21

DNA Telomerases

Carol W. Greider, Kathleen Collins, and Chantal Autexier
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

Telomerase is a specialized DNA polymerase that synthesizes telomeric DNA sequences onto chromosome ends. Linear DNA genomes pose a special problem for the DNA replication machinery. Because DNA polymerases function in the 5′ to 3′ direction and require a primer, part of the lagging strand is predicted to be lost at each round of division. For most eukaryotic chromosomes, sequence loss is balanced by the de novo addition of telomeric sequence by telomerase (for review, see Blackburn 1991).

Telomere DNA sequences in ciliates consist of simple GT-rich tandem repeats, and this motif is generally conserved in eukaryotes, although the exact repeat sequence is species-specific. For example, *Tetrahymena* contains tandem TTGGGG repeats, whereas mammals have TTAGGG repeats (for review, see Blackburn 1991; Henderson 1995). Telomerase synthesizes these short repeats by using an integral RNA component of the enzyme as a template (Fig. 1) (Greider and Blackburn 1989). Telomerase activity has been identified from *Tetrahymena*, *Euplotes*, *Oxytricha*, yeast, *Xenopus*, and mammals. In each case, the sequence synthesized by telomerase in vitro corresponds to the telomere sequence of the organism from which it was isolated (Table 1), and the RNA template contains more than one repeat of the sequence (Table 2) (Greider and Blackburn 1985; Zahler and Prescott 1988; Morin 1989; Shippen-Lentz and Blackburn 1989; Prowse et al. 1993; Lingner et al. 1994; Mantell and Greider 1994; Cohn and Blackburn 1995; Lin and Zakian 1995; Lue and Wang 1995).

TETRAHYMENA TELOMERASE

The telomerase mechanism has been best studied in *Tetrahymena*, where enzyme activity was first identified. A typical assay utilizes a synthetic DNA d(TTGGGG)$_3$ primer to mimic the end of a chromosome,

DNA Replication in Eukaryotic Cells

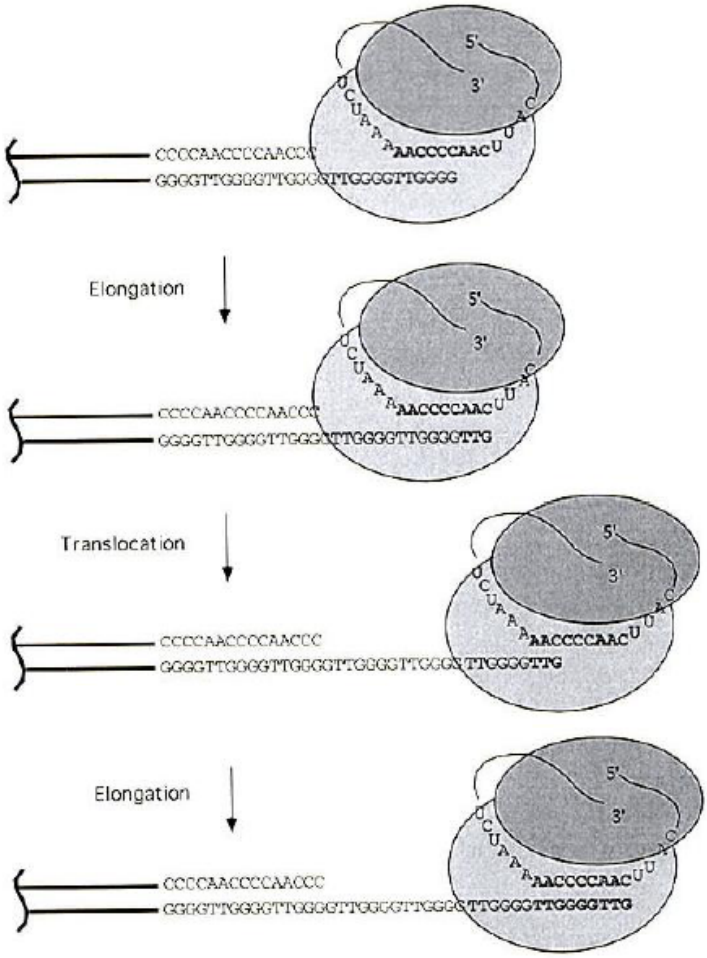

Figure 1 Telomerase elongation model. (1) Telomerase recognizes the telomere substrate, and the terminal d(TTGGGG) repeat is base-paired with the CAACCCCAA RNA template. (2) The RNA is copied to the end of the template region. (3) Translocation repositions the terminal TTGGGGTTG sequence and exposes additional template sequences. (4) Another round of template copying produces additional TTGGGG repeats. (Adapted from Greider and Blackburn 1989.)

[^{32}P]dGTP, and TTP. After incubation with telomerase extracts, the radiolabeled products are resolved on a denaturing gel or precipitated and counted. When resolved on a gel, the extension of the input 18-mer oligonucleotide by the addition of hundreds of d(TTGGGG) repeats can be directly visualized (Greider and Blackburn 1985). The nucleotides are

Table 1 Telomerase activity in various eukaryotes

Organism	Telomere sequence	RNA identified?	Reference
Tetrahymena	TTGGGG	yes	Greider and Blackburn (1985)
Oxytricha	TTTTGGGG	yes	Zahler and Prescott (1988)
Euplotes	TTTTGGGG	yes	Shippen-Lentz and Blackburn (1989)
Human	TTAGGG	yes	Morin (1989)
Mouse	TTAGGG	yes	Prowse et al. (1993)
Xenopus	TTAGGG	no	Mantell and Greider (1994)
S. cerevisiae	$TG_{(1-3)}$	yes	Cohn and Blackburn (1995); Lin and Zakian (1995)
S. castellii	$TCTGGG(TG)_{1-4}$	no	Cohn and Blackburn (1995)

added one base at a time, and the products show a distinct six-base periodicity. This periodicity is due to both pausing and enzyme dissociation during the addition of each repeat predominantly at the first G within the sequence TTGGGG (Greider 1991; Lee and Blackburn 1993). Thus, the sequence added in vitro can be deduced directly from the products of the reaction. Elongation of circularly permuted telomere sequence oligonucleotides results in banding patterns that are offset: The primer $d(TTGGGG)_3$ generates bands at positions primer +3, +9, +15, etc., whereas the primer $d(GTTGGG)_3$ generates bands at positions +4, +10, +16, etc. Telomerase binds and elongates telomere sequence oligonucleotide primers and, at a lower affinity, nontelomeric sequence primers (Greider and Blackburn 1987; Blackburn et al. 1989; Harrington and Greider 1991). A recent modification to the telomerase assay reaction in-

