Research Article

Functional Evidence for an RNA Template in Telomerase

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The RNA moiety of the ribonucleoprotein enzyme telomerase from the ciliate *Euplotes crassus* was identified and its gene was sequenced. Functional analysis, in which oligonucleotides complementary to portions of the telomerase RNA were tested for their ability to prime telomerase in vitro, showed that the sequence 5′ CAAAACCCCAAA 3′ in this RNA is the template for synthesis of telomeric TTTTGGGG repeats by the *Euplotes* telomerase. The data provide a direct demonstration of a template function for a telomerase RNA and demarcate the outer boundaries of the telomeric template. Telomerase can now be defined as a specialized reverse transcriptase.

REVERSE TRANSCRIPTASES, ONCE THOUGHT TO BE CONfined exclusively to retroviruses, appear to be widespread among eukaryotes and possibly prokaryotes (*1–4*). Eukaryotic genomes, especially those of higher eukaryotes, contain numerous sequences generated by reverse transcriptases that were introduced by RNA-mediated transposition of viral and nonviral retroposons (*1–3, 5*). These repeated sequences may constitute a significant proportion of their resident genome; however, they have as yet no identifiable function. In contrast, another class of repeated sequences, chromosomal telomeres, are essential components of genomes. We show here that telomeres are synthesized by a novel type of reverse transcriptase activity.

Telomeres, the specialized structures at the ends of linear chromosomes, are required for the stable maintenance of chromosomes (*6, 7*). Many features of telomeric DNA are highly conserved among eukaryotes. Consisting exclusively of simple repeated sequences, telomeric DNA's all exhibit a strand composition bias, with a G-rich strand always oriented 5′ to 3′ toward the end of the chromosome (*8–15*). In telomeres whose terminal structures have either been analyzed directly or can be inferred (*8, 9, 16*), the 3′ end of the G-rich strand protrudes by 12 to 16 nucleotides. In vitro, DNA oligonucleotides consisting of the protruding G-rich strand sequences assume novel higher order structures, involving non–Watson-Crick base pairing (*17*).

Accumulating evidence suggests that the conservation of structure and function of telomeric DNA reflects its mode of synthesis. A ribonucleoprotein (RNP) enzyme called telomerase extends the 3′ end of G-rich telomeric oligonucleotide primers by the de novo addition of tandem telomeric repeat sequences. First identified in the holotrichous ciliate *Tetrahymena* (*18, 19*), telomerase activity appears to be widespread among eukaryotes, as it was subsequently found in the hypotrichous ciliates *Oxytricha* (*20*) and *Euplotes* (*21*) and recently in extracts from HeLa cells (*22*). These activities synthesize the telomeric repeats of the organism from which they are derived. Thus, the *Tetrahymena* telomerase adds TTGGGG repeats (*18, 19, 23*), the *Euplotes* and *Oxytricha* telomerases, TTTTGGGG repeats (*20, 21*), and the human telomerase, TTAGGG repeats (*22*).

Greider and Blackburn previously identified the RNA moiety of the *T. thermophila* telomerase (*19, 23*). This RNA, 159 nucleotides in length, contains the sequence 5′ CAACCCCAA 3′. DNA oligonucleotides complementary to a region overlapping this sequence competed with telomeric primers for telomerase. Enzymatic inactivation by selective cleavage of this sequence indicated that its integrity is required for telomerase activity. On the basis of these results, the 5′ CAACCCCAA 3′ sequence in the *Tetrahymena* telomerase RNA was postulated to serve as a templating domain for the addition of the *Tetrahymena* telomeric repeat sequence TTGGGG to DNA primers (*23*).

A prediction of this model is that the RNA moieties of telomerases from other organisms will contain templates that correspond to their species-specific telomeric repeat sequence. We now report the identification of the RNA component of the telomerase from the ciliate *Euplotes crassus*. This RNA contains the sequence 5′ CAAAACCCCAAAACC 3′, which lies in a position directly corresponding to the 5′ CAACCCCAA 3′ sequence of the *Tetrahymena* telomerase RNA. We show that 13 of the 15 nucleotides in this telomere-complementary RNA sequence (5′ CAAAACCCCAAAA 3′) function as a template strand domain for the synthesis of tandem repeats of the *Euplotes* telomeric sequence TTTTGGGG.

A telomeric template region in a candidate telomerase RNA from *Euplotes*. We showed previously that the telomerase from *Euplotes* contains an essential RNA component (*21*). To identify this species among total nuclear RNA's, we reasoned that, like the telomerase RNA from *Tetrahymena thermophila*, the *Euplotes* telomerase RNA may also contain a sequence complementary to the telomeric repeats synthesized, in this case TTTTGGGG repeats. Macronuclear RNA from *Euplotes* was hybridized to a synthetic DNA oligonucleotide, d(TTTTGGGG)$_4$, complementary to the predicted templating sequence, r(CCCCAAAA)$_n$, and incubated with ribonuclease (RNase) H, an enzyme that specifically cleaves RNA that is paired in an RNA-DNA hybrid. A single prominent RNA band was lost in the RNase H–treated preparations compared

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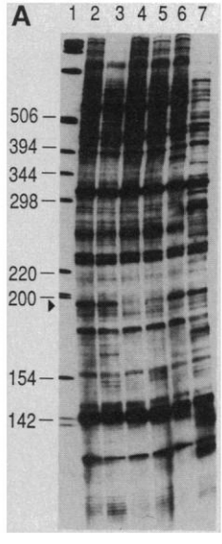
with the untreated controls (arrowhead in Fig. 1A).

