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Identification of telomerase from Kluyveromyces lactis and analysis of the roles of non-template RNA sequences

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Identification of telomerase from *Kluyveromyces lactis* and analysis of the roles of non-template RNA sequences

by

Tracy Boswell Fulton

DISSERTATION

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This thesis is dedicated to

Mom and Dad
for bringing me into the world.
Now I understand.

Scott
for riding with me on this roller coaster.
Your love and support make me who I am.

Acknowledgements

There are so many people I want to thank. Good thing this isn't the Oscars--I'd surely get booted off the stage.

I first want to thank my advisor, Liz Blackburn. Liz has an abundance of energy, patience, and enthusiasm. On several occasions, she managed to single-handedly rekindle my passion for my thesis project when I was feeling particularly weary and uninterested. Liz also was incredibly supportive of me pursuing interests outside of the bench, even those that took me away from said bench. Without this freedom, I wouldn't have worked as hard and probably wouldn't have made it this far. Another wonderful quality of Liz's is an amazing ability to make me feel like what I say matters. Finally, Liz took a couple of guinea pigs off of my hands, for which my husband will be forever grateful.

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Jen Frazier has been my closest friend for the last six years. It's a huge understatement to say we went through a lot together. She is involved in some way in all of my favorite exploits including: discovering how sleep deprivation affects your core body temperature, expounding on the 'Jughead' protein for our Macromolecules exam, my bachelorette party (thanks also to Antoine), accidentally wearing the same outfit almost every day, road trips, jaunts to Noah's (I'll never eat Sun-Dried Tomato Shmear again), countless hours at Spinelli's (oh why did they forsake us?), and a recent three hour nine course lunch at the French Laundry...the list goes on and on. Of course there are also sad memories. Jen was there too--usually with her arm around my shoulders. I hope I've been as good a friend to her. Here's to us.

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Acknowledgements (Published Materials)

The text of Chapter Two of this dissertation is a reprint of the material as it appears in *Molecular and Cellular Biology*.

The text of Chapter Three is a reprint of the material as it appears in *Genes and Development*. I contributed to this work by carrying out all *in vitro* assays (the majority of which are not published here), including preparation of partially purified telomerase from each strain assessed. The essence of this work is shown in Figure 9.

The text of Chapter Four is a reprint of the material as it appears in *Science*. Aside from the initial phylogenetic analysis (on which Dudy had been working for some time on his own), the first co-author and I contributed to this work quite equally. We both made strains, passaged cells, and analyzed cloned telomere sequences. In general, he carried out *in vivo* telomere analyses, and I prepared partially purified telomerase and conducted the *in vitro* assays.

E. H. Blackburn, the last co-author listed on each publication, directed and supervised the research that forms the basis for the dissertation.

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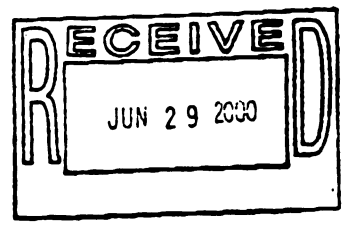

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Identification of telomerase from *Kluyveromyces lactis* and analysis of the roles of non-template RNA sequences

Tracy Boswell Fulton

Abstract

Telomerase, a ribonucleoprotein reverse transcriptase, carries its own template located within the integral RNA component of the enzyme. Telomerase copies the sequence of the RNA template, thereby replenishing and maintaining telomeric DNA. To further understand the action of telomerase and its relationship to telomere dynamics, I have undertaken a biochemical analysis of telomerase from the budding yeast *Kluyveromyces lactis*. Telomerase was partially purified from cells and analyzed *in vitro*, in conjunction with *in vivo* analyses of telomerase behavior and telomere dynamics. Telomerase from *K. lactis* catalyzes only a single round of telomeric repeat synthesis and remains bound to the elongation products. *K. lactis* telomerase exhibits an unusual behavior: stalling at specific positions along the template. Stalling is exacerbated by increasing the amount of complementarity between the DNA primer or product and the RNA template, suggesting stalling is influenced by extensive base-pairing between the enzyme and its substrate. We also investigated the influence of specific telomerase RNA sequences on telomerase activity and telomere maintenance. Four small deletions completely abolish activity *in vitro* and *in vivo*. In addition, we discovered a pairing region composed of sequence just 5' of the template and a region several hundred nucleotides away that comprises the template boundary of telomerase. This structure restricts reverse transcription to the template portion of telomerase RNA.

Elizabeth H. Blackburn, Thesis Advisor

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CHAPTER ONE

Introduction

Telomeres and the end replication problem

The genetic material of eukaryotes is organized into linear chromosomes, unlike the circular chromosomes of bacteria and many viruses. Though it seems unnecessary to state, eukaryotic chromosomes have 'ends.' Not much attention was paid to these ends until well into the 20th century, when in 1938 Hermann J. Muller, a *Drosophila* geneticist, coined the term 'telomere,' referring to the end-part of the chromosome. Implicit in Muller's term were two definitions: the physical end of the chromosome, and also a 'gene' or structure with special properties, able to keep the end from fusing with other ends or with internal breaks. Barbara McClintock, through her own work in maize in the 1930s, also came to the conclusion that chromosome ends have special characteristics distinguishing them from internal regions of DNA.

Studies on telomeres were strictly cytogenetic until the 1960s, when the structure of DNA had been revealed, and the mechanism for its replication was beginning to be elucidated. Watson pointed out that the complete replication of linear DNA poses a special problem (Watson 1972). Standard DNA polymerases catalyze synthesis in the 5' to 3' direction, primed by short RNA primers. Replication is completed when RNA primers are removed and the gaps filled in by DNA polymerase. However, when the terminal RNA primers are removed, DNA polymerase is left without a primer to initiate replication of the 3' end portion of the parent strand, resulting in an unreplicated portion of DNA. Without some way to replenish the terminal portions of DNA, the problem would presumably be compounded upon each cell division, resulting in progressive loss of the genetic material.

Several suggestions for how the 'end replication problem' could be avoided were proposed. Concatemerization, which would effectively eliminate ends, was suggested as a means for complete replication of T7 DNA (Watson 1972). Implicit in this model was the presence of repetitious sequences at the chromosome termini. Cavalier-Smith suggested that terminal palindromic sequences at the 3' ends of chromosomes could fold back and self-prime DNA synthesis on the opposite strand (Cavalier-Smith 1974). Both models emphasized the hypothesis that chromosome ends, at least in a given organism, might have a common sequence. To analyze the end replication problem any further, it would be necessary to study and understand the sequence and structure of chromosome ends, which seemed a difficult problem to surmount, given the low concentration of ends in typical eukaryotic organisms.

The organism in which telomeric sequences were first analyzed, the ciliate *Tetrahymena thermophila*, contains a genome that is developmentally broken into hundreds of very small pieces, including a high copy amplified linear rDNA. Thus, in this system, a high concentration of ends exists relative to the rest of the genomic material. Using the highly abundant *T. thermophila* rDNA, Blackburn and Gall were the first to actually observe telomeric fragments molecularly, visualized as fragments of heterogeneous length on agarose gels (Blackburn and Gall 1978). They also were able to use direct chemical methods to determine the *Tetrahymena* telomere sequence. The rDNA terminus contained a tandemly repeated six base-pair sequence, TTGGGG. For convenience, this sequence, along with the corresponding subsequently discovered sequences from different species, is termed the 'G strand', oriented 5' to 3' extending away from the centromere. Such repetitive TG-rich sequences were also identified in the ciliates *Oxytricha* and *Stylonychia* (Oka et al. 1980; Klobutcher et al. 1981) but questions were

raised as to whether these sequences would bear any resemblance to telomeres of higher eukaryotes. It was soon shown that the termini of *T. thermophila* chromosomes could function as telomeres in yeast, and that yeast had its own similar TG-rich repeat at chromosome termini (Szostak and Blackburn 1982; Shampay et al. 1984). Telomeric repeats have now been identified in numerous species by either direct sequencing or by cross-hybridization to known telomere sequences (for review, see (Henderson 1995))

While all telomeric sequences identified so far adhere to the rule that they are arranged as tandem copies, the length, precision, and total number of repeats vary considerably from species to species. In fact, telomere repeat number is often variable within one organism (although length within that range is regulated). For example, any given *T. thermophila* telomere may have 50-70 repeats. The length heterogeneity results in telomere restriction fragments having a "fuzzy" appearance on Southern blots. Interestingly, initial analysis of telomere length dynamics revealed that when *T. thermophila* cells are kept in log-phase growth in culture, an overall telomere lengthening occurs (Larson et al. 1987). These results substantiated earlier observations in trypanosomes (Bernards et al. 1983), and clearly demonstrated that the so-called end replication problem was being surmounted. To explain these phenomena, the equilibrium model for telomere replication was proposed, according to which sequences are lost from chromosome ends during conventional DNA replication, but replenished by the addition of sequences by a special telomere replication or addition mechanism (Shampay and Blackburn 1988).

A model for telomere maintenance by telomerase

Two models were proposed to explain how telomere addition could occur. The first model posited that recombination or polymerase slippage on repetitive sequences could account for net addition at telomeres (Walmsley et al. 1984). The second model arose from observations that yeast telomeric repeats are added to introduced *T. thermophila* chromosome ends without mixing of sequences, arguing against recombination as a method for generating the new telomere. This led to the proposal that an unidentified terminal transferase could add sequences to telomeres de novo (Shampay et al. 1984).

The terminal transferase model was supported in 1985 with the discovery of an enzyme from *T. thermophila* that synthesizes telomeric repeats *in vitro* (Greider and Blackburn 1985). This organism was wisely chosen as a source of this enzyme, since during macronuclear development telomeres are added to many thousands of chromosome ends. The *in vitro* assay used to identify the enzyme consisted of incubating partially purified *T. thermophila* extracts with synthetic single stranded oligonucleotides (TTGGGG oligomers) designed to mimic the telomere end. An activity was found that incorporated radiolabeled dGTP and dTTP onto the primer with 6-base periodicity, producing a characteristic 'ladder' of products when visualized on a sequencing gel. Telomeric primers were required for polymerization to take place, but the input primer could consist of repeats from other species. For example, an *S. cerevisiae* telomeric repeat primer (a $G_{1\text{-}3}T$ oligomer) was elongated quite efficiently by *T. thermophila* cell extracts. Interestingly, the sequence added to the yeast telomeric primer appeared to be the *T. thermophila*-specific ladder and not the product predicted if the irregular yeast sequence had been added. This indicated that the telomeric sequence to be added was not determined by the input primer. Several experiments supported the idea that the

enzyme activity elongated primers with a specific sequence one nucleotide at a time (as opposed to ligating large sequence blocks onto the primer). But how was such specific addition templated?

The abolishment of this terminal transferase activity from *T. thermophila* cell extracts by RNase, micrococcal nuclease, or proteinase K suggested the enzyme was an RNP (Greider and Blackburn 1987). A 159 nt-long RNA that copurified with the activity was sequenced and was found to contain an internal 5'-CAACCCCAA-3', complementary to one and a half telomeric repeats. RNase H-mediated cleavage of this region, directed by complementary oligonucleotides, inactivated telomerase activity (Greider and Blackburn 1989). These results suggested this enzyme carried an RNA template with it--a novel finding for any polymerase. Mutations in the proposed template region were copied onto telomeres *in vivo*, confirming the templating function of this region, and that telomerase is indeed responsible for telomere maintenance (Yu et al. 1990).

On the basis of these experiments, the model for telomeric repeat synthesis by telomerase was proposed (Greider and Blackburn 1989). In this model (Fig. 1), telomerase interacts with the 3' end of the chromosome through base-pairing with the template region. The end is then elongated by the addition of nucleotides in the 5' to 3' direction, until the 5' end of the template is reached. At this point telomerase pauses, which would account for the more pronounced *in vitro* products at 6-base intervals. Next a translocation step repositions the DNA at the 3' end of the template, again through base-pairing, so that initiation of another round of repeat synthesis can occur.

Telomerase activity has been identified and analyzed in many other systems to date, supporting many aspects of the above model. Polymerization activities from the ciliates *Oxytricha nova* and *Euplotes crassus* were both characterized by RNase sensitivity, addition of species-specific telomeric sequences to a telomeric oligonucleotide, and banding patterns corresponding to the length of the organism's repeat (Zahler and Prescott 1988; Shippen-Lentz and Blackburn 1989). These traits have become the defining behavioral characteristics of the telomerase reaction *in vitro*, and have led to the identification of telomerase activity from numerous other eukaryotes including human cell lines (Morin 1989), mouse (Prowse et al. 1993), chicken (Venkatesan and Price 1998), *Xenopus* (Mantell and Greider 1994), several yeast species (Cohn and Blackburn 1995; Lue and Peng 1997), *Paramecium* (McCormick-Graham and Romero 1996), plants (Fitzgerald et al. 1996), *Plasmodium* (Bottius et al. 1998) and parasitic protozoa (Cano et al. 1999).

Processivity: a trait not shared among all telomerases

The conservation of the telomerase mechanism in such diverse organisms underscores the importance of this enzyme. However, certain aspects of the telomerase model have not been conserved in all species. The ability of telomerases from most species to catalyze the addition of multiple repeats onto a single primer has been attributed to *in vitro* processivity (Greider 1991). However, the interspersion of repeats in *T. thermophila* cells containing wild-type and mutant alleles of the telomerase RNA gene suggested non-processive or distributive repeat addition *in vivo* (Yu et al. 1990; Yu and Blackburn 1991). Similar results were seen in *S. cerevisiae* cells: telomeres cloned from heteroallelic strains carrying mutant and wild-type telomerase RNA genes contained mixed repeats, arguing against processive addition of multiple repeats (Prescott and

Blackburn 1997b). *T. thermophila* telomerase clearly has the ability to dissociate from elongated products under specific conditions *in vitro* (Collins and Greider 1993; Lee and Blackburn 1993). However, *S. cerevisiae* telomerase remains bound to post-elongation reaction products *in vitro*, neither dissociating nor translocating to catalyze multiple rounds of repeat synthesis (Prescott and Blackburn 1997b). This aspect of telomerase action is explored further in this thesis.

The highly processive nature of *Tetrahymena* telomerase *in vitro* is thought to be influenced by at least two parameters: the nature of the contact between the primer or product with what is termed the 'lagging product site,' and the existence of a minimal template RNA/product DNA hybrid during the reaction (Fig. 1). The lagging product site (also called anchor or second site) is thought to interact with primer or product DNA residues 5' of the template-binding region (Collins and Greider 1993; Lee and Blackburn 1993; Benjamin et al. 2000; Ware et al. 2000). Cross-linking studies show that *Euplotes* telomerase has an anchor site for the DNA primer that is distinct from the binding site for the DNA (Hammond et al. 1997). The shunting of elongated DNA into this site could account for the ability of telomerase to remain bound to its product despite having to peel the template away with each round of repeat synthesis in order to begin anew. Processivity is also thought to depend on there being only a minimal (2-3 bp) RNA-DNA hybrid formed during the elongation reaction (Hammond and Cech 1998).

Processivity may also depend on telomerase multimerization and/or mechanisms influenced by the developmental state of an organism. Higher order telomerase complex formation is developmentally regulated in *Euplotes crassus* (Greene and Shippen 1998). When gentle purification methods are used to fractionate macronuclear

telomerase complexes from this organism, telomerase can exist in complexes of different sizes. Interestingly, the larger complexes exhibit a greater degree of processivity than the smaller ones. *S. cerevisiae* telomerase functions non-processively as a dimer (Prescott and Blackburn 1997b; Prescott and Blackburn 1997a), but it is unclear whether other complexes with varying degrees of processivity exist in the cell. The link between processivity and telomerase complex formation is further explored in this thesis (see appendix to Chapter 2).

The RNA component of telomerase

As described, the first telomerase RNA (TER) subunit was identified from *T. thermophila* (Greider and Blackburn 1987). Subsequently, a TER was identified in *Euplotes crassus* (Shippen-Lentz and Blackburn 1990) suggesting that the use of an internal RNA template was a conserved mechanism for telomere addition. However, the two RNAs share little sequence identity. The cloning of telomerase RNAs from six more *Tetrahymena* species and *Glaucoma chattoni* by cross-hybridization with the *T. thermophila* gene revealed surprisingly low overall sequence similarity even among very closely related organisms. For example, although a 23 nucleotide region of sequence identity centered on the template was conserved, only 72% telomerase RNA similarity was found between two ciliated protozoa with 98% similar rDNA sequences (Romero and Blackburn 1991). This work led to a conserved secondary structure prediction, which has been supported by solution probing of isolated RNA using both chemical probes and specific nucleases (Bhattacharyya and Blackburn 1994; Zaug and Cech 1995). The model predicts the presence of several stem-loop structures, and places the template in a single stranded region, consistent with the need for the template to be accessible for primer alignment and polymerization. A sequence upstream of the

template is conserved among ciliates, and may play a role in template boundary demarcation (see below and Chapter 4). Since that time, over 20 ciliate telomerase RNAs have been identified which share features of the secondary structure prediction (Lingner et al. 1994; McCormick-Graham and Romero 1995)

Telomerase RNAs have been identified in numerous other species, including seven budding yeasts (Singer and Gottschling 1994; McEachern and Blackburn 1995; Tzfati and Blackburn 2000), birds, amphibians, fishes, mouse, human, and other mammals (Blasco et al. 1995; Feng et al. 1995; Tsao et al. 1998; Chen et al. 2000). All of these RNAs contain a species-specific templating domain that is complementary to an entire telomeric repeat plus an additional portion of this sequence, resulting in small direct repeats at either end of the domain. Before the yeast TERs were identified, some controversy existed as to whether yeast telomeres would be specified by telomerase. The repeats of *Saccharomyces cerevisiae* telomeres are imprecisely repeated, which seemed in conflict with the precise nature of telomerase in vitro. However, two yeast TERs were identified around the same time, using two very different approaches. The TER gene from the budding yeast *Kluyveromyces lactis* was identified by direct probing of a BAL31-digested genomic Southern blot with the known telomeric repeat from this organism (McEachern and Blackburn 1995). This approach was made possible by the fact that telomeric repeats from *K. lactis* are unusually long, 25 base pairs (McEachern and Blackburn 1994). The fragment identified using this method contained a 30 nt-long potential template sequence, bordered by direct repeats. To verify that this fragment was part of telomerase RNA, a mutation was introduced into the putative template region designed to incorporate a *Bgl*II restriction site at telomeres. Indeed, the sequence of telomeric repeats in this mutant were altered: the *Bgl*II-containing repeats could be

detected by hybridization, and could be removed by digestion with the *Bgl*II enzyme. The *S. cerevisiae* telomerase RNA gene was identified in a screen designed to isolate genes that when overexpressed, alleviated telomeric silencing (Singer and Gottschling 1994). This gene contained a ~17 nt-long potential templating domain. When mutated, altered telomeric sequences were incorporated in vivo, confirming the templating function of this RNA.

Non-ciliate TERs diverge widely in terms of length as well as primary sequence. Vertebrate telomerase RNAs are considerably larger than ciliate TERS, ranging from 400 to 450 nucleotides, and share only 58% similarity (Blasco et al. 1995; Feng et al. 1995; Chen et al. 2000). Yeast TERs are even larger than the vertebrate TERs, around 1.3 kb, raising interesting questions about the potential functions of such a large portion of non-templating RNA. Since these RNAs vary remarkably in their size and sequence, comparison of the telomerase RNA sequences from mammals, yeast, and ciliates initially revealed no obvious similarity among these three distantly related groups. However, a recent phylogenetic comparison of 34 vertebrate telomerase RNAs revealed a core structure similar to that of ciliates. In addition, the vertebrate TER structure contains domains characteristic only to vertebrate TERs (Chen et al. 2000). Work currently being done suggests budding yeast TERs have a common core structure, but it is not yet apparent if it shares elements of the ciliate and vertebrate core structures (Tzfati and Blackburn 2000). Interestingly, the ciliate, yeast, and mammalian RNAs each have a domain responsible for binding a stabilization protein unique to that species (Lingner et al. 1994; Mitchell et al. 1999; Seto et al. 1999).

Functional analyses of non-template portions of telomerase RNA

Up until my thesis work began, we only had specific information about the role of the template portion of telomerase RNA in the mechanism of telomere addition. However, it was always assumed, and has become quite clear since then, that non-template portions of telomerase RNA play very important roles in telomerase activity. The first indication of this came from functional reconstitution experiments, in which *in vitro* transcribed wild-type or mutant *Tetrahymena* TER was introduced to MNase-treated cell extracts (Autexier and Greider 1995). In this partially reconstituted system, most analysis focused on internal template mutations. One non-template mutation, however, underscored the importance of a conserved sequence element 5' of the template. Mutations in this region resulted in polymerization beyond the template boundary, suggesting a role for telomerase RNA in limiting DNA synthesis. Similar behavior was later observed in an *S. cerevisiae* telomerase mutant, and is discussed at length in Chapter 4. An interspecies substitution of telomerase RNA from the ciliate *Glaucoma chattoni* into *Tetrahymena thermophila* cells produced functional telomerase, despite the lack of primary sequence similarity between the two RNAs (except for a 23 nucleotide region of identity centered on the template). However, this chimeric enzyme had aberrant endonuclease activity and exhibited weak activity *in vitro* and in vivo. This experiment demonstrated the importance of RNA secondary structure in telomerase function, and highlighted the importance of non-template domains (Bhattacharyya and Blackburn 1997).

The functional reconstitution assay used by Autexier and Greider (described above) revealed several different conserved regions of the RNA important for levels of telomerase activity. However, elimination of a highly conserved pseudoknot had

surprisingly little effect on *in vitro* activity (Autexier and Greider 1998). Licht and Collins used an alternative approach, with *Tetrahymena* telomerase subunits (*in vitro* transcribed RNA and *in vitro* translated reverse transcriptase component p133) expressed and assembled in rabbit reticulocyte lysates (Licht and Collins 1999). They showed that both RNA sequence and sequence positioning in a small region 5' of the template is critical for RNP assembly and consequently telomerase activity. Deletion of this region did not significantly alter telomerase activity in the partially reconstituted system, suggesting significant differences between the two systems. Interestingly, results in an *in vivo* system for *Tetrahymena* telomerase analysis contradicts the pseudoknot findings from the reconstituted systems: elimination of the pseudoknot structure abrogates activity, and prevents stable *in vivo* assembly of the telomerase RNA with the p133 catalytic subunit (Gilley and Blackburn 1999).

Functional analysis of non-template portions of *K. lactis* telomerase RNA have been carried out and are described in Chapter 3.

The telomerase reverse transcriptase component

While scientists in the telomerase field gained much information about the telomerase RNA subunit, catalytic protein components eluded discovery for close to ten years. Isolation of telomerase from *Tetrahymena* resulted in the identification of two proteins that initially looked promising as catalytic component candidates, p80 and p95, that co-purified with activity (Collins et al. 1995). p80 demonstrated telomerase RNA binding capabilities (Collins et al. 1995; Gandhi and Collins 1998), and homologs were subsequently identified from rat (Nakayama et al. 1997), mouse, and human (Harrington et al. 1997). p95 demonstrated telomeric primer-binding activity (Collins et al. 1995;

Gandhi and Collins 1998), but no homologs have been identified in other systems. These subunits have since been foundt to be non-essential for *in vitro* telomerase activity (Gandhi and Collins 1998; Licht and Collins 1999), and are currently thought of as 'accessory factors,' necessary perhaps for assembly of a 'holoenzyme' *in vivo*.

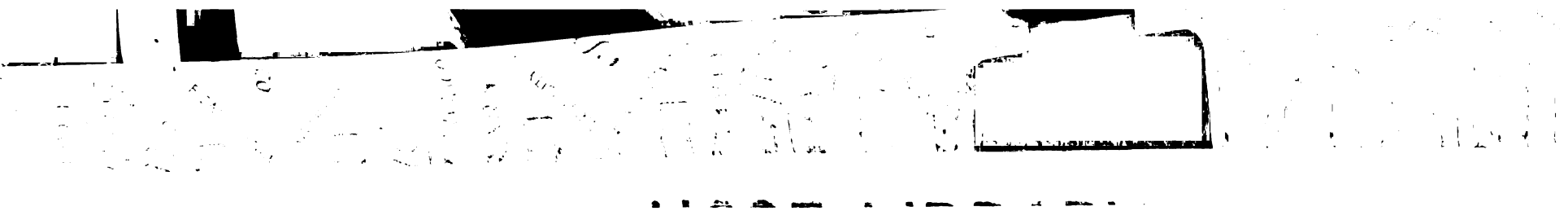
Success was finally met in work with (yet again) a ciliate, when biochemical purification of *Euplotes aediculatus* telomerase revealed two proteins of 123 kDa and 43 kDa (Lingner and Cech 1996). The gene encoding the 123 kDa subunit was found to be the homolog of an *S. cerevisiae* gene, *EST2* (Lingner et al. 1997), which was identified in a screen for genes involved in telomere maintenance (Lendvay et al. 1996). The amino acid sequences of p123 and Est2p contain motifs common to all reverse transcriptases, as expected for an enzyme that directs RNA-templated DNA polymerization. Three invariant aspartate residues appear in conserved regions of both proteins, which are highly conserved among RT families and are known to form the catalytic pocket of RTs (Poch et al. 1989; Xiong and Eickbush 1990). Mutation of these residues in the *Euplotes* and yeast proteins resulted in loss of telomerase activity, telomere shortening, and cellular senescence in yeast (Lingner et al. 1997), providing evidence that these proteins are responsible for telomerase polymerization activity.

Genes encoding homologous reverse transcriptase subunits have been identified in fission yeast, mice, humans, and other ciliates (Harrington et al. 1997; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Bryan et al. 1998; Collins and Gandhi 1998; Greenberg et al. 1998). The proteins are known collectively known as TERTs (for telomerase reverse transcriptases). TERT subunits all contain a set of seven characteristic sequence motifs common to all reverse transcriptases: motifs 1, 2, A, B',

C, D, and E. The similarity between the RT motifs of different TERTs is generally high, but is higher between closely related organisms, such as mouse and human, or within the ciliate TERTs. Unlike other RTs, TERTs contain a large, basic amino terminus. Within this region, TERTS share a unique sequence feature, an N-terminal 'T motif' (Nakamura et al. 1997) which is required for telomerase activity in a human reconstituted system, and has been proposed to facilitate the protein's interaction with RNA (Weinrich et al. 1997). TERT proteins from ciliated protozoa have an additional region of homology of unknown function called the CP motif (Bryan et al. 1998). Recent mutational analysis of *S. cerevisiae* TERT specifically implicates the amino terminus in telomerase RNA binding (Friedman and Cech 1999), demonstrating that regions outside of the catalytic motifs are important for telomerase activity.

K. lactis as a model system

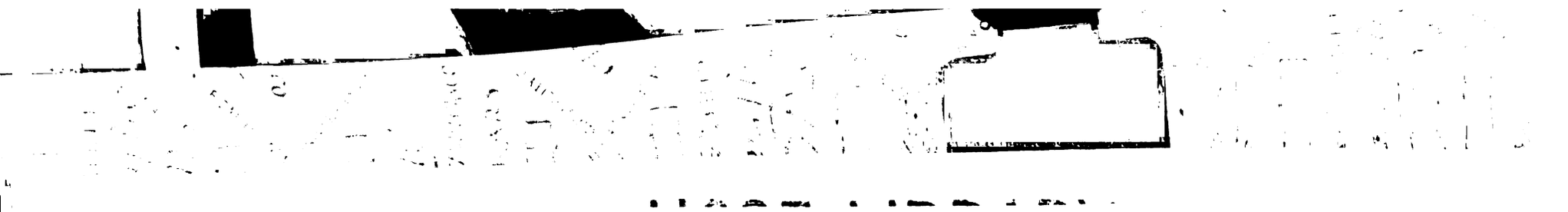
K. lactis has emerged as an extremely useful system for the study of telomeres and telomerase. Like *S. cerevisiae*, *K. lactis* is a genetically amenable budding yeast. As described earlier, the telomerase RNA gene has been cloned from *K. lactis* (TER1), and the templating domain identified (McEachern and Blackburn 1994). In various species, mutations in the template domain of the telomerase RNA moiety have resulted in telomerases that synthesize mutant telomere sequences in vivo. However, *K. lactis* offers certain advantages that have facilitated unambiguous analysis of these results. *K. lactis* telomeres are composed of long, perfectly repeated 25 base pair repeat units, unlike the degenerate, imprecise repeats at *S. cerevisiae* telomeres, and the extremely short (6-8 bp) repeats at ciliate and mammalian telomeres (summarized in Table 1). The length and precision of the telomeric repeats make telomere detection simple in *K. lactis*, and mutant repeats are readily distinguishable from wild type repeats. The long repeat



may also allow separation of telomere functions within the repeat that overlap in the shorter repeat units of many other organisms. In addition, the *K. lactis* genome lacks internal telomeric sequences that complicate telomere behavior and sometimes confound telomere analysis in *S. cerevisiae*. For these reasons, analysis of telomere dynamics in a multitude of *K. lactis* telomerase RNA mutants have led to a higher level of understanding of telomere dynamics.

The length of *K. lactis* telomeres is normally tightly controlled (McEachern and Blackburn 1994) but mutations in the telomerase template can result in dramatic changes in telomere length (McEachern and Blackburn 1995). The phenotypes of these mutants can in principle be attributed to either 1) alterations in the functionality of mutant telomeric DNA, or 2) changes in the enzymatic properties of mutant telomerase. The former concept is thought to explain the phenotype of one class of mutants with abnormally long telomeres. The phenotypic severity of these mutants is directly proportional to the loss of binding of the RAP1 protein to the mutant sequence produced at the telomere (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996). The conclusion from these studies is that RAP1 binding at telomeric repeats limits access of telomerase to the telomere terminus, thereby regulating telomere length (Krauskopf and Blackburn 1996). Other mutant phenotypes, however, are not readily explained by telomere sequence alterations. Specific template mutations outside of the RAP1 binding site produce extreme alterations of telomere length, some resulting in extremely long telomeres after a delayed period, and others resulting in short telomeres (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996).