Table 2 Telomerase RNA components

Organism	Telomere sequence	RNA template sequence	Size	References
Tetrahymena	TTGGGG	CAACCCCAA	160	1
Euplotes	TTTTGGGG	CAAAACCCCAAAACC	190	2
Oxytricha	TTTTGGGG	CAAAACCCCAAAACC	190	3,4
Human	TTAGGG	CUAACCCUAAC	450	5
Mouse	TTAGGG	CCUAACCCU	450	6
S. cerevisiae	$TG_{(1-3)}$	CACCACACCCACACAC	1300	7
K. lactis	TTTGATTAGGTATG	UCAAAUCCGUACACCAC	1300	8
	TGGTGTACGGA	~AUACCUAAUCAAA		

References: (1) Greider and Blackburn (1989); (2) Shippen-Lentz and Blackburn (1990); (3) Lingner et al. (1994); (4) Melek et al. (1994); (5) Feng et al. (1995); (6) Blasco et al. (1995); (7) Singer and Gottschling (1994); (8) McEachern and Blackburn (1995).

volves the detection of telomerase products by polymerase chain reaction (PCR) amplification (Kim et al. 1994). This assay is sensitive and can detect low levels of telomerase activity.

Identification and characterization of the *Tetrahymena* telomerase RNA component showed that the RNA sequence serves as a template for the synthesis of telomeric repeats in vitro and in vivo (Greider and Blackburn 1987, 1989; Yu et al. 1990; Autexier and Greider 1994). The *Tetrahymena* telomerase RNA was identified by its copurification with telomerase activity in a series of six chromatographic steps. The RNA contains within it the sequence 5′-CAACCCCAA-3′, which is complementary to one and a half repeats of the *Tetrahymena* telomere sequence d(TTGGGG). Using RNase H and oligonucleotides complementary to different regions of the RNA, this 159-nucleotide RNA was shown to be essential for telomerase function (Greider and Blackburn 1989).

SECONDARY STRUCTURE OF THE TETRAHYMENINE TELOMERASE RNAs

To identify potential functional domains in the *Tetrahymena* telomerase RNA, a secondary structure model was derived from a phylogenetic sequence comparison of telomerase RNAs from different tetrahymenine ciliates (Romero and Blackburn 1991; McCormick-Graham and Romero 1995). A potential secondary structure for the *Tetrahymena* telomerase RNA was proposed that includes four conserved helices, numbered from 5′ to 3′ as helices I–IV (Fig. 2). Telomerase RNAs were subsequently cloned from hypotrichous ciliates and shown to have a secondary structure similar to the proposed *Tetrahymena* structure, although, strikingly, the primary sequence is not conserved (Shippen-Lentz and Blackburn 1990; Lingner et al. 1994; Melek et al. 1994). In the proposed structure, helix I involves long-range base-pairing and is the most conserved of the helices. Abolishing this potential stem only partially decreased telomerase activity reconstituted in vitro, suggesting that these interactions are not essential (C. Autexier and C. W. Greider, unpubl.). Helix II is not conserved in all ciliates (Lingner et al. 1994; McCormick-Graham and Romero 1995). An unstructured region is predicted between helices II and III and includes the template region. Nucleotides in the loop of helix III can base-pair with a single-stranded region just upstream of the helix III stem to form a pseudoknot (ten Dam et al. 1991). Part of helix II contains a conserved sequence, 5′-(C)UGUCA-3′, which appears to play a role in defining the 5′ boundary of the template domain (Lingner et al. 1994; Autexier and Greider 1995). Chemical modification studies

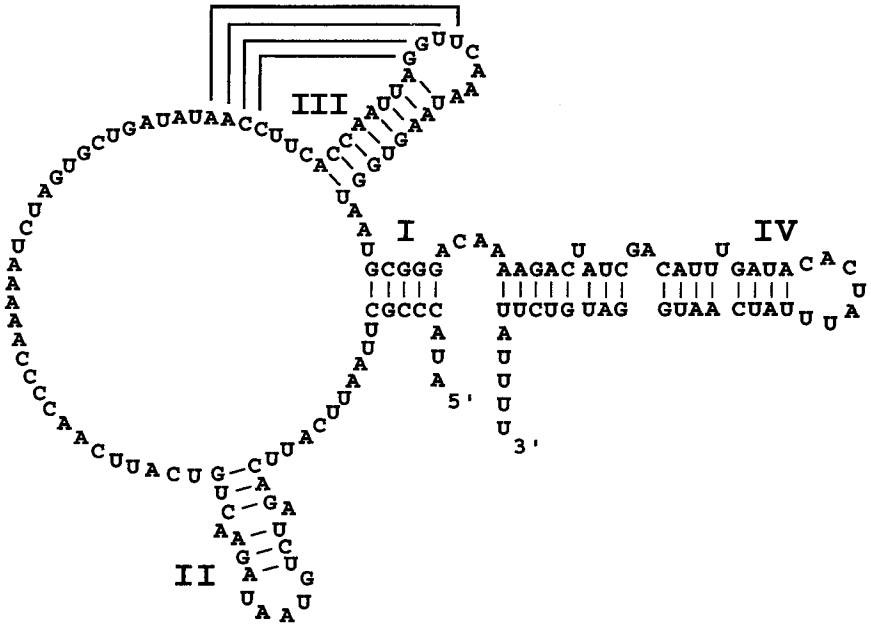

Figure 2 A secondary structure model was proposed for the *Tetrahymena* telomerase RNA based on conserved RNA sequences in different *Tetrahymena* species (Romero and Blackburn 1991). This figure shows the sequence of the *Tetrahymena thermophila* RNA. Other ciliate RNAs also fold into a similar secondary structure, although the primary sequence varies (Lingner et al. 1994). It is not yet known if other telomerase RNAs adopt a similar secondary structure.

of telomerase RNA in vivo and in vitro support the phylogenetically derived model, although some differences are seen between naked RNA and the RNP complex (Bhattacharyya and Blackburn 1994; Zaug and Cech 1995). In addition to conserved sequences within the *Tetrahymena* telomerase RNAs, there are conserved promoter regions found upstream of the telomerase RNA genes (Shippen-Lentz and Blackburn 1990; Romero and Blackburn 1991; Lingner et al. 1994).

