains two telomeres. The telomeric fragment marked with an asterisk in *B* contains a *Bcl*I restriction site between the subtelomeric *Eco*RI site and the telomere end. The arrowheads demonstrate examples of telomeric fragment shortening with *Bcl*I digestion. Each passage represents ~25 cell doublings.

porated into newly synthesized telomeric repeats at the telomere termini, as described previously for other *K. lactis* and *S. cerevisiae* telomerase RNA template mutants (McEachern and Blackburn 1995; Prescott and Blackburn 1997b). The *Bcl*I mutant repeats were detected by hybridization of *Eco*RI-digested genomic DNA to a radio-labeled oligonucleotide probe specific for the *Bcl*I repeat (Fig. 1C, lanes 1,3). The hybridizing bands were lost when the DNA was digested secondarily with *Bcl*I restriction enzyme (Fig. 1C, lanes 2,4). As seen by hybridization with a wild-type telomeric probe, this second digestion with *Bcl*I resulted in the expected shortening of *Eco*RI telomeric fragments (Fig. 1B, lanes 2,4, arrowheads), demonstrating that the *Bcl*I sites were at the ends of the telomeres, and were incorporated onto every chromosome. When *7BΔTER1* yeast cells were cotransformed with two plasmids, one expressing wild-type *TER1* and one expressing *TER1–BclI*, we estimated that equal numbers of wild-type and *Bcl*I marked repeats were incorporated onto telomeres (data not shown).

Various nontemplate mutations were created in the *TER1–BclI* allele (Fig. 2). We anticipated that both telomere length and the extent of incorporation of *Bcl*I-marked telomeric repeats would provide an accurate readout of varying degrees of function of the mutant alleles in vivo. Test plasmids with a *HIS3* marker, containing mutant *TER1–BclI* genes, unmutagenized *TER1–BclI*, or no *TER1* gene, were transformed into *K. lactis* strain *7BΔTER1* expressing the *URA3* marked plasmid pTERWT. Subsequently, pTERWT was selected against by plating cells on medium containing 5-fluoro-orotic acid (5-FOA). We compared telomere length and incorporation of *Bcl*I-marked repeats onto telomeres both before and after loss of the pTERWT plasmid. Thus, the function of each mutant RNA in vivo was examined both when it was coexpressed with wild-type telomerase RNA and when it was expressed alone.

In control experiments, when the *HIS3* plasmid contained no *TER1* gene, loss of the pTERWT plasmid resulted in the expected shortening of telomeres followed by telomere lengthening by the recombination pathway (Fig. 3A, lanes 1–6), and no *Bcl*I repeat incorporation (Fig. 3B, lanes 1–6). With the unmutagenized *TER1–BclI* gene, telomere length remained unchanged before and after transformation and after 5-FOA selection (Fig. 3A, lanes 7,9; data not shown). *Bcl*I repeats were incorporated at the earliest times observable after transformation (~25 cell doublings). Similarly, we determined the extent to

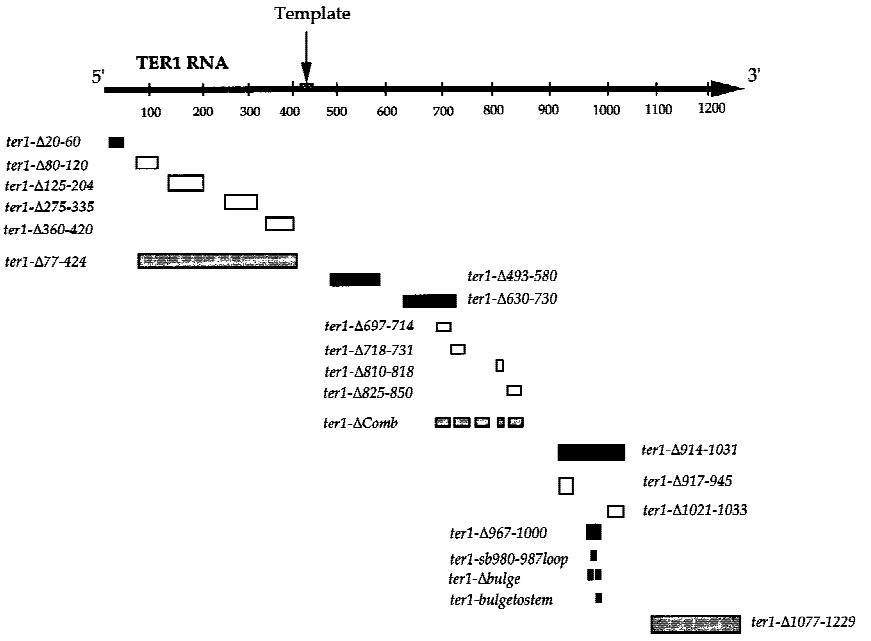

Figure 2. A schematic diagram of the *TER1* RNA. The template region is contained within residues 435 and 464. The boxed areas represent regions removed or substituted in mutant alleles.

which each deletion mutant was able to provide *TER1* function in vivo. The deletion mutants assayed are shown schematically in Figure 2 and the in vivo results obtained are summarized in Table 1.

Several regions of the TER1 *RNA are fully dispensable for telomerase activity in vivo*

Several small deletions of telomerase RNA provided wild-type levels of *TER1* function in vivo. The deletions between the 5′ end of the RNA and the template (*ter1-Δ80–120*, *ter1-Δ125–204*, *ter1-Δ275–335*, and *ter1-Δ360–420*) were almost indistinguishable from the *TER1–BclI* gene in terms of incorporation of *Bcl*I-marked telomeric repeats and the length of telomeres (Table 1; examples are shown in Fig 3C,D). With the *ter1-Δ125–204* and *ter1-Δ275–335* mutants, telomeres shortened slightly (<50 bp; Fig. 3C,D, lanes 5,7). However, the amount of *Bcl*I incorporation in these mutants both in the presence and absence of pTERWT was comparable to *TER1–BclI* (Table 1). We suggest that telomerase function is only slightly reduced in these two mutants. Four small deletions in the central part of the RNA downstream of the template (*ter1-Δ697–714*, *ter1-Δ718–731*, *ter1-Δ810–818*, and *ter1-Δ825–850*) also had telomere lengths and *Bcl*I repeat incorporation indistinguishable from *TER1–BclI* (Table 1; examples are shown in Fig. 3C,D). *Bcl*I repeats were also incorporated when each of these mutant RNAs was coexpressed with wild-type *TER1* RNA (Table 1). On the basis of these criteria, these deleted regions of *TER1* RNA are dispensable for its function in vivo.

We also tested, by Northern blotting analysis, whether the deletions affected the steady-state levels of these mutant RNAs. Total RNA was extracted from the *7BΔTER1* strain expressing mutant *TER1* alleles or the *TER1–BclI* control and hybridized to a *TER1* probe (Fig. 4). A second probe for an unrelated mRNA, ribosomal protein 59 (RP59), was used as a control for RNA loading in each lane. As expected, no detectable *TER1* RNA was found in the *7BΔTER1* strain transformed with *HIS3* plasmid only (Fig. 4; lane 6). The level of each of the essentially fully functional mutant RNAs was comparable to the level of *TER1–BclI* RNA. Two of these mutant RNAs (*ter1-Δ697–714* and *ter1-Δ718–731*) were in fact present at twofold higher levels than the *TER1–BclI* RNA. Therefore, these deletions do not decrease the synthesis or stability of *TER1* RNA.

Deleting three large domains of TER1 *results in partial telomerase function*

Three larger deletions that remove substantial portions of the RNA were tested for in vivo telomerase action: *ter1-Δ77–424*, a 347-nucleotide deletion between the 5′ end of *TER1* RNA and the template; *ter1-ΔComb*, a combination of five small deletions in the central part of the RNA removing a total of 129 nucleotides; and *ter1-Δ1077–1229*, a 154-nucleotide deletion near the 3′ end of the RNA (Fig. 2; Table 1).