This RNA is a homogeneous species, 191 nucleotides in length, and is present in approximately 10^5 molecules per macronucleus. We sequenced this RNA and then, using oligonucleotide probes derived from the RNA sequence, cloned and sequenced the 650-bp macronuclear DNA molecule containing the 191-nt RNA gene from a macronuclear genomic library (Fig. 1B). Because the macronuclear genome of hypotrichous ciliates such as *Euplotes* consists of gene-sized, free linear DNA molecules (*24*), this DNA sequence presumably contains all of the cis-acting upstream and downstream transcriptional control signals associated with the 191-nt RNA gene. Features of the 191-nt RNA gene indicating that it may be transcribed by RNA polymerase III include the observations that the RNA is not capped and that its 3′ end corresponds to the second in a stretch of five T residues (*25*). However, unlike the *T. thermophila* telomerase RNA (*23*) and the 5*S* rRNA gene from *Euplotes woodruffi* (*26*), the *Euplotes crassus* 191-nt RNA coding region does not contain the obvious A box consensus sequence characteristic of many genes transcribed by RNA polymerase III (*27*).

The most striking feature of the 191-nt RNA is a 15-nt sequence, 5′ CAAAACCCCAAAACC 3′, located at position 35 to 49, which could serve as a template for the synthesis of TTTTGGGG repeats. The *Euplotes* 191-nt RNA and the *Tetrahymena* telomerase RNA share little overall primary sequence similarities (Fig. 1B), a finding consistent with our inability to detect a cross-hybridizing species of *Euplotes* DNA with a *Tetrahymena* telomerase RNA gene probe. However, despite their divergent primary structures, the *Euplotes* 191-nt RNA, and the telomerase RNA's from *T. thermophila* and three other *Tetrahymena* species, can all be folded into similar secondary structures with the putative telomeric templating domains for each RNA lying in a corresponding position (*28*). In this respect, the telomerase RNA may be like the RNA moiety of the RNase P, an RNA molecule whose nucleotide sequence has also diverged extensively between species while its secondary structure has been conserved and is required for activity (*29, 30*). The presence of a telomeric sequence, and the potential for the *Euplotes* 191-nt RNA to assume a secondary structure similar to the *Tetrahymena* telomerase RNA, suggested that the 191-nt RNA is a component of the *Euplotes* telomerase.

Specificity of DNA oligonucleotides complementary to the 191-nt RNA as primers for TTTTGGGG repeat synthesis. Conclusive evidence that the 191-nt RNA is part of the *Euplotes* telomerase RNP was obtained from experiments with a series of oligonucleotides complementary to the 191-nt RNA over the region adjacent to the putative template sequence, and extending just 3′ to, into, or beyond it (Fig. 2A, top). We anticipated that the duplex formed by these complementary oligonucleotides with the RNA would immobilize the primer 3′ terminus on the putative templating domain and adjacent sequences. Although the interaction of these complementary oligonucleotides with telomerase may not mimic the action of natural telomeric primers, these studies allowed the boundaries of the template to be defined. Each complementary oligonucleotide was tested for its ability to act as a primer for telomerase, or to inhibit the telomerase elongation reaction (Fig. 2). These oligonucleotides exhibited a striking specificity for the *Euplotes* telomerase, several of them priming the addition of large numbers of TTTTGGGG repeats, as seen by the characteristic banding pattern with an eight-base periodicity in the upper portion of the gel (Fig. 2A) (*21*). In contrast, complementary oligonucleotides directed at other regions of the 191-nt RNA failed to act as primers for TTTTGGGG repeat synthesis.

Reactions primed by the complementary oligonucleotides generated large amounts of short elongation products (Fig. 2A, lanes 8,