TELOMERASE TEMPLATE FUNCTION

To dissect the functional regions of the *Tetrahymena* telomerase RNA, an in vitro reconstitution assay was developed (Autexier and Greider 1994). Telomerase extract is treated with micrococcal nuclease to digest endogenous telomerase RNA. After inactivation of the nuclease, the ex-

tract is incubated with in-vitro-transcribed *Tetrahymena* telomerase RNA to restore telomerase activity. The elongation products of telomerase reconstituted with telomerase RNAs mutated in the template domain were analyzed to determine whether all nine nucleotides of the template region serve to direct the synthesis of telomeric repeats. The analysis of several mutants suggested that the first six nucleotides of the sequence 5′-CAACCCCAA-3′ serve as template residues and that the 3′-most three residues serve to align substrate and product sequences. Under certain conditions, residue 49 can be used as a template nucleotide (Gilley et al. 1995; Autexier and Greider 1995). For instance, if residue 43 is deleted, telomerase can still synthesize wild-type GGGGTT repeats by using residue 49 as a template nucleotide (Autexier and Greider 1995).

The template region boundaries of the telomerase RNAs from hypotrichous ciliates have been analyzed using template-complementary primers and analysis of the elongation product patterns. Initially, in *Euplotes crassus*, oligonucleotides complementary to the telomerase RNA were tested for their ability to act as primers for telomerase or to inhibit the telomerase elongation reaction (Shippen-Lentz and Blackburn 1990). Preincubation with oligonucleotides that could base-pair to the putative template and adjacent sequences inhibited telomerase reactions, presumably by blocking access of the $d(TTTTGGGG)_4$ primer to the template sequence. These results were similar to the results obtained with analogous primers complementary to the *Tetrahymena* telomerase RNA (Greider and Blackburn 1989).

Later studies with *Oxytricha nova* reached different conclusions about which regions of the template provide sequence information. Using template-complementary oligonucleotides, Lingner et al. (1994) defined the template regions of the *O. nova* and *Stylonychia* species at positions 37–44 and 38–45, respectively, 5′-C/GAAAACCCCAAAACC-3′ and that of *Euplotes aediculayus* at positions 36–44, 5′-CAAAACCCC AAAACC-3′. Melek et al. concluded that the template domain of the *O. nova* telomerase RNA ranged from position 42 to 50, 5′-CAAAAC CCCAAAACC-3′ (Melek et al. 1994). The resolution of which positions specify nucleotide addition awaits mutational analysis of the telomerase RNA in these species.

YEAST TELOMERASE RNAs

A gene encoding the telomerase RNA component from *Saccharomyces cerevisiae* was recently cloned in a screen for genes affecting telomeric silencing (Singer and Gottschling 1994). Telomeric silencing is a

phenomenon whereby genes placed near telomeres in yeast are transcriptionally repressed (Gottschling et al. 1990). A screen was developed to identify high-copy suppressors of telomere silencing. One of the genes identified, *TLC1*, contained an expressed RNA but no open reading frame. However, *TLC1* did contain a potential yeast telomerase RNA template motif predicted from de novo telomere addition during chromosome healing (Kramer and Haber 1993). To confirm that *TLC1* encodes the telomerase RNA, a *Hae*III site was engineered into the putative template domain, and this mutant allele was used to replace one of the *TLC1* alleles in a diploid strain. To determine if the altered sequence was incorporated into newly formed telomeres, genomic DNA was digested with *Hae*III. Specific digestion of telomere repeats was seen in the mutant but not the wild-type strains, showing that *TLC1* encodes the telomerase RNA. Northern blot analysis indicated that the wild-type strain of yeast contains a relatively abundant 1.3-kb RNA that hybridizes to a *TLC1* probe.

The telomerase RNA was also cloned from a distinct species of budding yeast (McEachern and Blackburn 1995). Unlike the short 6- to 8-bp telomeric repeats in many eukaryotes, the telomeric repeats of certain yeasts are relatively long. For instance, telomere repeats in *Candida albicans* and *Kluveromyces lactis* are 23 bp and 25 bp in length, respectively (McEachern and Hicks 1993; McEachern and Blackburn 1994). It was hypothesized that the template region of the telomerase RNAs from these species would be at least the length of the telomeric repeats and that it should be possible to detect these RNAs using repeats of the telomere sequences as probes in hybridization experiments. However, to identify such genes, the abundant telomere repeats had to first be removed. This was done by BAL 31 nuclease digestion. BAL 31 is an exonuclease that removes the terminal sequence from genomic DNA. With this method, telomerase RNA gene candidates in *K. lactis*, *C. albicans*, and *Candida glabrata* were identified (McEachern and Blackburn 1994). To clone the gene from *K. lactis*, libraries were made from BAL-31-digested genomic DNA, and a potential RNA gene clone was identified. To verify that the cloned gene encoded the telomerase RNA, the sequence of the putative template domain was changed by the introduction of a *Bgl*II site. Yeast cells in which the wild-type locus was replaced by the *Bgl*II-containing mutation had the altered sequence at their telomeres, indicating that the cloned gene encodes the telomerase RNA component. Northern blot analysis showed that the *K. lactis* telomerase RNA is 1.3 kb, like the *S. cerevisiae* telomerase RNA. Due to the large size of these RNAs, it is not yet clear if the conserved secondary struc-

tures found in the ciliate telomerase RNAs are conserved in either of the yeast telomerase RNAs (Singer and Gottschling 1994; McEachern and Blackburn 1995). Recently, a telomerase activity assay has been reported from *S. cerevisiae* cell extracts (Cohn and Blackburn 1995; Lin and Zakian 1995; Lue and Wang 1995). The availability of this in vitro assay will allow a detailed analysis of yeast telomerase activity and components.