All three mutant RNAs functioned in vivo by the criterion of incorporation of *Bcl*I mutant repeats (Fig. 5A,B). However, fewer *Bcl*I-marked repeats were incorporated into the ends of the telomeres than in the *TER1–BclI* control, particularly in the case of the *ter1-ΔComb* mutant (Fig. 5B, cf. lanes 1, 3, 5, and 7). Furthermore, digestion of telomeres with *Bcl*I restriction enzyme resulted in less shortening than in *TER1–BclI* cells, indicating the presence of fewer *Bcl*I repeats at the ends (Fig. 5A, arrowheads). Thus, we categorized these mutant RNAs as partially functional (Table 1). The telomeric fragments in these strains were also shorter than wild-type by ~200 nucleotides (i.e., equivalent to eight 25-bp telomeric re-

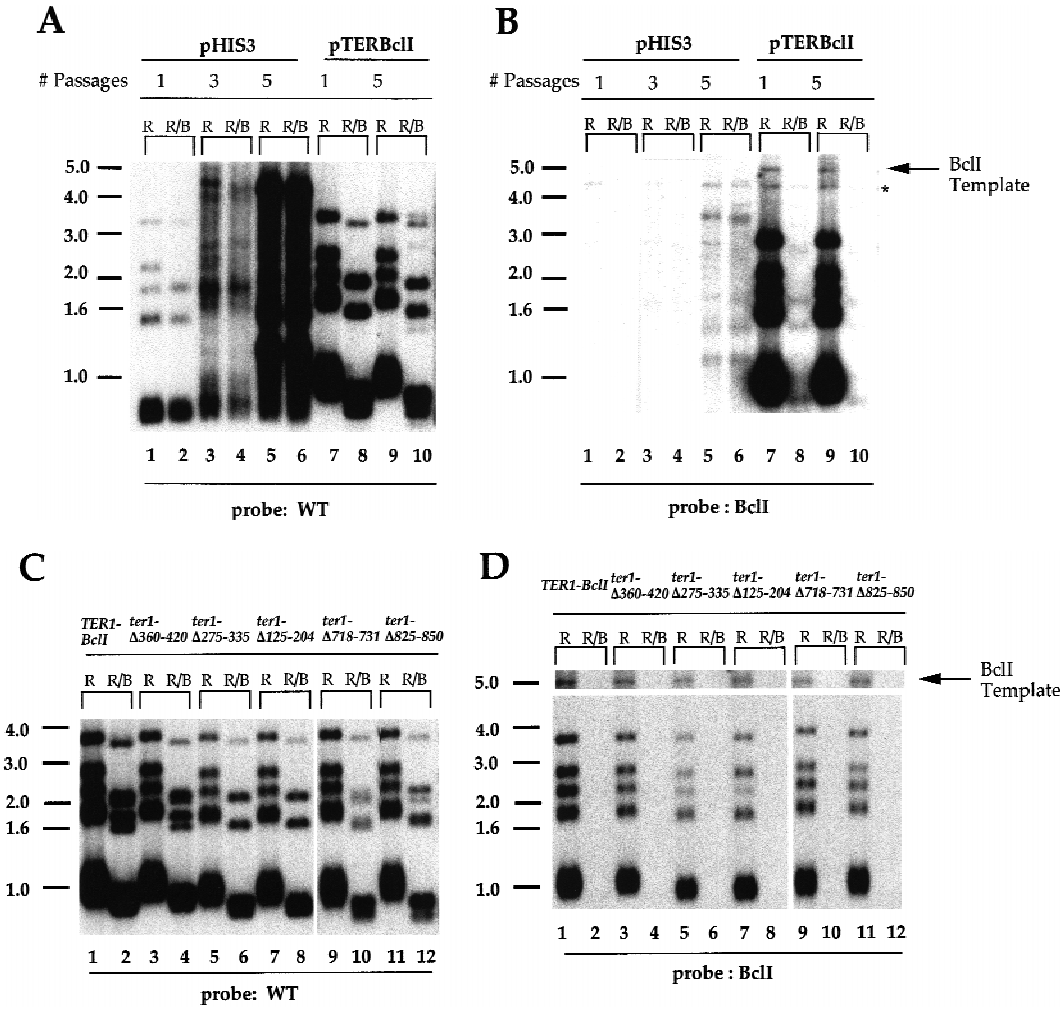

Figure 3. Telomeric profile of genomic DNA from the Δ*TER1* strain transformed with control pHIS3 and pTERBclI plasmids (*A*,*B*) or fully functional *TER1* deletion mutants (*C*,*D*). Each pair of lanes contains DNA digested with *Eco*RI (R) and *Eco*RI plus *Bcl*I (R/B) enzymes, respectively. Each passage represents ~25 doublings after the loss of the pTERWT plasmid. The DNAs in *C* and *D* were prepared from cells after five passages. The same filters were probed first with the *Bcl*I probe (*B*,*D*) and subsequently with the wild-type probe (*A*,*C*). The asterisk in *B* is a nonspecific band.

peats) for the *ter1-ΔComb* and *ter1-Δ1077–1229* mutants, and ~100–150 nucleotides (i.e., four to six 25-bp telomeric repeats) for the *ter1-Δ77–424* mutant. Interestingly, in all these cases the shortened telomeres were maintained stably at their new length, with no further change in telomere length occurring for up to 250 generations (data not shown).

When these mutant genes were coexpressed with wild-type *TER1* and assayed for *Bcl*I repeat incorporation, no *Bcl*I-specific hybridization was obtained (Fig. 5C). Because we were able to detect the *Bcl*I template of the mutant gene with the *Bcl*I-specific probe, we conclude that the probe can detect approximately one to three repeats per telomere. Hence, in contrast to the essentially fully functional mutants, these deleted RNAs, although functional when expressed alone, were unable to act at telomeres to cause incorporation of telomeric repeats in the presence of the wild-type *TER1* RNA.

We tested whether the decreased telomerase function of these partially functional mutants reflected decreased steady-state RNA levels. Levels of the *ter1-Δ1077–1229* and *ter1-ΔComb* RNAs were comparable to the wild-type *TER1–BclI* RNA (Fig. 4, lanes 7,9,14). Thus, the *Δ1077–1229* and *ΔComb* deletions do not affect the synthesis and stability of the mutant RNAs. In contrast, the *ter1-Δ77–424* mutant RNA was markedly less abundant than *TER1–BclI* (Fig. 4, lane 10). In Northern blots using a probe that is completely complementary to both RNAs, the *ter1-Δ77–424* RNA was found to be approximately eightfold less abundant than *TER1–BclI* (Fig. 6C; data not shown).

Overexpression of a partially functional RNA restores telomere length

We tested directly whether the lower steady-state levels of *ter1-Δ77–424* mutant RNA caused the decreased *Bcl*I repeat incorporation and shortened telomeres. The *ter1-*

Table 1. *Summary of* TER1 *mutant alleles assayed in vivo*

TER1 allele	*Bcl*I repeat incorporation in presence of pTERWT	*BCl*I repeat incorporation after loss of pTERWT	Telomere length after loss of pTERWT	Telomerase function in vivo
Δter1	N/A	N/A	short, followed by lengthening in survivor cells (S)	–
TER1–BclI[a]	+	++	wild type	++ (fully functional)
ter1-Δ80–120[b]	+	++	wild type	++
ter1-Δ125–204	+	++	marginally shorter than wild type	++
ter1-Δ275–335	+	++	marginally shorter than wild type	++
ter1-Δ360–420	+	++	wild type	++
ter1-Δ697–714	+	++	wild type	++
ter1-Δ718–731	+	++	wild type	++
ter1-Δ810–818	+	++	wild type	++
ter1-Δ825–850	+	++	wild type	++
ter1-Δ77–424	–	+	~100–150 bp shorter than wild type	+ (partially functional)
ter1-Δ1077–1229	–	+	~200 bp shorter than wild type	+
ter1-ΔComb[c]	–	+	~200 bp shorter than wild type	+
GAL1–TER1[d]	+	++	wild type	++
GAL1–ter1-Δ77–424[d]	+	++	wild type	++
ter1-Δ20–60	–	–	S	–
ter1-Δ493–580	–	–	S	–
ter1-Δ630–730	–	–	S	–
ter1-Δ914–1031	–	–	S	–
ter1-Δ917–945	+	++	wild type	++
ter1-Δ967–1000	–	–	S	–
ter1-Δ1021–1033	+	++	wild type	++
ter1-sb980–987loop[e,h]	–	–	S	–
ter1-Δ bulge[f,h]	–	–	S	–
ter1-bulgetostem[g,h]	–	–	S	–