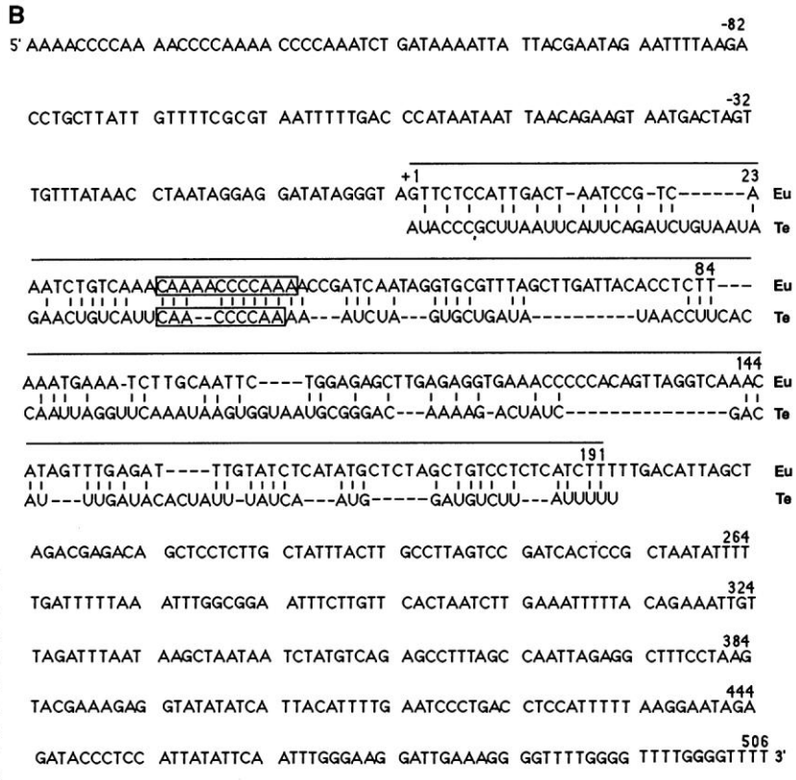


Fig. 1. (**A**) Identification of the *Euplotes* telomerase RNA. RNA was isolated from *E. crassus* macronuclei gradient preparations with guanidinium and CsCl (*37*). The RNA was end-labeled with [^{32}P]pCp and incubated with various oligonucleotides (lane 3, A+T rich oligonucleotide; lane 4, $(TTTTGGGG)_4$; lane 5, $(TTGGGG)_4$; lane 6, oligonucleotide complementary to the 3′ terminus of the *T. thermophila* telomerase RNA; lane 7, $(CCCCAAAA)_4$ in the presence of 1.6 units of RNase H per 20 μl of reaction mixture. The products were resolved on a 6 percent sequencing gel. Lane 1, [γ-^{32}P]dATP labeled molecular size markers; lane 2, untreated *E. crassus* RNA. (**B**) Nucleotide sequence of the *E. crassus* telomerase RNA gene and alignment with the *T. thermophilia* telomerase RNA. The *Euplotes* telomerase RNA was sequenced by means of a combination of the direct chemical method (*38*) and the dideoxynucleotide primer extension technique. DNA blot analysis with an oligonucleotide probe derived from the RNA sequence localized the telomerase RNA gene to a 650-bp macronuclear DNA molecule. The macronuclear genomic clone was obtained from a λgt10 library containing *E. crassus* macronuclear DNA and was subsequently subcloned into a pUC vector. Both strands of the insert were sequenced by dideoxy-primer extension. The *E. crassus* telomerase RNA gene was aligned with the *T. thermophila* telomerase RNA sequence on the basis of conserved domains in the proposed secondary structure models for these RNA's (*28*). The overline denotes the *E. crassus* 191-nt RNA coding domain. The functional telomeric template for the *Euplotes* RNA and the putative telomeric template for the *Tetrahymena* telomerase RNA are boxed. Te, *Tetrahymena*; Eu, *Euplotes*.

10, 12, 14, and 16) which correspond to the addition of approximately one-and-a-half to two TTTTGGGG repeats (see below). Whether the accumulation of these short products results from pausing before the addition of the next nucleotide, chain termination, or a combination of the two, is not known, but for brevity we will refer to it as pausing. This characteristic pausing profile was not seen with the standard telomeric repeat oligonucleotide, $d(TTTTGGGG)_4$, or other telomeric oligonucleotide primers (Fig. 2A, lane 19) and (*21*). Quantitation of the radioactivity from [α-^{32}P]dGTP incorporated into the telomerase reaction products indicated that reactions primed by the complementary oligonucleotides accumulated up to ten times more label than the standard reaction primed by $d(TTTTGGGG)_4$.

The specificity of the complementary oligonucleotides for the *Euplotes* telomerase was further demonstrated in experiments in which extracts were first incubated with $d(TTTTGGGG)_4$, with subsequent addition of a complementary oligonucleotide primer, and vice versa. Oligonucleotides 31 to 59 (Fig. 2A, lane 18) and 31 to 51, which completely cover and extend beyond the 5′ end of the putative template, did not serve as primers for telomerase. Moreover, these two oligonucleotides inhibited the standard telomerase reaction primed by $d(TTTTGGGG)_4$ (Fig. 2B, lanes 7 and 8), presumably by blocking access of the TTTTGGGG repeat primer to the telomeric RNA sequence. Oligonucleotide 50 to 79 (Fig. 2B, lanes 1 and 2) and control noncomplementary oligonucleotides had little or no effect. Some of the complementary oligonucleotide primers competed efficiently with $d(TTTTGGGG)_4$. The products of such reactions either exhibited a pausing pattern characteristic of elongation of the complementary primer superimposed on the standard $d(TTTTGGGG)_4$ profile (compare lanes 3 and 4 of Fig. 2B with lane 9), or in the case of oligonucleotide 37 to 65, a pattern that completley replaced the typical profile generated from the $(TTTTGGGG)_4$ primer alone (Fig. 2B, lanes 5, 6, and 9).

The available evidence suggests that the strength of the early pauses seen with the complementary primers reflects the inability of these oligonucleotides to dissociate from the putative telomeric template and the neighboring RNA sequence. First, the time course of label incorporation into the reactions with the complementary primers showed that most of the accumulation of short products occurred during the first few minutes of the typical 1-hour reactions shown in Fig. 2A. This finding suggests that the large amounts of short products are not due to extensive recycling of the complementary primers by telomerase (*31*) but rather, these primers remain bound, with the longer elongation products representing the small proportion of primers that were successfully disengaged. Second, decreasing the length of the complementary oligonucleotide decreased the magnitude of the pausing. Comparing pairwise oligonucleotides having the same 3′ terminus (a 24 *vs.* 29 nucleotide pair, a