Project rationale and thesis overview



I initially chose to study telomerase in *K. lactis* because it had been established as such a useful system for analysis of telomere length regulation. At the time, several classes of telomeric repeat mutants existed in *K. lactis* for which there were no counterparts in *S. cerevisiae* (which, for at least one class of mutants, is still the case). I reasoned that the identification of telomerase from *K. lactis* and subsequent analysis of the activity of these template mutants would be another step towards dissecting *in vivo* telomere dynamics. My initial question was whether aberrant activity of telomerase itself was contributing to changes in telomere length. It later became clear, however, that correlations between changes in telomerase activity and *in vivo* telomere length alterations in template mutants were only applicable in certain cases, specifically when telomerase activity was destroyed or partially compromised (Prescott and Blackburn 1997b; Roy et al. 1998).

K. lactis telomerase has the largest templating domain ever identified (30 nt). As described earlier, telomeric repeats in *K. lactis* are perfectly repeated 25-bp units, suggesting that the entire length of the templating domain is faithfully copied. As time went on, I became more interested in the obvious uniqueness of this enzyme in terms of its large template and *in vivo* precision. I set out to examine whether the behavior of *K. lactis* telomerase could reveal features of the telomerase mechanism that would be less pronounced, or perhaps nonexistent in more typical telomerases. To this end, I identified *K. lactis* telomerase activity and characterized some of its interesting and unique features, as described in Chapter 2. Later, I participated in two fruitful collaborations, both of which relied on the use of the *K. lactis* telomerase *in vitro* system that I had established. These collaborations both focused on the roles of non-template regions of telomerase RNA in telomerase activity *in vivo* and in vitro, the results of which appear in Chapters 3 and 4.

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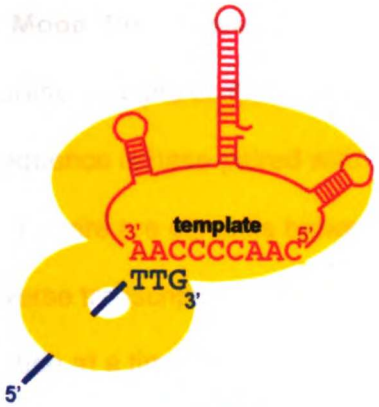
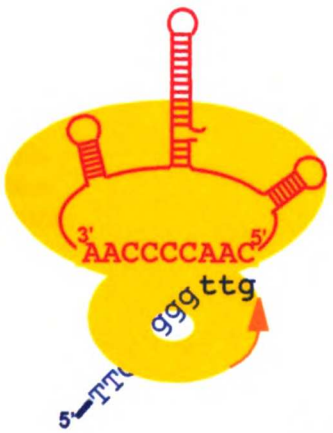
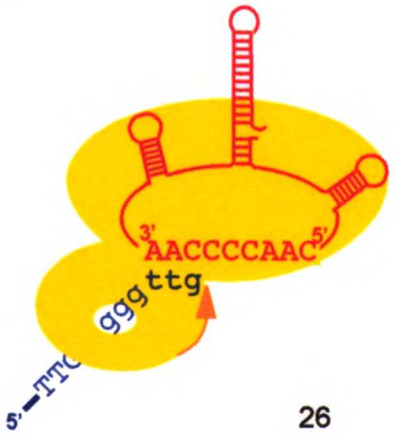


1. Substrate binding

2. Elongation



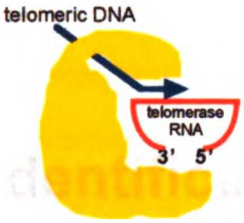
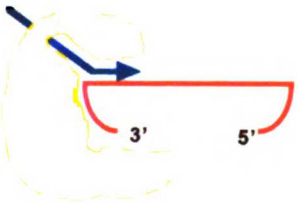
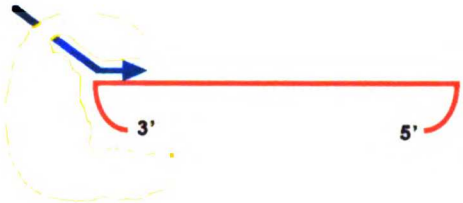
3. Pause at end of template

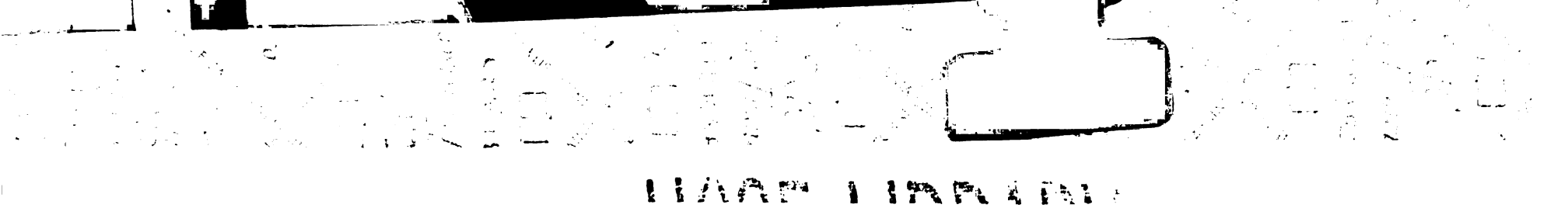


4. Translocation

Figure 1. Model for polymerization by *Tetrahymena* telomerase.

(1) Telomerase recognizes the DNA substrate, which in this case ends in TTG. This terminal sequence is base-paired with the 5' sequence of the RNA template. Presumably, there are contacts between protein and the DNA substrate also. (2) The reverse transcriptase protein component copies the RNA template, adding one nucleotide at a time to the 3' end of the DNA substrate. There is thought to be only a minimal RNA/DNA hybrid, and the remainder of the DNA product is displaced into another site in a protein component. (3) Polymerization stops when the end of the template domain is reached. (4) Translocation repositions the terminal sequence at the 3' end of the template, and additional template sequences can be added. Protein components are shown in yellow . RNA component is shown in red. Substrate DNA is shown in blue. The site of polymerization is marked by the orange arrow.

T. thermophila	*S. cerevisiae*	*Kluyveromyces lactis*	
159 nt	~1.3 kb	~1.3 kb	TELOMERASE RNA SIZE
9 nt	~17 nt	30 nt	TEMPLATING DOMAIN SIZE
			
short (6bp), precise	degenerate	long (25bp), precise	TELOMERIC REPEATS
multiple rounds of processive repeat synthesis or single round of dissociative repeat synthesis	single round of non-dissociative repeat synthesis	single round of non-dissociative repeat synthesis	IN VITRO REACTION



CHAPTER TWO

Identification of *Kluyveromyces lactis* telomerase: discontinuous synthesis along the thirty nucleotide-long templating domain

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ABSTRACT

Telomeres in the budding yeast *Kluyveromyces lactis* consist of perfectly repeated 25 base pair units, unlike the imprecise repeats at *Saccharomyces cerevisiae* telomeres and the short (6-8 bp) telomeric repeats found in many other eukaryotes. Telomeric DNA is synthesized by the ribonucleoprotein telomerase, which uses a portion of its RNA moiety as a template. *K. lactis* telomerase RNA, encoded by the *TER1* gene, is ~1.3 kb long and contains a 30 nucleotide templating domain, the largest ever examined. To study the mechanism of polymerization by this enzyme, here we identify and analyze telomerase activity from *K. lactis* whole cell extracts. This activity is shown to be telomerase based on its dependence on primer, specificity of the sequence synthesized, and telomerase RNA template dependency. In this study we exploit the length of the template and the precision of copying by *K. lactis* telomerase to examine primer elongation within one round of repeat synthesis. Under all *in vitro* conditions tested, *K. lactis* telomerase catalyzes only one round of repeat synthesis and remained bound to reaction products. We demonstrate that *K. lactis* telomerase polymerizes along the template in a discontinuous manner, and stalls at two specific regions in the template. Increasing the amount of primer DNA/template RNA complementarity results in stalling, suggesting that the RNA/DNA hybrid is not unpaired during elongation *in vitro*, and that lengthy duplexes hinder polymerization through particular regions of the template. We suggest that these observations provide an insight into the mechanism of telomerase and its regulation.

INTRODUCTION

Telomeres, the essential protein-DNA elements at the ends of most eukaryotic chromosomes, confer chromosome stability and constitute protective terminal caps for the genetic material of the cell (for review, see (45)). Telomeric DNA typically consists of tandem arrays of a precisely repeated 5-8 bp sequence (for review, see (17)). However, the telomeric repeat units of yeast species have greatly diverged in precision and length (4, 27). The budding yeast *Kluyveromyces lactis*, with a perfectly repeated 25 bp telomeric repeat unit (28), is one of several budding yeasts with exceptionally large telomeric repeats (27). In *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* the telomeric sequences are shorter, imprecisely repeated units (5'-TG_{1-3} -3' and primarily 5'-GGTTACA-3' respectively; (18, 36)). Despite their variability between species, all these telomeric sequences are specified by the enzyme telomerase.

Telomerase, a ribonucleoprotein (RNP) reverse transcriptase, facilitates the complete biosynthesis and maintenance of telomeres. Telomerase activity, as shown initially for the ciliate *Tetrahymena thermophila* (12) and subsequently for many other eukaryotes (3, 24, 25, 31, 35, 38, 44), is dependent on an integral telomerase RNA component (13, 14) which contains a sequence that serves as a template for telomeric DNA synthesis (14, 43). While in *T. thermophila* the templating domain is 5'-CAACCCCAA-3', complementary to one and a half telomeric repeats (14), in *K. lactis* it is a 30-nucleotide (nt) RNA sequence complementary to one and a fifth telomere repeat units (28). Specific mutations within the templating domain of both *T. thermophila* and *S. cerevisiae* telomerase drastically reduce or alter telomerase activity *in vitro* and *in vivo*, suggesting that bases in the template are not simply copied but play crucial roles in active site functions (8, 9, 34). Active site functions are also carried by the reverse transcriptase

protein component of telomerase (the hTERT gene product), which has been identified in *Euplotes aediculatus*, yeasts, and human cells (16, 23, 30, 32), confirming that telomerase requires both reverse transcriptase protein and RNA components.

Unlike the extensive RNA genome copying carried out by the more typical reverse transcriptases of viruses and retroposons, the polymerization activity of telomerase is restricted to copying the discrete template portion of the telomerase RNA. *In vitro*, telomerase elongates a telomeric DNA primer substrate, which aligns within the templating domain via Watson-Crick base pairing (14). The sequence of the oligonucleotide primer determines the positioning of the 3' end and therefore the site of initiation. Telomerase then extends the primer by polymerization of one nucleotide at a time along the RNA template to the 5' end boundary. In *T. thermophila* and *S. cerevisiae*, mutating the RNA sequence adjacent to the templating domain allows polymerization to proceed beyond the normal template. Hence telomerase RNA structures or interactions outside of the template also appear to prevent polymerization beyond the template boundary (1, 34). It is currently unknown whether during primer elongation a constant length of template RNA/product DNA hybrid is maintained (monotonic polymerization), or if the RNA/DNA duplex builds up, although it has been proposed that *T. thermophila* telomerase maintains a minimal 3-4 bp hybrid during elongation (21). During *in vitro* reactions, telomerases from most organisms catalyze multiple rounds of telomere repeat synthesis, and two modes of synthesis have been distinguished: distributive and translocative. In the distributive synthesis mode, *T. thermophila* telomerase dissociates from its DNA product, then binds a new primer to repeat the cycle (5, 21). Translocative synthesis, catalyzed by telomerase from *T. thermophila*, *E. aediculatus, Saccharomyces castellii* and human cells, involves repositioning of the 3' end of the newly elongated primer at the beginning of the template

without release of the product (for review, see (10)). Such translocation and the resulting processive synthesis of multiple repeats on a single primer *in vitro* is influenced by interactions of the 5' end of the primer with an anchor site within a protein and/or RNA component of telomerase (5, 15, 20).

Synthesis of small repeats (such as the 6 nt repeat of *T. thermophila*), requires minimal relative movement within the telomerase RNP of the built-in RNA template as it crosses the catalytic site of the TERT protein (41). However, the templating domains of the yeast telomerase RNAs that have been identified, *TLC1* RNA from *S. cerevisiae* and *TER1* RNA from *K. lactis*, are considerably larger, posing interesting mechanistic challenges to telomere repeat synthesis. *TLC1* RNA contains a templating domain maximally 17 nt long (39). An 11 nt portion of this domain has been shown to be copied (34), although it is rarely copied in its entirety *in vitro*, resulting in a series of incomplete single round extension products (3, 23, 33, 34). Since the frequent stalling exhibited *in vitro* produces variable 3' end sequences, alignment of the partially redundant template sequence at telomeric ends can presumably take place in multiple registers, and may underlie the degeneracy of the telomeric repeat sequences *in vivo* (3, 34). The templating domain of *K. lactis TER1* RNA is theoretically 30 nt, longer than any examined to date, and in contrast to the irregular repeats of *S. cerevisiae* telomeres, the telomeric repeat units in *K. lactis* are tandem arrays of perfect copies of a 25 bp sequence (28). These features suggest that *K. lactis* telomerase faithfully copies its entire template.

We predicted that the properties that enable *K. lactis* telomerase to combine a precise mode of copying with an exceptionally long template would be important for understanding general mechanistic features of telomerase action. In particular, we

anticipated that analysis of telomerase polymerization along a lengthy template would allow in-depth dissection of the steps in primer elongation occurring within a round of repeat synthesis. In addition, *K. lactis* has already been established as a highly informative and experimentally advantageous model system for studies on telomere maintenance and length regulation (19, 26, 28).

Here we report the identification and characterization of telomerase activity from *K. lactis*. We show that *in vitro*, *K. lactis* telomerase catalyzes no more than a single round of repeat synthesis, remains bound to its elongated DNA products, and stalls at specific positions along the template. Stalled complexes result from position-specific arrest and pausing; both are exacerbated by increased complementarity between DNA product and the RNA template. These observations provide new insights into the mechanism of polymerization by telomerase, and have implications for the *in vivo* functioning of the enzyme.

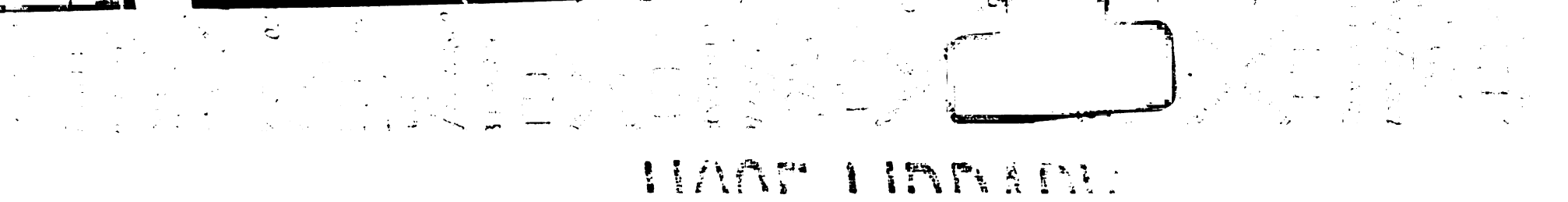
MATERIALS AND METHODS

Strain construction.

The mutant telomerase RNA yeast strains used in this study (29) were constructed in a *K. lactis* strain 7B520 background using procedures described previously (28).

DNA oligonucleotides.

All oligonucleotides (Cruachem) were purified on denaturing polyacrylamide gels (15% polyacrylamide, 8M urea) run in 1xTBE. DNA was eluted from excised gel fragments by overnight incubation at 30° in distilled water with nutation. Purified oligonucleotides were desalted on Sep-Pak C18 columns (Waters). The concentration of purified



oligonucleotides was calculated based on 1 OD_{260} = 30 µg of DNA and the molecular weight of the individual oligonucleotide.

Extract preparation and fractionation.

K. lactis cells were harvested at OD_{595} of 1.2-1.5 and resuspended in TMG buffer [10 mM Tris-HCl pH 8, 1.2 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM dithiothreitol, 1 µM pepstatin A, and 1x EDTA-free COMPLETE protease inhibitor tablet (Boehringer Mannheim)]. Whole cell extracts and S-100 supernatants were prepared essentially as described (3), except for extract used in the experiment shown in Fig. 1A, lanes 1-5, which was prepared by disrupting cells using a mortar and pestle under liquid nitrogen (7). For partial purification of telomerase, S-100 supernatants at protein concentrations of 10-15 mg/ml were adjusted to 0.5 M sodium acetate pH 8.0 and loaded onto 5 ml disposable columns (Iso-Lab) packed with 2 ml DEAE-agarose (Bio-Rad) equilibrated in the same buffer. Columns were washed with TMG containing 0.5 M sodium acetate, and telomerase eluted with 3 ml TMG containing 0.7 M sodium acetate. Fractions were desalted by dialysis (Dispo-dialysers; Spectra/Por) against TMG for 2 hours, then aliquoted and stored at -80°. Mutant cell extracts were prepared alongside wild type cell extracts, and equivalent amounts of protein (by Bradford assay, 10-15 mg/ml) were loaded onto DEAE-agarose columns. *TER1* RNA levels were compared by dot blot and hybridization with a labeled *TER1* probe to ensure that extracts contained similar telomerase RNA concentrations.

***In vitro* telomerase reactions.**

Unless otherwise indicated, standard 20 µl telomerase reactions contained 50% (v/v) DEAE fraction, 50 mM Tris-HCl pH 8, 1 mM spermidine, 1 mM DTT, 50 µM dTTP, 50 µM dATP, 50 µM dCTP, 3.75 µM [α-^{32}P] dGTP (800 Ci/mmol), and 1 µM gel-purified

oligonucleotide. Reactions were incubated at 30°C for 20 minutes, stopped with the addition of 2.5 µl stop buffer (2% SDS, 250 mM Tris-HCl pH 8, 250 mM EDTA) and 2.5 µl proteinase K (20 mg/ml), and incubated at 65°C for 25 minutes. An equal amount of terminal transferase-labeled 10-mer was added to each reaction after the stop to monitor the recovery of the products. Reactions were extracted with phenol:chloroform:iso-amyl alcohol (25:24:1) and precipitated with 1/10 volume 3M sodium acetate pH 5.2, 2.5 volumes ethanol, and 10 µg tRNA or glycogen as a carrier. For the pulse/chase and time course reactions, a nucleotide mix and a mix of the other reaction components were prewarmed separately at 30°C for 5 minutes before being mixed together, aliquots were removed at various time points, and were stopped with the addition of stop buffer and proteinase K. Reaction products were resolved on denaturing 15% acrylamide (20:1 acrylamide:bisacrylamide), 8M urea gels in 1xTBE and visualized by autoradiography. Reaction products were quantified either by using a Molecular Dynamics phosphorimager and ImageQuant or by scanning and using the NIH Image program.

Sephacryl S-300 gel filtration, native gel electrophoresis, and Northern analysis. A scaled-up standard 260 µl telomerase reaction with primer KL13(12) was incubated at 30°C for 7 minutes, mixed with a terminal transferase-labeled 30-mer (TGGGTGTGGTGTGTGGGTGTGGTGTGTGGG), then separated on Sephacryl S-300 (Pharmacia) essentially as described (33). The reaction was loaded onto a 2 ml column, and eluted with TMG buffer in 150 µl fractions. A 15 µl portion of each fraction was Cerenkov counted. A 30 µl portion was further processed as described for the standard telomerase reaction. Two 30 µl aliquots from each fraction were loaded onto separate native gels composed of 3% acrylamide (80:1 acrylamide:bis-acrylamide) and 0.6% agarose and electrophoresed in 50 mM Tris-acetate at 200V for 3 hours. One gel was exposed to film immediately for 2 hr. The other gel was soaked in 50% (w/vol) urea for

20 minutes, and transferred to a Hybond Plus membrane (Amersham) in 0.5xTBE. Since the transferred labeled products generated considerable background signal, the membrane was set aside for approximately two months to allow the radioisotope to decay to a level that would not interfere with hybridization. To ensure low background levels, the membrane was exposed to film for 2 hours. The membrane was then hybridized to a mixture of two ^{32}P-labeled probes prepared by random-prime labeling (Amersham) of PCR fragments amplified from the *TER1* gene. The fragments span nt 26-277 and 701-1273 of the *TER1* gene, which exclude the template region. The membrane was then re-exposed to film for 2 hours.

RESULTS

Identification of telomerase activity from *Kluyveromyces lactis*.

To identify *K. lactis* telomerase *in vitro*, we fractionated S-100 extracts prepared from wild-type cells by ion-exchange chromatography on a DEAE-agarose column (see Materials and Methods), and assayed for telomerase activity. In these experiments, DEAE fractions were incubated with one [α-^{32}P] radiolabeled dNTP, three unlabeled dNTPs, and an oligonucleotide complementary to part of the previously identified *K. lactis TER1* RNA templating domain (28). After incubation of the reaction for 20 minutes at 30°C, reactions were terminated and products were purified and separated on 15% denaturing polyacrylamide gels. Initially, a 17 nt-long oligonucleotide (17-mer) (KL22(17); see Fig. 1A), was used as the DNA primer to detect polymerization activity in extracts. ^{32}P-labeled products longer than the input primer by up to eight nt (+1 to +8 products) were expected if the primer was elongated all the way along the template. Such products were revealed, with the majority of the labeled products appearing at +6 (Fig. 1B, lane 1; arrowhead), corresponding to a position near the presumed end of the template (Fig. 1A; arrowhead). Through the remainder of this paper, we refer to these

prominent products as 'near-terminal' products. The high molecular weight products seen at the top of the gel were, as described previously for comparable *S. cerevisiae* extracts (3, 34), independent of RNase pretreatment or telomerase RNA as described below, and were therefore not attributable to telomerase activity.

Telomerase activity requires input of a DNA primer and is dependent on both protein and RNA components. *K. lactis* polymerization activity fulfilled these essential criteria, as no products were detected in the absence of a primer (Fig. 1B, lane 5), and the activity was abolished by preincubation of the extract with either proteinase K or RNase A (Fig. 1B, lanes 2 and 3). RNase pretreated extracts sometimes produced bands (Fig. 1B, lane 3), but these were often variable in appearance, and have never been attributable to telomerase activity. Specific polymerization still occurred when the telomerase preparation was incubated with RNase inhibitor (RNasin) before pretreatment with RNase A (Fig. 1B, lane 4). Hence RNase inhibition of the activity was due to RNA digestion rather than other aspects of the preincubation conditions.

Further support that this activity was telomerase came from testing its ability to prime synthesis from another telomeric oligonucleotide, KL12(17). This 17-mer was designed to align on the template 10 nt 3' of KL22(17) (Fig. 1A). The longest major near-terminal product produced in assays with KL12(17) (Fig. 1B, lane 6; +16 product, arrowhead) was 10 nt longer than the major product from KL22(17) (Fig. 1B, lane 1; +6 product). This result is in agreement with the predicted alignment of the primers on the *TER1* RNA template. The RNase sensitive products seen with KL12(17) included both the near-terminal products as well as a set of shorter products (Fig. 1B, lane 7). The shorter products, which we will refer to as 'mid-template' products, are further characterized below.

To test the prediction for *TER1* RNA template-directed synthesis, we performed assays in which each unlabeled dNTP was substituted by its ddNTP analog. With both primers, this resulted in premature chain termination at positions predicted from the template sequence (Fig. 1B, lane 8; Fig. 1C, lanes 4-6). In addition, omission of unlabeled dNTPs from the KL22(17)-primed reaction resulted in the expected strong stop at +2, after the addition of two ^{32}P-dG residues to the input primer (Fig. 1C, lane 7).

As a direct test for dependence on the telomerase RNA template, extracts were prepared from strains in which the telomerase RNA gene was mutated. First, extracts were assayed from the *ter1-Δ7* strain (28), in which a 300 bp fragment of the *TER1* gene, including the template, is deleted. As predicted, these extracts had no RNase sensitive polymerization activity with either primer, demonstrating that the template region is required for synthesis of both mid-template and near-terminal products (Fig. 1D, lanes 3 and 6, and data not shown). Confirmation of template-directed synthesis came from mutations introduced in the template region. The *TER1-SnaBI* strain (29) was engineered to contain a C to A mutation within the *TER1* RNA template (boxed residue in Fig. 1A). Fractionated extracts prepared from this strain produced the predicted patterns of incorporation: specifically, in assays with KL22(17), chain termination due to incorporation of ddTTP occurred at +2 instead of +4 (Fig. 1C, compare lanes 5 and 12), and polymerization ended at +1 instead of +2 in ^{32}P-dGTP only reactions (Fig. 1C, compare lanes 7 and 14). Thus the SnaBI mutation, which is incorporated into telomeres *in vivo*, produced the expected changes in dNTP incorporation *in vitro*. In addition, polymerization was specifically altered in extracts prepared from both the *TER1-SnaBI* mutant and another template mutant, *TER1-TaqI*

(data not shown) (29). Together, these results showed that telomerase is responsible for the *K. lactis* polymerization activity.

***K. lactis* telomerase catalyzes a single round of telomere repeat synthesis *in vitro*.** Under a variety of *in vitro* conditions, *S. cerevisiae* telomerase remains bound to its DNA reaction products in a manner that prevents further elongation (33). With many different primers, polymerization by *K. lactis* telomerase *in vitro* was also found to be confined to one round of synthesis across the template, which was often incomplete (Fig. 1B and (7)). To address whether these properties of the *K. lactis* telomerase reaction have a basis similar to that of *S. cerevisiae* telomerase, we first characterized the kinetics of *K. lactis* telomerase by varying the amounts of extract and primer (KL22(17)) under standard assay conditions. Product yield increased linearly with increasing enzyme concentration, indicating that telomerase was limiting (Fig. 2A). Reactions with various primer concentrations demonstrated that the 1 μM concentration used in standard assays was well above the apparent K_m for the reaction (Fig. 2B). In fact, maximal telomerase activity was observed at primer concentrations as low as 0.04 μM, with no change in product distribution (data not shown). At 1 μM primer, despite the large excess of primer and dNTP substrates used in all the experiments described (see below), product yield reached a plateau by ~3 minutes (Fig. 2C; quantitation shown in Fig. 2D), which would not be expected if repeated cycles of extension occurred. For example, in similar experiments with *T. thermophila* telomerase, total product yield increases over a period of up to 60 minutes (11, 20). In a pulse-chase experiment, excess unlabeled dGTP was added to a standard reaction after 45 seconds and incubated for a total of 25 minutes. The shorter (+1 to +5) products were chased into longer (+6 and +7) products (Fig. 2C; compare 45 sec lane to P/C lane), consistent with telomerase remaining bound to the +1 to +5 products during elongation. Notably, the

chase did not stimulate synthesis of multiple consecutive 25 bp repeats (Fig. 2C, in which second round products would have been visible in the top quarter of the autoradiogram portion shown). These results suggested that *K. lactis* telomerase consistently catalyzes only a single round of synthesis.

K. lactis telomerase remains bound to reaction products.

The early plateau in product yield seen in Fig. 2 was similar to that previously observed with *S. cerevisiae* telomerase (33), and could have been attributed to 1) dissociation and inactivation of telomerase at the completion of the reaction, or 2) telomerase remaining bound to its reaction products, thus precluding binding and extension of the excess free primer present in the reaction. To test directly whether telomerase enzyme remains bound to its products, we used size-exclusion chromatography to determine whether *K. lactis* telomerase products co-eluted with the large telomerase RNP complex, as has been shown for *S. cerevisiae* telomerase (33). Reactions were primed with the 12-mer oligo KL13(12), which produces a product profile similar to KL12(17), including both mid-template and near-terminal products. After a 7 minute incubation, the reaction was size-fractionated on a Sephacryl S-300 column. A portion of each fraction was electrophoresed on a native gel, transferred to a membrane, and hybridized with a radiolabeled probe for the *TER1* RNA subunit of telomerase to determine the elution profile of the telomerase complex (Fig. 3A). The hybridization was performed after the ^{32}P label incorporated into the products had decayed for two months, to prevent interference from any signal resulting from products of the telomerase reaction bound to telomerase complexes. The ^{32}P labeled DNA products of the telomerase reaction, purified from a second aliquot of each fraction from the sizing column (see Materials and Methods), were separated by denaturing polyacrylamide gel electrophoresis (Fig. 3B). The remainder of each fraction was also electrophoresed on a separate native gel and

exposed to film directly, to visualize large complexes carrying ^{32}P label after the telomerase reaction. Although a large ^{32}P-labeled complex in this directly exposed native gel co-fractionated and co-migrated with the *TER1* RNA (data not shown), it was partially obscured by a background of the high molecular weight, non-telomerase ^{32}P-labeled products shown in Figure 1B. However, quantitation of the results shown in Figure 3A and B clearly showed that after Sephacryl S-300 chromatography, both mid-template and near-terminal products co-fractionated with the *TER1* RNA, peaking in fractions 4 and 5 (Fig. 3C). Reaction products that had dissociated from the telomerase RNP would have eluted in a peak following the *TER1* RNA/telomerase product peak. This was demonstrated by loading a marker 30-mer (previously ^{32}P-labeled by terminal transferase) together with the telomerase reaction mix onto the Sephacryl S-300 column; the 30-mer eluted after the telomerase product/*TER1* RNA peak (Fig. 3B; top panel). A small population of telomerase products eluted after the peak (Fig. 3B, C), which may be attributable to the stability of the enzyme/product complex. These results indicated that *K. lactis* telomerase remained bound to the majority of both near-terminal and mid-template products of the extension reaction, and suggested that each telomerase active site carries out only one round of extension on a given primer molecule.

Telomerase stalls at specific template regions in arrested and paused conformations.