[a]*TER1* nucleotide A458 is changed to G. All subsequent *ter1* mutant alleles also contain this mutation.
[b](*Δa–b*) A deletion of nucleotides a through b.
[c]The *ΔComb* deletion is a combination of the *Δ697–714, Δ718–731, Δ750–785, Δ810–818, and Δ825–850* deletions.
[d]In the *GAL1–ter1* alleles nucleotides 1–20 of *TER1* are replaced with *GAL1* RNA leader sequences.
[e]*TER1* nucleotides 980–987 are substituted with TTCG.
[f]*TER1* nucleotides 971–975 and 992–996 are deleted.
[g]*TER1* nucleotides 992–996 are replaced with TGAGG.
[h]Loop, bulge, and stem refer to putative secondary structural elements.

Δ77–424 gene was placed under the control of the inducible *GAL1* promoter resulting in ~50-fold higher levels of the induced *GAL1-ter1-Δ77–424* RNA (Fig. 6C). This restored both the extent of *Bcl*I repeat incorporation in telomeric ends and the length of the telomeric fragments to wild-type levels (Fig. 6A). In contrast, overexpression of the wild-type *TER1–BclI* RNA from the *GAL1* promoter (Fig. 6C, cf. lanes 1 and 3) caused no change in the length of telomeric fragments or *Bcl*I repeat incorporation (Fig. 6B). Thus, increasing the expression of the poorly expressed or unstable *ter1-Δ77–424* RNA restores full in vivo function to this telomerase RNA. Hence, we conclude that the decreased *Bcl*I repeat incorporation by the *ter1-Δ77–424* mutant is attributable to the lower steady-state level of the mutant RNA.

Deletion mutants identify four regions of TER1 *outside the template that are crucial for telomerase function*

Deletions of four regions of the RNA (*ter1-Δ20–60*, *ter1-Δ493–580*, *ter1-Δ630–730*, and *ter1-Δ914–1031*) comprising 40, 87, 100, and 117 residues, respectively, abolished detectable telomerase action in vivo. *Bcl*I repeats were not incorporated in the telomeres of cells expressing these RNAs, even up to 100 generations after the loss of the pTERWT plasmid (Fig. 7). We detected clear hybridization of the *Bcl*I-specific probe to the *Bcl*I template of the mutant genes, which is estimated to be present at one to three copies per cell (Fig. 7A). This hybridization signal was specific because unlike nonspecific hybridization, it was completely lost upon digestion of genomic DNA with *Bcl*I enzyme. Thus, our inability to detect any specific hybridization to telomeric DNA indicated that *Bcl*I repeats are not incorporated into the telomeres in these strains. Furthermore, the telomeres in the mutant cells exhibited the typical behavior of telomeres in the absence of telomerase activity; they shortened to a critical minimal length, and then in the population of surviving cells were generally lengthened, presumably by recombinational events (Fig. 7B). Consistent with a lack of telomerase activity, the colony phenotype of these

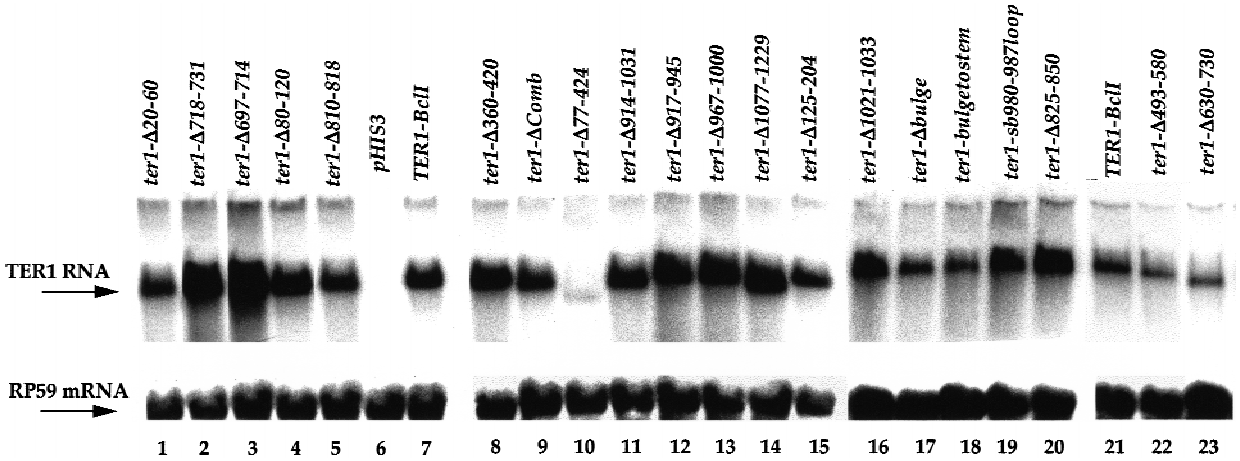

Figure 4. Steady-state levels of wild-type and mutant *TER1* RNAs. Northern blot of RNA extracted from the *ΔTER1* strain expressing pHIS3, *TER1–BclI* or deletion mutants of *TER1* and probed with a *TER1* DNA probe (*top*) or a probe for RP59 mRNA (*bottom*).

cells was identical to the *ΔTER1* strain in which *TER1* is deleted completely.

These mutant RNAs were expressed and stably maintained in cells at levels comparable to the *TER1–BclI* RNA, as judged by Northern blotting analysis (see Fig. 4, lanes 1,11,22,23). Thus, RNA residues in each of these four deleted regions (see Fig. 2) are essential for an aspect of telomerase activity other than the synthesis and stability of the RNA.

Small mutations in a domain of TER1 *RNA abolish telomerase function*

We examined in more detail the region of the *TER1* RNA between residues 914 and 1031. This part of the RNA was predicted to form a discrete structural domain, on the basis of a computer folding program (see Materials and Methods) and phylogenetic comparisons of *TER1* RNA sequences of four different *Kluyveromyces* species (Y. Tzfati and E.H. Blackburn, unpubl.). This computer-predicted RNA secondary structure contains three putative stable stem-loops and was used to guide construction of three small deletion mutants (*ter1-Δ917–945*, *ter1-Δ967–1000*, and *ter1-Δ1021–1033*; Fig. 8A). The steady-state levels of all three mutant RNAs were similar to *TER1–BclI* RNA (see Fig. 4, lanes 12,13,16). The *ter1-Δ917–945* and *ter1-Δ1021–1033* mutants incorporated *Bcl*I repeats into telomeres and were fully functional in vivo (Fig. 8B,C, lanes 1,2,5,6). In contrast, the *ter1-Δ967–1000* mutant RNA was completely nonfunctional in vivo; there was no incorporation of *Bcl*I repeats into telomeres (Fig. 8B,C, lanes 3,4), and the telomeres behaved similarly to those in strain *7BΔTER1*. Hence,