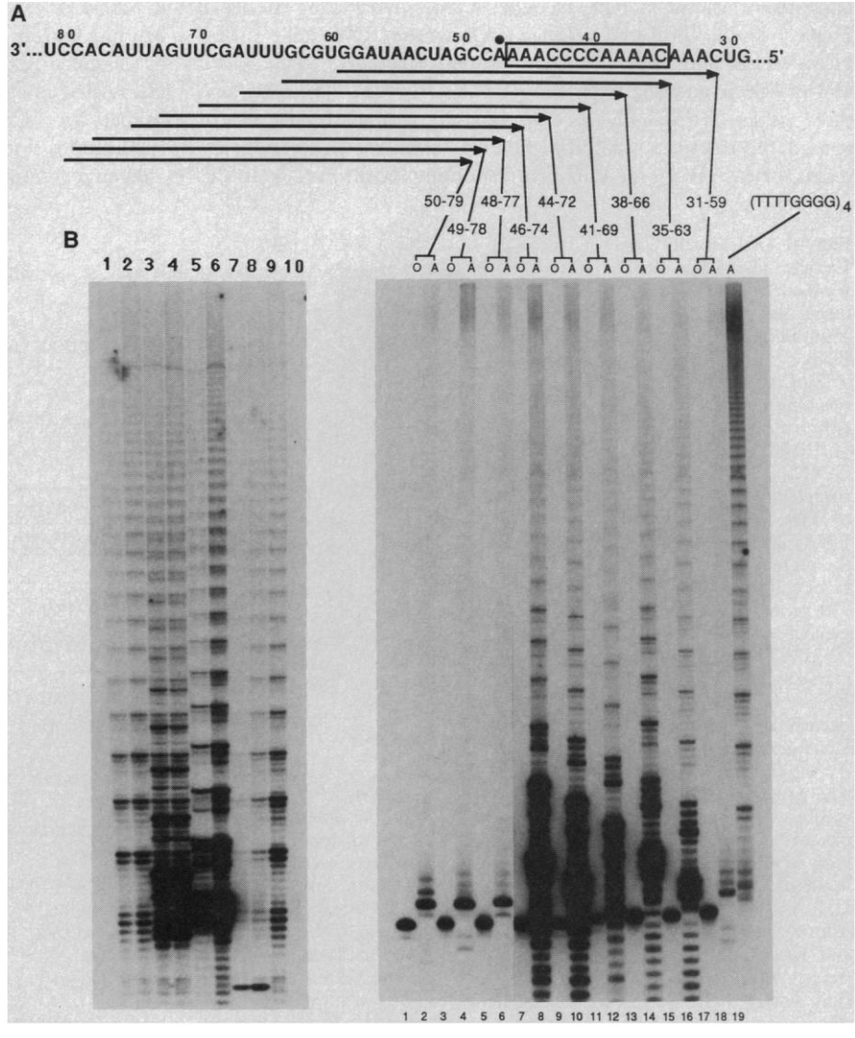


Fig. 2. (**A**) Priming activity of oligonucleotides complementary to the *Euplotes* telomerase RNA in telomerase assays. Macronuclear telomerase extracts were prepared from mated *E. crassus* (*21*). For the assay, 0.2 μg of oligonucleotide was incubated with 8 to 15 μl of the telomerase extract along with an equal volume of 2× reaction mixture containing 200 m*M* TTP, (thymidine triphosphate) 200 m*M* sodium acetate, 100 m*M* tris, *p*H 8.0, RNasin at 2 unit/μl, and 10 to 20 μCi of [α-^{32}P]dGTP at 400 Ci/mmol. Reactions were carried out at 30°C for 60 minutes, then extracted with phenol and precipitated with ethanol, and the products were resolved on a 6 percent polyacrylamide sequencing gel. Arrows at the top portion correspond to the oligonucleotide sequences, with the arrowhead marking their 3′terminus. Boxed region in the RNA sequence denotes nucleotides that are copied into telomeric DNA. The dot above position 47 indicates the outermost nucleotide that can position the 3′ end of a primer for elongation on the template. O, [γ-^{32}P]dATP-labeled oligonucleotide marker; A, telomerase assay with the corresponding oligonucleotide. (Lanes 1 and 2) Oligonucleotide 50 to 79; (lanes 3 and 4) oligonucleotide 49 to 78; (lanes 5 and 6) oligonucleotide 48 to 77; (lanes 7 and 8) oligonucleotide 46 to 74; (lanes 9 and 10) oligonucleotide 44 to 72; (lanes 11 and 12) oligonucleotide 41 to 69; (lanes 13 and 14) oligonucleotide 38 to 66; (lanes 15 and 16) oligonucleotide 35 to 63; (lanes 17 and 18) oligonucleotide 31 to 59; (lane 19) $(TTTTGGGG)_4$. (**B**) Order of addition experiment. Telomerase extracts were first incubated for 5 minutes at room temperature with various oligonucleotides. A second oligonucleotide was added with the reaction mixture (as described above), and the reactions were incubated at 30°C for an additional 60 minutes. The samples were extracted with phenol, precipitated with ethanol, and analyzed on a 6 percent polyacrylamide sequencing gel. For each lane description, the initial incubation and second oligonucleotides are indicated. (Lane 1) Extract first incubated with oligonucleotide 50 to 79; second oligonucleotide $(TTTTGGGG)_4$. (Lane 2) $(TTTTGGGG)_4$; 50 to 79. (Lane 3) 41 to 69; $(TTTTGGGG)_4$. (Lane 4) $(TTTTGGGG)_4$; 41 to 69. (Lane 5) 37 to 65; $(TTTTGGGG)_4$. (Lane 6) $(TTTTGGGG)_4$; 37 to 65. (Lane 7) 31 to 51; $(TTTTGGGG)_4$. (Lane 8) $(TTTTGGGG)_4$; 31 to 51. (Lane 9) Prior incubation and assay with $(TTTTGGGG)_4$. (Lane 10) Incubation and assay without an oligonucleotide.