As described above, the majority of the products of *K. lactis* telomerase were shorter than would be expected if polymerization reached the end of the template (Figures 1 to 3). To investigate the influence of the primer on the progression of polymerization along the template, we primed telomerase reactions with a series of template-complementary 12-mers which align step-wise across the template (Fig. 4A). Each primer was designed to anneal completely within the templating domain, with 3' ends at template positions 12

through 3R (see numbering of template, Fig. 4A). Two distinct regions of product accumulation emerged from this data set: region M (mid-template) at positions 18-22, and region NT (near-terminal) at positions 2R-4R (Fig. 4). A somewhat altered set of mid-template products were also seen with *TER1-SnaBI* mutant extracts (data not shown), again confirming that the mid-template products as well as the near-terminal products were generated by telomerase activity. As shown in Fig. 4B for the wild-type enzyme, as primers aligned closer to the 3' end of the template, the amount of products ending in the M region increased (compare bands in Fig. 4B marked with stars in lanes 12 and 17) while the amount of products ending in the NT region decreased proportionally (Fig. 4B; compare bands marked with arrowheads in lanes 12 and 20). Quantitation of the individual products and correction for the number of labeled incorporated nucleotides substantiated this trend, and also confirmed that the amounts of total products formed were similar for all primers. These results suggest that mid-template products accumulate at the expense of near-terminal products. Because, as shown above, *K. lactis* telomerase remains bound to the majority of its extension products (Fig. 3), we hypothesized that product accumulation in the M and NT regions represents stalled telomerase-DNA complexes. Interestingly, stalling was not uniform along the template, but instead occurred specifically at these two preferred regions.

At limiting dNTP concentrations, telomerases from other species pause at template positions just prior to incorporation of that particular dNTP (20, 31, 37). Similarly, a subset of the stalls in the *K. lactis* telomerase reactions resulted from the low ^{32}P-dGTP concentration used in standard *in vitro* reactions, as shown by titrating unlabeled dGTP into telomerase reactions with primer KL12(12) and monitoring product formation (Fig. 5A and B). Along with the expected quantitative decrease in total incorporated ^{32}P label as the unlabeled dGTP concentration was increased, the distribution of products also

changed, suggesting that stalling was overcome at some positions along the template. However, strikingly, products at template positions 19, 20, and 21 (M stalls) remained visible even at 50 μM dGTP (Fig. 5A; boxed stars). This result is in agreement with the accumulation of products at these same template positions in the experiment shown in Fig. 1B , in which dTTP was the limiting radiolabeled limiting nucleotide and dGTP was not limiting (Fig. 1B, lane 6; +7 to +9 products). After correction for both the decreasing specific activity and the number of radiolabeled dG residues incorporated into each product, total product formation was similar at all dGTP concentrations, confirming that higher dGTP does not stimulate turnover. Product yields at 18 (data not shown) and 22 (Fig. 5B; small open circles) dropped as the dGTP concentration was increased, as expected for positions preceding incorporation of dG ('pre-G' positions). This suggests that telomerase stalled in the M region at positions 18 and 22 was paused, awaiting incorporation of a dG residue. Yields of the longer products at 3R and 4R in the NT region increased as the dGTP concentration was raised (Fig. 5B; filled and open triangles, respectively). However, high dGTP did not promote their extension to the extreme terminus of the template (Fig. 5A; 5R position). Strikingly, the high dGTP concentrations only slightly affected the product yields for positions 19, 20, and 21 (Fig. 5B; large circles), with the observed minor increase being attributable to depletion of smaller intermediates. Hence we conclude that the strong stalls at positions 19-21 in the M region and 3R and 4R in the NT region are functionally distinct from the pauses at pre-G positions 18 and 22. As we have shown that *K. lactis* telomerase remains associated with its products, we will refer to the stalls which are not elongated in response to high dGTP concentrations (at positions 19-21, 3R, and 4R) as arrested telomerase/product complexes.

The assignment of stalled complexes as either paused or arrested was confirmed by a pulse chase experiment with the same primer. Excess unlabeled dGTP was added to a 45 second reaction and the incubation continued for a total of 25 minutes (Fig. 5C). Consistent with the findings from the dGTP titration, mid-template pre-G intermediates at positions 18 and 22 that had accumulated by 45 seconds (Figure 5C, non-boxed stars) were chased into 3R and 4R (NT region) products (Figure 5C, arrowheads). In contrast, product yield at positions 19, 20, and 21 was unaffected by the unlabeled dGTP chase (Fig. 5C, starred bands), confirming a steady-state level of arrested complexes. The lack of elongation to 5R during the chase provided further evidence that most 3R and 4R products were also arrested.

Increased primer/template complementarity exacerbates stalling.

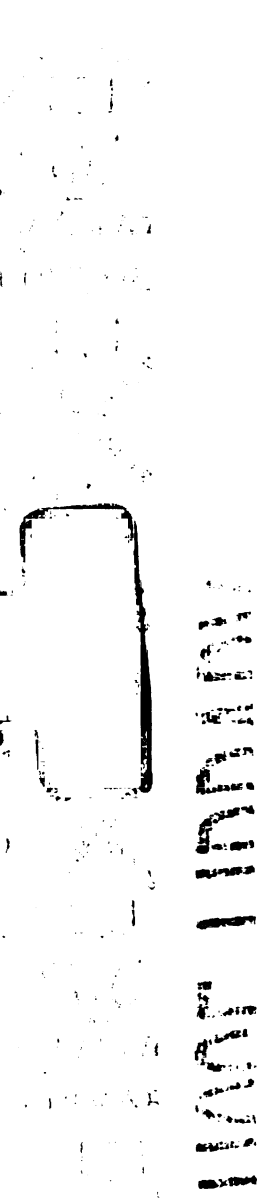
The frequency of telomerase stalling in the M region increased as primers annealed further toward the 3' end of the template (Fig. 4A and B). This could have resulted from the increased length of 1) just the DNA added to the primer by the enzyme, 2) total potential RNA/DNA duplex created during polymerization, or 3) the DNA product itself (primer plus added nucleotides) when polymerization reaches the stall region, regardless of its degree of pairing to the template. To test which of these parameters influenced stalling, we performed telomerase reactions using two sets of primers, each set having a common 3' end, but with variable 5' ends. The 3' end of the first set of primers was at template position 16, and with such a 12-mer primer little stalling occurred in the M region (Fig. 4B, lane marked 16 and Fig. 6, lane 1). However, extending the amount of primer/template complementarity with extra nucleotides on the 5' end of the primer dramatically increased the ratio of mid-template products (Fig 6, starred bands) to near-terminal products (Fig. 6, arrowhead, lanes 1-5). In contrast, extra non-complementary 5' nucleotides did not exacerbate stalling (Fig. 6, lanes 6 and 7). In additional

experiments, M region stalling was also alleviated when the length of potential RNA/DNA duplex of a 12-mer ending at position 15 was reduced to 11 nt, by changing the 5' nucleotide from the complementary dG to a dA, dT, or dC residue (data not shown). Comparable results were seen with a second set of primers with a common 3' end at position 22, designed to examine stalling in the NT region. Stalling at position 3R relative to 4R was exacerbated when the 5' end of a 12-mer was extended with five template-complementary nucleotides (Fig. 6, open and closed arrowheads, compare lanes 8 and 9), and alleviated when the 5' end was made non-complementary (Fig. 6, lanes 10 and 11).

These results demonstrated that stalling by *K. lactis* telomerase *in vitro* is not determined by the total length *per se* of the product, or by the length of only the newly synthesized stretch of DNA. Instead, in the M and NT regions of the template, stalling is exacerbated by increasing the length of the potential DNA/RNA template duplex. Interestingly, the class of stalled complexes influenced by limiting dGTP concentrations (at positions 18 and 22; top and bottom starred bands) also accumulated in response to increased primer/template complementarity (Fig. 6), suggesting an interplay between dGTP concentration and complementarity in contributing to stalling.

DISCUSSION

The telomerases described heretofore use a short template to synthesize either short, precise repeats or irregular repeats (3, 24, 25, 31, 35, 38, 44). However, *K. lactis* telomerase synthesizes 25 base pair repeats from a 30 nucleotide templating domain, the largest described to date (28). Here we have reported the identification and characterization of *in vitro* telomerase activity from this budding yeast. This study reveals intriguing properties of *K. lactis* telomerase, including stalling at specific regions



of the template, and the dependence of stalling on increased primer/template complementarity. The establishment of an *in vitro K. lactis* telomerase system will allow other questions about telomere biology to be addressed. For example, certain previously characterized template mutations in *K. lactis* cause major effects on telomere length and maintenance *in vivo* (19, 28). It will now be possible to distinguish whether these effects result from altered binding of telomere-associated proteins (for example, Rap1p) or altered telomerase activity.

We found that *K. lactis* telomerase catalyzes only a single round of primer elongation *in vitro*, like telomerase from *S. cerevisiae* (3, 23, 33, 34). In *K. lactis* and *S. cerevisiae* cells lacking intact telomerase RNA, telomeres shorten by an average of only 5 bp per cell division (28, 39). Hence in wild-type *K. lactis* cells the copying of even one-fifth of a telomeric repeat per cell division on average would be sufficient to maintain telomere length equilibrium. Thus, single-round repeat synthesis by *K. lactis* telomerase could maintain telomeres *in vivo*. The *in vitro* behavior of *K. lactis* telomerase reported here is consistent with a model in which telomerase acts in each cell cycle to synthesize a small amount of telomeric DNA. Synthesis of only small increments per cell cycle was proposed previously for *S. cerevisiae* based on analysis of the observed small length fluctuations of its telomeres *in vivo* (36). Another property of *K. lactis* telomerase activity reported here, its non-dissociativity from reaction products, may indicate a structural role for telomerase in a telomere-capping complex, as suggested previously for *S. cerevisiae* telomerase (33).

An unusual property of the *K. lactis* telomerase activity is that it stalls during the synthesis of a telomeric repeat in at least two specific regions of the template. The mechanistic basis for this stalling is unknown, but it may be influenced by the

constrained nature of the template combined with the potential to create a lengthy RNA/DNA hybrid during polymerization. In *Tetrahymena* telomerase, the regions of telomerase RNA outside the template region are apparently buried in the RNP (14). Therefore, the movement of the templating domain across the catalytic center of telomerase at each polymerization step is necessarily constrained by the anchoring of the template in the RNP by RNA-protein associations. The exacerbation of stalling we observed upon increasing the DNA/template RNA complementarity supports the idea that base pairing between template RNA and DNA contributes to stalling. Building up a long, presumably rigid RNA/DNA hybrid duplex during elongation *in vitro* is likely to interfere with the constrained movement of the template through the catalytic center. Furthermore, such a duplex may also prevent the active site of the RNP from assuming the correct conformation necessary for polymerization. Hence, as the hybrid lengthens, steric restriction may become sufficient to prevent further polymerization.

The formation of lengthy template RNA/primer or product DNA hybrids during polymerization *in vitro* would imply that telomerase lacks the ability to unpair the duplex, which as discussed above, may contribute to stalling. Other telomerases analyzed to date, including those from ciliated protozoa, human cells, and the budding yeast *S. castellii*, catalyze multiple cycles of synthesis *in vitro* (3, 11, 15, 31), which requires unpairing of the product DNA/RNA template hybrid. Telomerase activity reconstituted from a human telomerase protein (hTERT) and the telomerase RNA component (hTR), as well as extensively purified *Tetrahymena* and *Euplotes* telomerase preparations are able to carry out translocative synthesis, indicating that such unpairing ability is intrinsic to these telomerase enzymes, and does not require other factors *in vitro* (6, 22, 42). It has been proposed that *Tetrahymena* telomerase unpairs the recently made DNA from the template through the energy of binding to another site in the RNP, and that this site

is analogous to that shown to bind the nascent transcript of RNA polymerase (21). The *K. lactis* telomerase preparations used in this study may lack telomeric binding proteins, other components of the telomerase complex, or accessory factors such as a helicase that could alleviate stalling *in vitro*. Alternatively, the build-up of an RNA/DNA hybrid and stalling may be inherent to the *in vivo* action of telomerase.

The available information is compatible with some occurrence of stalling *in vivo*. *S. cerevisiae* telomerase has been suggested to stall frequently *in vitro* as copying proceeds along its template, and in this yeast, degenerate repeats are found *in vivo* in a pattern consistent with the *in vitro* properties of this enzyme (3, 34). Stalling by other polymerases appears to be exploited *in vivo*. For example, pausing by RNA polymerase in bacterial cells coordinates transcription and other processes including translation, possibly the loading of regulatory proteins important for antitermination, and the formation of appropriate RNA structure during rRNA transcription (for review see (40)). The stalling we have reported here for *K. lactis* telomerase, which often results in only partial synthesis of a repeat *in vitro*, is compatible with synthesis of the perfectly repeated telomeric sequence of this species *in vivo* as long as primer alignment on the template is correct before the next round of synthesis. Since the template lacks internal redundancy, primers with 3' ends in the M or NT regions can align in a unique register, and thus incomplete extension could be tolerated *in vivo*. Hence stalling by telomerase is a potential step at which telomerase action could be regulated *in vivo*.

The diversity of telomeric repeat lengths and sequences in budding yeasts suggests differences among the polymerization properties and, potentially, the evolutionary stages of their telomerases. Long-template telomerases may have been derived sporadically in budding yeasts, arising from short template telomerases copying beyond their normal

template boundaries. In this case, it is conceivable that the *K. lactis* telomerase template positions where stalling occurs may represent the ancient boundaries of a shorter template. It has been suggested that the ancestor to telomerase was a catalytic self-replicating RNA. This ancestor is proposed to have acquired a reverse transcriptase protein component, but retained RNA with at least a portion still functioning as a template (2). By this model, the long-template telomerases of budding yeasts may represent an intermediate stage in the evolution from a ribozyme that completely copied itself to the typical short template telomerases which copy a small discrete region of RNA. Indeed, stalling by the more 'primitive' telomerases is one possible means that may have helped initiate the eventual evolution into copying progressively shorter regions of telomerase RNA. Further analysis of telomerases from budding yeasts will be valuable for our understanding the origins of their diverse telomere repeats and the general mechanism of telomerase.

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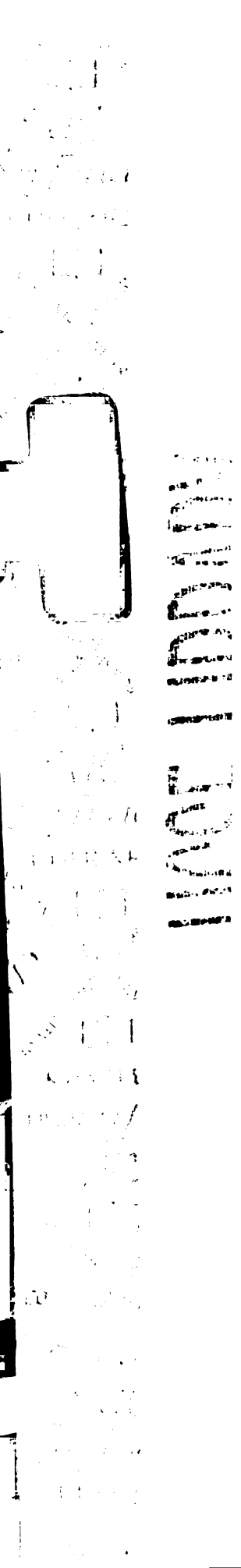

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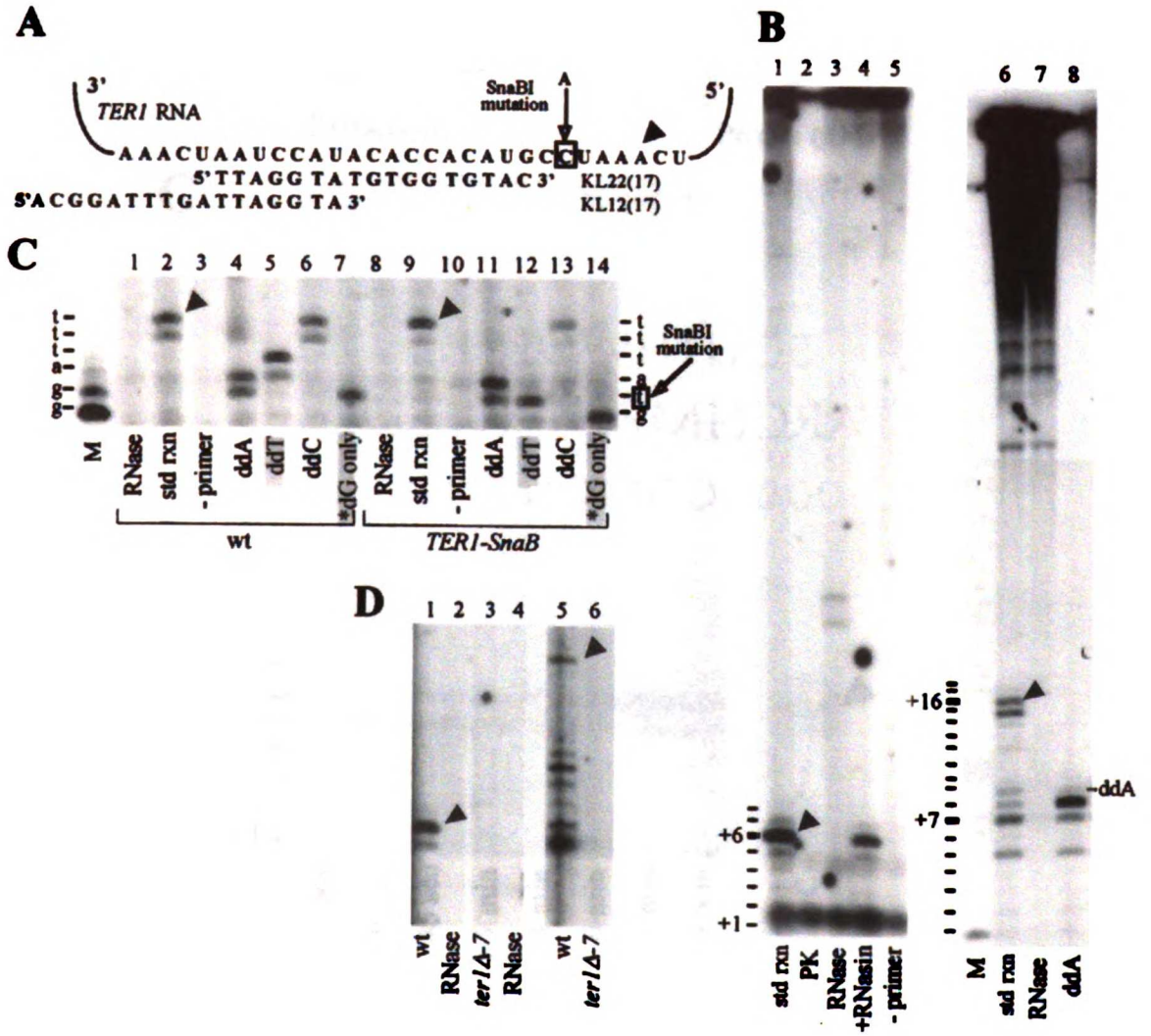
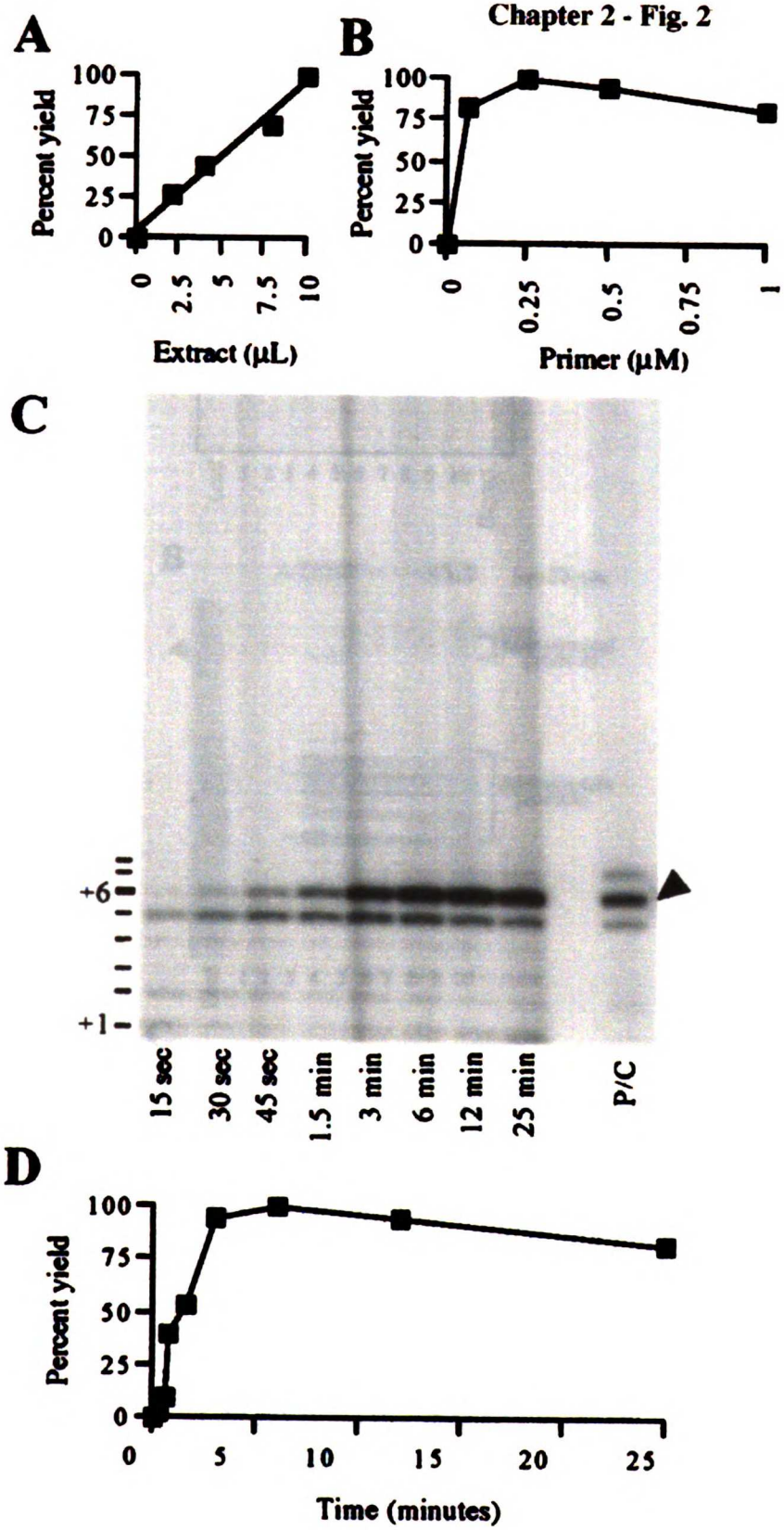
Chapter 2 - Fig. 1

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50
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5
7.5
10
Extract (µL)
B
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75
50
25
0
0
0.25
0.5
0.75
1
Primer (µM)
C
+6
+1
15 sec
30 sec
45 sec
1.5 min
3 min
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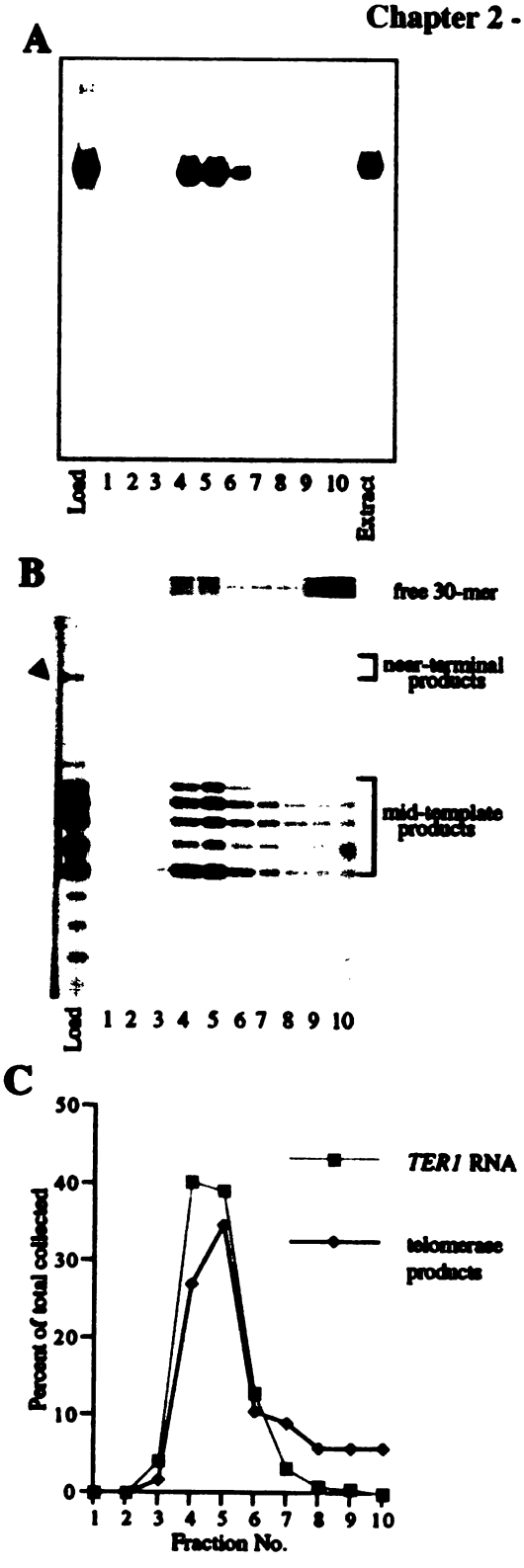
Time (minutes)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near-terminal products
mid-template products
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Percent of total collected
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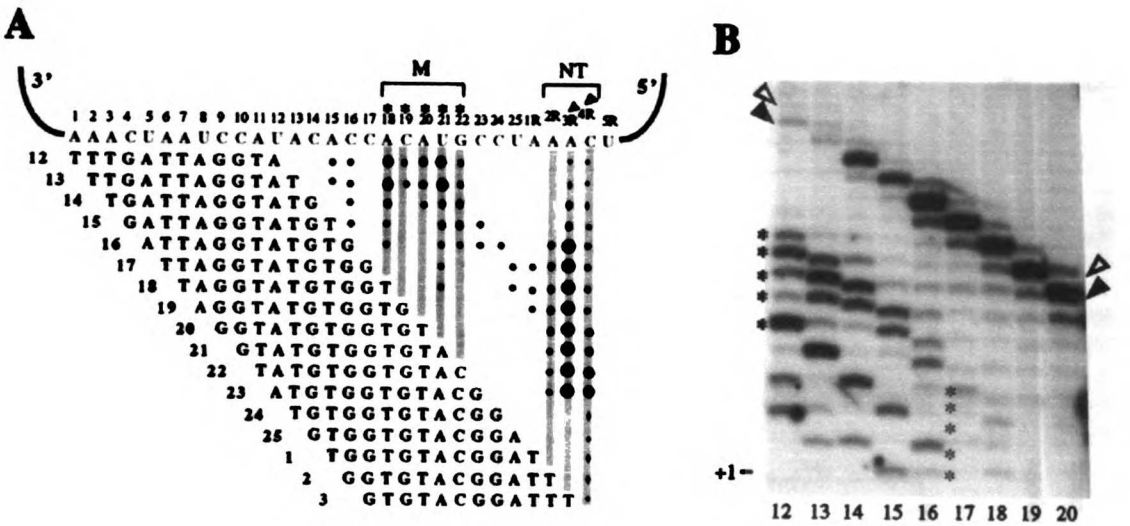
1 2 3 4 5 6 7 8 9 10
Fraction No.
TER1 RNA
telomerase products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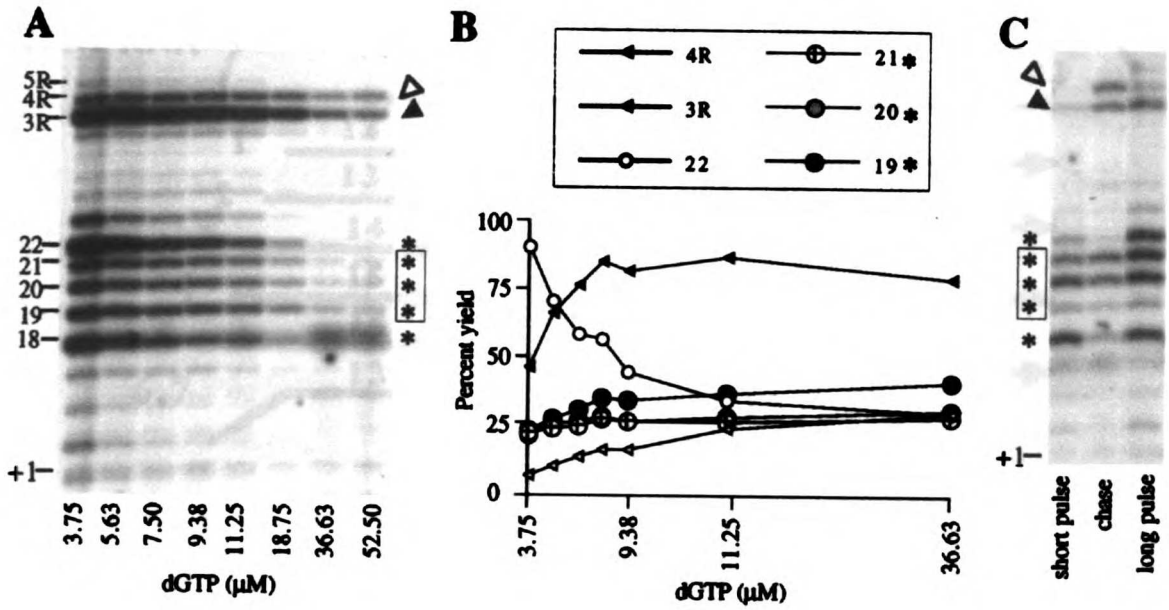
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9.38
11.25
18.75
36.63
52.50
dGTP (μM)
B
4R
3R
22
21*
20*
19*
100
75
50
25
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Percent yield
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+1
short pulse
chase
long pulse