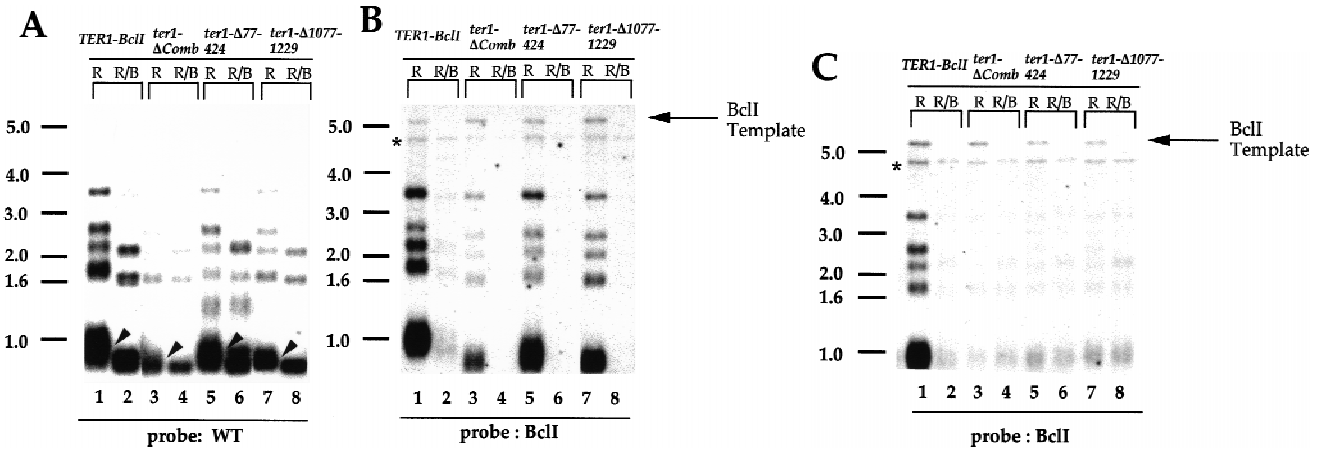

Figure 5. *Bcl*I repeat incorporation and telomere shortening in partially functional *TER1* mutants. Telomeric profile of DNAs prepared from the fifth passage of the *ΔTER1* strain expressing *TER1–BclI* or partially functional *TER1* alleles alone (*A*,*B*) or together with the pTERWT plasmid (*C*). Each pair of lanes contains DNA digested with *Eco*RI (R) and *Eco*RI plus *Bcl*I (R/B) enzymes, respectively. The same filter was probed first with the *Bcl*I probe (*B*) and subsequently with the WT probe (*A*). The asterisk in *B* and *C* is a nonspecific band.

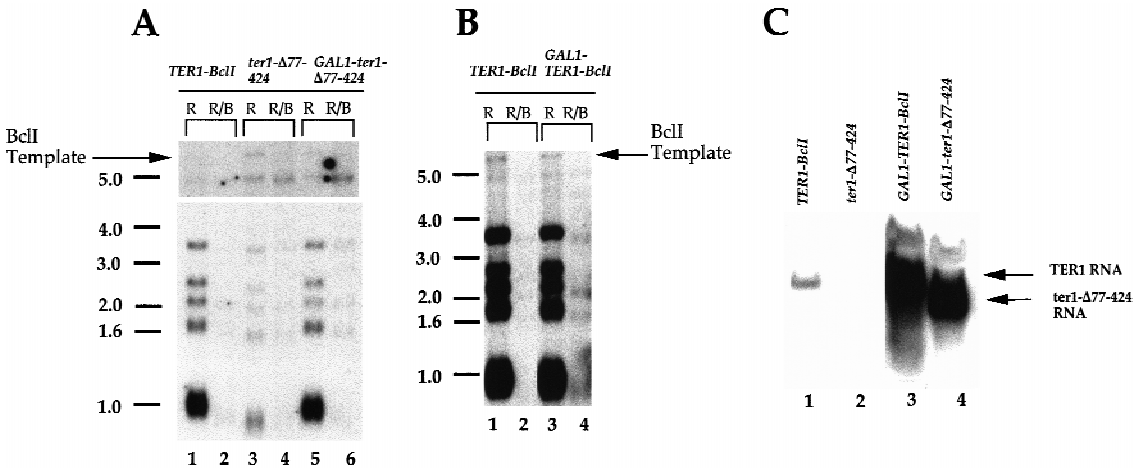

Figure 6. Overexpression of a partially functional *TER1* mutant restores telomere length. (*A*,*B*) Telomeric profile of DNAs prepared from the fifth passage of the Δ*TER1* strain expressing *TER1* alleles on plasmids as indicated. The cells were maintained in medium containing galactose as the carbon source. Each pair of lanes contains DNA digested with *Eco*RI (R) and *Eco*RI plus *Bcl*I (*R*/*B*) enzymes, respectively. The blots are probed with the *Bcl*I probe. (*C*) Northern blot of RNA prepared from the Δ*TER1* strain expressing *TER1* alleles on plasmids. The cells were grown in galactose-containing medium. The blot is probed with radiolabeled *TER1* DNA. Equal amounts of RNA were loaded in each lane.

RNA sequences critical for telomerase function lie between nucleotides 967 and 1000 of *TER1*.

We then constructed three smaller deletion and substitution mutants between residues 967 and 1000 to further narrow down the crucial residues. The first, *ter1–sb980–987loop*, substitutes the proposed 8-base 'loop' of this stem–loop region (residues 980–987) with a stable tetraloop sequence (UUCG). The second mutant, *ter1–Δbulge*, deleted the putative 'bulge/internal loop' in the stem region (deletion of residues 971–975 and 992–996). The third mutant, *ter1–bulgetostem*, was designed to convert the putative bulge to a stem and was a substitution of residues 992–996, making these positions complementary to residues 971–975 (see Table 1).

All three mutants were completely nonfunctional in vivo. They showed no *Bcl*I repeat incorporation for up to

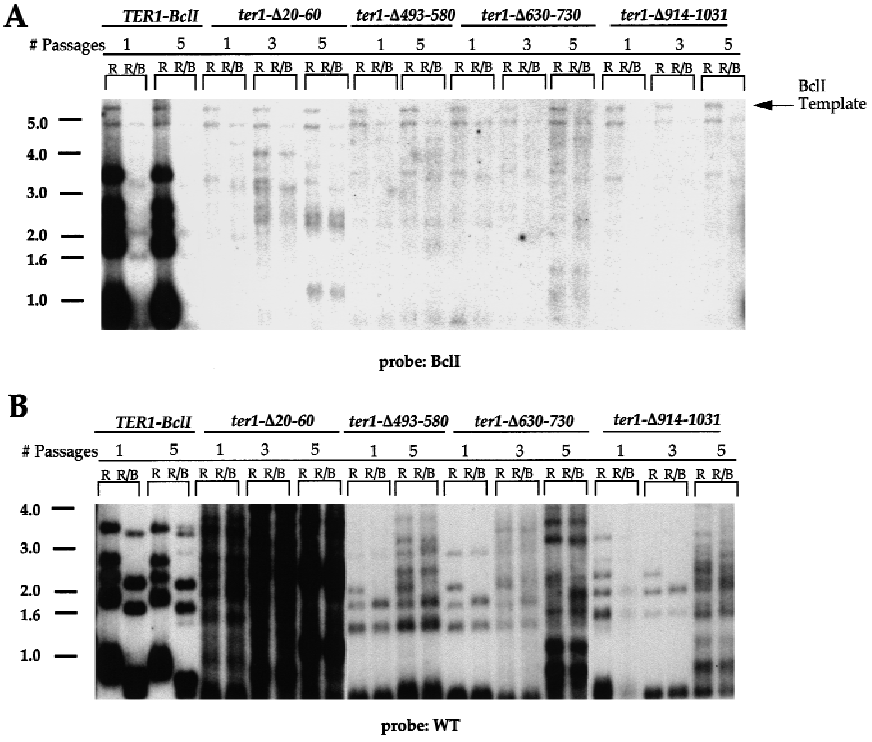

Figure 7. *Bcl*I repeats are not incorporated in telomeres of nonfunctional *TER1* mutants. Telomeric profiles of DNAs prepared from the Δ*TER1* strain expressing *TER1–BclI* or *TER1* deletion alleles, as indicated. Each pair of lanes contains DNA digested with *Eco*RI (R) and *Eco*RI plus *Bcl*I (R/B) enzymes, respectively. Each passage represents ~25 doublings after the loss of the pTERWT plasmid. The same filter was probed first with the *Bcl*I probe (*A*) and subsequently with the wild-type (WT) probe (*B*).