MAMMALIAN TELOMERASE RNAs

In mammals, both the number of telomeres and the level of telomerase activity are lower than in ciliates, suggesting telomerase components might not be abundant. To clone the gene encoding the human telomerase RNA, a strategy was employed to amplify the telomerase RNA (Feng et al. 1995). A randomly primed cDNA library was prepared from either partially purified telomerase fractions or size-selected total RNA. The cDNA library was subjected to several rounds of selection for sequences encoding a putative telomerase RNA template domain 5′-CUAACCCUA-3′, using a biotinylated telomeric oligonucleotide and PCR. The telomerase RNA candidates were evaluated by determining whether they copurified with telomerase activity and whether antisense oligonucleotides inhibited telomerase activity. The genomic copy of the best candidate was cloned, and the putative template domain was mutated to encode d(TTGGGG) or d(TTTGGG) instead of d(TTAGGG) repeats. In vitro, telomerase assays using telomerase extracted from cells stably transfected with the altered gene indicated that the altered telomeric repeats were synthesized, which showed that this gene encodes the human telomerase RNA (hTR) component.

The hTR is approximately 450 nucleotides long, in contrast to both the shorter ciliate telomerase RNAs and the longer yeast RNAs. Also in contrast to the ciliate RNAs, evidence suggests that the hTR is an RNA polymerase II, not a polymerase III, transcript (Greider and Blackburn 1989; Yu et al. 1990; Avilion 1995; Feng et al. 1995). Using the human hTR gene as a probe in hybridization experiments, the mouse telomerase RNA (mTR) was identified, and its gene was cloned (Blasco et al. 1995). To confirm that this RNA is an essential component of mouse telomerase, oligonucleotide inhibition experiments and template alteration experiments were done. Interestingly, the mouse telomerase RNA has only 61% sequence identity to the human telomerase RNA, in contrast to other small RNAs that are more highly conserved between human and mouse. For example, the U6 snRNA has 100% and U7 has 85% identity

between the two organisms (Shumyatsky and Reddy 1992). The template regions of the two mammalian telomerase RNAs also differ; the mouse has a shorter template domain than the human. This shorter template domain in the mouse RNA could in part explain the decreased processivity of the mouse enzyme compared to human (Prowse et al. 1993), although there are also other biochemical differences between telomerases in the two species, such as the affinity for dGTP (see below).

IN VIVO ANALYSIS OF MUTANT TELOMERASE RNAs

In vivo expression of mutant telomerase RNA genes in *Tetrahymena*, and the corresponding alteration of telomere sequences, demonstrated that telomerase synthesizes telomeric repeats in vivo (Yu et al. 1990). The telomerase RNA gene template region was mutated and introduced into *Tetrahymena* cells. The RNA template was changed from 5′-CAACCCCAA-3′ to 5′-CGACCCCAA-3′, resulting in the synthesis of d(GGGGTC) repeats; whereas changing the template domain to 5′-CAACCCCCAA-3′ resulted in the synthesis of d(GGGGGTT) repeats at telomeres. A more efficient method of transformation by electroporation has recently been developed. Thus, it is now possible to make extracts from *Tetrahymena* cells expressing mutant telomerase RNAs and to analyze their elongation properties in vitro (Gilley et al. 1995).

In addition to causing changes in telomeric sequences, overexpression of mutant telomerase RNAs in *Tetrahymena* also causes changes in telomere length and in cell morphology and viability (Yu et al. 1990). In the transformants expressing telomerase RNA specifying d(GGGGGTT) or d(GGGGTC) telomere repeats, telomere lengths were longer than in wild-type cells. In one transformant in which mutant telomere sequences were not detected, telomere length was shorter. Expression of each of the three mutant RNAs eventually caused striking morphological changes and cell death. Before the cells died, most were large and arrested in cell division, had rounded or irregular shapes, and had enlarged and irregularly shaped macronuclei. Only loss of the mutant telomerase gene caused a reversion of these phenotypes. This was the first evidence establishing an essential role for telomerase in vivo. A more extensive analysis of cells expressing mutant telomerase RNAs indicates the mutants fall into three classes: some that have a wild-type phenotype, some that are early lethals, and some that are delayed lethals (Gilley et al. 1995).

A gene disruption of *TLC1* in yeast causes telomere shortening, as predicted for cells that can no longer synthesize telomeric DNA de novo (Singer and Gottschling 1994). In addition, these cells display a gradual

increase in division time by generation 65, accompanied by a 50% drop in viability after 75 generations. These phenotypes are similar to those reported for *est1* strains (Lundblad and Szostak 1989). *tlc1* cultures survive only when the population is overtaken by faster-growing cells. Presumably, these cells are similar to the *est1* survivors, where telomere recombination provides some level of telomere maintenance (Lundblad and Blackburn 1993).

Mutants in the template region of *K. lactis* telomerase RNA are divided into three classes: Some have wild-type telomere length, some have telomeres that are short and stable, and some manifest dramatic telomere lengthening (McEachern and Blackburn 1995). Mutations that fall into each of these categories are interspersed throughout the 23-bp template domain. Complete deletion of the telomerase RNA gene also has *est1*-like phenotypes in *K. lactis*. A strain overexpressing the *Bgl*II-containing telomerase RNA showed a decreasing number of wild-type repeats with increasing rounds of cell division. At about 400 generations, when all wild-type sequence may have been replaced with mutant repeats, there was a drastic increase in telomere length and the cells died, suggesting that sequence alteration of all telomere repeats has a drastic effect on both telomere function and cell viability (McEachern and Blackburn 1995).

BIOCHEMISTRY OF TELOMERASES

Telomerase activity has been assayed in vitro using extracts from a variety of organisms and cell lines including ciliates (*Tetrahymena*, *Euplotes*, and *Oxytricha*), yeasts (*Saccharomyces*), vertebrates (*Xenopus*), and mammals (human and mouse) (Table 1). Although all telomerase activities are sensitive to pretreatment of the extract with RNase, other requirements for DNA synthesis vary. Currently the interpretation of differences in these telomerase activities is complicated by the lack of purified enzyme preparations. However, despite this limitation, clear differences are found in processivity, and primer and nucleotide specificity, between telomerases from *Tetrahymena*, hypotrichs, and mammalian cells.

Primer Specificity

The sequence requirements for primer elongation by *Tetrahymena* telomerase are minimal. Although telomere sequences are preferred, at very high oligonucleotide concentrations, telomerase elongates almost any oligonucleotide primer (Greider and Blackburn 1987; Blackburn et

al. 1989; Harrington and Greider 1991; Lee and Blackburn 1993). The addition of telomeric repeats onto nontelomeric primers greatly increases the affinity of telomerase for a synthetic primer. In fact, the presence of only four guanosine residues at the primer 3′ end is sufficient to promote efficient elongation of a nonspecific oligonucleotide (Harrington and Greider 1991). Similar results are observed for human (Morin 1989), *Euplotes* (Shippen-Lentz and Blackburn 1990; Melek et al. 1994), and *Oxytricha* (Lingner et al. 1994; Melek et al. 1994) telomerases. These results suggest that in vitro, primer recognition is relatively promiscuous for telomerase enzymes.