Chapter 2 - Fig. 6

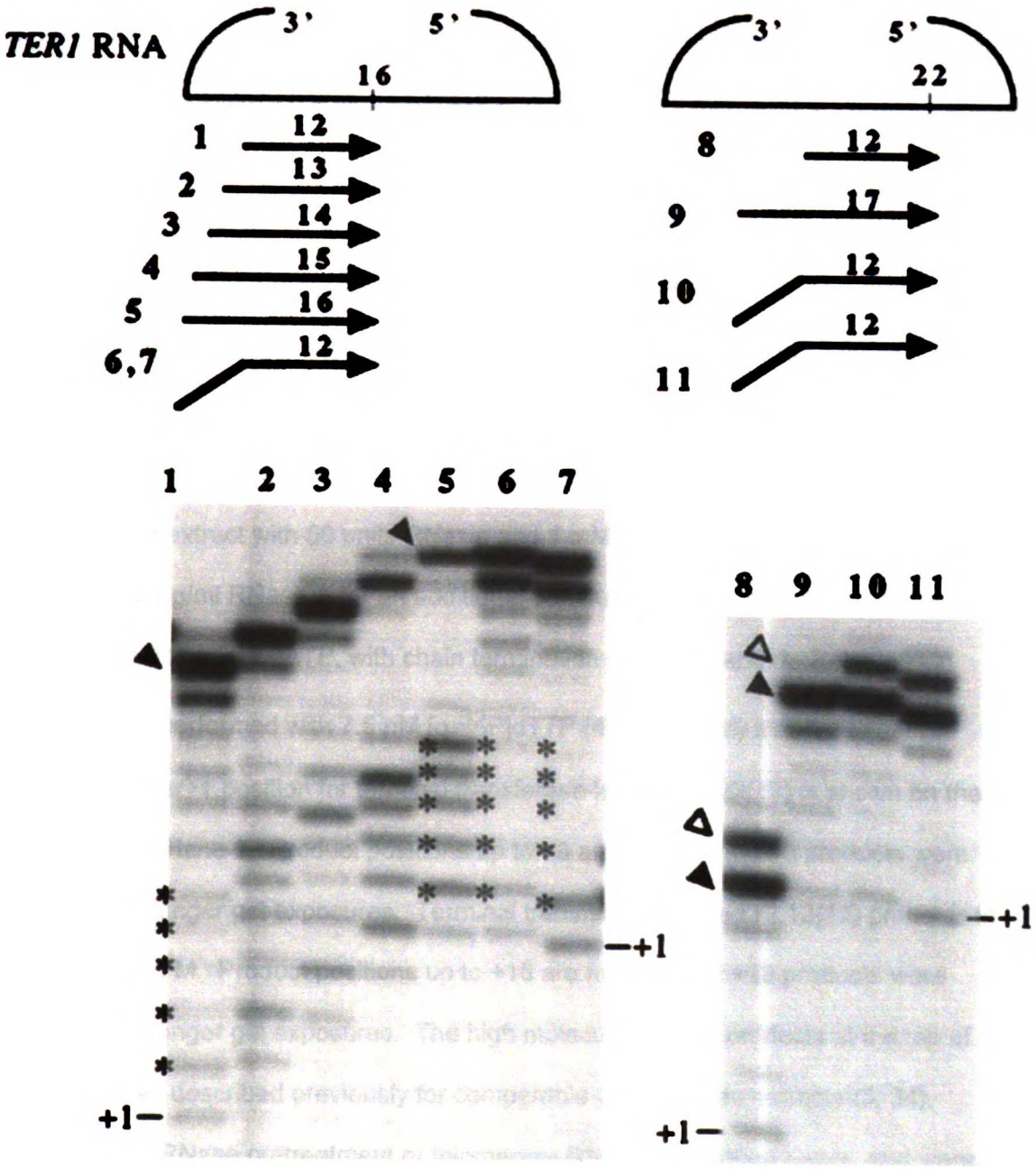

FIGURE LEGENDS

Figure 1. Identification of *K. lactis* telomerase activity *in vitro*. (A) Schematic of *K. lactis* telomerase RNA (*TER1*) template region and the expected alignment of the primers KL22(17) and KL12(17). Arrowhead corresponds to the 3' end position of correspondingly marked products in (B) and (C). Boxed template residue corresponds to the site of the C to A mutation in the *TER1-SnaB* strain examined in (C). (B) Telomerase reactions were carried out with primers KL22(17) (lanes 1-5) and KL12(17) (lanes 6-8) as described in Materials and Methods, with the following changes: lane 2, pretreatment of extract with 0.5 mg/ml proteinase K for 5 minutes at 25°C; lanes 3 and 7, pretreatment of extract with 10 µg/ml RNase A at 25°C for 5 minutes (lane 3, followed by an additional 5 minute incubation with 50 units RNasin and 1 mM DTT); lane 4, pretreatment of extract with 50 units RNasin and 1 mM DTT at 25°C for 5 minutes, followed by 10 µg/ml RNase A for an additional 5 minutes; lane 5, no input primer; lane 8, ddATP substituted for dATP, with chain termination product marked. Reactions in lanes 6-8 were performed with 7.5 µM [α-^{32}P]dTTP (400 Ci/mmol) as the radioactive label. The primer+1 position for terminal transferase-labeled KL22(17) is shown on the lower left side of lane 1. Product positions up to +8 are marked, but +8 products were visible only on longer gel exposures. Terminal transferase-labeled KL12(17) primer is in the lane marked M. Product positions up to +18 are marked, but +18 products were visible only on longer gel exposures. The high molecular weight products at the top of the gel were, as described previously for comparable *S. cerevisiae* extracts (3, 34), independent of RNase pretreatment or telomerase RNA as described below, and were therefore not attributable to telomerase activity. Also, the diffuse band between +1 and +2 in lanes 1-5 was not produced by telomerase, as it formed independent of primer, and was insensitive to RNase A and proteinase K. (C) Reactions with DEAE-

fractionated extracts from wild type (lanes 1-7) and *TER1-SnaB* (lanes 8-14)-strains were carried out with primer KL22(17) as described in Materials and Methods with the following changes: lanes 1 and 8, pretreatment with RNase A as described above; lanes 3 and 10, no input primer; lanes 4-6 and 11-13, substitution of each indicated ddNTP for its partner dNTP; lanes 7 and 14, [α-^{32}P]dGTP as the sole dNTP substrate. Shaded lanes highlight nucleotide incorporation differences between the wild type and mutant extracts. Terminal transferase-labeled KL22(17) primer (lane M) marks the primer+1 position. The nucleotides predicted to be incorporated by wild-type and *TER1-SnaB* telomerase are marked on the left and right sides of the panel, respectively. (D) Reactions with DEAE-fractionated extracts from wild type (lanes 1, 2, and 5) and *ter1*-Δ*7* (lanes 3, 4, and 6) were carried out with primer KL22(17) (lanes 1-4) and primer KL12(12) (lanes 5, 6). Extract in lanes 2 and 4 was pretreated with RNase A as described above.

Figure 2. Kinetics of *K. lactis* telomerase activity. Reactions were performed using wild type DEAE-fractionated extract and primer KL22(17). (A) Plot of product yield from reactions containing 0, 2, 4, 8, or 10 µl of wild type DEAE-fractionated cell extract. The sums of the +1 to +7 products for each reaction were quantified, and are presented as percentages of the yield with 10 µl extract. (B) Plot of product yield from reactions containing 0, 0.125, 0.25, 0.5, and 1 µM primer. Product yield was quantified as in (A). (C) Reaction with 10 µl extract and 1 µM primer incubated for various time periods. Products from a 45 second reaction followed by a 24 minute chase with 100 µM unlabeled dGTP, shown in the lane marked P/C. Any products resulting from translocative synthesis would have appeared in the top quarter of the gel region shown. (D) Plot of product yield from reactions shown in (C). The sums of the +5 and +6

(marked by arrowhead) products were quantified from a scanned autoradiogram using the program NIH Image, and the results are presented as percentages of the yield at 3 minutes.

Figure 3. Association of *K. lactis* telomerase with elongation products. (A-C) A completed *in vitro* telomerase reaction with primer KL13(12) was performed and size-fractionated on Sephacryl S-300 as described in Materials and Methods. (A) Aliquots of each fraction were separated on a native gel composed of 3% polyacrylamide (80:1 acrylamide:bis-acrylamide) and 0.6% agarose (lanes 1-10, numbers correspond to fraction numbers), along with a portion of the non-fractionated telomerase reaction (load lane) and 10 µl of the partially purified *K. lactis* extract that was used in the reaction (extract lane). The gel was transferred to a Hybond Plus membrane (Amersham) and hybridized to a mixture of two ^{32}P-labeled fragments of the *TER1* gene that exclude the template region. (B) Aliquots of each fraction were separated on a denaturing 15% polyacrylamide (20:1 acrylamide:bis-acrylamide) and 8M urea gel (lanes 1-10), along with a portion of the non-fractionated telomerase reaction (load lane). Arrowhead corresponds to products described in Fig. 1B, and positions of mid-template and near-terminal products are bracketed. The upper portion of the panel shows the fractionation pattern of a telomeric 30-mer, ^{32}P-labeled by terminal transferase and loaded on the S-300 column with the telomerase reaction. This portion of the panel is from an exposure of the gel 30-fold longer than that used to show telomerase products. (C) Plot of the relative amounts of TER1 RNA (A) and telomerase products (B) recovered in each fraction. Products in each fraction were quantified and are represented as a percentage of the total collected.

Figure 4. Primer-dependent stalling by *K. lactis* telomerase. (A) Schematic of results from assays using 12-mer oligonucleotides aligning at different positions along the template. Numbers next to primers reflect the positions of the primers' 3' end on the template, as indicated by numbers above the template. Product bands are denoted at their appropriate template positions with black dots. The size of each dot represents the relative intensity of signal from products stalled at each position, and shaded columns of dots indicate the two preferred regions of stalling. Stars above positions 18, 19, 20, 21, and 22 and the arrowheads above positions 3R and 4R mark 3' end positions of products shown in (B). (B) Reactions using primers 12-20, as described in (A). Since all primers are the same length but align step-wise across the template, elongation of primers to the same position resulted in products varied by one nucleotide in length (compare positions of arrowheads and stars).

Figure 5. Dependence of *K. lactis* telomerase stalling on nucleotide concentration. (A) Reactions using the primer KL13(12) and dGTP concentrations equaling 3.75, 5.63, 7.50, 9.38, 11.25, 18.75, 35.63, and 52.50 μM (each reaction contains 50 μCi [α-^{32}P] dGTP and varying amounts of unlabeled dGTP). Products of particular interest are marked on the left side by their 3' end position. Stars and arrowheads mark products corresponding to those shown in Fig. 4. (B) Plot of telomerase products at various dGTP concentrations. At each concentration, products stalled at template positions 18, 19, 20, 21, 22, 3R and 4R were quantified, corrected for specific activity differences, and divided by the number of dG residues incorporated into each product. Results for 18 were omitted due to interference of the diffuse background band visible in the last two lanes, which corresponds to the non-telomerase-generated product seen in Fig. 1B, lanes 1-5. (C) A scaled-up reaction with 3.75 μM [α-^{32}P] dGTP

was incubated for 1.5 minutes, then divided into three aliquots: one part was stopped (short pulse lane) with proteinase K and SDS (see Materials and Methods); the second aliquot was mixed with 100 μM unlabeled dGTP and incubated for an additional 23.5 minutes (long chase lane); the third aliquot was incubated for a total of 25 minutes with no chase (long pulse lane). Stars and arrowheads mark mid-template and near-terminal products respectively, and boxed stars show unchaseable products.

Figure 6. Dependence of telomerase stalling on primer/template complementarity. Lanes 1-7, products from reactions with primers 1-7 as illustrated above (lane numbers correspond to the numbers at the left of the primers). All primers have 3' ends aligning at template position 16. Primers' amounts of template complementarity increase by one nucleotide at a time from 12 to 16 nt (numbers above each primer). Note that the overall length of the primers also increases, and therefore the +1 position changes on the gel correspondingly. Primers numbered 6 and 7 are both 16 nt long, with 12 nt of complementarity to the template. The 4 nts at the 5' end of primer 6 are the same as those of primer 5, but scrambled so that none of them are complementary to the template. The nucleotide composition of the 5' end of primer 7 is non-telomeric (ATAT). Lanes 8-11, products from reactions with primers, as illustrated. Primers in this set share 3' ends that align at position 22. Again, the numbers above each primer reflect their degree of complementarity to the template. Primers 10 and 11 are both 17 nt long, and both have 12 nucleotides of complementarity to the template. The 5 nts at the 5' end of primer 10 are as in primer 9, but scrambled (as with primer 6). The 5' end of primer 11 is non-telomeric (ATATA). Stars and arrowheads correspond to products previously discussed.

Appendix to Chapter Two

This appendix contains experiments that further characterize the stalled products of *K. lactis* telomerase, but did not fit into the scope of the previous chapter.

As described in the previous chapter, the *K. lactis* telomerase reaction is characterized by the production of a curious set of products that are the result of stalling by the enzyme at specific positions along the template. Increasing the extent of primer or product DNA/template RNA complementarity exacerbates stalling, supporting the hypothesis that extensive base pairing contributes to this phenomenon. The formation of lengthy DNA/RNA hybrids during polymerization in this system would imply that telomerase lacks the ability to unpair the duplex *in vitro*. Two (not mutually exclusive) explanations can be considered for this behavior. First, stalling could be a property of the *in vitro* assay: the telomerase preparations used here may lack accessory factors or other components of the telomerase complex. Alternatively, the build-up of a lengthy RNA-DNA hybrid and the consequent stalling are inherent to the in vivo action of *K. lactis* telomerase.

The fractionation scheme utilized to partially purify *K. lactis* telomerase involves elution from an ion exchange column at a high salt concentration (0.7M NaOAc). Such conditions could 1) potentially result in the stripping away of accessory components that may only weakly interact with telomerase, or 2) interfere with the native conformation of the core enzyme. To examine this issue, we fractionated *K. lactis* telomerase by glycerol gradient centrifugation in salt concentrations that were more physiological, a much more gentle method for partial purification.

K. lactis S100 supernatant was fractionated on a 25%-45% glycerol gradient alongside the TER1-containing 0.7M salt fraction from a DEAE column. Fractions from the glycerol gradient were analyzed for the presence of telomerase by detection of TER1 RNA by dot blot hybridization. TER1-containing complexes from the high salt DEAE eluate exhibited a sedimentation coefficient of approximately 20-23S after glycerol gradient centrifugation (Figure 1A). This result is in agreement with the sedimentation coefficient of *S. cerevisiae* telomerase (24S) obtained using similar methods (3). Interestingly, TER1 RNA-containing complexes that had not been previously subject to DEAE-fractionation sedimented considerably faster, with a coefficient of 30-33S (Figure 1A). This result was not unexpected, given that telomerase complexes from S100 were observed to migrate considerably slower in native gels than DEAE-fractionated complexes (4). However, S100 telomerase from *S. cerevisiae* shows only a negligibly different sedimentation coefficient than that of DEAE fractionated material (2).

The difference in complex sizes was verified by analysis of the glycerol gradient fractions by native gel electrophoresis. As expected, TER1-containing complexes from the high salt DEAE eluate exhibited faster mobility than complexes from S100 supernatant (Figure 1B). Glycerol gradient fractionation did not alter the mobility of high salt DEAE complexes, suggesting that the conformation and/or content of the telomerase complex was not affected (Figure 1B, compare DEAE fractions to load). However, glycerol gradient sedimentation did alter the mobility of TER1-containing complex from S100, resulting in complexes that migrated significantly faster (Figure 1B, compare S100 fractions to load). It is not clear at this point if the size of the S100 complex reflects a telomerase-specific interaction that is disrupted by subsequent fractionation (via glycerol gradient or ion exchange). Alternatively, the large complex detected could be the result

of weak, non-specific interactions between telomerase and other proteins or nucleic acid in the crude extract that would not affect telomerase activity.

A crucial question was whether telomerase fractionated by the gentler glycerol gradient centrifugation conditions exhibited stalling in an *in vitro* assay. For example, lack of stalling by the larger telomerase complex would suggest a factor is missing from the typical DEAE-fractionated telomerase. Glycerol gradient fractions containing peak amounts of TER1 RNA were assessed for the ability to extend a telomeric primer in the standard *in vitro* assay for *K. lactis* telomerase activity. The primer utilized, 12(12), had been previously shown to result in stalling by telomerase (see Chapter 2 and (1)). The fraction that contained the peak amount of TER1 RNA from the fractionated S100 extract exhibited the same banding pattern of radiolabeled extension products as that produced by the high salt DEAE eluate (Figure 2, compare S100 fraction 14 to DEAE load). Unexpectedly, the DEAE fraction with peak activity (around fraction 23) did not correspond to the fraction with peak RNA levels (fraction 25). However, this matter was not pursued further.

Because preparation of telomerase by glycerol gradient sedimentation did not result in an alleviation of stalling, we propose that the stalling exhibited by DEAE-fractionated telomerase does not result from the stripping away of components necessary for complete elongation. Clearly, DEAE fractionation does result in a smaller complex than that detected in the starting material or in glycerol gradient-fractionated material, but this change does not obviously alter the core *in vitro* enzymatic properties of *K. lactis* telomerase. These results suggest that stalling could be an inherent property of *K. lactis* telomerase, which perhaps serves a regulatory role during telomerase elongation in vivo.

We propose a specific model for *K. lactis* telomerase behavior that involves stalling as a potential regulatory step. During this step, a conformational change may take place to facilitate polymerization to the end of the template. The conformational change would presumably facilitate unpairing of the RNA/DNA hybrid formed during polymerization to that point. This unpairing could take place as a result of displacement of the 5' portion of the product into a second site in the enzyme. The conformational change could also involve formation of an alternative RNA structure, such that a portion of the TER1 template would then pair with another part of the RNA, preventing it from interacting with the growing product. In an effort to explore the possibility of a conformational change occurring at stall positions during elongation, we examined the temperature dependence of formation of *K. lactis* telomerase products. Telomerase reactions were incubated at 23, 30, and 37°C and aliquots of the reaction mix were stopped at various time points, up to 20 minutes. The ratio of mid-template intermediates to near-terminal products decreased as the temperature of the reaction was increased. At early time points, total labeled products accumulated at similar rates at each temperature, suggesting that the eventual differences in 20 minute reaction profiles were not simply due to a temperature dependent increase in the polymerization rate. This data supported the hypothesis that a conformational change takes place to facilitate escape from stalling. Of course, much more information is needed to be able to draw any solid conclusions. In the future, direct investigation into 1) the length of the RNA/DNA hybrid formed, 2) RNA structure, and 3) contact of product with protein components of telomerase during polymerization would help clarify this issue.

MATERIALS AND METHODS

Glycerol gradient centrifugation

100 µl of TER1-containing DEAE fraction and 100 µl of S100 supernatant (both prepared as described in previous Chapter) were loaded onto separate 12 ml 25%-45% glycerol gradients containing 0.1% Triton X-100. Gradients were centrifuged at 40 krpm for 24 hours in an SW41 rotor at 4°C. TER1 RNA from each fraction was detected by dot blot hybridization with a *TER1* gene probe and quantitated using a STORM phosporimaging system. The sedimentation coefficients were determined relative to marker proteins (thyroglobulin, 19.3S; ferritin, 17.3S; catalase, 11.3S; and aldolase, 7.6S) that were run in a parallel gradient. Marker proteins were loaded on a 5-15% polyacrylamide gradient gel (acrylamide: bisacrylamide, 29:1) and run for 1.5 hr in 0.1% SDS, 25mM Tris-glycine buffer, followed by staining with Coomassie brilliant blue.

Native gel electrophoresis

Glycerol gradient fractions were run on 0.45% agarose/3.5% 76:1 acrylamide:bisacrylamide in 50mM Tris-Acetate for 11 hours at 240 V (15V/cm). The gels were electroblotted to Hybond N^+ membrane in 0.5X TBE buffer for 2 hours at 70V and hybridized to random-primed *TER1* probes as above.

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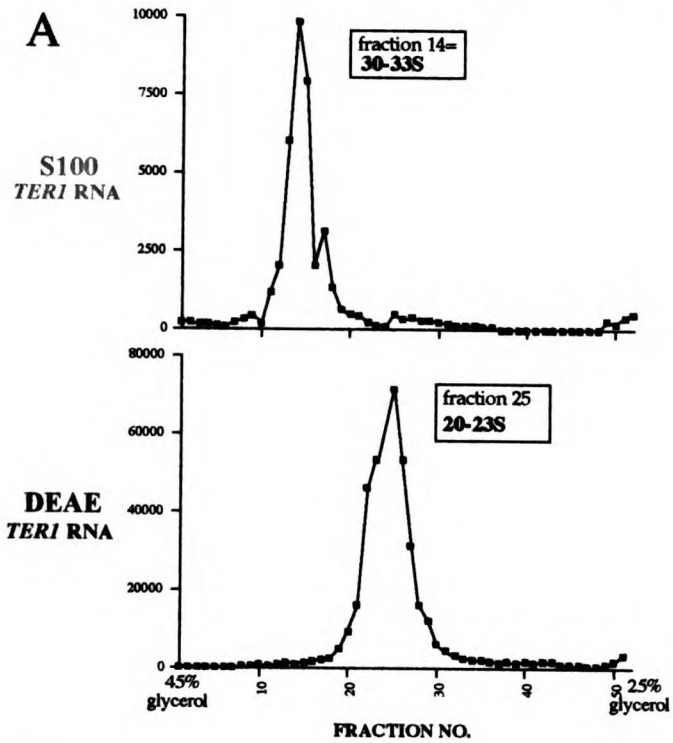
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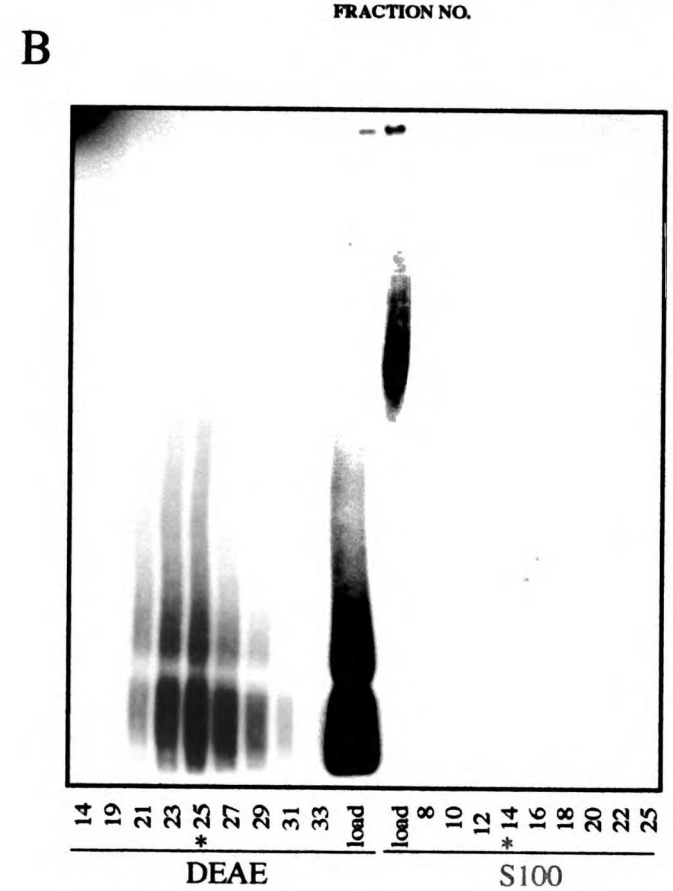
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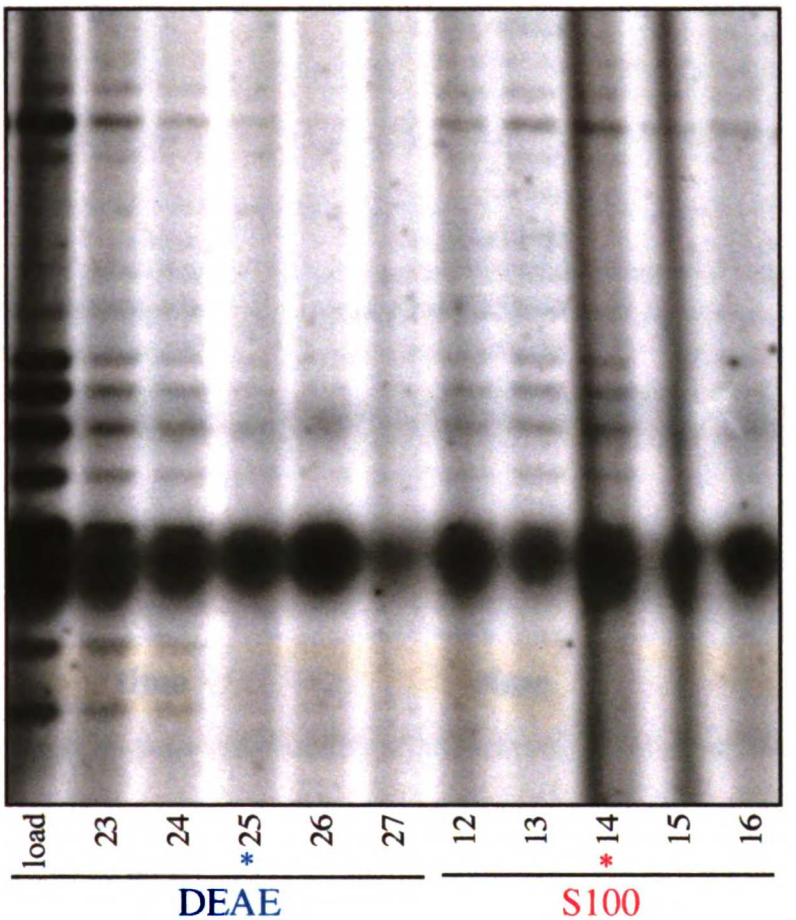
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Appendix to Chapter 2 - Figure 3

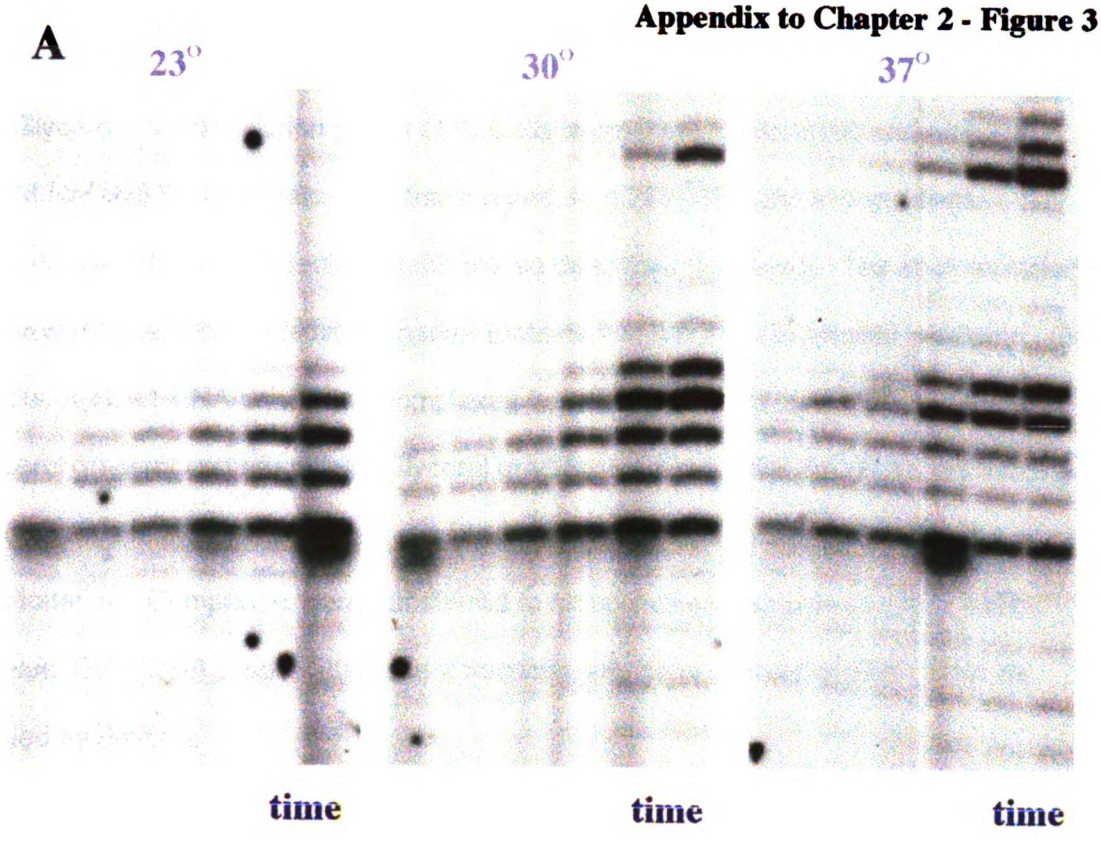

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FIGURE LEGENDS

Figure 1. DEAE-fractionation results in a smaller telomerase-containing complex. (A) Glycerol gradient elution profile of *K. lactis* telomerase. Both S100 and high salt (0.7M NaOAc) DEAE eluate were fractionated on a 25%-45% glycerol gradient. Telomerase RNA was detected by dot blot as described previously. The sedimentation value was determined relative to marker proteins that were run in parallel gradients. (B) Profile of TER1 RNA-containing complexes from S100 and high salt DEAE eluate after glycerol gradient centrifugation. Glycerol gradient fractions were run on nondenaturing gels. Lanes marked load contain samples that had not been subject to glycerol gradient fractionation. Complexes were transferred to nitrocellulose and probed with a radio-labeled *TER1* DNA probe. Stars mark fractions with peak amount of TER1 RNA as judged by dot blot.