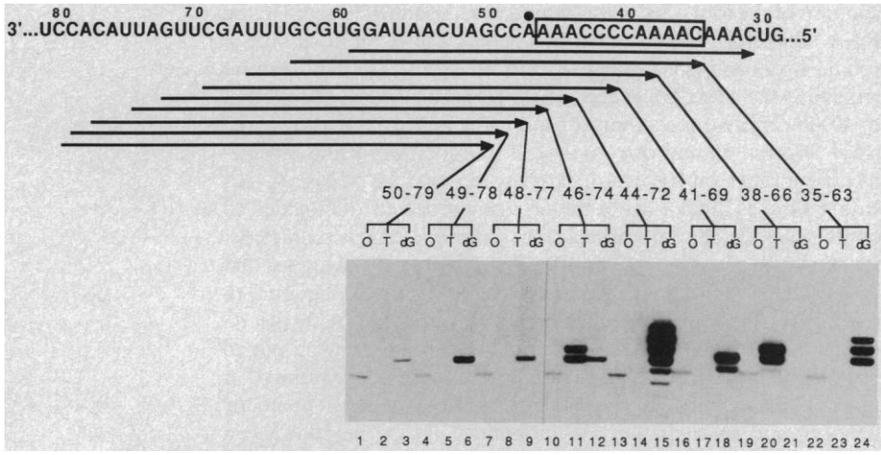


Fig. 3. Identification of the first nucleotides added by telomerase. Telomerase reactions were carried out with various complementary oligonucleotides in the presence of either dGTP or TTP. For dGTP reactions, extracts were incubated in reaction mixture containing 12.5 μCi of [α-^{32}P]dGTP (400 Ci/mmole) at a final dGTP concentration of 1.25 μ*M*. For TTP reactions, extracts were incubated with 25 μCi of [α-^{32}P]TTP (400 Ci/mM) plus 7.5 μ*M* unlabeled TTP (final TTP concentration 10 μ*M*). The products were resolved on a 12 percent sequencing gel. For each group of three lanes: O, [γ-^{32}P]dATP labeled oligonucleotide; T, TTP reaction; dG, dGTP reaction. (Lanes 1 to 3) Oligonucleotide 50 to 79; (lanes 4 to 6) 49 to 78; (lanes 7 to 9) 48 to 77; (lanes 10 to 12) 46 to 74; (lanes 13 to 15) 44 to 72; (lanes 16 to 18) 41 to 69; (lanes 19 to 21) 38 to 66; (lanes 22 to 24) 35 to 63.

23 *vs.* 29 nucleotide pair, and a 15 *vs.* 29 nucleotide pair), the shorter oligonucleotide of each pair produced a pausing pattern of reduced intensity compared to the longer oligonucleotide (*31*), consistent with the expected lower stability of the shorter DNA-RNA duplex. The specificity of these complementary oligonucleotides provides compelling evidence that the 191-nt RNA is a component of the *Euplotes* telomerase. The results also suggested that it is the telomeric sequence within the RNA that is involved in telomerase activity.

Identification of the boundaries of the functional template strand domain in the *Euplotes* telomerase RNA. We define the template strand domain to include nucleotides that can form base pairs with the 3′ terminus of a primer, such that it can be extended by copying the nucleotides of the template to synthesize TTTTGGGG repeats. The boundaries of this domain were demarcated by analyzing the reaction products generated by the complementary oligonucleotides. Oligonucleotides 50 to 79, 49 to 78, and 48 to 77, whose 3′ termini, respectively, correspond to the rG residue at nucleotide 50 and the two rC residues at positions 49 and 48, did not prime TTTTGGGG repeat addition (Fig. 2A, lanes 2, 4, and 6). In contrast, the oligonucleotides complementary to nucleotides 47 to 75 and 46 to 74 (Fig. 2A, lane 8) were efficient primers. We infer from these data that the rA residue at position 47 (marked with a dot above the nucleotide in Fig. 2A) can position the 3′ end of a complementary primer for TTTTGGGG repeat synthesis and therefore marks the 3′ boundary of the *Euplotes* telomeric template strand domain. However, the first nucleotide that can be copied into DNA, and which therefore marks the 3′ end of the template itself, at least for elongation of a complementary primer, is the rA residue at position 46. By this definition the two rC residues at positions 49 and 48 are not part of the strand template domain.

The rC at position 35 is the outermost nucleotide possible in the RNA sequence for the 5′ boundary of the template, because copying the next nucleotides would not produce a repeated TTTTGGGG sequence. Accordingly, oligonucleotide 31 to 59, whose 3′ terminus corresponds to the rC at nucleotide 31, did not serve as a primer (Fig. 2A, lane 18). The observation that this oligonucleotide inhibited elongation of a $(TTTTGGGG)_4$ primer (Fig. 2B, lane 7) is also consistent with the idea that this complementary oligonucleotide blocks the entire template strand domain. In contrast, oligonucleotide 35 to 63 (whose 3′ terminus corresponds to the rC at position 35) was active as a primer (Fig. 2A, lane 16). This disparate utilization of oligonucleotides 31 to 59 and 35 to 63, which have the same 3′ terminal sequence ...TTTG, suggested that the rC at position 35 is part of the functional template.