The K_m values measured for *Tetrahymena* telomerase primer binding vary with the purity of the extract, the length and sequence of the primers, and the assay used. When processive products were measured, the apparent K_m was around 100 nM (Greider 1991). However, in this processive assay, multiple catalytic events are occurring. If elongation assays are used to determine K_m, measuring the first nucleotide added is most accurate, since only one catalytic step is required after binding for product formation. Using this assay, the $K_{m\ (app)}$ values for the 18-nucleotide primers $(GGGGTT)_3$ and $(GGGTTG)_3$, were measured at 1.5 μM. However, the $K_{m\ (app)}$ for dideoxy-terminated higher products in these reactions was between 10 nM and 50 nM (Collins and Greider 1993). This suggests that terminal dG addition onto these primers may represent several different steps. Lee and Blackburn were careful to show telomerase turnover under their reaction conditions, indicating that elongation to the first nucleotide did not represent stalled telomerase product complex. In these experiments measuring the addition of the first dT residue onto oligonucleotide primers, the $K_{m\ (app)}$ for $(TTGGGG)_3$ was 20 nM (Lee and Blackburn 1993).

To more directly assay primer binding to *Tetrahymena* telomerase, a direct primer-binding assay was developed (Harrington et al. 1995). This assay has the advantage that catalysis of dNTP addition is not required to measure primer binding. In this gel shift assay, the sequence specificity of primer binding is similar to that previously determined for primer elongation, suggesting that elongation specificity is determined in part at the level of binding.

Despite the different results with different assays, in all assays, the affinity for primers increases as primer length decreases: 18 nucleotides > 12 nucleotides > 10 nucleotides >> 6 nucleotides, such that a 6-nucleotide primer requires 1 μM to 5 μM concentration for elongation (Collins and Greider 1993; Lee and Blackburn 1993). If the inefficient 6-nucleotide telomeric primer is extended by additional specific or non-

specific sequence at the primer 5′ end, elongation is improved. In contrast, if nonspecific sequence is added to the 3′ end of 6 nucleotides of telomeric sequence, elongation is reduced. Thus, for optimal binding, the primer 3′ end should be complementary to the template RNA; however, the sequences in the primer 5′ region also affect elongation (Harrington and Greider 1991; Collins and Greider 1993; Lee and Blackburn 1993).

These results and others suggest that *Tetrahymena* telomerase contains two (or more) distinct primer-binding sites (Collins and Greider 1993; Lee and Blackburn 1993). One primer-binding site, termed the template site, aligns the primer 3′ end with the template RNA; primer binding at this site increases with complementarity to the template RNA. The second site, termed the anchor site, binds a more 5′ region of the primer (Fig. 3). Binding at the anchor site is enhanced with telomeric or G-rich sequences, but other sequences can serve as an anchor for processive elongation (Collins and Greider 1993; Lee and Blackburn 1993). It is unlikely that a folded primer structure such as a hairpin or a G-quartet is recognized by the anchor site (Blackburn et al. 1989). Such structures inhibit priming by *Oxytricha* telomerase (Zahler et al. 1991), and a 24-nucleotide-long primer containing only six 3′ terminal d(TTGGGG) bases is elongated efficiently, although it cannot form intramolecular hairpins (Harrington and Greider 1991). In addition, G-rich sequence primers with dispersed G residues are also elongated (Blackburn et al. 1989; Harrington 1993).

Analysis of primer K_m values for telomerases other than *Tetrahymena* is complicated by the presence of competing primer-binding activities in crude extracts. However, it is clear from primer titrations (Morin 1989) and the standard conditions used in most laboratories that the affinity of mammalian telomerases for telomeric primers is likely to be at most 10-fold higher than that of the *Tetrahymena* enzyme. For human telomerase, longer primers are more efficiently elongated than primers of 1–1.5 repeats. At saturating primer concentrations, nonspecific sequence added to the 5′ end of inefficient primers increases elongation, but nonspecific sequence added to a telomeric primer 3′ end is inhibitory (Morin 1989). These similarities of human and *Tetrahymena* telomerase primer specificity suggest that human telomerase also utilizes a second primer-binding site in addition to the template for optimal elongation (Morin 1989).

Nucleotide Specificity

The specificity of nucleotide incorporation by *Tetrahymena* telomerase can be altered in vivo or in vitro by mutation of the RNA template sequence. Thus, a large component of the nucleotide specificity of

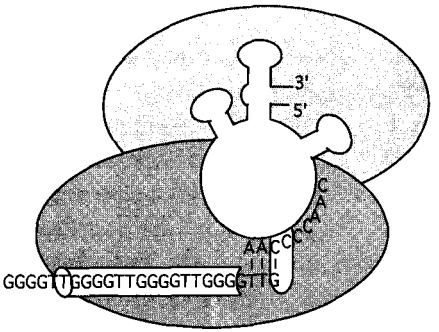

Figure 3 Two-site model of telomerase primer binding and elongation. Telomeric primer oligonucleotides interact with telomerase at two distinct sites, the 3′ end aligns and base-pairs with the template region allowing additions in the catalytic site (white oval), and the body of the primer is bound by a second site, the anchor site (white tube). This two-site model is similar to that proposed for RNA polymerases, to allow for processive elongation. To utilize the 9-nucleotide template regions, there must be some conformational change in the RNP complex. Either the catalytic site moves along the template or the template RNA moves relative to the catalytic site.

telomerase is determined by base-pairing with the template (Yu et al. 1990; Autexier and Greider 1994). However, quantitation of telomerase activity with titrations of individual nucleotides, assayed in partially purified extract (Greider and Blackburn 1987; Greider 1991; Lee and Blackburn 1993) or with highly purified telomerase (Collins and Greider 1995), showed *Tetrahymena* telomerase prefers guanosine nucleotides. Only 1 μM to 10 μM dGTP is required for optimal elongation, but TTP is required at 100 μM to 500 μM. This represents a 50- to 100-fold difference in the affinity of telomerase for its two natural dNTP substrates. Template-complementary ddNTPs are efficiently incorporated at concentrations of 100 μM to 500 μM (Greider 1991; Autexier and Greider 1994).