Figure 2. Stalling is an inherent property of telomerase. Products of telomerase before and after DEAE fractionation. Glycerol gradient fractions (corresponding to those in Figure 1) were assayed for telomerase activity in vitro with primer 12(12). Lane marked load contains products form high salt DEAE eluate prior to glycerol gradient centrifugation. Stars mark fractions that contained peak amounts of tER1 RNA s judged by dot blot.

Figure 3. Higher temperatures favor formation of near-terminal products by telomerase. Products of telomerase reactions at different temperatures. Telomerase reactions were incubated at 23, 30, and 37 degrees. (A) Aliquots were stopped at 15 seconds, 30 seconds, 1.5 minutes, 6 minutes,and 20 minutes. The ratio of mid-template

Chapter Three

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

Jagoree Roy, Tracy B. Fulton, and Elizabeth H. Blackburn

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ABSTRACT

The reverse transcriptase telomerase is a ribonucleoprotein complex that adds telomeric repeats to chromosome ends, utilizing a sequence within its endogenous RNA component as a template. Though 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 non-template 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 analysed 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.

INTRODUCTION

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 telomerase is also an RNP complex (for reviews see (Blackburn 1992; Blackburn 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 reverse transcriptase 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 ~150-200 nucleotides in ciliates (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990) and ~400-450 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 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 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 1997b; Prescott and Blackburn 1997a). Hence, as the template region of the RNA must be within or close to the active site of the enzyme, mutating a residue may interfere with active site functions. The large size of

the yeast telomerase RNAs is intriguing, because the roles of the non-template regions of telomerase RNA are not well understood. An exciting possibility is that the RNA component of telomerase may contribute to the catalytic properties of the enzyme. Additionally, 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 non-template residues (Bhattacharyya and Blackburn 1997). When the non-template 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 non-template 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 non-template residues in the telomerase RNA (TER1) of the budding yeast *Kluyveromyces lactis*. Each of the 12 *K. lactis* telomeres contains an average of fifteen tandem 25-bp repeats, with the range being 10-20 repeats. TER1 RNA contains a 30 nucleotide template region that is complementary to one and a fifth telomeric repeat units (McEachern and Blackburn 1995) and an *in vitro* telomerase activity in *K. lactis* has recently been described (Fulton

and Blackburn 1998). We report that though 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 mutant telomerase RNA provides a marker for assaying *in vivo* functions of TER1 RNA.

In order 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 base pair 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 and 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 which had regenerated long tracts of telomeric DNA by an alternative *RAD52*-dependent recombination pathway ((McEachern and Blackburn 1996), Fig. 1A, lanes 7 and 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 and 10), each passage representing approximately 25 cell doublings.

To differentiate between telomeric repeats added by telomerase containing the mutant RNAs and the pre-existing 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 BclI restriction site (M. McEachern and E. H. Blackburn, unpublished results). Telomeric repeats synthesized by *TER1-BclI* incorporate the single base change without otherwise altering the telomeric repeat sequence (D. Hager and M. McEachern, personal communication). 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 and data not shown). The BclI mutation was incorporated 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 BclI mutant repeats were detected by hybridization of EcoRI-digested genomic DNA to a radio-labeled oligonucleotide probe specific for the BclI repeat (Fig. 1C, lanes 1 and 3). The hybridizing bands were

lost when the DNA was secondarily digested with BclI restriction enzyme (Fig. 1C, lanes 2 and 4). As seen by hybridization with a wild-type telomeric probe, this second digestion with BclI resulted in the expected shortening of EcoRI telomeric fragments (Fig. 1B, lanes 2 and 4, arrowheads), demonstrating that the BclI sites were at the ends of the telomeres, and were incorporated onto every chromosome. When *7BΔTER1* yeast cells were co-transformed with two plasmids, one expressing wild-type *TER1* and one expressing *TER1-BclI*, we estimated that equal numbers of wild-type and BclI marked repeats were incorporated onto telomeres (data not shown).

Various non-template mutations were created in the *TER1-BclI* allele (Fig. 2). We anticipated that both telomere length and the extent of incorporation of BclI 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. pTERWT was subsequently selected against by plating cells on medium containing 5-FOA. We compared telomere length and incorporation of BclI 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 co-expressed 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 BclI 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 and data not shown). BclI repeats were incorporated at the earliest times observable after transformation (approximately 25 doublings). Similarly, we determined the extent to which each deletion mutant was able to provide *TER1* function *in vivo*. The deletion mutants assayed are shown schematically in Fig. 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 BclI marked telomeric repeats and the length of telomeres (Table 1; examples are shown in Fig 3 C and D). With the *ter1-Δ125-204* and *ter1-Δ275-335* mutants, telomeres shortened slightly (< 50 bps; Fig. 3C and D, lanes 5 and 7). However, the amount of BclI 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 BclI repeat incorporation indistinguishable from *TER1-BclI* (Table 1; examples are shown in Fig. 3C and D). BclI repeats were also incorporated when each of these mutant RNAs was co-expressed with wild-type TER1 RNA (Table 1). Based on 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 two fold higher levels than the TER1-BclI RNA. Thus 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 and Table 1).

All three mutant RNAs functioned *in vivo* by the criterion of incorporation of BclI mutant repeats (Fig. 5A and B). However, fewer BclI 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, compare lanes 1, 3, 5 and 7). Furthermore, digestion of telomeres with BclI restriction enzyme resulted in less shortening than in *TER1-BclI* cells, indicating the presence of fewer BclI 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 approximately 200 nucleotides (i.e. equivalent to eight 25-bp telomeric repeats) for the *ter1-ΔComb* and *ter1-Δ1077-1229* mutants, and approximately 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 stably maintained 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 co-expressed with wild-type *TER1* and assayed for BclI repeat incorporation, no BclI-specific hybridization was obtained (Fig. 5C). Since we were able to detect the BclI template of the mutant gene with the BclI- specific probe, we conclude that the probe can detect approximately 1-3 repeats per telomere. Hence, in contrast to the essentially fully functional mutants, these deleted RNAs, though 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 and 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 eight-fold 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 BclI repeat incorporation and shortened telomeres. The *ter1-Δ77-424* gene was placed under the control of the inducible *GAL1* promoter resulting in approximately 50-fold higher levels of the induced GAL1-ter1-Δ77-424 RNA (Fig. 6C). This restored both the extent of BclI 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, compare lanes 1 and 3) caused no change in the length of telomeric fragments or BclI 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 BclI 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*. BclI 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 BclI specific probe to the BclI 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 non-specific hybridization, it was completely lost upon digestion of genomic DNA with BclI enzyme. Thus our inability to detect any specific hybridization to telomeric DNA

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

These mutant RNAs were expressed and stably maintained in cells at levels comparable to the TER1-BclI RNA, as judged by Northern blotting analysis (Fig. 4 lanes 1, 11, 22 and 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 4 different *Kluyveromyces* species (Y. Tzfati and E. H. Blackburn, unpublished results). 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 (Fig. 4, lanes 12, 13 and 16). The *ter1-Δ917-945* and *ter1-Δ1021-1033* mutants incorporated BclI repeats into telomeres and were fully functional *in vivo* (Fig. 8B and C,

lanes 1, 2, 5 and 6). In contrast, the ter1-Δ967-1000 mutant RNA was completely non-functional *in vivo*; there was no incorporation of BclI repeats into telomeres (Fig. 8B and C, lanes 3 and 4), and the telomeres behaved similarly to those in strain *7BΔTER1*. Hence RNA sequences critical for telomerase function lie between nucleotides 967 and 1000 of *TER1*.

We next 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 to 987) with a stable tetraloop sequence (UUCG). The second mutant, *ter1-Δbulge*, deleted the putative '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 through 996, making these positions complementary to residues 971 through 975 (see Table 1).

All three mutants were completely non-functional *in vivo*. They showed no BclI repeat incorporation for up to 10 passages (Fig. 8B, lanes 7-12), and telomeres shortened for the first 2-3 passages and then lengthened as in strain *7BΔTER1* (Fig. 8C, lanes 7-12 and data not shown). The levels of the ter1-Δbulge and ter1-bulgetostem mutant RNAs were about 30% lower than the control TER1-BclI RNA (Fig. 4 lanes 7, 17 and 18). Thus residues 971 to 975 and 992 to 996 are important for both telomerase action and stability and/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 through 987 of the RNA specifically abolished telomerase action *in vivo*.

The telomerase 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 non-functional TER1 mutant RNAs: a catalytically inactive telomerase enzyme and/or 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 non-functional *TER1* mutants, *ter1-Δ20-60*, *ter1-Δ493-580*, *ter1-Δ630-730* and *ter1-sb980-987loop*, were directly tested for telomerase activity *in vitro*.

An *in vitro* telomerase assay for *K. lactis* cell extracts has recently been developed (Fulton and Blackburn 1998). This activity extends telomeric oligonucleotides by the incorporation of deoxy-nucleotides (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 and 3), producing the characteristic pattern of mid-template and near-terminal products previously described for wild-type telomerase reactions with this primer (Fulton and Blackburn 1998). The activity was sensitive to RNase A (Fig. 9A, lanes 2 and 4). No significant difference in activity was found between the extracts containing TER1 and TER1-BclI RNA under the conditions used, confirming that the BclI 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 di-deoxy TTP at the expected position along the

template (Fig. 9B, lanes 1 and 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 non-functional mutant RNAs, when synthesis was examined in the presence of all four dNTPs (Fig. 9A, lanes 5 and 6 and 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). Since telomerase activity was detected in the *TER1-BclI* extract when it was diluted up to five fold (data not shown), we estimated that the mutant extracts contained less than 20% of wild-type telomerase activity. The *in vivo* lack of function suggests that activity may in fact be completely abolished.

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, though 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 results 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 and see Materials and Methods). Fractions from *TER1* or *TER1-BclI* cell extracts contained a single major RNP complex (Fig. 9C, lanes 1 and 3). The mobility of the hybridizing species increased upon pre-treatment with proteinase K or phenol/chloroform extraction, indicating that the complex contains at least one protein component (Fig. 9C, lanes 2 and 4 and data not shown). As expected, the RNP complex was also sensitive to RNase A treatment (data not shown). For the non-functional mutants *ter1-Δ493-580* and *ter1-Δ630-730*, a single major hybridizing species was observed, whose mobility remained unchanged upon 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 non-functional *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 and 12). Thus, though 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 non-functional *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-Bcl1* counterparts (Fig. 9C, compare lanes 3 and 5, 4 and 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 though it is still assembled with at least some protein(s). The protein composition of this enzymatically inactive mutant complex may be similar to that of the wild-type complex, since 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, compare lanes 3, 5, 7 and 4, 6, 8). Extracts from these strains also contained undetectable levels of telomerase activity *in vitro* (data not shown). Thus 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 co-expressed 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 co-expression we would also be able to detect co-operativity 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 non-template 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. Since this complex is functional, it must contain the reverse transcriptase (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 through 580 and 630 through 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. Thus another possibility is that the mutant RNAs do not achieve proper secondary, tertiary and/or quaternary structural conformation, and this prevents correct RNP assembly.

Critical non-template 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 non-template 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, unpublished results). 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 non-denaturing electrophoretic conditions, indicative of a significantly different conformation. However, this does not prevent the mutant RNAs from interacting with protein(s). Thus these mutated nucleotides are likely to be involved in functions other than protein binding. These could include recognition and binding of DNA and/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 co-expression 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 (reviewed in (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 (reviewed in (Nilsen 1998). Based on these precedents and the results reported above, we suggest that the 971-996 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*. Further, 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 state 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 reverse transcriptase (RT) protein subunit, these mutants would be expected to demonstrate defects in telomerase RNP assembly and/or decreased *in vitro* telomerase activity. Alternatively, the mutant RNAs may be unable to properly bind 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 analysed 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 non-dissociative *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 XhoI-EcoRI fragment containing *TER1* was cloned into the *URA3* marked yeast integrating vector YIP5. A 1.3 kb PmeI- SphI 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 BamHI-Sau3A DNA fragment containing the *K. lactis CEN2* was ligated to a 2.5 kb BamHI-SalI DNA fragment containing the *K. lactis ARS1B*. The resulting 4.5 kb *CEN-ARS* cassette was cloned into the AatII 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 non-selective 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 XhoI-EcoRI 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 BclI 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 BamHI site was introduced in the *TER1* sequence of pTERBclI at position 21 by site-directed mutagenesis. The BamHI-EcoRV fragment containing *TER1* was removed from this construct and cloned into pGAL-Kl313 (BamHI and SmaI digested) to result in the *GAL-TER1* allele. This places nucleotide 22 of *TER1* approximately 50 nucleotides 3' of the RNA start site in the *GAL-TER1* construct. Thus the *GAL-TER1* construct contains the BclI 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 1X 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 hours whereas for random primed DNA probes hybridization was carried out overnight. The 5' radio-labeled oligonucleotide Klac1-25 (ACGGATTTGATTAGGTATGTGGTGT) was hybridized to blots at 55°C to visualize mutant and wild-type telomeric repeats. To detect BclI marked telomeric repeats specifically, the 5' radio-labeled oligonucleotide KTelBcL (GATCAGGTATGTGG) was hybridized to the blots at 40°C. Two 5 minute 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 and/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, 1XTBE gels containing 7% formaldehyde and blotted to Hybond Nytran^{+} in 10 X SSC buffer. The membrane was subjected to cross linking and hybridized to random primed DNA probes as above. For the gels shown in Fig. 5, 9B and 9C 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 Fig. 6C only the *TER1-704-1273* 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 3-4 litres of cells, at optical density (OD_{600}) of 2.0, for each plasmid bearing strain (approximately 10-14g of cell pellet for each strain). Cells were grown in selective medium to OD_{600} of 2.0, then diluted 4-5 fold 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 pre-chilled mortar and pestle by grinding under liquid nitrogen for approximately 20 minutes. 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 0.35M 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 0.5M, 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 0.7M 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 minutes. RNase pretreatment consisted of incubation of the DEAE fractions with 10 μg/ml RNase A at 25°C for 5 minutes prior to 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 15V/cm for 10-13 hours at 4°C. The gels were electroblotted to Hybond N^+ membrane in 0.5X TBE buffer for 2 hours at 70V and hybridized to random-primed *TER1* probes as above. Fig. 9 shows samples electrophoresed in the presence of heparin. Omitting heparin caused no obvious changes in the mobility of complexes, though 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).

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Table 1

Summary of *TER1* mutant alleles assayed *in vivo*

TER1 allele	BclI repeat incorporation in presence of pTERWT	BclI 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 [1]	+	++	wild-type	++ (fully functional)
ter1-Δ80-120 [2]	+	++	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[3]	-	+	~200 bp shorter than wild-type	+
GAL1-TER1[4]	+	++	wild-type	++
GAL1-ter1-Δ77-424 [4]	+	++	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[5]*	-	-	S	-
ter1-Δ bulge [6]*	-	-	S	-
ter1-bulgetostem[7]*	-	-	S	-

[1] *TER1* nucleotide A458 is changed to G. All subsequent *ter1* mutant alleles also contain this mutation.

[2] Δ a-b represents a deletion of nucleotides a through b.

[3] The *ΔComb* deletion is a combination of the Δ697-714, Δ718-731, Δ750-785, Δ810-818 and Δ825-850 deletions.

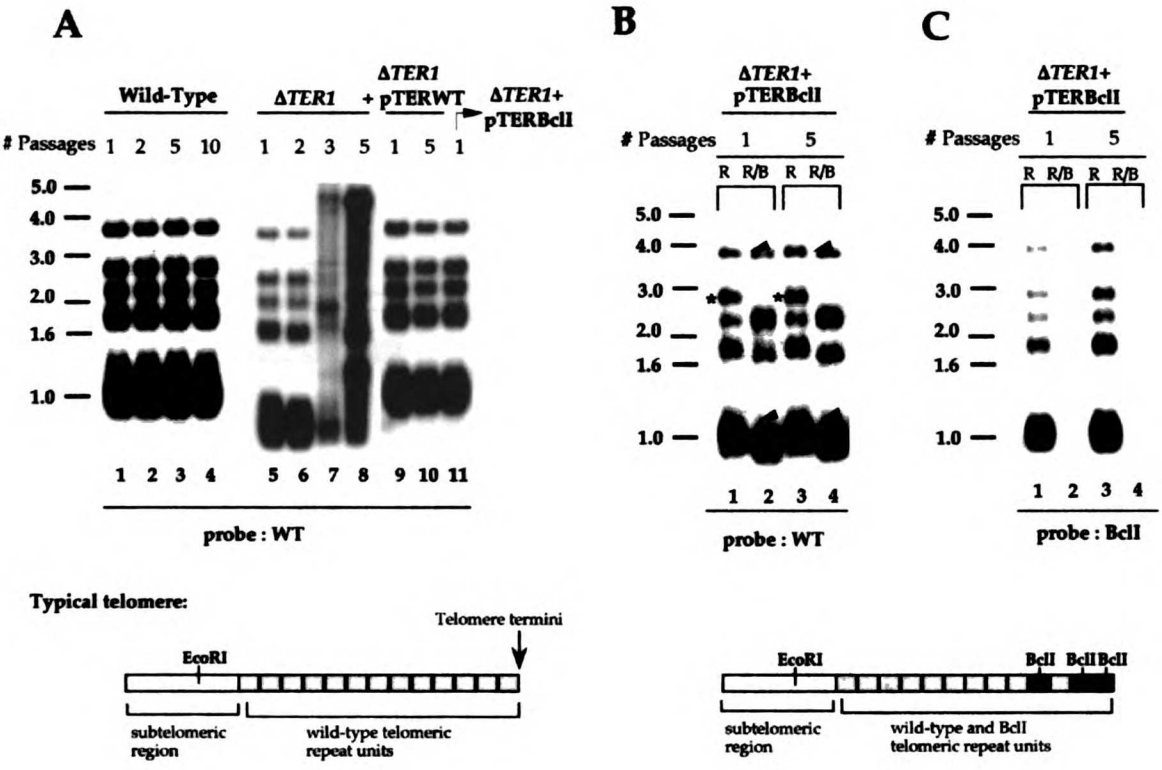
[4] In the *GAL1-ter1* alleles nucleotides 1-20 of *TER1* are replaced with GAL1 RNA leader sequences.

[5] *TER1* nucleotides 980 through 987 are substituted with TTCG.

[6] *TER1* nucleotides 971 through 975 and 992 through 996 are deleted.

[7] *TER1* nucleotides 992 through 996 are replaced with TGAGG.

* loop, bulge and stem refer to putative secondary structural elements.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Wild-Type
ΔTER1
ΔTER1 + pTERWT
ΔTER1+ pTERBclI
Passages 1 2 5 10 1 2 3 5 1 5 1
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4.0
3.0
2.0
1.6
1.0
1 2 3 4 5 6 7 8 9 10 11
probe : WT
B
ΔTER1+ pTERBclI
Passages 1 5
R R/B R R/B
5.0
4.0
3.0
2.0
1.6
1.0
1 2 3 4
probe : WT
C
ΔTER1+ pTERBclI
Passages 1 5
R R/B R R/B
5.0
4.0
3.0
2.0
1.6
1.0
1 2 3 4
probe : BclI
Typical telomere:
Telomere termini
EcoRI
subtelomeric region
wild-type telomeric repeat units
EcoRI
BclI BclI BclI
subtelomeric region
wild-type and BclI telomeric repeat units

5'

ter1-Δ20-60

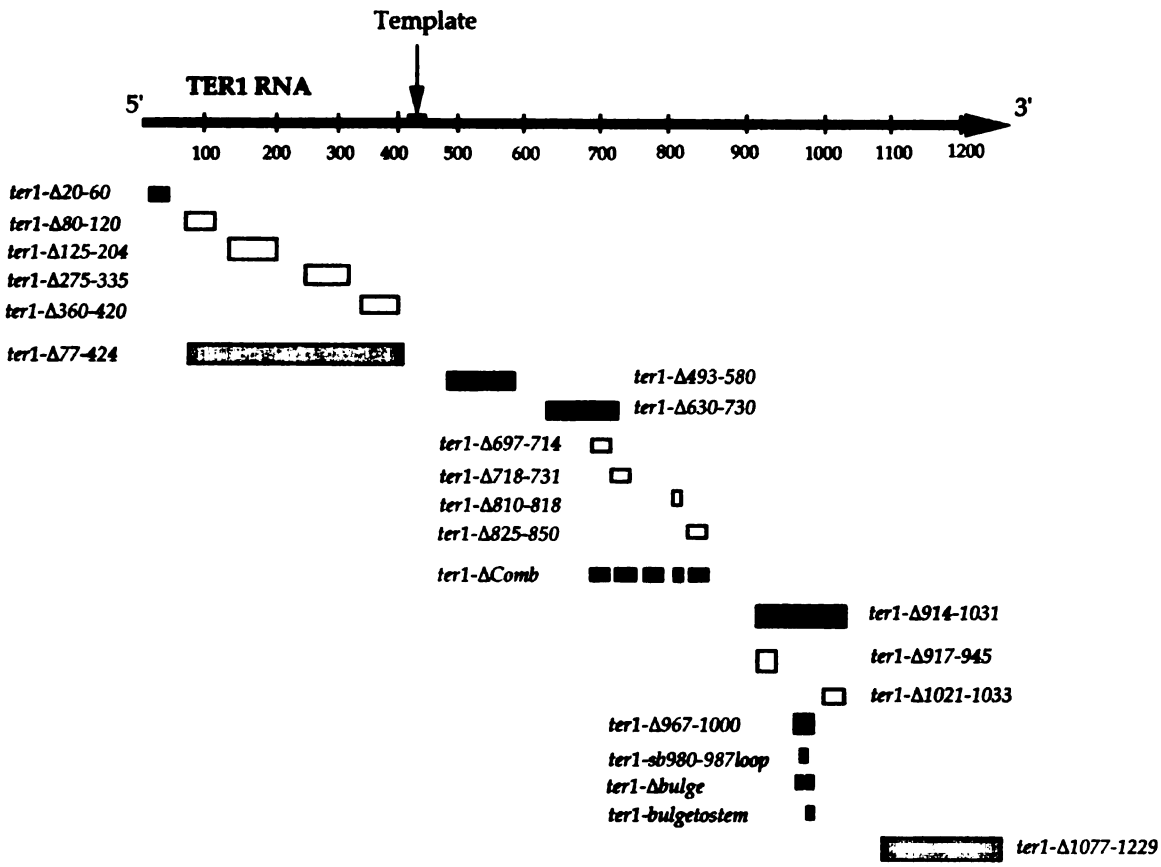
ter1-Δ80-120

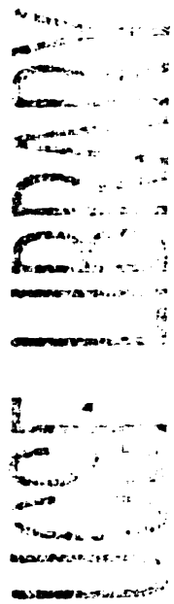
ter1-Δ125-204

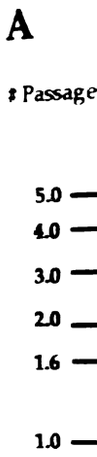
ter1-Δ275-335

ter1-Δ360-420

ter1-Δ77-424


Template
TER1 RNA
5'
3'
100
200
300
400
500
600
700
800
900
1000
1100
1200
ter1-Δ20-60
ter1-Δ80-120
ter1-Δ125-204
ter1-Δ275-335
ter1-Δ360-420
ter1-Δ77-424
ter1-Δ493-580
ter1-Δ630-730
ter1-Δ697-714
ter1-Δ718-731
ter1-Δ810-818
ter1-Δ825-850
ter1-ΔComb
ter1-Δ914-1031
ter1-Δ917-945
ter1-Δ1021-1033
ter1-Δ967-1000
ter1-sb980-987loop
ter1-Δbulge
ter1-bulgetostem
ter1-Δ1077-1229

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Passage
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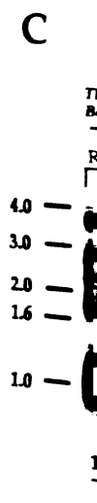
3.0
2.0
1.6
1.0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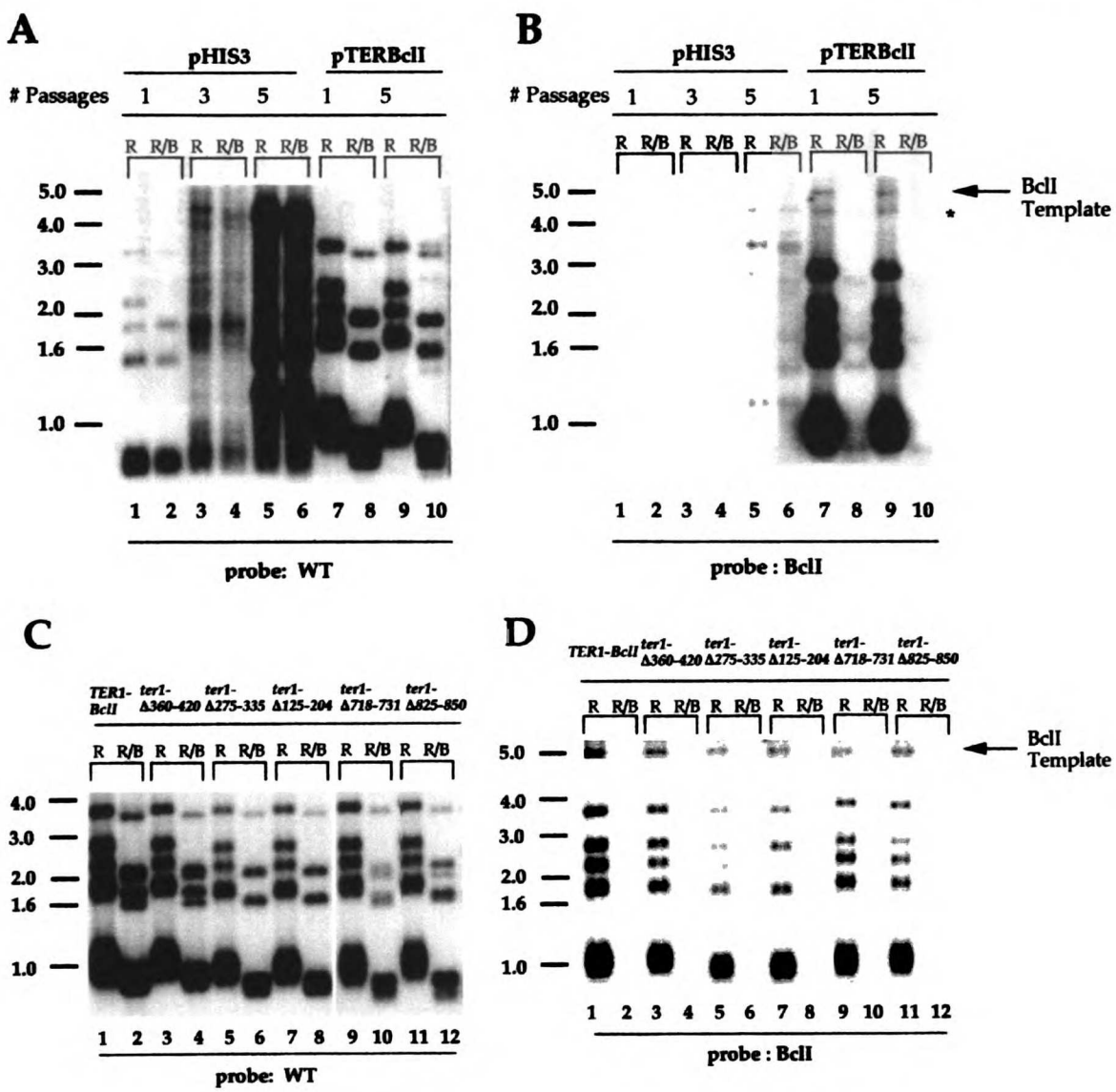
2.0
1.6
1.0