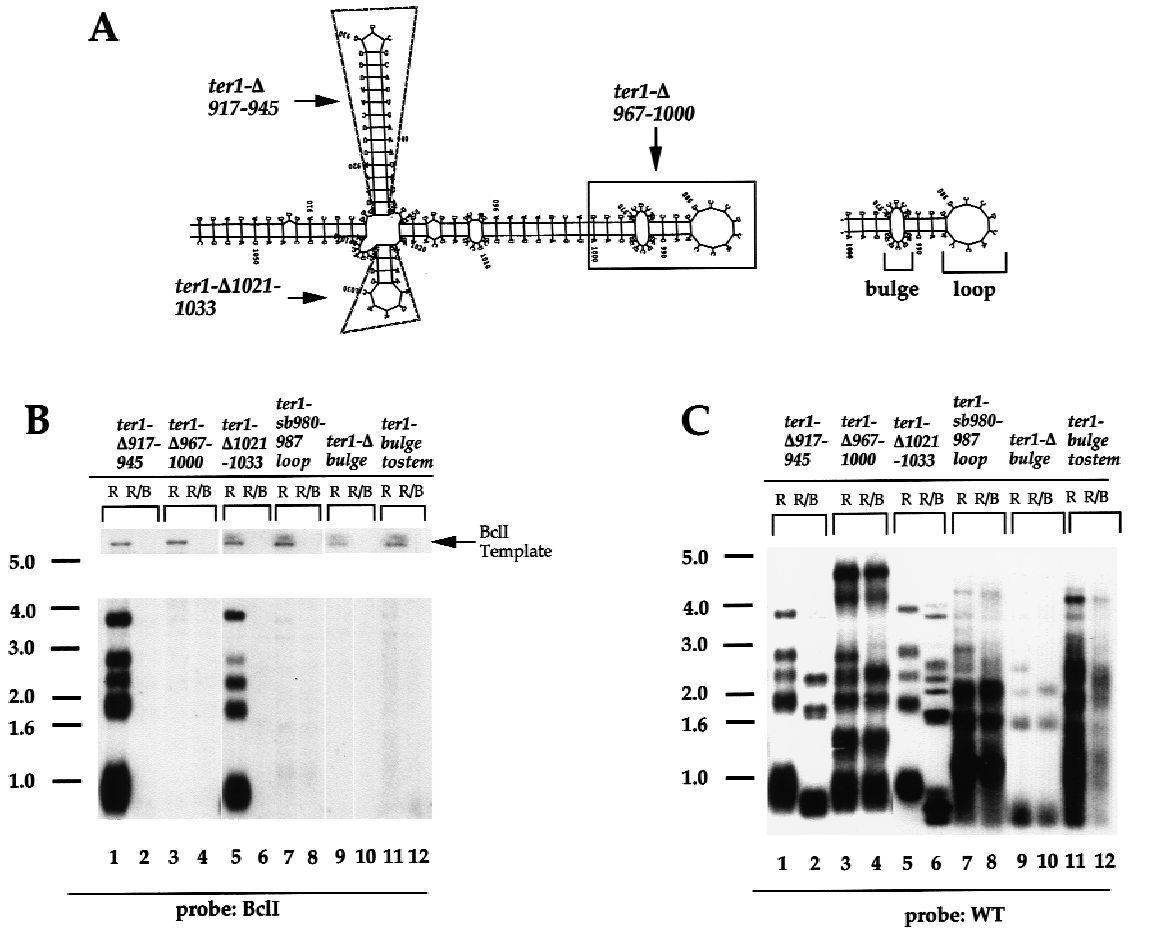

Figure 8. Mutations in a small region of *TER1* abolish telomerase function in vivo. (*A*) Predicted secondary structure of the *TER1* RNA between residues 901 and 1054. (*B*,*C*) Telomeric profiles of DNAs prepared from the 10th passage of the Δ*TER1* strain expressing *TER1* deletion alleles. Each pair of lanes contains DNA digested with *Eco*RI (R) and *Eco*RI plus *Bcl*I (R/B) enzymes, respectively. The same filter was probed first with the *Bcl*I probe (*C*) and subsequently with the wild-type (WT) probe (*B*).

10 passages (Fig. 8B, lanes 7–12), and telomeres shortened for the first two to three passages and then lengthened as in strain *7BΔTER1* (Fig. 8C, lanes 7–12; data not shown). The levels of the *ter1–Δbulge* and *ter1–bulgetostem* mutant RNAs were ~30% lower than the control *TER1–BclI* RNA (Fig. 4, lanes 7,17,18). Thus, residues 971–975 and 992–996 are important for both telomerase action and stability or expression. The level of the *ter1–sb980–987loop* RNA (Fig. 4, lane 19) was identical to the *TER1–BclI* control. Thus, altering residues 980–987 of the RNA specifically abolished telomerase action in vivo.

The TER1 *RNA mutants lacking function in vivo have undetectable telomerase activity in vitro*

We tested two possibilities for the lack of telomerase action in vivo for the nonfunctional *TER1* mutant RNAs: (1) a catalytically inactive telomerase enzyme and (2) defective interactions between a catalytically active enzyme and other components of the telomere maintenance pathway that prevent telomerase from adding telomeric repeats to telomeres. The latter, for example, is true for the *S. cerevisiae est1*, *est3*, and *est4* mutants, in which telomerase is active in vitro (Cohn and Blackburn 1995; Lingner et al. 1997a). Four of the nonfunctional *TER1* mutants, *ter1-Δ20–60*, *ter1-Δ493–580*, *ter1-Δ630–730*, and *ter1–sb980–987loop*, were tested directly for telomerase activity in vitro.

An in vitro telomerase assay for *K. lactis* cell extracts has been developed recently (Fulton and Blackburn 1998). This activity extends telomeric oligonucleotides by the incorporation of deoxynucleotides (one of which is radio-labeled) and can undergo one round of telomeric repeat synthesis. Control wild-type *TER1* or *TER1–BclI* cell extracts extended a telomeric DNA oligonucleotide primer (Fig. 9A, lanes 1,3), producing the characteristic pattern of mid-template and near-terminal products described previously for wild-type telomerase reactions with this primer (Fulton and Blackburn 1998). The activity was sensitive to RNase A (Fig. 9A, lanes 2,4). No significant difference in activity was found between the