Euplotes and *Oxytricha* telomerases processively elongate telomeric primers at nucleotide concentrations similar to those used for *Tetrahymena* (Zahler and Prescott 1988; Shippen-Lentz and Blackburn 1989; Lingner et al. 1994). Like the *Tetrahymena* enzyme, *Oxytricha* telomerase requires significantly less dGTP than TTP for elongation (Zahler and Prescott 1988). Although human telomerase assays use 10-fold higher dNTP levels than the ciliates, human telomerase also appears to have a different affinity for dGTP versus TTP and dATP (Morin 1989). The dideoxynucleotides ddATP, ddTTP, and ddGTP are also incorporated by mouse telomerase (Prowse et al. 1993).

Processivity

The processivity of a polymerase represents the probability of dissociation after each successive nucleotide addition. Using telomeric $(TTGGGG)_n$ primers with three or more repeats, the *Tetrahymena* telomerase is highly processive (Greider 1991). Primers with shorter length and those that diverge from the telomeric $d(TTGGGG)_n$ sequence are elongated less processively (Collins and Greider 1993; Lee and Blackburn 1993). Nucleolytic cleavage has been observed for *Tetrahymena* telomerase, and a role for cleavage in normal processive elongation is suggested by analogy with DNA-dependent RNA polymerases (for review, see Kassavetis and Geiduschek 1993). The primer length requirements for processive elongation by *Tetrahymena* telomerase support the two-site model for primer binding. Products not bound to the anchor site are more likely to dissociate during repositioning of the product 3′ end. This model is similar to that proposed for processive elongation by RNA polymerase (Chamberlin 1992).

In contrast to the processive telomerases from ciliates and human cells, mouse and *Xenopus* telomerases are relatively nonprocessive. Using the reaction conditions originally described, hundreds of repeats are added onto a primer by human telomerase, whereas predominantly 1–3 repeats are added by mouse and *Xenopus* telomerases (Morin 1989; Prowse et al. 1993; Mantell and Greider 1994). Some of this difference arises from the requirement of the nonprocessive enzymes for higher concentrations of dGTP: When 10- to 100-fold higher concentrations of dGTP are used, synthesis of at least 10 repeats is observed for *Xenopus*, rat, and mouse telomerases (L.L. Mantell et al., unpubl.). In addition, the shorter template region in mouse telomerase RNA may affect processivity (Blasco et al. 1995).

TETRAHYMENA RIBONUCLEOPROTEIN STRUCTURE

The protein components of telomerase have recently been identified and the genes have been cloned from *Tetrahymena* (Collins et al. 1995). Two polypeptides of 80 kD and 95 kD were identified that reproducibly copurified with telomerase activity. Using peptide sequence from these proteins, the genes were both cloned from *Tetrahymena*. To determine if p80 and p95 were essential components of telomerase, antipeptide antibodies were generated against these two proteins. Telomerase activity was immunoprecipitated using an antibody directed against p80, but an

unrelated antibody that did not recognize p80 or p95 did not immunoprecipitate telomerase activity. These experiments indicated that p80 is a functional component of telomerase. Western analysis of the pellet fraction showed that p95 coprecipitated with p80 in fractions that had activity. In addition, the p80 and p95 proteins copurified with each other over all columns tested, comigrated in a native gel, and cofractionated in a glycerol gradient even after RNase treatment. These data suggest that p80 and p95 bind to each other directly and are required for telomerase activity. Based on the predicted mass of telomerase, the active enzyme is likely to consist of one subunit each of the telomerase RNA, p80, and p95 (Collins et al. 1995).

The sequences of p80 and p95 do not show a high degree of similarity with other proteins in available databases. The p80 protein contains a zinc finger that may be involved in RNA or DNA binding. A region in the p95 protein can be aligned with the active-site motifs from two different classes of polymerases: RNA-dependent RNA polymerases and DNA polymerases α and β (Poch et al. 1989; Delarue et al. 1990). The lack of any other sequence homology with known polymerases, together with these motif regions, suggests that telomerase represents a new class of polymerase (Collins et al. 1995). RNA-dependent RNA polymerases are thought to be some of the earliest polymerases (Gesteland and Atkins 1993). If telomerase is indeed most closely related to this class of polymerase, it suggests that telomerase may have evolved from an ancestral polymerase.

Protein components have not yet been identified from organisms other than *Tetrahymena*. Nevertheless, it is possible to compare RNP composition through information obtained during enzyme purification. *Tetrahymena* telomerase fractionates during glycerol gradient sedimentation and gel filtration with a molecular weight of 270,000 or 200,000 (Greider and Blackburn 1987; Collins and Greider 1993; Harrington 1993; Collins et al. 1995). In contrast, telomerase from human cells fractionates by velocity sedimentation or gel filtration with a molecular weight around 750,000, and the mouse enzyme appears to fractionate even slightly larger than the human (K. Collins et al., unpubl.). Although differences in the sizes of telomerase RNAs may contribute somewhat to the increased molecular mass of the mammalian enzymes, there may be additional (or larger) protein components as well. Human and mouse telomerases appear to be less stable to column chromatography than *Tetrahymena*, perhaps due to the increased mass of the mammalian enzymes. Detailed comparison of telomerase RNP structures awaits the purification and cloning of additional telomerase protein components.

TELOMERASE ACTIVITY IN HUMAN CANCER CELLS

Unlike telomeres in most single-cell eukaryotes, telomeres in human somatic tissues are not maintained at a constant average length. In fibroblasts, blood cells, and other tissues, telomeres shorten with each round of cell division both in vitro and in vivo, whereas germ-line cells are immune to this progressive shortening (Cooke and Smith 1986; Harley et al. 1990; Hastie et al. 1990; Lindsey et al. 1991; Allsopp et al. 1992; Vaziri et al. 1993). Telomerase activity was not detected in many primary fibroblast cultures, suggesting that telomere shortening is due to the absence of telomerase (Counter et al. 1992, 1994; Kim et al. 1994). Telomere shortening may be due to the inability of conventional polymerase to replicate chromosome ends. Using a recently developed sensitive PCR assay, telomerase activity was also not detected in a number of primary human tissue samples (Kim et al. 1994). However, recent evidence indicates that some primary cells such as white blood cells do contain telomerase activity (Broccoli et al. 1995; Counter et al. 1995; Hiyama et al. 1995).