A
pHIS3
pTERBclI
Passages
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R R/B R R/B R R/B R R/B R R/B
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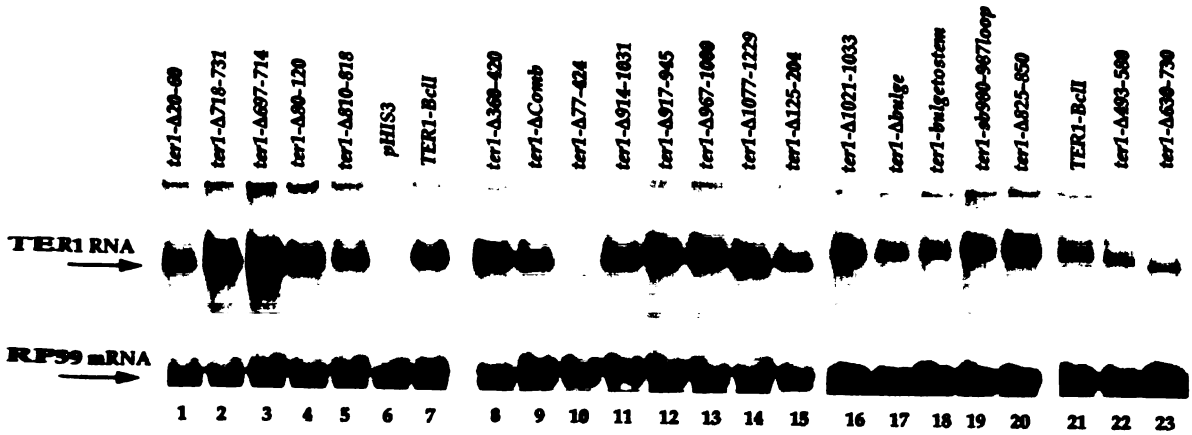
4.0
3.0
2.0
1.6
1.0
1 2 3 4 5 6 7 8 9 10
probe: WT
B
pHIS3
pTERBclI
Passages
1 3 5 1 5
R R/B R R/B R R/B R R/B R R/B
5.0
4.0
3.0
2.0
1.6
1.0
BclI Template
*
1 2 3 4 5 6 7 8 9 10
probe : BclI
C
TER1-BclI
ter1-Δ360-420
ter1-Δ275-335
ter1-Δ125-204
ter1-Δ718-731
ter1-Δ825-850
R R/B R R/B R R/B R R/B R R/B R R/B
4.0
3.0
2.0
1.6
1.0
1 2 3 4 5 6 7 8 9 10 11 12
probe: WT
D
TER1-BclI
ter1-Δ360-420
ter1-Δ275-335
ter1-Δ125-204
ter1-Δ718-731
ter1-Δ825-850
R R/B R R/B R R/B R R/B R R/B R R/B
5.0
4.0
3.0
2.0
1.6
1.0
BclI Template
1 2 3 4 5 6 7 8 9 10 11 12
probe : BclI

ter1-Δ20-60
TER1 RNA
RP59 mRNA
1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ter1-Δ80-120
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pHIS3
TER1-BclI
ter1-Δ368-420
ter1-ΔComb
ter1-Δ77-424
ter1-Δ914-1031
ter1-Δ917-945
ter1-Δ967-1000
ter1-Δ1077-1229
ter1-Δ125-204
ter1-Δ1021-1033
ter1-Δbulge
ter1-bulgetostem
ter1-ab900-967loop
ter1-Δ825-850
TER1-BclI
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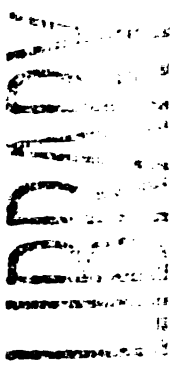
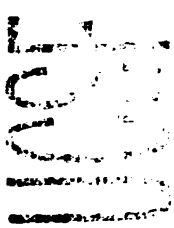
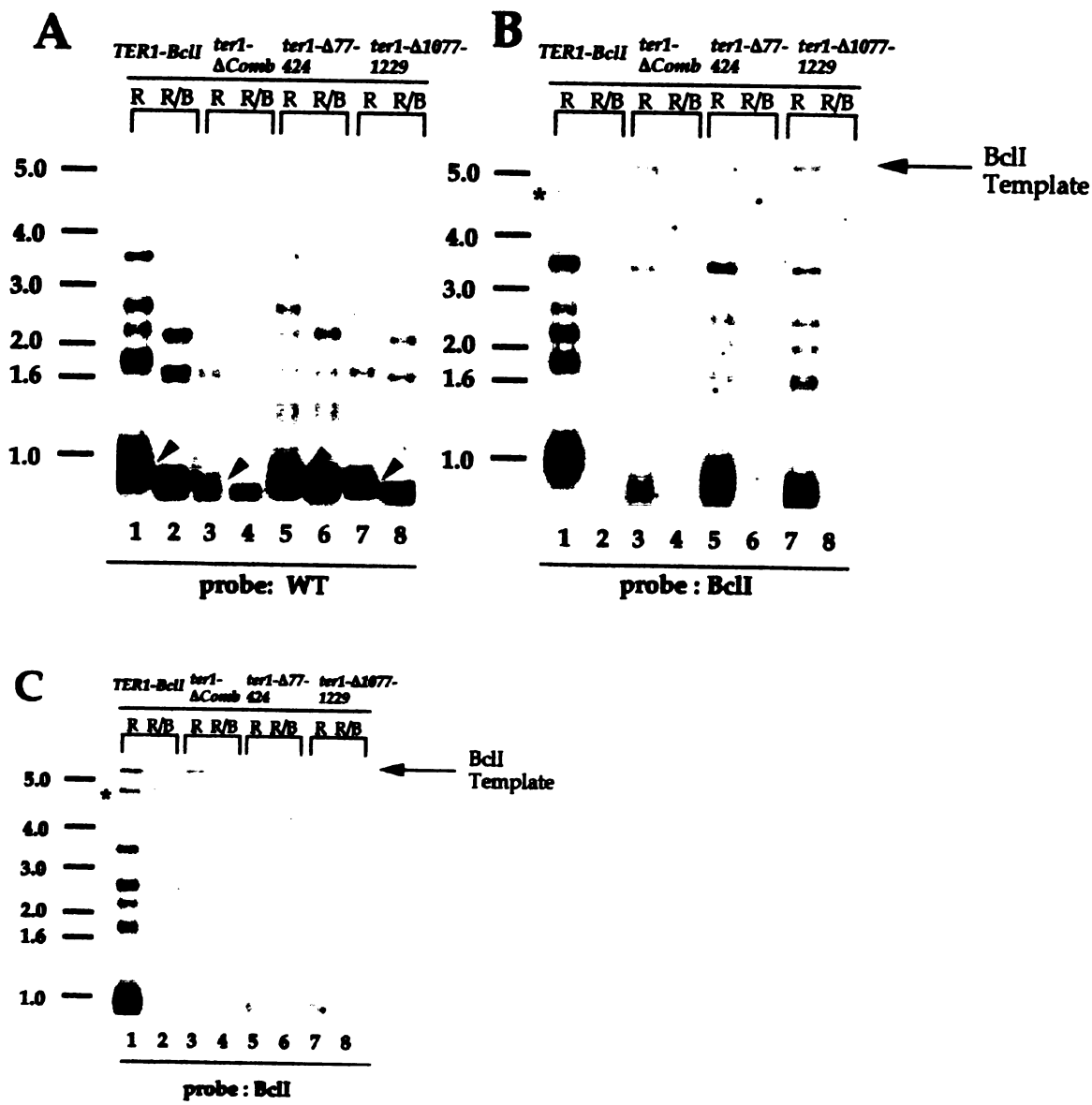
ter1-Δ630-730
TER1 RNA
RP39 mRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23






A
TER1-BclI
ter1-ΔComb
ter1-Δ77-424
ter1-Δ1077-1229
R R/B R R/B R R/B R R/B
5.0
4.0
3.0
2.0
1.6
1.0
1 2 3 4 5 6 7 8
probe: WT
B
TER1-BclI
ter1-ΔComb
ter1-Δ77-424
ter1-Δ1077-1229
R R/B R R/B R R/B R R/B
BclI Template
5.0
4.0
3.0
2.0
1.6
1.0
1 2 3 4 5 6 7 8
probe : BclI
C
TER1-BclI
ter1-ΔComb
ter1-Δ77-424
ter1-Δ1077-1229
R R/B R R/B R R/B R R/B
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2.0
1.6
1.0
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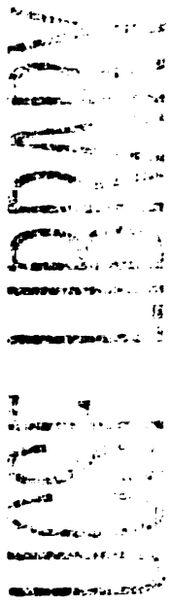
probe : BclI



Bdl
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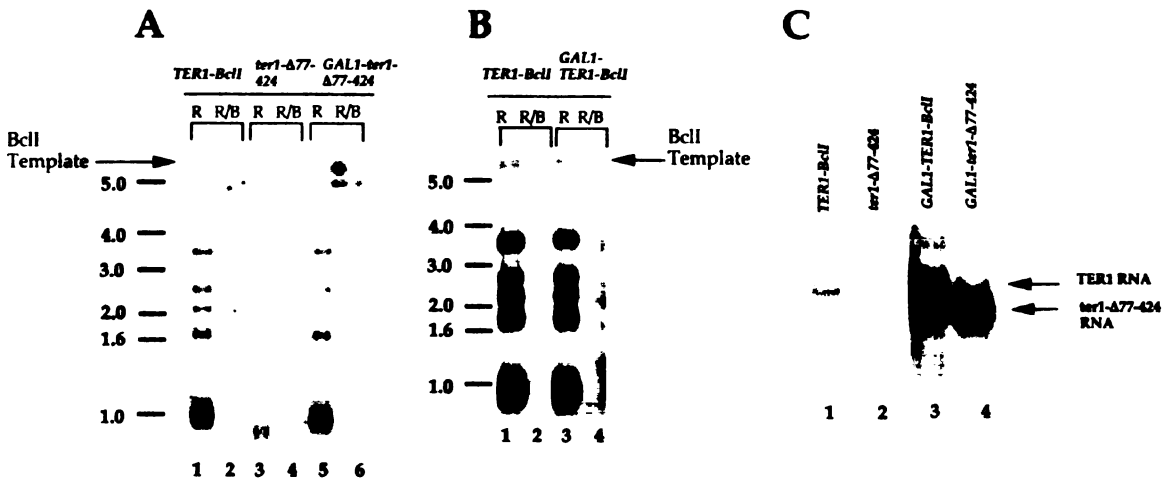
2.
1.
1.0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GAL1-ter1-Δ77-424
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2.0
1.6
1.0
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TER1-BclI
GAL1-TER1-BclI
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C
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ter1-Δ77-424 RNA
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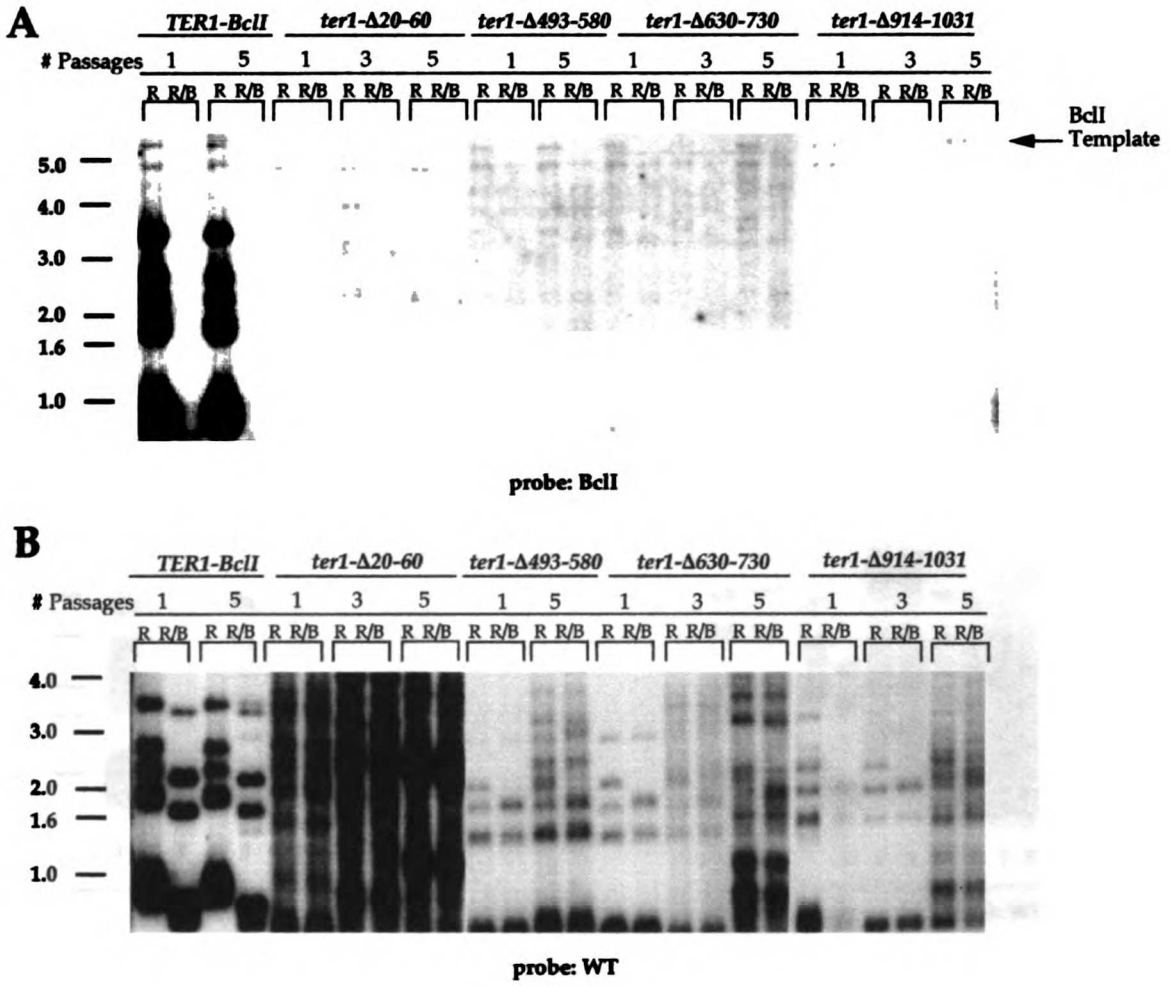
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ter1-Δ20-60
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ter1-Δ630-730
ter1-Δ914-1031
Passages
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R R/B
BclI Template
5.0
4.0
3.0
2.0
1.6
1.0
probe: BclI
B
TER1-BclI
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ter1-Δ493-580
ter1-Δ630-730
ter1-Δ914-1031
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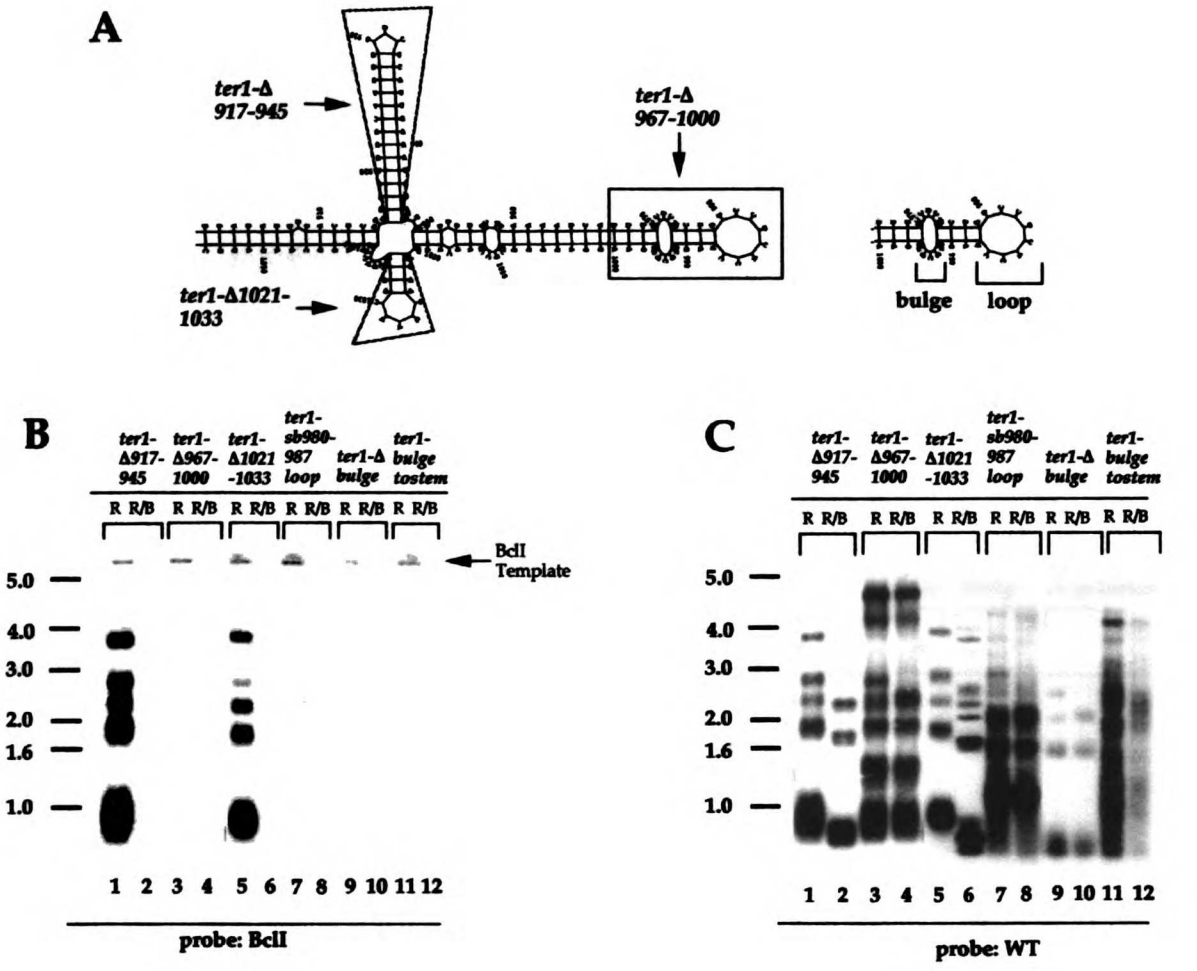
probe: WT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ter1-Δ1021-1033
bulge
loop
B
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ter1-Δ967-1000
ter1-Δ1021-1033
ter1-sb980-987 loop
ter1-Δ bulge
ter1-bulge tostem
R R/B
BclI Template
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1.6
1.0
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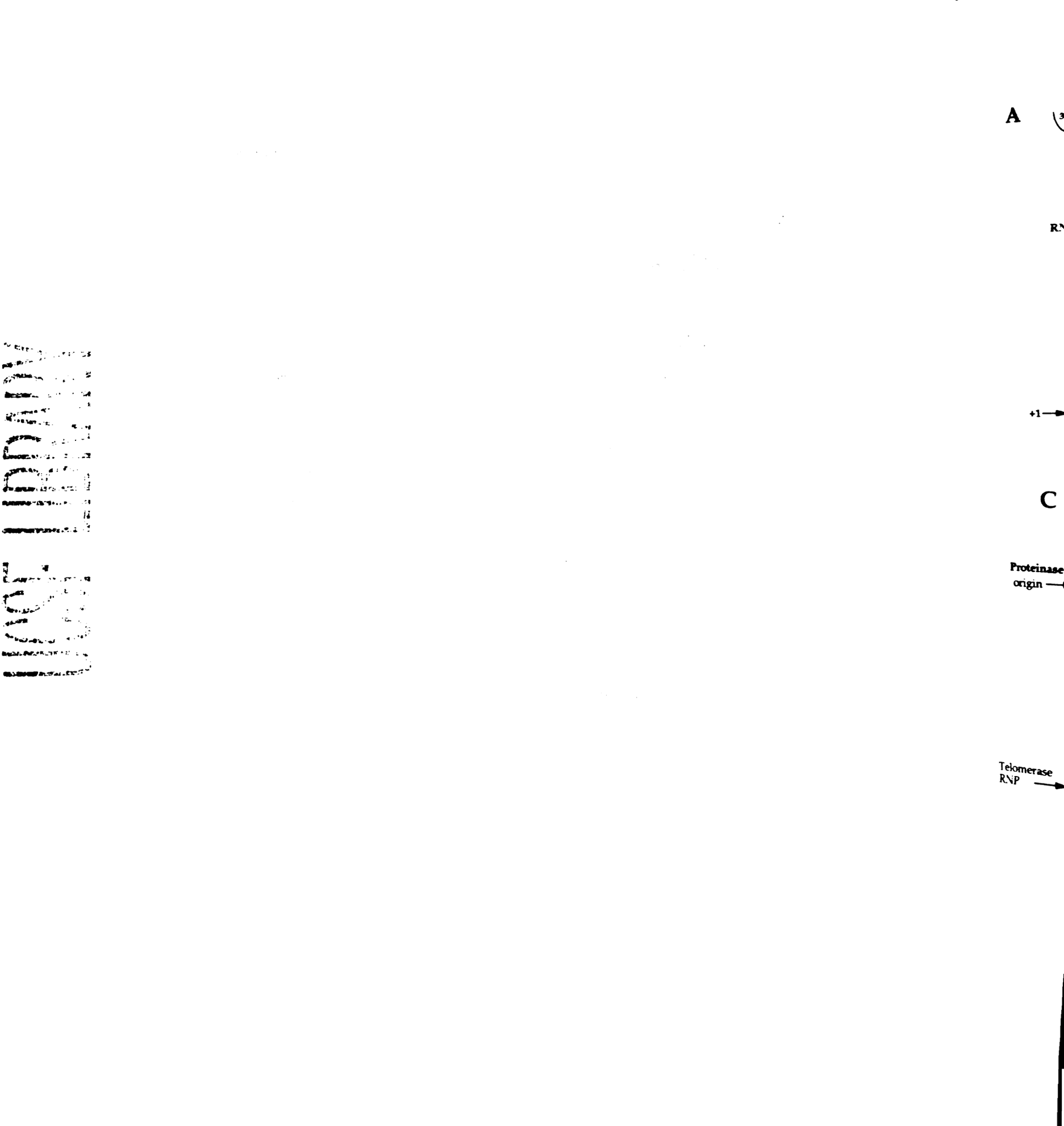
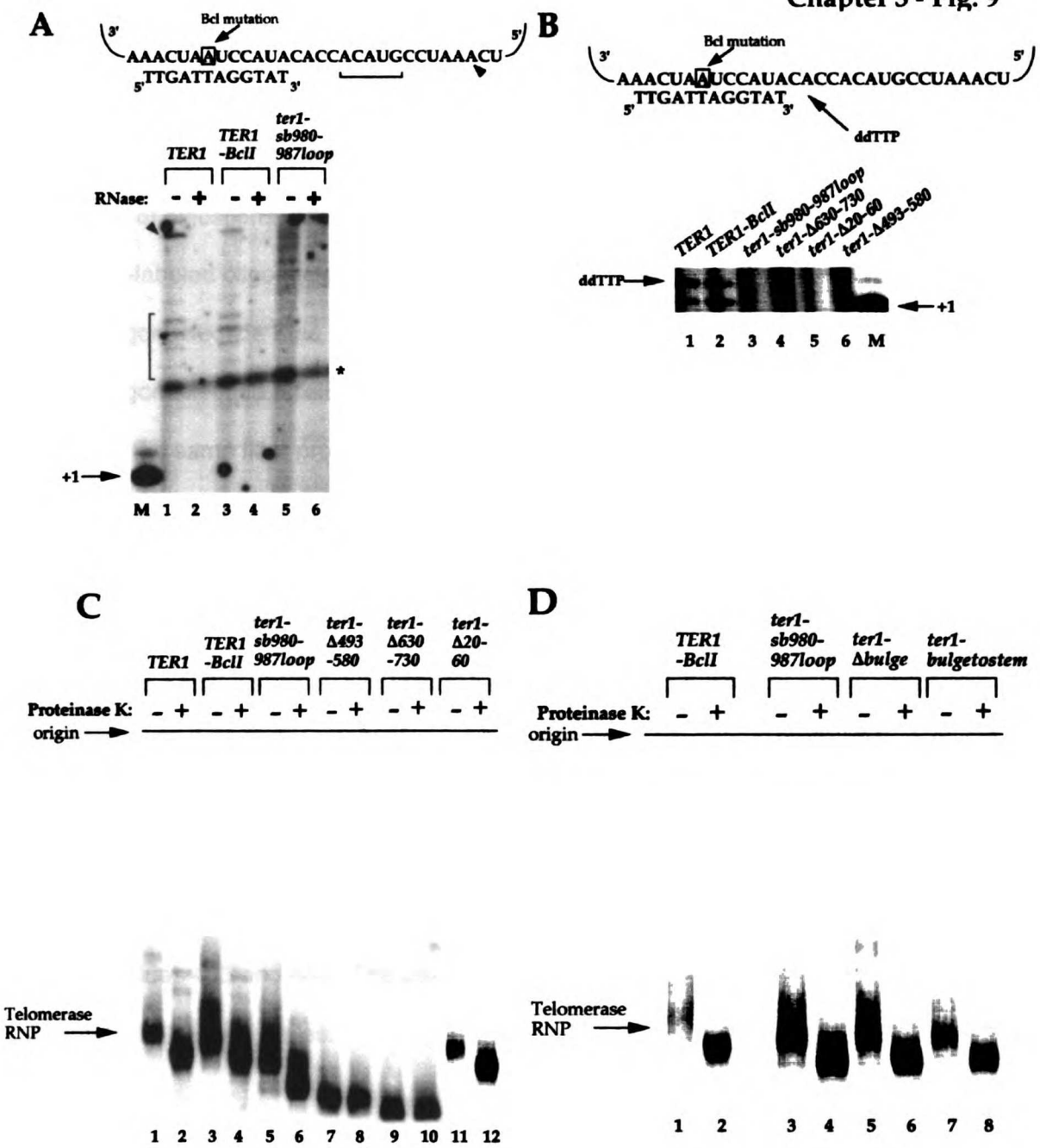
probe: BclI
C
probe: WT


A
RN
+1
C
Proteinase
origin
Telomerase
RNP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ter1-sb980-987loop
RNase: - + - + - +
+1
M 1 2 3 4 5 6
B
Bcl mutation
3'
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5'
5'TTGATTAGGTAT3'
5'
ddTTP
TER1
TER1-BclI
ter1-sb980-987loop
ter1-Δ630-730
ter1-Δ20-60
ter1-Δ493-580
ddTTP
+1
1 2 3 4 5 6 M
C
TER1
TER1 -BclI
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origin
Telomerase RNP
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D
TER1 -BclI
ter1-sb980-987loop
ter1-Δbulge
ter1-bulgetostem
Proteinase K: - + - + - + - +
origin
Telomerase RNP
1 2 3 4 5 6 7 8

FIGURE LEGENDS

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, *ΔTER1* or derivatives thereof. DNAs are digested with EcoRI (A) or in pairs of digestions with EcoRI (R) and EcoRI plus BclI (R/B) (B and C) and probed with radio-labeled oligonucleotide probes for telomeric repeats as indicated. The WT probe (oligonucleotide Klac 1-25) hybridizes to wild-type and mutant repeats. The BclI probe (oligonucleotide KTelBcl) hybridizes only to BclI marked repeats. Panels B & C represent the same filter probed first with the BclI probe (C) and subsequently with the WT probe (B). DNA markers in kilobases are indicated to the left of each panel. All twelve 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 '*' in (B) contains a BclI restriction site between the subtelomeric EcoRI site and the telomere end. The arrowheads demonstrate examples of telomeric fragment shortening upon BclI digestion. Each passage represents approximately 25 cell doublings.

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.

Figure 3. Telomeric profile of genomic DNA from control and mutant strains. The *ΔTER1* strain was transformed with control pHIS3 and pTERBclI plasmids (A and B) or fully functional *TER1* deletion mutants (C and D). Each pair of lanes contains DNA digested with EcoRI (R) and EcoRI plus BclI (R/B) enzymes, respectively. Each passage represents about 25 doublings after the loss of the pTERWT plasmid. The

DNAs in (C) and (D) were prepared from cells after 5 passages. Panels A&B and C&D represent the same filters probed first with the BclI probe (B, D) and subsequently with the WT probe (A, C). The '*' in (B) is a non-specific band.

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 (upper panel) or a probe for RP59 mRNA (lower panel).

Figure 5. BclI repeat incorporation and telomere shortening in partially functional *TER1* mutants. Telomeric profile of DNAs prepared from the 5th passage of the *ΔTER1* strain expressing *TER1-BclI* or partially functional *TER1* alleles alone (A and B) or together with the pTERWT plasmid (C). Each pair of lanes contains DNA digested with EcoRI (R) and EcoRI plus BclI (R/B) enzymes, respectively. Panels A & B represent the same filter probed first with the BclI probe (B) and subsequently with the WT probe (A). The '*' in (B) and (C) is a non-specific band.

Figure 6. Overexpression of a partially functional *TER1* mutant restores telomere length. (A, B) Telomeric profile of DNAs prepared from the 5th passage of the *ΔTER1* 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 EcoRI (R) and EcoRI plus BclI (R/B) enzymes, respectively. The blots are probed with the BclI probe. (C) Northern blot of RNA prepared from the *ΔTER1* strain expressing *TER1* alleles on plasmids. The cells were grown in galactose containing

medium. The blot is probed with radio-labeled *TER1* DNA. Equal amounts of RNA were loaded in each lane.

Figure 7. BclI repeats are not incorporated in telomeres of non-functional *TER1* mutants. Telomeric profiles of DNAs prepared from the *ΔTER1* strain expressing *TER1-BclI* or *TER1* deletion alleles, as indicated. Each pair of lanes contains DNA digested with EcoRI (R) and EcoRI plus BclI (R/B) enzymes, respectively. Each passage represents about 25 doublings after the loss of the pTERWT plasmid. Panels A & B represent the same filter probed first with the BclI probe (A) and subsequently with the 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 *ΔTER1* strain expressing *TER1* deletion alleles. Each pair of lanes contains DNA digested with EcoRI (R) and EcoRI plus BclI (R/B) enzymes, respectively. Panels B & C represent the same filter probed first with the BclI probe (C) and subsequently with the WT probe (B).

Figure 9. Telomerase activity and RNP complex profiles of non-functional mutants. (A,B) Non-functional *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 to 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 minutes. 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 '*' marks a non-telomerase generated background band described previously (Fulton and Blackburn 1998). (B) Reactions with DEAE-fractionated extracts from *TER1* (lane 1), *TER1-BcI* (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 non-denaturing gels and probed with a radio-labeled *TER1* DNA probe. The gel in (C) was run for 13 hours whereas the gel in (D) was run for 10 hours.

CHAPTER FOUR

RNA structure in a yeast telomerase specifies the template boundary

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ABSTRACT

The telomerase ribonucleoprotein has a phylogenetically divergent RNA subunit, which contains a short template for telomeric DNA synthesis. To understand how telomerase RNA participates in mechanistic aspects of telomere synthesis, we studied a conserved secondary structure adjacent to the template. Disrupting this structure caused DNA synthesis to proceed beyond the normal template boundary, resulting in altered telomere sequences, telomere shortening and cellular growth defects. Compensatory mutations restored normal telomerase function. Thus, the RNA structure, rather than its sequence *per se*, specifies the template boundary. This study reveals the first specific function for an RNA structure in the enzymatic action of telomerase.

Telomerase, a ribonucleoprotein reverse transcriptase (RT), replenishes telomeric DNA that would otherwise be lost with each round of eukaryotic DNA replication (1). The telomerase complex contains an RNA subunit (TER), a catalytic RT protein (TERT) and several additional protein components (2). Telomerase is activated in most human cancers and its ectopic expression can greatly extend the lifespan of normal human cells in culture (3, 4).