Further evidence that the 5′ boundary of the template extends up to position 35 came from detailed analysis of the short elongation products. We first assayed all of the complementary oligonucleotides in the presence of either [α-^{32}P]dGTP or [α-^{32}P]TTP, in order to identify the first nucleotides added by telomerase (Fig. 3). Oligonucleotides 46 to 74 and 38 to 66, which both contain two T residues at the 3′ terminus, were elongated by the two additional T residues expected from the template sequence (Fig. 3, lanes 11 and 20). Similarly, oligonucleotide 44 to 72 was elongated by the expected four additional dG residues (Fig. 3, lane 15). Note, however, oligonucleotide 35 to 63 was extended by the addition of three dG residues (Fig. 3, lane 24). This finding is only readily explainable by the 3′ terminus of the 35 to 63 oligonucleotide translocating and hybridizing to another portion of the telomeric template sequence and then being extended by telomerase (see below). This conclusion is supported by the pausing patterns in the short products primed by the complementary oligonucleotides (Fig. 4). A characteristic band intensity corresponds to addition at each position on the template. When we use the data from Fig. 3 to make the assignments of the added nucleotides, we see that the most predominant pauses in the profiles occur after the incorporation of dG residues (Fig. 4). In the reactions primed by oligonucleotides 46 to 74 and 44 to 72, addition of the dG's corresponding to positions 43 to 40 results in a pattern of four bands with gradually increasing intensities proceeding from position 43 to 40 (Fig. 4, lanes 8 and 10). However, after

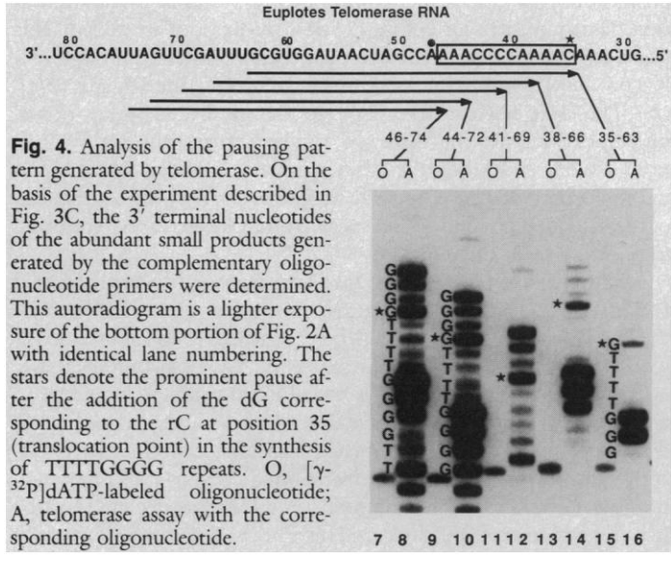


Fig. 4. Analysis of the pausing pattern generated by telomerase. On the basis of the experiment described in Fig. 3C, the 3′ terminal nucleotides of the abundant small products generated by the complementary oligonucleotide primers were determined. This autoradiogram is a lighter exposure of the bottom portion of Fig. 2A with identical lane numbering. The stars denote the prominent pause after the addition of the dG corresponding to the rC at position 35 (translocation point) in the synthesis of TTTTGGGG repeats. O, [γ-^{32}P]dATP-labeled oligonucleotide; A, telomerase assay with the corresponding oligonucleotide.

addition of the four T's corresponding to rA residues 39 to 36 in the RNA template, a dG is added by copying the rC at position 35, producing a band (stars in Fig. 4, lanes 8, 10, and 12) that is strong relative to the next dG's added, which correspond to rC residues 42 to 40. Therefore this prominent band is characteristic of copying the rC at position 35 and correlates with translocation of the complementary primer on the RNA template. In lane 14 of Fig. 4, this strong band is missing from the first group of four dG's added to oligonucleotide 38 to 66; instead, the pausing profile is characteristic of copying residues 43 to 40. This observation suggests that before the first nucleotides are added, the 3′ region of oligonucleotide 38 to 66 folds back, aligning its 3′ nucleotide with the rC residue at position 43. After the primer has been extended out to position 35, a strong pause is seen. Thus, we conclude that the rC at position 35 demarcates the 5′ end of the functional telomeric template and specifies the last nucleotide, dG, to be added in a round of templates copying.

We noted (Fig. 3) that several of the complementary oligonucleotide primers were elongated by a single dG, as well as by the next appropriate nucleotide in the RNA sequence (Fig. 3, lanes 3, 6, 9, and 12). Additional weak bands corresponding to the incorporation of further dG's as well as T's were visible on longer autoradiographic exposures. Interestingly, the nonpriming complementary oligonucleotides with 3′ termini corresponding to the region just 3′ of the functional template were also extended by a single dG residue, in the presence of [α-^{32}P]dGTP plus TTP or [α-^{32}P]dGTP alone (Fig. 2A, lanes 2, 4, and 6; Fig. 3, lanes 3, 6, and 9). In contrast, oligonucleotides complementary to other portions of the *Euplotes* telomerase RNA as well as standard telomeric oligonucleotide primers were not elongated by a similar prominent dG residue. These data suggest that slippage in the template copying step, which may involve inaccurate positioning of the primer on the template, can occur under certain conditions in vitro.