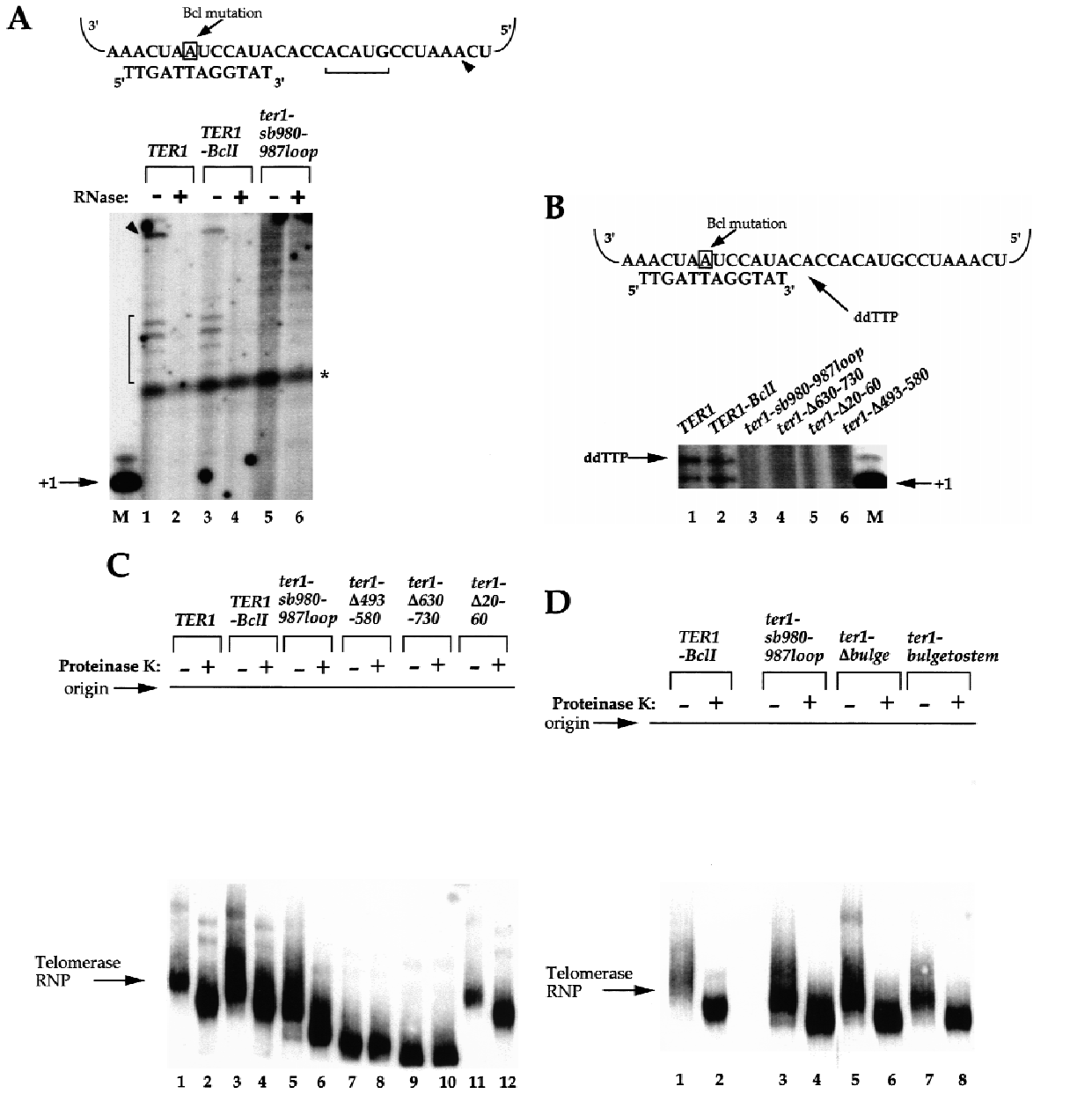

Figure 9. (*A*,*B*) Nonfunctional *TER1* mutants contain undetectable telomerase activity in vitro. DEAE fractions of extracts from strains expressing *TER1* alleles were assayed for *K. lactis* telomerase activity in vitro with primer KL13(12). The sequence of the *TER1* templating domain and the predicted alignment of the primer are shown. The boxed template residue corresponds to the site of the A → G mutation in the *TER1–BclI* strains examined. Terminal transferase-labeled KL13(12) primer is shown in M lanes, and the positions of the +1 products are marked correspondingly. (*A*) Telomerase reactions with DEAE fractionated extracts were carried out with all four dNTPs. RNase pretreatment (lanes *2*,*4*,*6*) consisted of incubation of extracts with 10 μg/ml RNase A at 25°C for 5 min. Mid-template products are denoted with brackets, and near-terminal products are marked with arrowheads. A background ladder of RNase A insensitive bands was detected in lanes *5* and *6* and is most likely caused by contaminating polymerases in the fractions assayed. The asterisk marks a nontelomerase generated background band described previously (Fulton and Blackburn 1998). (*B*) Reactions with DEAE fractionated extracts from *TER1* (lane *1*), *TER1–Bcl* (lane *2*), *ter1–sb980–987loop* (lane *3*), *ter1-Δ630–730* (lane *4*), *ter1-Δ20–60* (lane *5*), and *ter1-Δ493–580* (lane *6*) strains were carried out as in *A* but with ddTTP substituted for dTTP. (*C*,*D*) Profiles of *TER1* RNA-containing complexes in wild-type and mutant cell extracts. DEAE fractions of extracts from strains expressing *TER1* alleles were fractionated on nondenaturing gels and probed with a radio-labeled *TER1* DNA probe. The gel in *C* was run for 13 hr, whereas the gel in *D* was run for 10 hr.

extracts containing *TER1* and *TER1–BclI* RNA under the conditions used, confirming that the *Bcl*I mutation does not obviously affect the enzymatic properties of telomerase.

Evidence for copying of the template domain of *TER1* RNA came from termination of synthesis with the incorporation of dideoxy-TTP (dd-TTP) at the expected position along the template (Fig. 9B, lanes 1,2). Such termination reactions provided a sensitive assay for telomerase activity in this system. Telomerase activity was undetectable in extracts prepared from cells expressing the four nonfunctional mutant RNAs, when synthesis was examined in the presence of all four dNTPs (Fig. 9A, lanes 5,6; data not shown) or with ddTTP substituted for dTTP (Fig. 9B, lanes 3–6). No activity was detected with these mutant extracts in assays using different concentrations of oligonucleotide primer, or primers aligning to different regions of the *TER1* template, or under conditions where the incorporation of even a single templated nucleotide would be detected (data not shown). The mutant extracts did not contain *trans*-acting factors that inhibited telomerase activity, because mixing equal amounts of mutant and wild-type extracts did not inhibit the activity of the wild-type extract (data not shown). Because telomerase activity was detected in the *TER1–BclI* extract when it was diluted up to fivefold (data not shown), we estimated that the mutant extracts contained <20% of wild-type telomerase activity. The in vivo lack of function suggests that activity may in fact be abolished completely.

These results showed that the *ter1-Δ20–60*, *ter1-Δ493–580*, *ter1-Δ630–730*, and *ter1–sb980–987loop* mutants are defective in core enzymatic properties of telomerase, although it is possible that they may also be defective for other aspects of telomerase function. Any one or more of the following steps could be affected: proper RNA folding and/or assembly of a functional telomerase RNP complex, binding of core telomerase to accessory proteins or to the oligonucleotide primer and/or nucleotide triphosphate substrates, and polymerization.

Mutations in the TER1 *971–996 region result in a major conformational change in the RNA but allow assembly into an RNP complex*

We examined telomerase RNA and RNP complexes by native gel electrophoresis (Fig. 9C; see Materials and Methods). Fractions from *TER1* or *TER1–BclI* cell extracts contained a single major RNP complex (Fig. 9C, lanes 1,3). The mobility of the hybridizing species increased with pretreatment with proteinase K or phenol/chloroform extraction, indicating that the complex contains at least one protein component (Fig. 9C, lanes 2,4; data not shown). As expected, the RNP complex was also sensitive to RNase A treatment (data not shown). For the nonfunctional mutants *ter1-Δ493–580* and *ter1-Δ630–730*, a single major hybridizing species was observed, whose mobility remained unchanged with proteinase K digestion or phenol extraction (Fig. 9C, lanes 7–10; data not shown). Hence these two mutants appear to be defective in telomerase RNP assembly, which we propose accounts for their failure to exhibit telomerase activity in vivo or in vitro.

The mobilities of the *TER1*-hybridizing species of the nonfunctional *ter1-Δ20–60* mutant, before and after proteinase K treatment, were identical to those of *TER1* and *TER1–BclI* cell extracts (Fig. 9C, lanes 11,12). Thus, although subtle changes may not be detected by mobility in these gels, the *ter1-Δ20–60* mutant does not appear to be impaired for stable telomerase RNP assembly or have a greatly changed RNA conformation. Because telomerase in this mutant was nonfunctional in vitro, we suggest that the *ter1-Δ20–60* mutation affects an aspect of enzymatic activity rather than RNP assembly.

The *ter1–sb980–987loop* mutant contained a single RNP complex (Fig. 9C, lane 5), which migrated more slowly than its proteinase K-treated counterpart (Fig. 9C, lane 6). Strikingly, both this RNP complex and the proteinase K-treated species migrated significantly faster than their *TER1* or *TER1–BclI* counterparts (Fig. 9C, cf. lanes 3 with lanes 5 and 4 with 6). This fast migration cannot be accounted for by the removal of only four nucleotides in this RNA mutant. Hence, we conclude that the *ter1–sb980–987loop* RNA has a different conformation from wild-type, even although it is still assembled with at least some proteins. The protein composition of this enzymatically inactive mutant complex may be similar to that of the wild-type complex, as proteinase K treatment caused approximately the same degree of change in mobility for both types of complexes.