Telomerase RNAs are extremely divergent in sequence, and vary in length from 146 nucleotides (nts) in the ciliate *Tetrahymena paravorax* (5) to 1,544 nts in the budding yeast *Candida albicans* (6). Unlike the extensive genome copying carried out by other reverse transcriptases, telomerase copies only a small portion (termed the 'template') of its TER moiety (7). This unique feature enables telomerase to synthesize onto telomeres a species-specific, 5 to 26 base-long, repeated sequence (8). How telomerase specifies its template boundaries (where DNA synthesis initiates and where it ends on the TER sequence) has yet to be understood.

To study how telomerase RNA participates in the mechanism and regulation of telomerase function, we searched for conserved sequences and structural elements in budding yeast telomerase RNAs. We cloned and analyzed TER genes from four *Kluyveromyces* species closely related to *K. lactis* (9). The mature RNAs ranged in length from 930 nts in *K. aestuarii* to 1,320 nts in *K. dobzhanskii*. Sequence identity between any given pair of genes ranged from insignificant to about 70% overall identity. The computer-program *mfold* (10) predicted extensive secondary structures for these RNA sequences, including a common feature shared by all five TERs: base pairing of the sequence immediately upstream of the template (pairing element B, Fig. 1A) with a

sequence 200-350 nts further upstream (pairing element A), located near the 5' end of the RNA. The region between the pairing elements (indicated by dashed line in Fig. 1A and dashed loop in Fig. 1B) was shown previously to be dispensable in *K. lactis* (11). The proximity of this conserved putative pairing region to the 5'-end of the template led us to hypothesize that its function is to limit DNA synthesis, thereby defining the downstream boundary of the template.

To test this hypothesis, we constructed a series of mutations in the putative pairing region of the *K. lactis* TER gene (Fig. 1C and 1D). We replaced the wild-type TER gene in *K. lactis* with the mutant genes by a vector-shuffling system described previously (11), and analyzed their effects *in vitro* and *in vivo*. In each of four disruption mutations (D1, D1', D2, D2'; Fig. 1C and 1D) a trinucleotide sequence within either strand of the pairing region was substituted with its complementary sequence. The D1 and D1' mutations, in pairing elements A and B, respectively, were designed to unwind the first 3 base pairs of the putative pairing region, adjacent to the template. The D2 and D2' mutations targeted 3 base pairs in the middle of pairing elements A and B, respectively, and were predicted to cause a more extensive disruption of the pairing. Pairing potential was restored in the double mutants D1/D1' and D2/D2'. In a full replacement mutant, R1, 10 nucleotides on each strand of the pairing region were replaced with unrelated sequences that maintained the original base composition and pairing potential (Fig. 1C and 1D).

To study the effects of the pairing mutations on telomerase function, partially purified extracts from wild type or mutant cells were assayed for telomerase activity *in vitro* (12). The characteristic pattern of elongation products synthesized by telomerase with a wild-type pairing region (Fig. 2A, lane 2) was described previously (13). It includes a faint

band corresponding to the longest product that can result from one round of synthesis along the maximal potential template (open arrowhead), and stronger bands, which are one to three nucleotides shorter. The activities from each of the various cell extracts were ribonuclease A-sensitive [Fig. 2A, lane 1 and (14)], a hallmark of telomerase activity.

Strikingly, all the pairing-disruption mutants synthesized longer products than wild type telomerase (Fig. 2A, compare lanes 4, 6, 12 to lane 2; and see Fig. 2B lanes 5 and 7). The extent of read-through synthesis beyond the normal boundary of the maximal template (indicated in Fig. 2 by closed and V-shaped arrowheads) correlated with the extent of the predicted disruption of the pairing region (Fig. 1D). The D1 and D1' mutations each resulted in detectable read-through of 2 nucleotides (Fig. 2A, lanes 4 and 6). Since extracts prepared from the D2 and D2' mutants had reduced telomerase activity (Fig. 2A, lanes 10-13), we integrated the D2 and D2' mutant TER genes into the genome by replacing the endogenous gene (15). The resulting strains, iD2 and iD2', exhibited stronger telomerase activity *in vitro*, generating detectable products with up to 7 and 4 read-through nucleotides (Fig. 2B, lanes 7 and 5), respectively. For all strains tested, the polymerization activity observed was specific to the telomerase template sequence, as indicated by omitting dATP from the reactions. The resulting –dATP products were of the length expected if telomerase elongated the correctly-aligned primer and stopped just prior to the uridine that is the last nucleotide in the maximal possible template (Fig. 2A, -dATP lanes). The read-through products were correctly copied from the TER sequence 5' of the template, as indicated by the pattern of bands when dCTP was omitted from the reactions. Telomerase activity of the D1', but not the D1 mutant, was limited by the omission of dCTP, as expected if the G-containing D1'

mutation was to be copied (Fig. 2B, see disappearance of V-shaped arrowhead in lane 2 compared to lane 1). The difference in mobility between the D1 and D1' read-through products (Fig. 2B, lanes 1 and 3) was another indication that these mutant enzymes copied different nucleotides. Synthesis by the D2 mutant was also limited by the omission of dCTP, as expected from copying the wild-type sequence adjacent to the template (Fig. 2B, see disappearance of bands indicated by V-shaped arrowheads in lane 6 compared to lane 7).

The presence of sequence covariation in the pairing elements of the TER genes (Fig. 1B) suggested that base pairing, rather than a specific sequence *per se*, is required to specify the template boundary. Two different experiments support this prediction. First, the D1/D1' and D2/D2' compensatory mutations reestablished an apparently wild-type template boundary (Fig. 2A, lanes 8 and 14). Second, R1 telomerase, with complementary but scrambled pairing sequences, also retained the normal boundary (Fig. 2A, lane 16). Together, the results showed that disrupting the secondary structure shifts the template boundary, and restoring base pairing with complementary mutations reestablishes the normal boundary.

Having shown that disrupting the pairing region caused template read-through *in vitro*, we tested the effects of these mutations *in vivo*. To distinguish telomeric repeats added by the mutant telomerases, from repeats synthesized previously by the wild-type enzyme, we used an additional single-nucleotide mutation producing a BclI restriction site within the template sequence. This mutation is phenotypically silent but results in the incorporation of telomeric repeats containing a BclI restriction site onto telomeres (11), thus marking the action of the mutant enzyme. All the pairing-mutant telomerases were

active *in vivo*, as evident by the incorporation of BclI-site-containing telomeric repeats. These repeats were detected by differential hybridization of a BclI-specific probe to Southern blotted genomic DNA cut with EcoRI restriction endonuclease (Fig. 3A). Secondary digestion with BclI endonuclease shortened the EcoRI telomeric restriction fragments, as detected by a wild-type probe (Fig. 3B), and eliminated the BclI-specific hybridization signal (Fig. 3A). The D1 and D1' mutations, as well as the D1/D1' double mutation, did not significantly affect telomere length or colony morphology (Fig. 3A). However, the D2 and D2' telomeres (Fig. 3A and B), and the iD2 and iD2' telomeres (16), were considerably shortened, containing significantly fewer total telomeric repeats than wild type telomeres, as indicated by the reduced hybridization intensity of the telomeric bands. This shortening correlated with rough colony appearance and longer population doubling times than that of the wild-type strain (14, 17). In addition, two telomeric restriction fragments disappeared in each of the D2 and D2' mutants (arrowheads, Fig. 3B) most likely through recombination in the sub-telomeric region, which has been shown to be associated with impaired telomere maintenance (17).

The D2/D2' and R1 mutants exhibited normal colony morphology and wild-type telomere length (Fig. 3A), consistent with their normal template boundary observed *in vitro* (Fig. 2, lanes 14 and 16). The lack of any apparent effect of the 20-base R1 substitution is striking in light of the severe defects in telomere maintenance and cell growth caused by the 3-base D2 or D2' substitutions. Thus, base pairing at this region, rather than sequence *per se*, is important for telomere maintenance *in vivo*, supporting the results obtained *in vitro*.

To test whether non-telomeric read-through sequences were incorporated onto telomeres *in vivo*, we cloned and sequenced telomeres from each of the TER mutants using a new method designed to preserve any 3'-overhang of the telomere (18). At least ten telomere clones were sequenced for each strain. In these clones the average telomere length and the extent of BclI incorporation (14) matched those observed by Southern analyses (Fig. 3B), indicating that the PCR products were representative of the telomere population.

In two of the D1 telomere clones (Fig. 3C), composed of the expected BclI-marked telomeric repeats (in green) distal to wild type repeats (in blue), an extra A residue (in red) was found, embedded between two BclI repeats. The position of this A residue is consistent with DNA synthesis proceeding one nucleotide beyond the normal template boundary *in vivo*, in agreement with the limited extent of read-through observed *in vitro* (see arrowheads, Fig. 2A, lane 4).

The D2 and D2' telomere clones contained up to 11 read-through nucleotides incorporated onto telomeric termini (in red, Fig. 3C). In each case, the additional sequence was that predicted from copying by the mutant enzyme beyond the normal template boundary, including the D2' mutation (boxed sequences). In contrast to the D1, D2 and D2' clones, no read-through sequences were detected in any of the D1' (19), D1/D1', D2/D2' and R1 clones (at least 10 different clones of each). Together, these results demonstrate that disrupting the pairing region causes template read-through not only *in vitro*, but also *in vivo*.

In summary, here we demonstrated that a phylogenetically conserved, long-range base-pairing interaction adjacent to the template in a yeast telomerase RNA, acts to specify one boundary of the telomerase template, thus determining the end of the telomeric repeat synthesized. It is not known yet whether this novel mechanism of demarcating the template boundary is used in other telomerases. In ciliate TERs, a conserved sequence immediately upstream of the template has been proposed to play a role in specifying the template boundary (20). In *T. thermophila*, mutations at this region resulted in alteration of the template boundary, as revealed by an *in vitro* activity reconstitution assay (21). In *Saccharomyces cerevisiae*, a trinucleotide substitution introduced adjacent to the template caused telomerase to copy one nucleotide beyond the normal boundary *in vitro* (22). Secondary structure predictions reveal a putative double-stranded element adjacent to the template in *S. cerevisiae* telomerase RNA (14). Whether this putative structure functions similarly to the one described here is yet to be explored.

Telomerase is unique among reverse transcriptases in that polymerization is restricted to a short template region within an intrinsic RNA moiety. RNA secondary structures have been shown to cause pausing in DNA synthesis by retroviral reverse transcriptases (23, 24). Here we report the utilization of a long-range RNA-RNA base-pairing interaction as a barrier for reverse transcription, specifying the telomerase template boundary. Hence, the special feature of telomerase—precisely limiting polymerization to the template—is achieved by an RNA-directed mechanism of the ribonucleoprotein (RNP) enzyme, and is not an inherent property of the telomerase RT protein. Such a direct function for TER in the enzymatic action of telomerase is consistent with an evolutionary scheme in which RNA enzymes, in an archaic RNA world, acquired protein components evolving into RNP enzymes (25). The RNA components of these RNP enzymes then gradually lost

their functional roles in catalysis and were subsequently dispensable. As has been suggested previously (26), telomerase RNP may represent an evolutionary relic—an intermediate in the transition from RNA replicases to protein reverse transcriptases. Further study of functional elements in TER will shed more light on telomerase evolution and function.

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9. *K. lactis* TER was cloned previously (15). The rest of the TER genes were cloned by a combination of PCR, RT-PCR and standard cloning methods, and will be described in details elsewhere.
10. Secondary structures were predicted by the computer program *mfold* version 2.3 (27). The analyses were performed on the full-length wild type and mutant TER sequences, using the default parameters of the program except for folding temperature, which was defined as 30°C.
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12. Cells were either grown in minimal media to an OD of 1 (plasmid-encoded TER alleles), diluted 1:4 in YPD and grown to an OD of 2, or grown directly in YPD to an OD of 2 (integrated TER alleles), and homogenized as described (11). Cell extracts were partially purified and assayed for telomerase activity as in (13). The primer used for all telomerase assays was 5'-GTGGTGTACGGA-3'.
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18. Cloning of telomere fragments was modified from the Ligation-Anchored PCR strategy to clone 5' ends of RNA (28) as follows. Genomic DNA (0.5 mg) was ligated to a 5'-phosphorylated, 3'-amino-modified anchor oligonucleotide (Operon, California), as described (28), except that the incubation was performed at 37°C for 2 hours. The ligation was followed by heat inactivation for 15 minutes at 70°C, digestion with EcoRI restriction endonuclease and purification using QIAquick PCR kit (Qiagen, California). The ligated DNA (150ng) was amplified by PCR using an upper primer containing a sub-telomeric sequence present internally to 11 out of the 12 *K. lactis* telomeres (14) and an ApaI restriction site (5'-GACCGGGCCCAGCAGGACCAAG-3'), and a lower primer complementary to the anchor primer and containing an EagI restriction site (5'-CGACGCGGCCGCTTATTAACCCT-3'). PCR products were extracted from an agarose gel, cloned into a Bluescript vector and sequenced.

19. In the D1' mutant, read-through of up to 3 nucleotides would copy the D1' mutation, resulting in the incorporation of 5'-TCA-3'. However, fortuitously, the same sequence would be incorporated at the beginning of a new round of synthesis by the BclI-marked telomerase (see Fig. 3C). Therefore, limited read-through by the D1' mutant enzyme may also have occurred *in vivo* but could not be distinguished from normal BclI telomeric repeats.

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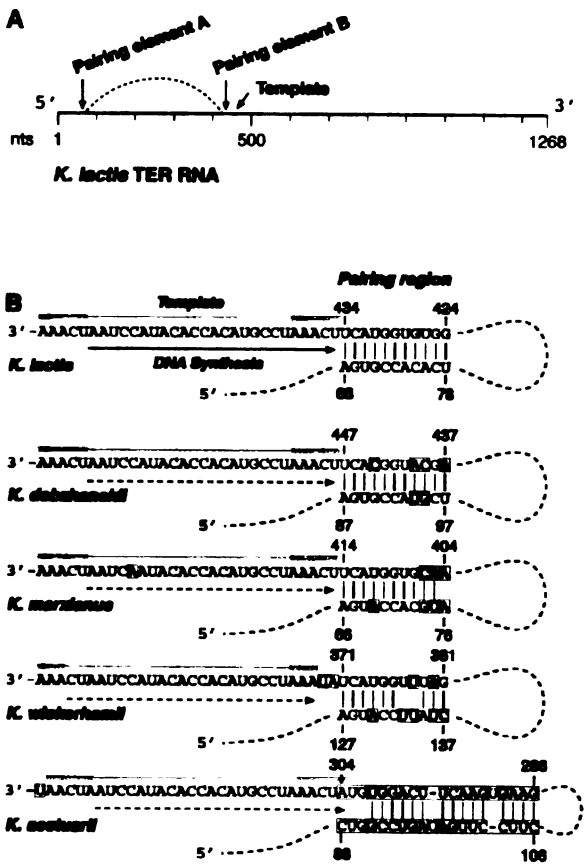
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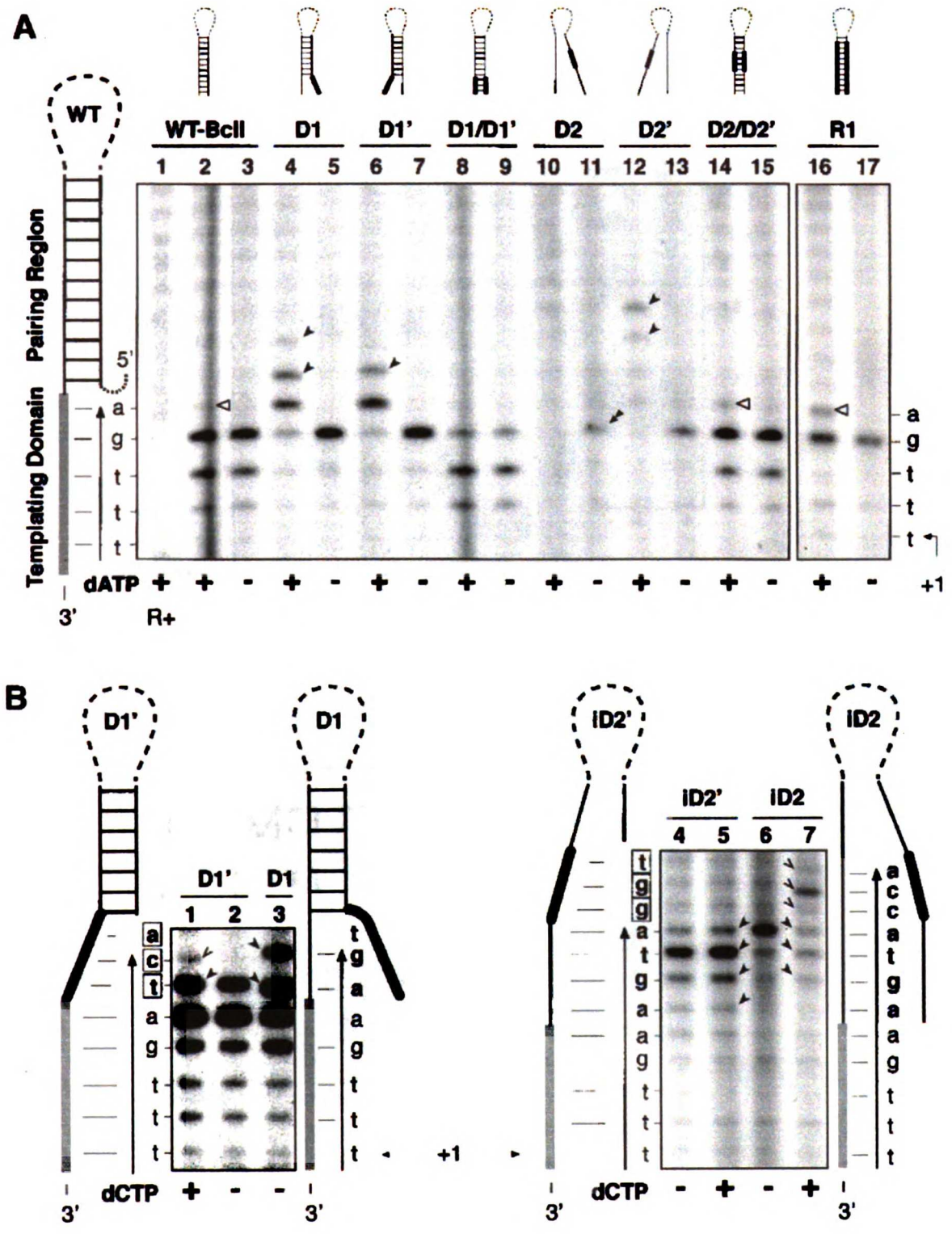
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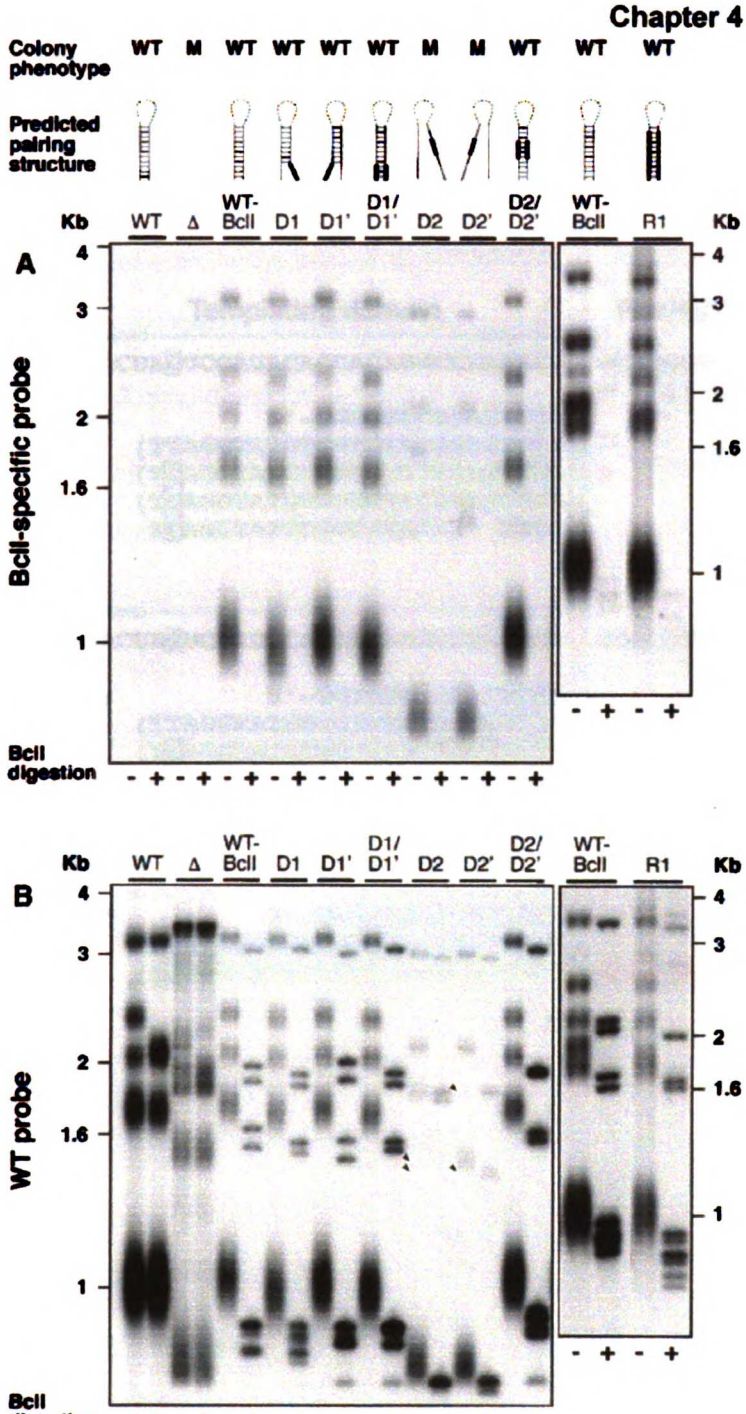
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Kb
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3
2
1.6
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BclI-specific probe
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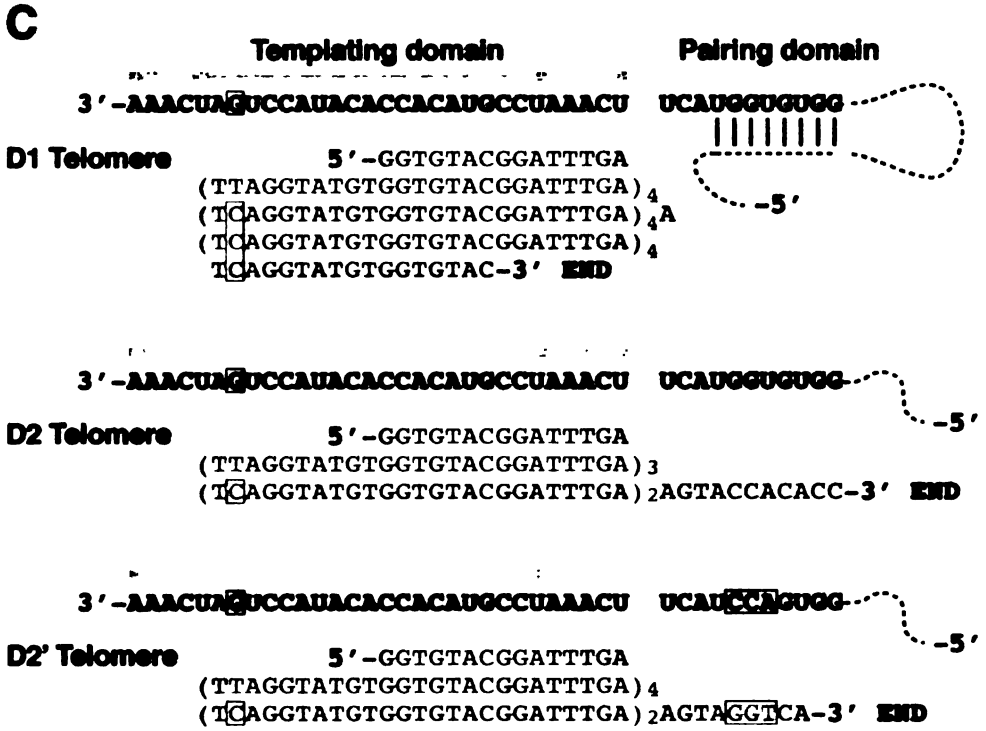
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UCAUGGUGUGG
5'-GGTGTACGGATTTGA
(TTAGGTATGTGGTGTACGGATTTGA)3
(TCAGGTATGTGGTGTACGGATTTGA)2AGTACCACACC-3' END
D2 Telomere
-5'
3'-AAACUAGUCCAUACACCACAUGCCUAAAACU
UCAUCCAGUGG
5'-GGTGTACGGATTTGA
(TTAGGTATGTGGTGTACGGATTTGA)4
(TCAGGTATGTGGTGTACGGATTTGA)2AGTAGGTCA-3' END
D2' Telomere
-5'

FIGURE LEGENDS

Figure 1. Prediction of a pairing region in budding yeast telomerase RNAs. (A) A linear map of *K. lactis* TER illustrating the location of the template and the pairing elements A and B. (B) A pairing region, predicted by the computer program *mfold* (10), is located immediately upstream of the telomerase RNA template in five *Kluyveromyces* species. For each species, the horizontal gray line denotes the maximal putative template sequence, including the short repeated sequence at both ends (thick gray lines) thought to be required for realignment and synthesis of multiple telomeric repeats. Arrows indicate the direction of DNA polymerization along the template (solid arrow for the known template of *K. lactis* and dashed arrows for the putative templates of the newly cloned genes). Boxed nucleotides, sequence variation from the *K. lactis* TER gene. Shaded boxed nucleotides, variations that retain the pairing potential. (C) Substitution mutations designed to test the pairing hypothesis in *K. lactis*. Shaded boxes, wild-type sequences that are substituted in the mutations. (D) Effects of mutations within the pairing region, as predicted by the *mfold* program (10). Thick lines represent mutated sequences.

Figure 2. Base-pairing disruption results in polymerization beyond the normal template boundary. *K. lactis* telomerase activity was assayed *in vitro* (12). All telomerases assayed contained a silent BclI mutation that is used to mark their action *in vivo* (see Fig. 3 and the respective text). (A) Telomerase reactions were incubated in the presence or absence of dATP, as indicated below the lanes. The predicted pairing region structures are illustrated above the lanes for each mutant. In lane 1 (R+), cell extract was pretreated with ribonuclease A (13). Open arrowheads, products ending at the last position of a template with a normal boundary. Double arrowhead, a product of

the D2 mutant enzyme observed upon omitting dATP. Closed arrowheads, read-through products. (B) Reactions were incubated in the presence or absence of dCTP, as indicated below the lanes. V-shaped arrowheads highlight read-through products that disappear upon omitting dCTP from the reaction. Schematics on the sides of the panels illustrate polymerization along the template (thick gray line) with vertical arrows ending at the positions where the longest corresponding wild-type (A) or mutant (B) telomerase products were detected. Thick black lines, mutated TER sequences. The schematics show the nucleotides predicted to be incorporated at each position, starting at the first nucleotide added to the primer terminus. Read-through nucleotides are shown in bold. Nucleotides resulting from the predicted incorporation of the TER mutations are boxed.

Figure 3. Base-pairing disruption causes impaired telomere maintenance *in vivo*. Genomic DNA was prepared from *K. lactis* strains (deleted for the chromosomal TER gene and carrying the different TER alleles on a plasmid) at their 15th passage. The control strains carry on a plasmid a wild-type TER gene (WT), a BclI-marked TER gene (WT-BclI), or no insert (Δ). DNA was digested with EcoRI restriction endonuclease (- lanes) or double-digested with EcoRI and BclI (+ lanes), separated on 1% agarose gel and vacuum blotted. Hybridization was carried out as described previously (11), first with a BclI-specific oligonucleotide probe (A), and then with a wild-type telomeric sequence probe (B). Arrowheads indicate the expected location of telomeric restriction fragments that disappeared during cell divisions. The predicted pairing region structures are illustrated above the lanes for each mutant. Yeast colonies of the corresponding strains were examined under the microscope. Strains exhibiting the smooth wild-type colony phenotype are labeled 'WT', while mutants exhibiting rough colony phenotype are labeled 'M' (see text). The observed phenotype remained unchanged from the 3rd

passage (60-75 cell divisions) to the 15th passage (300-375 cell divisions).

(C) Incorporation of BclI-marked repeats and read-through sequences onto telomeres *in vivo.* Sequence examples of telomere clones (18) are shown. Black, telomerase RNA sequences. Blue, wild-type repeats. Green, BclI-marked repeats. Red, read-through sequences. Boxed, the BclI and the D2' mutations in the RNA, and the corresponding incorporated DNA sequences.

APPENDIX TO CHAPTER FOUR

Described in Chapter 4 are studies in which it was demonstrated that an RNA structure serves as the telomerase template boundary. In those experiments, a series of mutations was constructed that were intended to disrupt the structure, and were predicted to result in polymerization beyond the template. As an extension of that analysis, we also constructed two *K. lactis* telomerase 'zip-up' mutants. These mutants were designed to extend base-pairing into the template region, resulting in a predicted polymerization boundary now located internal to the template. Initially, we replaced the wild-type *TER* gene with the mutant genes by the vector-shuffling system described previously (1) and analyzed the effects in vitro and in vivo. In both mutants, the RNA strand opposite the template was altered to extend the pairing region (Figure 1). The mutation Z1, in which two nucleotides in the strand opposite the template were replaced, was designed to result in pairing of the last two template nucleotides. Z2 consisted of an insertion of three nucleotides opposite the template, and was predicted to 'zip up' the three last template residues. The Mfold program (2) predicted the expected local changes in structure, without any other alterations to regions outside the template and pairing region (data not shown).