The mechanism of complementary primer elongation by *Euplotes* telomerase. Taken together, our data suggest the following mechanism for elongation of the complementary oligonucleotide primers (Fig. 5). (i) The 3′ end of the primer hybridizes to the telomerase RNA template and is anchored by pairing with the RNA region 3′ to the template. (ii) RNA-templated synthesis extends the hybridized primer out to the rC residue at position 35, the end of the template. (iii) The newly polymerized 3′ terminus of the elongated oligonucleotide translocates, becoming aligned so that its 3′ terminal dG now hybridizes to the rC residue at position 43 in the template. The pause after the addition of the dG residue at position 35, the end of the template, corresponds to this translocation, which must occur before the next nucleotides are added. This pause is seen with all of the complementary primers, although it is less prominent with telomeric primers [Fig. 2A, lane 19, and (*21*)]. (iv) The next nucleotides added to the elongated, repositioned oligonucleotide are the three dG residues corresponding to positions 42, 41, and 40 on the RNA template, followed by extension out to position 35, the end of the template. (v) The new 3′ terminus of the extended oligonucleotide is again translocated and hybridized to position 43 in the template.

Each translocation step must involve unpairing the newly formed DNA-RNA helix. We propose that this unpairing is facilitated by the known ability of telomeric G-rich sequences to form intramolecular non–Watson-Crick G·G base pairs (*17, 32*). Engaging the newly unpaired region in such a G·G pair–stabilized structure would be an energetically favorable alternative to leaving the DNA unpaired as it dissociated from the RNA template. With telomeric G-rich primers, and with the complementary primers after a few rounds of elongation-translocation, such a structure could form, as depicted for the last elongation-translocation cycle in Fig. 5. Repeated slippage of

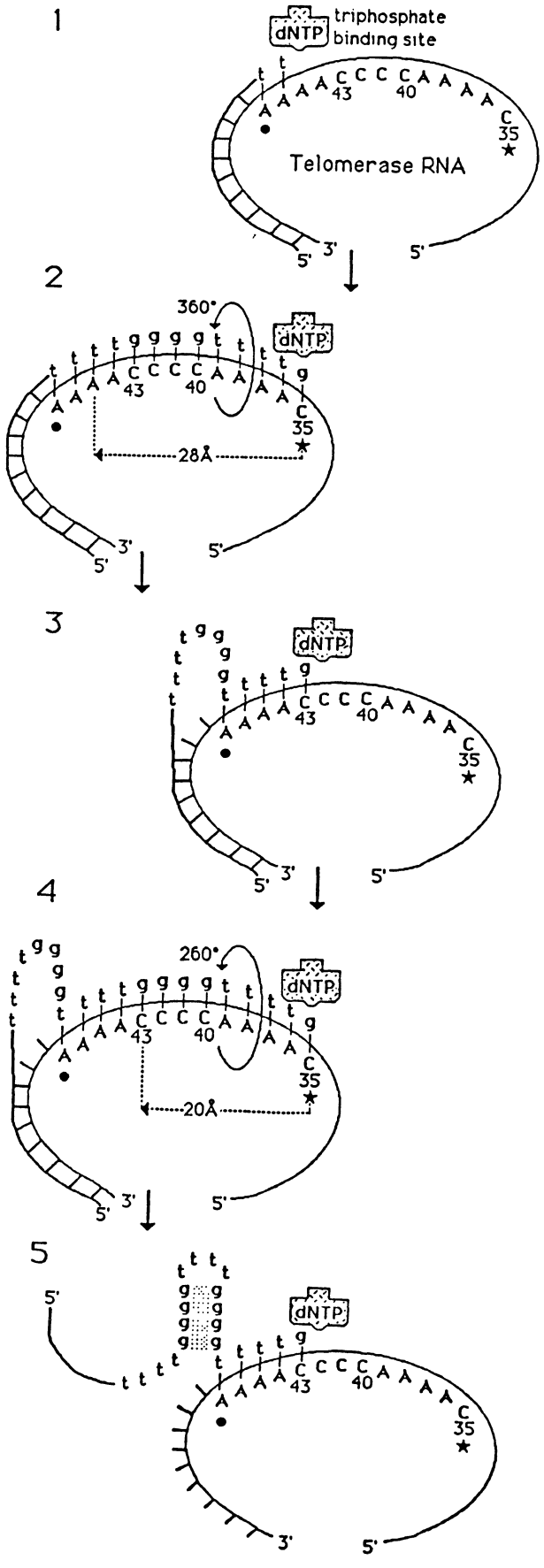


Fig. 5. Proposed mechanism for telomere synthesis by telomerase from *Euplotes*. (Step 1) Bind complementary primer; (step 2) polymerize; (step 3) translocate and rehybridize extended primer; (step 4) polymerize; and (step 5) translocate and rehybridize extended primer.

the G·G paired structures could occur with each subsequent translocation-elongation cycle, as the most recently synthesized 3′ portion of the primer formed G·G pair-stabilized structures with the previously synthesized (or pre-existing) 5′ neighboring region of the primer. The resulting inchworm mechanism could account for the high processivity of the in vitro reaction that results in large numbers of telomeric repeats being added to telomeric primers (*18, 19, 21, 32*). Moreover, the occurrence of the strong stops or pauses after the addition of the first one or two TTTTGGGG repeats supports the above inchworm model, because at the first translocation and rehybridization step, the newly synthesized 3′ terminus will not have sufficient telomeric sequences to assume structures that favor the unpairing of the oligonucleotide from the RNA template (Fig. 5, step 3). Finally, extension of this model to the *Tetrahymena* telomerase may explain why $(TTGGGG)_2$ is active as a primer whereas the hexanucleotide TTGGGG sequence is not (*19*).