Results similar to those with the *ter1–sb980–987loop* mutant were found with the *ter1–Δbulge* and *ter1–bulgetostem* mutants (Fig. 9D, cf. lanes 3–8). Extracts from these strains also contained undetectable levels of telomerase activity in vitro (data not shown). Therefore, for each of these three mutants, both the mutant RNA itself and its RNP complex differed in conformation from wild type.

Discussion

Here we have determined the effects of mutations in *K. lactis* yeast telomerase RNA on the properties of telomerase in vivo and in vitro. A silent mutation marking the template of the mutant RNAs created a sensitive assay for the ability of mutant telomerases to add the marked telomeric repeats in vivo. By using *TER1* RNAs in a yeast strain completely deleted for wild-type *TER1*, any telomerase activity observed in vivo and in vitro was attributable only to the mutant RNA. By also examining the function of mutant *TER1* RNAs when they were coexpressed in cells with wild-type RNA, we tested the ability of mutant RNAs to compete with wild-type RNA for assembly with telomerase proteins, or other factors involved in the biogenesis and function of the telomerase complex. Conversely, by such coexpression we would also be able to detect cooperativity between mutant and wild-type RNAs, as was found for the 476GUG mutant of *S. cerevisiae* telomerase RNA (Prescott and Blackburn 1997b).

Small regions of TER1 *are critical for enzyme activity*

Four specific *TER1* sequences several hundred residues

from the template were critical for telomerase function in vivo and in vitro. This is the first example of specific nontemplate telomerase RNA residues that are essential for enzyme activity both in vivo and in vitro.

With wild-type enzyme, we observed a single RNP complex containing *TER1* RNA. Because this complex is functional, it must contain the RT protein subunit and may also contain other components such a homolog of Est1p. Two of the essential *TER1* RNA regions, defined by residues 493–580 and 630–730, map to the central part of the RNA (Fig. 2). Deletion of these regions result in a lack of telomerase RNP assembly; the simplest interpretation of our results is that these mutant RNAs are unable to assemble with the RT protein subunit. *S. cerevisiae* telomerase functions as a dimer with two RNAs (Prescott and Blackburn 1997a) suggesting that the *K. lactis* enzyme may be similar. Therefore, another possibility is that the mutant RNAs do not achieve proper secondary, tertiary, or quaternary structural conformation, and this prevents correct RNP assembly.

Critical nontemplate RNA residues may provide active site functions

The phenotypes of the *ter1-Δ20–60* mutant and the mutations in the *TER1* 971–996 region create the most compelling argument for a direct role of nontemplate telomerase RNA residues in active site functions. Interestingly, recent phylogenetic comparisons of closely related *Kluyveromyces* species have indicated that these two regions include some of the most conserved *TER1* sequences (Y. Tzfati and E.H. Blackburn, unpubl.). These mutations, which remove or substitute very small regions of the RNA, abolish detectable enzyme activity in vitro and enzyme function in vivo but are able to interact with at least some telomerase proteins. The *ter1-Δ20–60* telomerase RNP particle appears similar in size and shape to the wild-type complex. Thus, this mutant is likely to be defective at a step after RNP assembly. The *ter1–sb980–987loop*, *ter1–Δbulge*, and *ter1–bulgetostem* mutants delete or substitute residues in the same region of *TER1*, resulting in mutant RNAs that migrate considerably faster than wild-type under nondenaturing electrophoretic conditions, indicative of a significantly different conformation. However, this does not prevent the mutant RNAs from interacting with proteins. Therefore, these mutated nucleotides are likely to be involved in functions other than protein binding. These could include recognition and binding of DNA or dNTP substrates, positioning of the template region in the active site, and incorporation of nucleotides. Alternatively, this RNA may also fail to form a dimeric/oligomeric telomerase. However, unlike the *S. cerevisiase* 476GUG template mutant (Prescott and Blackburn 1997b), the *ter1–sb980–987loop*, *ter1–Δbulge*, and *ter1–bulgetostem* mutants are not rescued by coexpression with wild-type *TER1*.

Unpaired RNA residues provide critical enzymatic functions in both the spliceosome and the ribosome (recently reviewed in Green and Noller 1997; Staley and Guthrie 1998). For example, the 530-loop region of the 16S rRNA interacts with the mRNA and EF-Tu (for review, see Powers and Noller 1994) and mutations in the conserved domain V loop of 23S rRNA affect proper use of the aminoacyl tRNA substrate (Green et al. 1997). In the spliceosome, mutations in a loop region of U5 snRNA affect exon recognition, highly conserved U6 snRNA residues are essential for catalytic activity, and extensive RNA–RNA interactions between U2 and U6 snRNA residues are also necessary for catalysis (for review, see Nilsen 1998). On the basis of these precedents and the results reported above, we suggest that the 971- to 996-region of *TER1* may be involved in RNA–RNA, RNA–protein, or RNA–DNA interactions that are critical for the functioning of the telomerase active site.

More than half of TER1 *is dispensable for in vivo telomerase function*

Several stretches of *TER1* sequences are apparently fully dispensable for telomerase action in vivo. Furthermore, large deletions of the RNA maintain partial function in vivo, suggesting peripheral roles for these regions. These results are consistent with the findings that more than half of the human telomerase RNA is not essential for in vitro-reconstituted activity (Autexier et al. 1996; Beattie et al. 1998).

Deletion mutants lacking large sections of the RNA, *ter1-Δ77–424*, *ter1-ΔComb*, and *ter1-Δ1077–1229*, had decreased telomerase function in vivo. This could result from decreased stability of the mutant RNA, partial impairment of enzyme activity, or defective ability of the enzyme to interact with telomeres. The reduced function of mutant *ter1-Δ77–424* was attributable to the reduction in the steady-tate levels of mutant RNA. This deleted region may be involved in binding factors that stabilize newly synthesized *TER1* RNA or aid in its assembly into telomerase RNP, failure of which leads to RNA degradation.

The *TER1* domains defined by nucleotides 1077–1229 and the *ΔComb* deletion are not involved in the synthesis and stability of the RNA, as deletion of these sequences did not influence steady-state expression levels. In both cases, the mutant RNAs did not function in the presence of wild-type RNA, suggesting that they are unable to compete with wild-type RNA for the binding of one or more limiting factors. If this factor were the RT protein subunit, these mutants would be expected to demonstrate defects in telomerase RNP assembly or decreased in vitro telomerase activity. Alternatively, the mutant RNAs may be unable to bind properly proteins that mediate the interaction between telomerase and the chromosome ends. Candidates for such proteins have been identified in other species; for example, the EST1 protein in *S. cerevisiae*, and p80 and p95 in *Tetrahymena*.

The phenotypes of the partially functional *TER1* mutants reveal interesting properties of telomere length control. In these mutants, telomeres are steadily maintained for at least 200 generations at a length 100–200

nucleotides shorter than wild type. Unlike previously observed short and stable mutant telomeres (McEachern and Blackburn 1995), the telomeric repeats analyzed here are phenotypically wild type and thus the binding to proteins that regulate telomere length (such as RAP1p) is expected to be unchanged. These results indicate that the decreased ability of the mutant telomerases to add repeats results in shorter telomeres, reflecting a new balance between telomere addition and loss of telomeric DNA. Thus, the mechanism for telomere length regulation can respond to a reduction in telomerase activity. Telomerase enzyme of *K. lactis*, like that of *S. cerevisiae*, is nondissociative in vitro and is proposed to be stably bound to the telomeric end after one round of repeat synthesis (Prescott and Blackburn 1997a; Fulton and Blackburn 1998). It is possible that telomerase is part of the structure that caps the ends of the chromosomes and the interaction between telomerase RNA and other components of this cap structure may be a target of telomere length regulation.