We analyzed the behavior of the mutants in vivo by telomere fragment analysis, probing specifically for telomeric repeats containing a BclI restriction site as described (see previous Chapter and (1)). Both mutant telomerases were active *in vivo* as demonstrated by their ability to maintain telomeres and incorporate BclI-marked telomeric sequences (Figure 2). However, the telomeres of both mutants were significantly shortened, suggesting the mutant enzymes were in some way impaired.

To directly test whether the template boundary had now shifted inward, we assayed partially purified extracts from the mutant cells for telomerase activity in vitro (3) with four different oligonucleotide 12-mers as primers (Fig. 4A). With primer 15(12), a strong +1 band was produced by the WT-BclI control extract and by both mutants in assays with a single nucleotide (Figure 4B, dG only lanes). Thus, as expected based on the in vivo data, Z1 and Z2 mutants were active in vitro. However, no discrete bands were observed near the predicted boundary (Figure 4B, see position of bracket) that were distinguishable from background. Interestingly, products were only observed with one of the two primers used in this experiment, primer 15(12). Primer 22(12), whose 3' end aligns closer to the 5' end of the template, was extended in the predicted manner by the control WT-BclI extract, but was not extended by the mutants, even in experiments designed to monitor incorporation of only one nucleotide (Fig. 4B, dG only lanes).

Previously, integrating mutant TER genes into the genome by replacing the endogenous gene resulted in greater telomerase activity than plasmid-borne TER genes in *K. lactis*, and was utilized as a means of analyzing mutants that did not exhibit robust activity (see previous Chapter). Therefore, we integrated one of the zip-up mutant genes into the genome, hoping to directly observe shifted boundary in the in vitro assay. Unfortunately, such a result was not observed, with no Z2 activity being detectable in the experiment shown (Figure 4C, compare Z2 to WT-BclI results).

In conclusion, we were unable to conclude with certainty whether 'zipping up' the template shifted the polymerization boundary inward, although the results in Figure 3 are consistent with this possibility. However, the striking finding was that small alterations to the strand opposing the template near the pairing region have severe effects on

telomere maintenance in vivo, and on telomerase activity in vitro. Specifically, the addition of a single nucleotide by the zip-up mutants becomes less efficient with primers aligning closer to the end of the template. This aberrant polymerization activity presumably contributes to the short telomere phenotype observed *in vivo*. Perhaps the additional base pairing creates a structural constraint or otherwise distorts the active site, preventing elongation of the 3' end of the primer in close proximity to the pairing region. Alternatively, the additional pairing potential created in the mutants could affect telomerase activity in an unpredicted manner.

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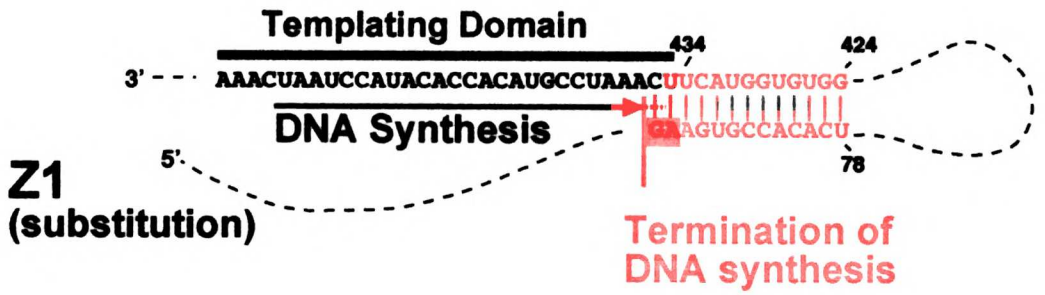
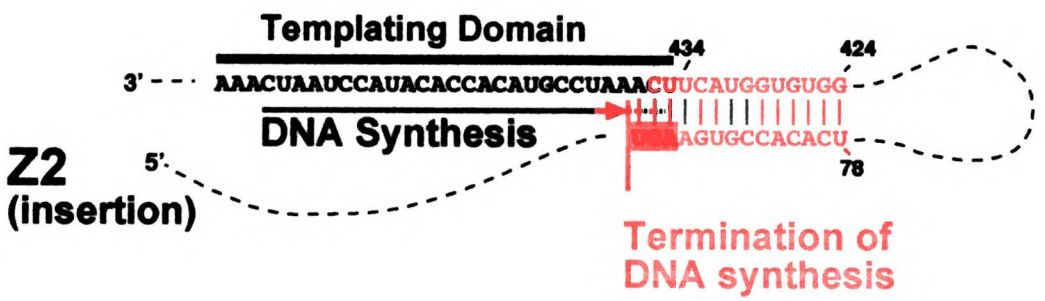
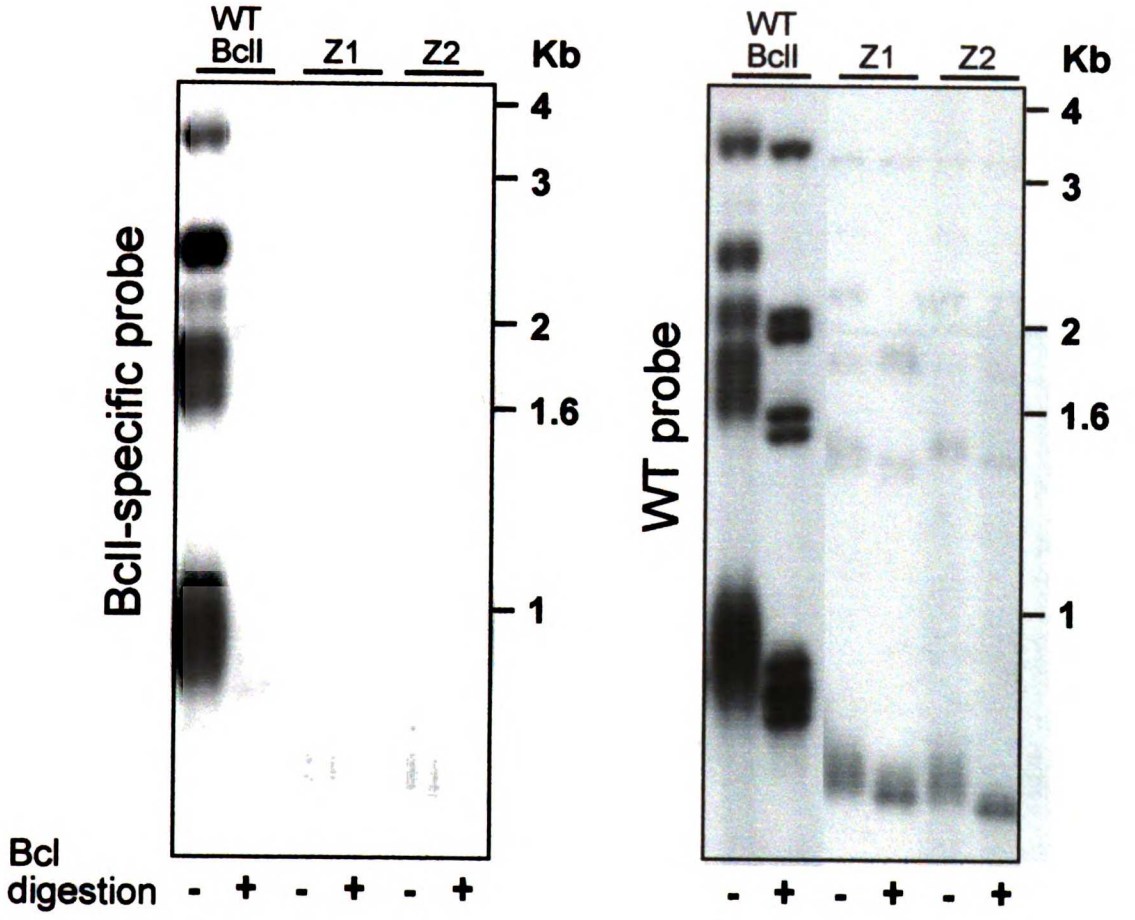
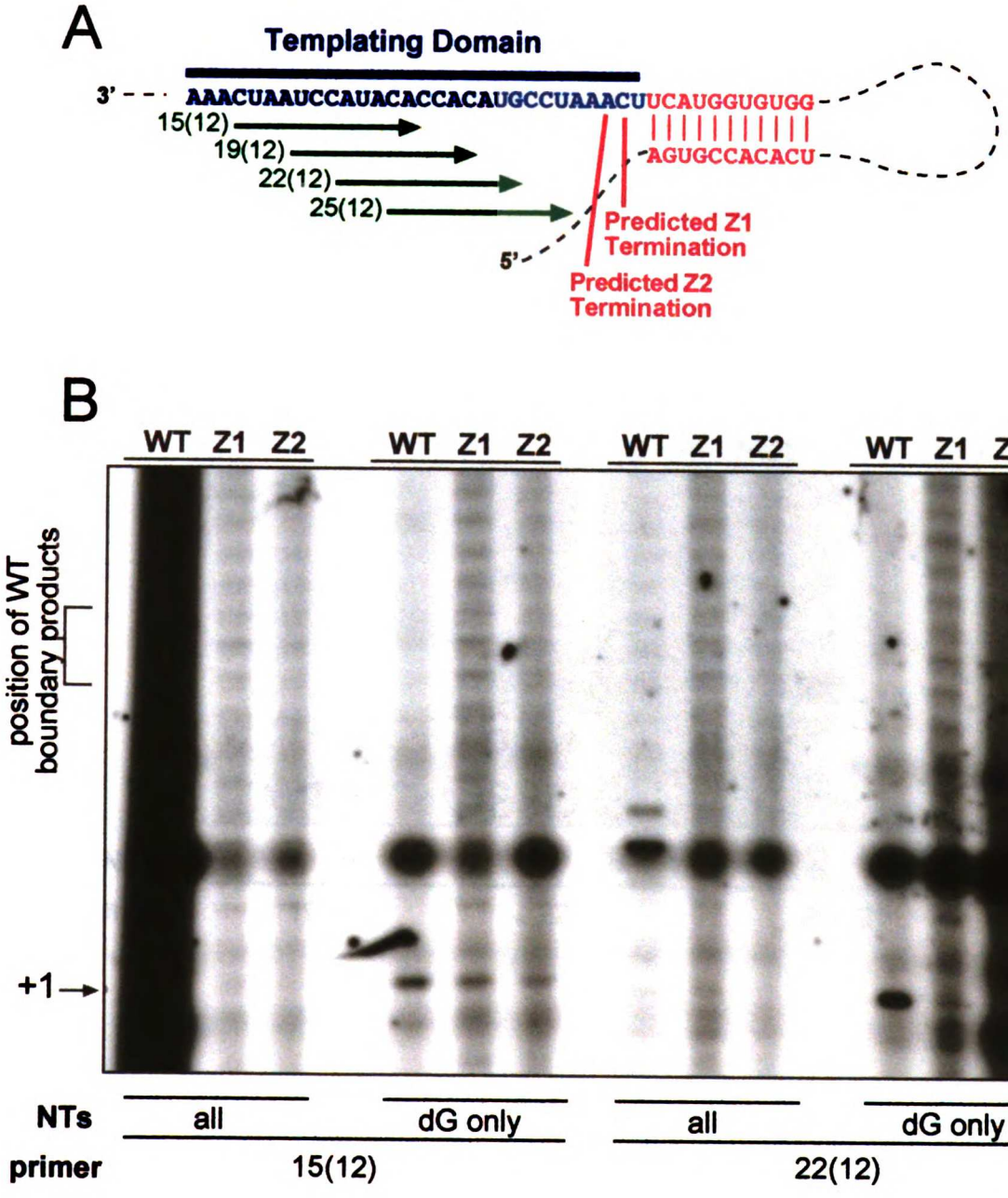

Templating Domain
434
424
3'
AAACUAAUCCAUACACCACAUGCCUAAACUUCAUGGUGUGG
DNA Synthesis
AGUGCCACACU
5'
78
Z1
(substitution)
Termination of
DNA synthesis

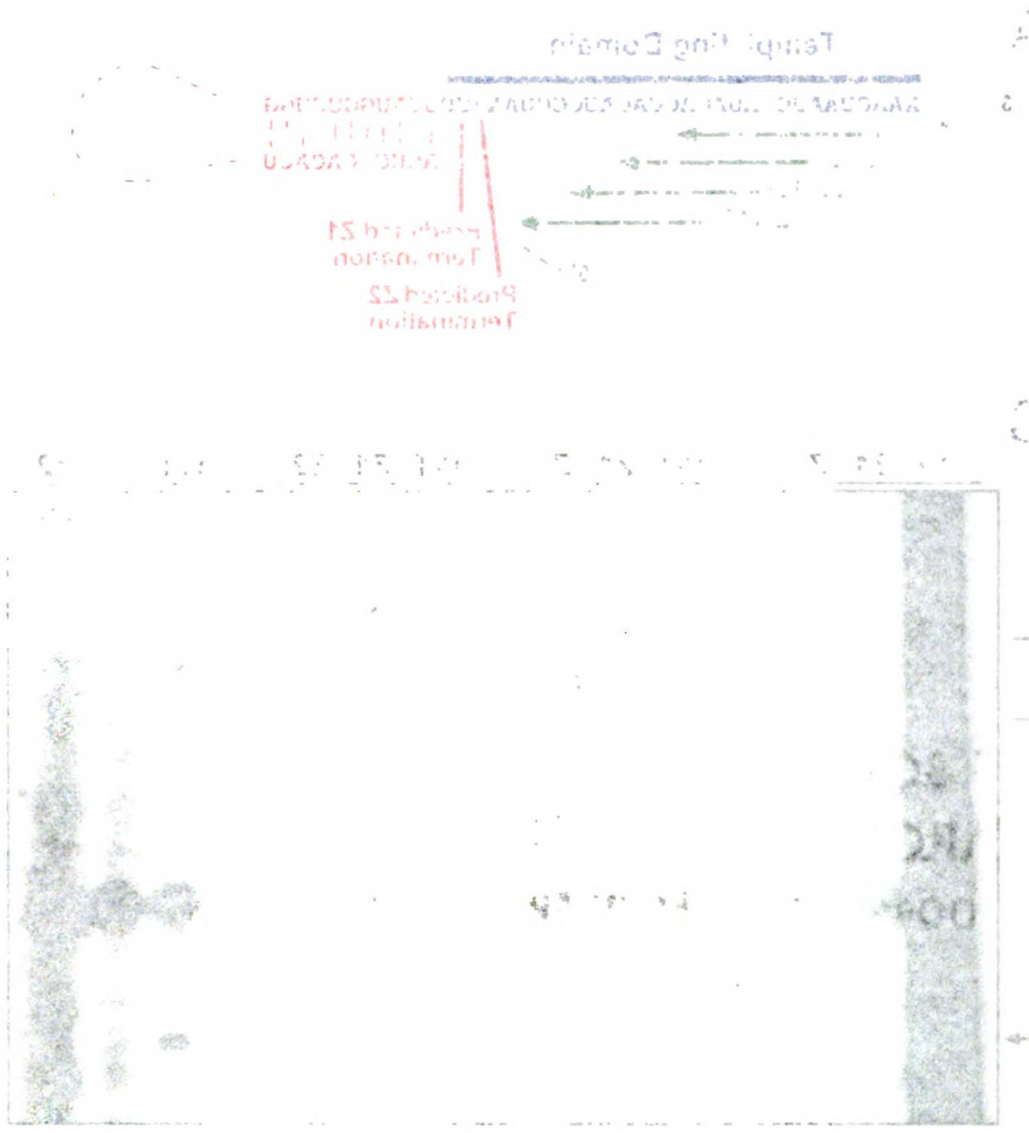

Templating Domain
434
424
3'
AAACUAAUCCAUACACCACAUGCCUAAACUUCAUGGUGUGG
DNA Synthesis
AGUGCCACACU
5'
78
Z2
(insertion)
Termination of
DNA synthesis

Appendix to Chapter 4 - Figure 2



position of WT


A
Templating Domain
3'
AAACUAAUCCAUACACCACAUGCCUAAACUUCAUGGUGUGG
AGUGCCACACU
15(12)
19(12)
22(12)
25(12)
5'
Predicted Z1 Termination
Predicted Z2 Termination
B
WT Z1 Z2
WT Z1 Z2
WT Z1 Z2
WT Z1 Z2
position of WT boundary products
+1
NTs
all
dG only
all
dG only
primer
15(12)
22(12)



C

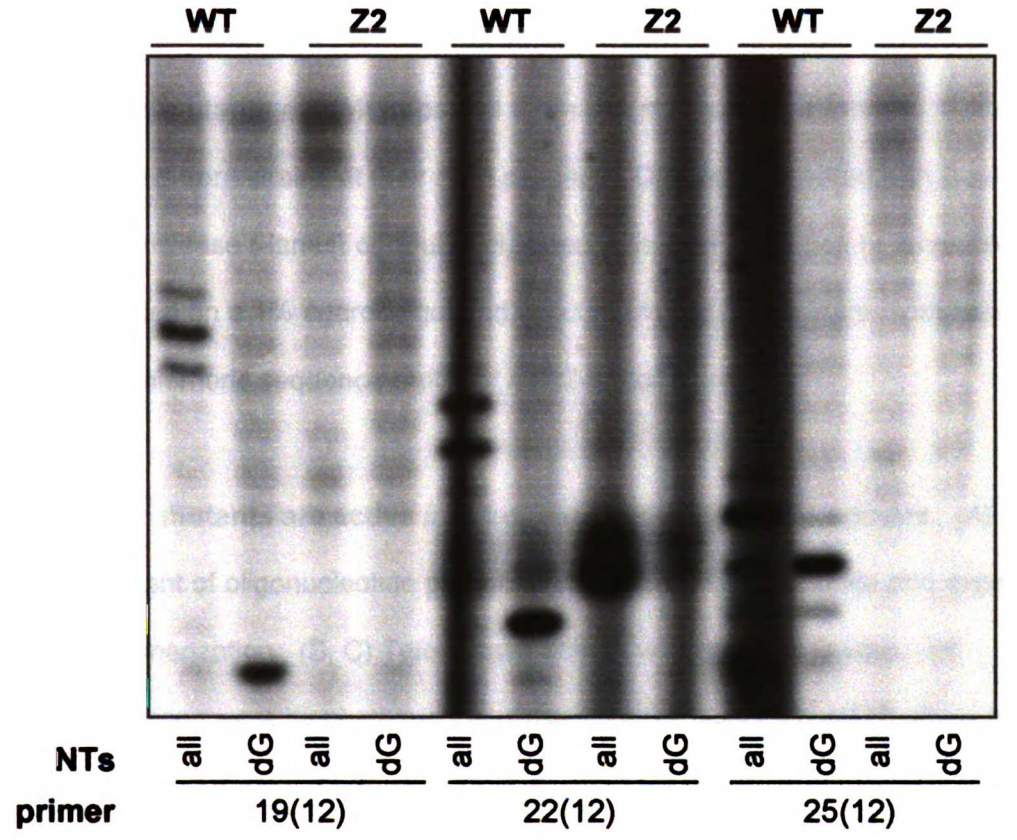

WT
Z2
WT
Z2
WT
Z2
NTs
all
dG
all
dG
all
dG
all
dG
all
dG
all
dG
primer
19(12)
22(12)
25(12)

FIGURE LEGENDS

Figure 1. Zip-up mutants and predicted effects on telomerase activity. Shaded nucleotides indicate location of mutations. Arrows indicate the direction of DNA polymerization along the templates of each mutant. Dashed lines indicate where polymerization would occur in wild-type telomerase. Effects of the mutations on termination are predicted by the Mfold program.

Figure 2. Zip-up mutants exhibit impaired telomere maintenance *in vivo*. Genomic DNA was prepared from strains at their sixth passage. DNA was digested with EcoRI restriction endonuclease (-lanes) or double digested with EcoRI and BclI (+ lanes) and was then separated on a 1% agarose gel and vacuum blotted. Blots were hybridized with a wild-type telomeric sequence probe or a BclI-specific probe.

Figure 3. Zip-up mutants are active *in vitro*, but only with certain primers. (A) Predicted alignment of oligonucleotide primers on the TER1 RNA template and expected outcome on polymerization. (B, C) Telomerase activity was assayed *in vitro*. Telomerase reactions were incubated in the presence of radiolabeled dGTP alone, or in addition to dATP, dTTP, and dCTP, as indicated below the lanes. In (B) bracket indicates position of products produced at the boundary of wild-type telomerase.

CHAPTER FIVE

Conclusions

When I began my thesis work, telomere studies in budding yeast were entering a new era. The RNA components of telomerase had recently been identified in *S. cerevisiae* and *K. lactis*. In addition, Marita Cohn had identified telomerase activity from *S. cerevisiae*, paving the way for a wealth of fruitful biochemical analysis. She had also determined that the Est1 protein was not required for in vitro function. Since this protein was initially thought to be the reverse transcriptase component, it was clear that another protein or proteins were responsible for telomerase activity, and were as of yet unidentified. *K. lactis* was in the process of being established as a highly useful model system for telomere analysis, work pioneered by Mike McEachern. He had discovered that specific mutations in the telomerase template resulted in dramatic changes in telomere length. I was interested in distinguishing whether these changes resulted from altered binding of telomere-associated proteins (for example, Rap1p) or altered telomerase activity. Based on studies in *Tetrahymena*, we knew that mutations in the RNA template had often drastic effects on the enzymatic properties of telomerase. It seemed likely that mutating the *K. lactis* telomerase template would produce similar effects, thereby influencing telomere dynamics in vivo. I chose to take a biochemical approach toward understanding telomere dynamics in *K. lactis*, and undertook as my thesis project the identification and analysis of *K. lactis* telomerase.

K. lactis telomerase catalyzes only one round of repeat synthesis in vitro

The crucial first step in this thesis project was the identification of *K lactis* telomerase activity in vitro. After having ascertained with numberous controls that the activity I observed was telomerase, I began to characterize the most intersting and obvious properties of the enzyme. First , *K. lactis* telomerase consistently catalyzes only one round of repeat synthesis in vitro and remains bound to the reaction products, as is also

the case with *S. cerevisiae* telomerase (Prescott and Blackburn 1997). At the time, this result was somewhat controversial: other researchers thought that we night be observing only a partial telomerase reaction, and that a factor could be missing that might promote the sort of multiple round processivity observed with the *Tetrahymena* enzyme, for example. I was also not convinced that *K. lactis* telomerase did not have the potential to synthesize multiple repeats. The enzyme remained bound to its products after each round of synthesis, so why didn't it translocate? The answer to this question still is not apparent, as the behavior remained non-translocative under conditions of varying temperature, primer length, and composition, and salt, nucleotide, and primer concentration. The fact that the activity was non-processive after very gentle glycerol gradient fractionation supported the idea that this mode of synthesis actually reflects an intrinsic property of the enzyme. In several cases telomerase has been found to be non-processive in vivo, suggesting that processivity may no be necessary in the cell (Yu et al. 1990; Yu and Blackburn 1991; Prescott and Blackburn 1997). Alternatively, perhaps in my in vitro system translocation cannot take place due to the build-up of a lengthy RNA/DNA hybrid during elongation (discussed below). Another interesting possibility that has not been addressed is that an auxiliary factor associated with *K. lactis* telomerase limits polymerization to a single round. This could be tested in the future by reconstituting ativity with only the RT component and TER RNA. Human and *Tetrahymena* telomerase activity reconstituted in a reticulocyte lysate system depend only on TERT and TER (Weinrich et al. 1997; Licht and Collins 1999). However, telomerase from *Euplotes crassus* exists in distinct complexes with different sizes and activities, suggesting that additional factors can modulate enzyme processivity (Greene and Shippen 1998).

Stalling: regulation of *K. lactis*telomerase by RNA structure?

I found *K. lactis* telomerase to exhibit an unusual property: stalling during the synthesis of a telomeric repeat in at lest two specific regions of the template. This enzymatic characteristic, like polymerization for only a single round of repeat synthesis, occurred regardless of assay conditions (although increased temperatures favored polymerization through stall regions, see discussion below). In addition, stalling occurs when telomerase is fractionated only by glycerol gradient sedimentation, suggesting that this behavior does not result form a missing factor.

In a predicted structure of *K. lactis* TER RNA, the template region appears to interact with another portion of of the RNA molecule (Tzfati and Blackburn 2000). A deletion that includes much of the region (terΔ20-60) abolishes telomerase activity in vitro and in vivo, but more specific analysis of the residues in this putative template-interacting region will be necessary to verify and assess the function of the predicted structure. Support for the predicted structure comes from the findings that the predicted pairing just outside of the template is indeed taking place. The prediction that the template is involved in a secondary structure was quite surprising given that ciliate and vertebrate telomerase RNA structure predictions place the template in a single stranded region (Romero and Blackburn 1991; Lingner et al. 1994; Chen et al. 2000). However, the *K. lactis* telomerase RNA template region is comparatively much larger, and perhaps it I energetically unfavorable for the entire region to exist unpaired. Strikingly, the regions where stalling occurs correspond to areas of the template predicted to be single stranded, and these bulges are separated by small double-stranded stems. This predicted structure appears to be conserved among other budding yeast telomerase RNAs.

We suggest a model for telomerase in which RNA double strandedness upstream of the active site limits polymerization, resulting in telomerase stalling and/or termination. This model is supported by the 3' boundary structure. Also, extending the pairing region inward severely limits polymerization at a distance away from the location of the stem. In the future, the template-pairing aspect of this model could be tested by mutating the strand opposite the template, and then restoring pairing potential with compensatory changes in the template strand. There are existing template mutants that are predicted to alter the putative template structure which could also be used to examine this question. Finally, it would be interesting to assess whether stalling occurs in other budding yeasts for which such template interactions have been predicted.

Primer-template pairing potential exacerbates stalling: evidence for discontinuous synthesis?

The exacerbation of stalling I observed upon increasing the product DNA/template RNA complementarity supports the idea that extensive base-pairing contributes to stalling. This result suggests the RNA/DNA hybrid formed during polymerization builds up and is maintained (discontinuous, as opposed to monotonic synthesis). It has been suggested that only a minimal RNA/DNA hybrid exists during telomerase action (Collins and Greider 1993; Hammond and Cech 1998). It would be extremely interesting to address this question in *K. lactis* by analyzing the length of the RNA/DNA hybrid during polymerization. this could presumably be done by chemical or enzymatic modification and probing of elongation complexes.

Stalling by *K. lactis* telomerase is alleviated at higher temperatures. If stalling is indeed dependent on the build-up of a rigid RNA/DNA hybrid, then higher temperatures could simply melt the base-paired structure, allowing polymerization to proceed. Alternatively, a more dynamic conformational change could take place to unpair the hybrid and allow further polymerization. This conformational change could potentially involve a change in RNA structure and/or displacement of the primer 5' end to a second site in a protein component of telomerase.

Small mutations in the large RNA can have drastic effects on telomerase activity

My thesis work contributed to our understanding that specific RNA residues outside of the telomerase template are directly important for both in vitro and in vivo activity. Four specific *TER1* deletions several hundred residues from the template abolished telomerase activity in vitro and telomerase function in vivo, and led to shortening of telomeric DNA invivo. Two of these mutants have significantly altered migration on native gels, implying defects in RNP assembly. It is unclear as to whether these defects are due to changes in protein binding, RNA structure, or both. The other two had apparently normal RNAP behavior, suggesting that they could affect some aspect of telomerase enzymatic activity.

Several mutations in the *K. lactis* template boundary pairing region and in the immediate vicinity also drastically alter telomerase activity. The phenotypes result at least in part from predicted RNA structure disruptions. However, these disruptions could affect polymerization above and beyond simply altering the location of the boundary. That is, RNA tertiary structure, protein binding, and/or multimerization could be affected as well.

The pairing region: an RNA structure that serves as the template boundary

As described in Chapter Four, we have found a long-range pairing interaction in *K. lactis* telomerase that serves as the 3' end template boundary. We suggest that the predicted pairing regions in the other budding yeasts also serve the same purpose. Experiments are underway in *S. cerevisiae* to address this question. Mutations in a double stranded region adjacent to the template result in telomere length changes that are suppresed when pairing potential is restored (Seto and Cech 2000). It is unknown whether such a mechanism functions in other organisms. A conserved sequence plays a role in limiting polymerization to the template in ciliate telomerases, but this sequence is predicted to lie in a single stranded region of the RNA (Autexier and Greider 1995).

This is the first budding yeast telomerase RNA structure to be identified. The pairing was predicted by the Mfold program as part of a much larger secondary structure, and the pairing region structure was conserved among several other budding yeasts. Much more work lies ahead in ascertaining experimentally which aspects of the predictions are valid, but verification of at least a portion of the structure is an important step. Having an understanding of the core RNA structure for telomerase RNA in such an advantageous genetic system would facilitate rapid, specific analyses of the telomerase mechanism. Establishment of a secondary structure for yeast telomerase RNA would also allow comparison with the ciliate and vertebrate structures, providing an even better understanding of which functional elements are important for telomerase function.

The pairing region is the first telomerase RNA structure found to play a direct role in regulating activity of the enzyme. There are other examples in which mutating telomerase RNA results in aberrant activity, but none with a known mechanism. The

pairing region directly influences telomerase activity by determining the extent of polymerization along the template. Opening up the pairing region in effect removes the template boundary, allowing telomerase to proceed into non-template portions of the RNA, copying non-telomeric sequence as it goes. As long as pairing potential is retained, the entire sequence can be scrambled and the boundary still functions properly. This result suggests that sequence-specific protein binding does not contribute to establishment of the template boundary. In fact, boundary establishment appears to be an inherent property of telomerase RNA.

In conclusion, I feel that a general theme to have emerged from my thesis work is the idea of RNA structure regulating polymerization by *K. lactis* telomerase. The pairing region serves as an example of this, and stalling may also be an example, though this still needs to be addressed experimentally. *K. lactis* has proved to be a favorable experimental system for these analyses, and future biochemical and genetic studies in this system could provide a great deal of interesting information about telomerase function.

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