In contrast to some normal human tissues, cancer cells from tissue culture and those taken directly from tumors contain detectable telomerase activity (for review, see Bacchetti and Counter 1995). Evidence suggests that telomerase may be required for the growth of tumor cells. Thus, telomerase inhibition is potentially a prime target for cancer chemotherapy (Harley et al. 1990; Hastie et al. 1990; Counter et al. 1992; Kim et al. 1994; Klingelhutz et al. 1994; Mehle et al. 1994).

Unlike many human tissues that appear to lack telomerase activity, many primary mouse tissues express telomerase activity (Chadeneau et al. 1995; Prowse and Greider 1995). One explanation for this difference may be that mouse cells lack a repressor or inhibitor of telomerase that is present in human cells. Primary human cells rarely, if ever, spontaneously immortalize, whereas mouse cells frequently spontaneously immortalize (Macieira-Coelho and Azzarone 1988). If human cells have additional negative controls on telomerase that mouse cells lack, it may take fewer "hits" to activate expression in mouse cells than in human cells (Prowse and Greider 1995).

What role telomerase activation plays in cellular immortalization and cancer is an important question for future studies. To determine the role of telomerase in cancer, and to help design potential inhibitors, it is essential to have a thorough understanding of the telomerase mechanism. With the detailed groundwork of telomerase biochemistry laid and with the recent cloning of the yeast and mammalian RNAs and the *Tetrahymena* proteins, the stage is set to explore the telomerase

polymerase mechanism and the role of telomerase in cell biology, development, and tumorigenesis.

REFERENCES

Allsopp, R.C., H. Vaziri, C. Patterson, S. Goldstein, E.V. Younglai, A.B. Futcher, C.W. Greider, and C.B. Harley. 1992. Telomere length predicts the replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci.* **89:** 10114–10118.

Autexier, C. and C.W. Greider. 1994. Functional reconstitution of wild type and mutant *Tetrahymena* telomerase. *Genes Dev.* **8:** 563–575.

———. 1995. Boundary elements of the *Tetrahymena* telomerase RNA template and alignment domains. *Genes Dev.* **15:** 2227–2239.

Avilion, A.A. 1995. *"Identification and characterization of human telomerase RNA."* Ph.D. thesis, State University of New York, Stony Brook.

Bacchetti, S. and C.M. Counter. 1995. Telomeres and telomerase in human cancer: Review. *Int. J. Oncol.* **7:** 423–432.

Bhattacharyya, A. and E.H. Blackburn. 1994. Architecture of telomerase RNA. *EMBO J.* **13:** 5521–5531.

Blackburn, E.H. 1991. Structure and function of telomeres. *Nature* **350:** 569–573.

Blackburn, E.H., C.W. Greider, E. Henderson, M. Lee, J. Shampay, and D. Shippen-Lentz. 1989. Recognition and elongation of telomeres by telomerase. *Genome* **31:** 553–560.

Blasco, M., W. Funk, B. Villepounteau, and C.W. Greider. 1995. Functional characterization and developmental regulation of mouse telomerase RNA. *Science* **269:** 1267–1270.

Broccoli, D., J.W. Young, and T. de Lange. 1995. Telomerase activity in normal and malignant haematopoietic cells. *Proc. Natl. Acad. Sci.* **92:** 9082–9086.

Chadeneau, C., P. Siegel, C.B. Harley, W. Muller, and S. Bacchetti. 1995. Telomerase activity in normal and malignant murine tissues. *Oncogene* **11:** 893–898.

Chamberlin, M.J. 1992. New models for the mechanism of transcription elongation and its regulation. *Harvey Lect.* **88:** 1–21.

Cohn, M. and E.H. Blackburn. 1995. Telomerase in yeast. *Science* **269:** 396–400.

Collins, K. and C.W. Greider. 1993. *Tetrahymena* telomerase catalyzes nucleolytic cleavage and non-processive elongation. *Genes Dev.* **7:** 1364–1376.

———. 1995. Elongation of RNA primers and incorporation of ribonucleotides by *Tetrahymena* telomerase. *EMBO J.* **14:** 5422–5432.

Collins, K., R. Koybayashi, and C.W. Greider. 1995. Purification of *Tetrahymena* telomerase and cloning of the genes for the two protein components of the enzyme. *Cell* **81:** 677–686.

Cooke, H.J. and B.A. Smith. 1986. Variability at the telomeres of human X/Y pseudoautosomal region. *Cold Spring Harbor Symp. Quant. Biol.* **51:** 213–219.

Counter, G.M., H.W. Hirte, S. Bacchetti, and C.B. Harley. 1994. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci.* **91:** 2900–2904.

Counter, C.M., J. Gupta, C.B. Harley, B. Leber, and S. Bacchetti. 1995. Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* **85:** 2315–2320.

Counter, C.M., A.A. Avilion, C.E. LeFeuvre, N.G. Stewart, C.W. Greider, C.B. Harley,

and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11:** 1921–1929.

Delarue, M., O. Poch, N. Tordo, and P. Argos. 1990. An attempt to unify the structure of polymerases. *Protein Eng.* **3:** 461–467.

Feng, J., W. Funk, S. Wang, S. Weinrich, A. Avilion, C.-P. Chiu, R. Adams, E. Chang, J. Yu, S. Le, M. West, C.B. Harley, W. Andrews, C.W. Greider, and B. Villeponteau. 1995. The human telomerase RNA component. *Science* **269:** 1236–1241.

Gesteland, R.F. and J.F. Atkins, eds. 1993. *The RNA world.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Gilley, D., M. Lee, and E.H. Blackburn. 1995. Altering specific telomerase RNA template residues affects active site function. *Genes Dev.* **9:** 2214–2226.

Gottschling, D.E., O.M. Aparicio, B.L. Billington, and V.A. Zakian. 1990. Position effect at *S. cerevisiae* telomeres: Reversible repression of Pol II transcription. *Cell* **63:** 751–762.

Greider, C.W. 1991. Telomerase is processive. *Mol. Cell. Biol.* **11:** 4572–4580.

Greider, C.W. and E.H. Blackburn. 1985. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* **43:** 405–413.

———. 1987. The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* **51:** 887–898.

———. 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* **337:** 331–337.

Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* **345:** 458–460.