Materials and methods

Strains, plasmids, and media

Yeast and bacteria were grown as described elsewhere (Sherman et al. 1986). The wild-type *K. lactis* strain used in this study is 7B520 (Ura^-, Trp^-, His^-, Ade^-) (Wray et al. 1987) and all other strains were derived from this. Transformation of *K. lactis* was done by electroporation, identical to the method described for *S. cerevisiae* (Becker and Guarente 1991). A deletion allele of *TER1* was constructed as follows. A 2.5-kb *Xho*I–*Eco*RI fragment containing *TER1* was cloned into the *URA3* marked yeast integrating vector YIP5. A 1.3-kb *Pme*I–*Sph*I fragment containing *TER1* was then removed from this construct and the remaining DNA was blunted with T4 DNA polymerase and circularized by ligation. This *TER1* deletion clone was used to transform *K. lactis* strain 7B520 to Ura^+ colonies. Transformants that had correctly 'looped in' the construct by homologous recombination were plated on medium containing 5-FOA and resulting 'loop out' colonies were screened for the Ura^-, *ΔTER* combination by Southern analysis.

Centromeric plasmids that can be maintained in *K. lactis* were constructed as follows. A 2.0-kb *Bam*HI–*Sau*3A DNA fragment containing the *K. lactis CEN2* was ligated to a 2.5-kb *Bam*HI–*Sal*I DNA fragment containing the *K. lactis ARS1B*. The resulting 4.5-kb *CEN–ARS* cassette was cloned into the *Aat*II restriction site of the *URA3* or *HIS3* containing yeast plasmids pRS306 and pRS303, to result in vectors pKL316 and pKL313, respectively. These plasmids are maintained stably in *K. lactis*; 0.1% of the cells per doubling lost the plasmids when maintained in nonselective medium. The copy number of the vectors was determined by Southern analysis to be an average of two copies per cell.

The 2.5-kb *Xho*I–*Eco*RI fragment of *TER1* was cloned into pKl316 to result in plasmid pTERWT. A similar *TER1* fragment, containing a mutation in the template region that changes nucleotide A458 to G was cloned into plasmid pKL313 to result in pTERBclI. The A458G mutation creates a *Bcl*I restriction site in the template of *TER1*. Mutant versions of pTERBclI were constructed by oligonucleotide mediated site-directed mutagenesis as described elsewhere (Kunkel et al. 1987). All mutant clones were confirmed by DNA sequencing of relevant fragments of the constructs.

A *GAL1-TER1* allele was constructed as follows. A 1.2-kb DNA fragment containing the *S. cerevisiae GAL1* promoter and *CYC1* terminator was cloned into pKL313 to result in pGAL–KL313. A *Bam*HI site was introduced in the *TER1* sequence of pTERBclI at position 21 by site-directed mutagenesis. The *Bam*HI–*Eco*RV fragment containing *TER1* was removed from this construct and cloned into pGAL–KL313 (*Bam*HI and *Sma*I digested) to result in the *GAL–TER1* allele. This places nucleotide 22 of *TER1* ~50 nucleotides 3′ of the RNA start site in the *GAL–TER1* construct. Thus, the *GAL–TER1* construct contains the *Bcl*I template mutation and replaces the first 20 nucleotides of *TER1* with 50 nucleotides of *GAL1* RNA sequence. The Δ77–424 deletion mutation was introduced into this plasmid by site-directed mutagenesis as above.

DNA analysis

Yeast genomic DNA was prepared from wild type or transformants grown in YPD or YP galactose media. Strains containing plasmids were grown in the appropriate minimal medium. The DNA was digested with restriction endonucleases, electrophoresed in 0.8% agarose gels in 1× TBE buffer, and blotted onto Hybond-N+ (Amersham) membrane. The DNA was cross-linked to the membrane using a Sratalinker 1800 (Stratagene). Hybridization of the blots to DNA probes was done according to Church and Gilbert (1984). For oligonucleotide probes, hybridization was carried out for 4 hr, whereas for random primed DNA probes hybridization was carried out overnight. The 5′ radio-labeled oligonucleotide Klac1–25 (ACGGATTTGATT-AGGTATGTGGTGT) was hybridized to blots at 55°C to visualize mutant and wild-type telomeric repeats. To detect *Bcl*I-marked telomeric repeats specifically, the 5′ radiolabeled oligonucleotide KTelBcl (GAT$\underline{C}$AGGTATGTGG) was hybridized to the blots at 40°C. Two 5-min washes were carried out at 55°C for the Klac1–25 oligonucleotide and 42°C for the KTelBcl oligonucleotide, respectively. Where applicable, DNA blots were first hybridized to the KTelBcl probe, stripped by washing with 0.4 N NaOH solution and then hybridized to the Klac1–25 probe. The hybridized blots were exposed to film or PhosphorImager screens. Quantitation of bands was done using the ImageQuant program.

RNA analysis

Total RNA extracted from *K. lactis* strains was electrophoresed in 1.0% agarose, 1× TBE gels containing 7% formaldehyde and blotted to Hybond-N^+ in 10× SSC buffer. The membrane was subjected to cross-linking and hybridized to random primed DNA probes as above. For the gels shown in Figures 5 and 9B,C the radio-labeled probe used was a random-primed mixture of two PCR fragments spanning nucleotides 26–277 and 704–1273 of *TER1*, whereas for Figure 6C only the *TER1-704–273* PCR fragment was used. For detecting RP59 mRNA, a DNA fragment containing the entire *RP59* coding region was used as the probe.

In vitro telomerase assays

Partially purified telomerase fractions were prepared as described previously (Fulton and Blackburn 1998) with the following modifications. Extracts were prepared from 3 to 4 litres of cells, at optical density (OD_{600}) of 2.0, for each plasmid bearing strain (~10–14 grams of cell pellet for each strain). Cells were grown in selective medium to OD_{600} of 2.0, then diluted four- to

fivefold in YPD medium and allowed to continue dividing for about three generations. Cells were harvested and frozen in liquid nitrogen as strings of noodles. Subsequently, frozen cell noodles were homogenized into a powder with a prechilled mortar and pestle by grinding under liquid nitrogen for ~20 min. The powdered cells were resuspended in two volumes of TMG [10 mM Tris-HCl (pH 8.0), 1.2 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA] buffer and S100 fractions were obtained as in Cohn and Blackburn (1995). S100 supernatants were adjusted to 0.35 M NaOAc (pH 8.0) and loaded onto 2-ml DEAE–agarose columns equilibrated in 0.35 M NaOAc (pH 8.0)/TMG. Elutions were collected in 0.5 M, 0.7 M, 1 M, and 2 M NaOAc (pH 8.0)/TMG, respectively. Fractions were assayed for *TER1* RNA by dot blot analysis. Peak fractions containing *TER1* RNA at 0.7 M NaOAC were pooled, dialyzed in TMG, and used for further analysis. Extracts from different mutant strains were normalized for the amount of *TER1* RNA by dilution with TMG. Assays for telomerase activity were carried out as previously described (Fulton and Blackburn 1998).

Analysis of RNP complexes

For observation of telomerase RNA-containing complexes, DEAE fractions were first incubated in 10 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$ and either 1 μg/ml proteinase K or water at 37°C for 45 min. RNase pretreatment consisted of incubation of the DEAE fractions with 10 μg/ml RNase A at 25°C for 5 min before proteinase K addition. The samples were then adjusted to 50 mM Tris-acetate (pH 8.0). Heparin was added to 1 μg/ml and the samples were loaded on a 3.5% polyacrylamide (acrylamide:bisacrylamide, 76:1), 0.45% agarose gel. Electrophoresis was carried out in 50 mM Tris-acetate (pH 8.0) at 15 V/cm for 10–13 hr at 4°C. The gels were electroblotted to Hybond-N^+ membrane in 0.5× TBE buffer for 2 hr at 70 V and hybridized to random-primed *TER1* probes as above. Figure 9 shows samples electrophoresed in the presence of heparin. Omitting heparin caused no obvious changes in the mobility of complexes, although resolution was decreased.

Sequence analysis

RNA secondary structures were predicted using the Mfold program available at the web site http://ibc.wustl.edu/~zuker/rna. All other DNA and RNA sequence analysis was carried out with the genetics computer group software package (version 9, University of Wisconsin, Madison).

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