Harrington, L.A. 1993. *Characterization and purification of Tetrahymena telomerase.* Ph. D. thesis, State University of New York, Stony Brook.

Harrington, L.A. and C.W. Greider. 1991. Telomerase primer specificity and chromosome healing. *Nature* **353:** 451–454.

Harrington, L.H., C. Hull, J. Crittenden, and C.W. Greider. 1995. Gel shift and UV crosslinking analysis of *Tetrahymena* telomerase. *J. Biol. Chem.* **270:** 8893–8901.

Hastie, N.D., M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green, and R.C. Allshire. 1990. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346:** 866–868.

Henderson, E.R. 1995. Telomeric DNA sequences. In *Telomeres* (ed. E.H. Blackburn and C.W. Greider) pp. 11–34. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hiyama, K., Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M.A. Piatyszek, J. Shay, S. Ishioka, and M. Yamakido. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.* **155:** 3711–3715.

Kassavetis, G.A. and E.P. Geiduschek. 1993. RNA polymerase marching forward. *Science* **259:** 944–945.

Kim, N.W., M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, and J.W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* **266:** 2011–2014.

Klingelhutz, A.J., S. Barber, P.P. Smith, K. Dyer, and J.K. McDougall. 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. *Mol. Cell Biol.* **14:** 961–969.

Kramer, K.M. and J.E. Haber. 1993. New telomeres in yeast are initiated with a highly selected subset of TG_{1-3} repeats. *Genes Dev.* **7:** 2345–2356.

Lee, M.S. and E.H. Blackburn. 1993. Sequence-specific DNA primer effects on telomerase polymerization activity. *Mol. Cell Biol.* **13:** 6586–6599.

Lin, J.-J., and V.A. Zakian. 1995. An in vitro assay for *Saccharomyces* telomerase requires *EST1*. *Cell* **81:** 1127–1135.

Lindsey, J., N.I. McGill, L.A. Lindsey, D.K. Green, and H.J. Cooke. 1991. In vivo loss of telomeric repeats with age in humans. *Mutat. Res.* **256:** 45–48.

Lingner, J., L.L. Hendrick, and T.R. Cech. 1994. Telomerase RNAs of different ciliates have a common secondary structure and a permuted template. *Genes Dev.* **8:** 1984–1998.

Lue, N.F. and J.C. Wang. 1995. ATP-dependent processivity of a telomerase activity from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270:** 21453–21456.

Lundblad, V. and E.H. Blackburn. 1993. An alternative pathway for yeast telomere maintenance rescues *est1*$^-$ senescence. *Cell* **73:** 347–360.

Lundblad, V. and J.W. Szostak. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* **57:** 633–643.

Macieira-Coelho, A. and B. Azzarone. 1988. The transition from primary culture to sponteaneous immortalization in mouse fibroblast populations. *Anticancer Res.* **8:** 669–676.

Mantell, L.L. and C.W. Greider. 1994. Telomerase activity in germline and embryonic cells of *Xenopus*. *EMBO J.* **13:** 3211–3217.

McCormick-Graham, M. and D.P. Romero. 1995. Ciliate telomerase RNA structural features. *Nucleic Acids Res.* **23:** 1091–1097.

McEachern, M.J. and E.H. Blackburn. 1994. A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts. *Proc. Natl. Acad. Sci.* **91:** 3453–3457.

———. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations in the yeast *K. lactis*. *Nature* **376:** 403–409.

McEachern, M.J. and J.B. Hicks. 1993. Unusually large telomeric repeats in the yeast *Candida albicans*. *Mol. Cell. Biol.* **13:** 551–560.

Mehle, C., B. Ljungberg, and G. Roos. 1994. Telomere shortening in renal cell carcinoma. *Cancer Res.* **54:** 236–241.

Melek, M., B. Davis, and D.E. Shippen. 1994. Oligonucleotides complementary to the *Oxytricha nova* telomerase RNA delineate the template domain and uncover a novel mode of primer utilization. *Mol. Cell. Biol.* **14:** 7827–7838.

Morin, G.B. 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59:** 521–529.

Poch, O., I. Sauvaget, M. Delarue, and N. Tordo. 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* **8:** 3867–3874.

Prowse, K.R. and C.W. Greider. 1995. Developmental and tissue specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci.* **92:** 4818–4822.

Prowse, K.R., A.A. Avilion, and C.W. Greider. 1993. Identification of a nonprocessive telomerase activity from mouse cells. *Proc. Natl. Acad. Sci.* **90:** 1493–1497.

Romero, D.P. and E.H. Blackburn. 1991. A conserved secondary structure for telomerase RNA. *Cell* **67:** 343–353.

Shippen-Lentz, D. and E.H. Blackburn. 1989. Telomere terminal transferase activity from *Euplotes crassus* adds large numbers of TTTTGGGG repeats onto telomeric primers. *Mol. Cell. Biol.* **9:** 2761–2764.

———. 1990. Functional evidence for an RNA template in telomerase. *Science* **247:** 546–552.

Shumyatsky, G. and R. Reddy. 1992. Compilation of small RNA sequence. *Nucleic Acids Res.* **20:** 2159–2165.

Singer, M.S. and D.E. Gottschling. 1994. *TLC1:* Template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266:** 404–409.

ten Dam, E., A. Van Belkum, and C.W.A. Pleij. 1991. A conserved pseudoknot in telomerase RNA. *Nucleic Acids Res.* **19:** 6951.

Vaziri, H., F. Schaechter, I. Uchida, L. Wei, Z. Xiaoming, R. Effros, D. Choen, and C.B. Harley. 1993. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am. J. Hum. Genet.* **52:** 661–667.

Yu, G.-L., J.D. Bradley, L.D. Attardi, and E.H. Blackburn. 1990. *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature* **344:** 126–132.

Zahler, A.M. and D.M. Prescott. 1988. Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules. *Nucleic Acids Res.* **16:** 6953–6972.

Zahler, A.M., J.R. Williamson, T.R. Cech, and D.M. Prescott. 1991. Inhibition of telomerase by G-quartet DNA structures. *Nature* **350:** 718–720.

Zaug, A.J. and T.R. Cech. 1995. Analysis of the structure of *Tetrahymena* nuclear RNAs in vivo: Telomerase RNA, self-splicing rRNA intron and U2 snRNA. *RNA* **1:** 363–374.