Steric implications of complementary primer elongation. Implicit in the synthesis of telomeric repeats by telomerase is the requirement that the RNA template-primer duplex and the active site (polymerization site) translocate relative to each other. In the first elongation cycle from a complementary primer such as oligonucleotide 46–74 (Fig. 4, lane 8) up to 11 base pairs of RNA-DNA helix are formed. If, as discussed above, this primer remains paired to the telomerase RNA (Fig. 5, step 2), the RNA template-primer duplex and the polymerization site of the *Euplotes* telomerase RNP must be capable of translocating relative to each other over a distance of approximately 28 Å with a rotation of 360° (one complete turn of an A helix).

The entire template (positions 46 through 35) may not normally be used in vivo; however, subsequent translocations following synthesis of each eight-nucleotide repeat (Fig. 5, steps 4 and 5), which may more accurately reflect in vivo telomere addition onto natural chromosome termini, would also require substantial relative movement. Steric considerations, and the precedents of *E. coli* DNA polymerase I (*33*) and possibly RNA polymerase (*34*), argue that it is the RNA template-primer duplex of telomerase that threads and rotates through a relatively fixed polymerization site. Movement of an RNA template with respect to the active site of an RNA enzyme has been established by Been and Cech (*35*), who showed that the internal guide sequence of the *Tetrahymena* group 1 intron can translocate over a distance of 10 Å with respect to other portions of the catalytic core of this ribozyme. Thus an inherent structural flexibility may be a necessary feature of polymerases with internal RNA templates.

An important consideration in interpreting the experiments with complementary oligonucleotide primers is the possibility that the extended RNA-DNA duplex formed with the telomerase RNA region 3′ to the template sequence distorts the RNP structure, as this is an unnatural interaction which may not occur in vivo. To address this question, in an additional experiment we compared the pausing patterns produced by priming with a long complementary oligonucleotide 41 to 69 compared to a short oligonucleotide 41 to 55 having the same 3′ terminus. Use of the short primer did not change the relative intensities of the short product bands, but reduced their overall intensity significantly compared with the longer primer. Adding a second oligonucleotide 56 to 70 to the reaction containing the short primer, to create the same region of potential RNA-DNA helix as with the longer oligonucleotide, did not change the banding pattern or increase the band intensities (*31*). Hence the strength of the pauses seen with the complementary oligonucleotides appears to correlate with the inability of the primer to dissociate from the RNA, rather than with the length of RNA engaged in a rigid RNA-DNA helix. Nevertheless, such helix formation may affect the relative intensities of the pauses characteristic of each position in the template. Inspection of the banding patterns generated by the complementary oligonucleotides revealed that after primers were elongated by one-and-a-half to two TTTTGGGG repeats (Fig. 2A), the pattern switched to the profile characteristic of telomeric primers, in which the strongest pause occurs after the addition of the fourth dG. This prominent pause, which corresponds to copying residue 40 in the template, has been noted in our initial characterization of the *Euplotes* telomerase (*21*); however, its origin remains unclear. We postulate that the switch occurs as the extended 3′ terminus begins to more closely resemble the normal telomeric substrate, and perhaps coordinately with the release of the complementary primer from the RNA-DNA duplex (Fig. 5, step 5).

Telomerase: a reverse transcriptase with an internal RNA template. We have shown here that a 13-nt telomeric sequence in the *Euplotes* telomerase RNA serves as a template strand domain for the addition of the *Euplotes*-specific telomeric repeats, and that 12 of these nucleotides can be copied into telomeric DNA by telomerase. Whether all 12 nucleotides are used in vivo as the template with telomeric primers is not known. On the basis of the requirement for the analogous sequence in the RNA of the telomerase from *Tetrahymena*, Greider and Blackburn proposed a mechanism for telomerase activity (*23*). Our data provide the first phylogenetic as well as functional evidence for this mechanism. These findings predict that by mutating the template of a telomerase RNA gene, and reintroducing that gene, a cell with new telomeres containing the corresponding altered sequence will be produced. This prediction has been confirmed with the use of two different mutated *Tetrahymena* telomerase RNA genes in *Tetrahymena* transformants (*36*).

The origin of the degenerate or irregular telomeric sequences found in several organisms (*12*, and references therein) presents an intriguing problem. The mechanism of translocation and repositioning on the RNA template usually appears to be precise for telomerases from species such as *Tetrahymena, Euplotes*, and humans, organisms that have tandem identically repeated telomeric sequences (*18, 19, 21–23*). For the *Euplotes* telomerase, the precision of this event may be aided by the last nucleotide incorporated before translocation being a single dG after a run of T's, which could promote correct repositioning of the primer on the template. Nevertheless, we did find evidence that, under some conditions in vitro, slippage in the template copying step can occur. Perhaps degenerate telomeric repeat sequences are produced by an analogous slippage in vivo.

In its ability to polymerize DNA nucleotides onto a DNA primer with an RNA template, telomerase can now be defined as a specialized reverse transcriptase. It differs from the usual reverse transcriptase in that the RNA template is an integral part of the enzyme, and the enzyme translocates efficiently when it reaches the end of the template after each round of template copying. Whether the function of the RNA moiety of telomerase extends beyond simply providing the template for telomere synthesis remains to be determined.

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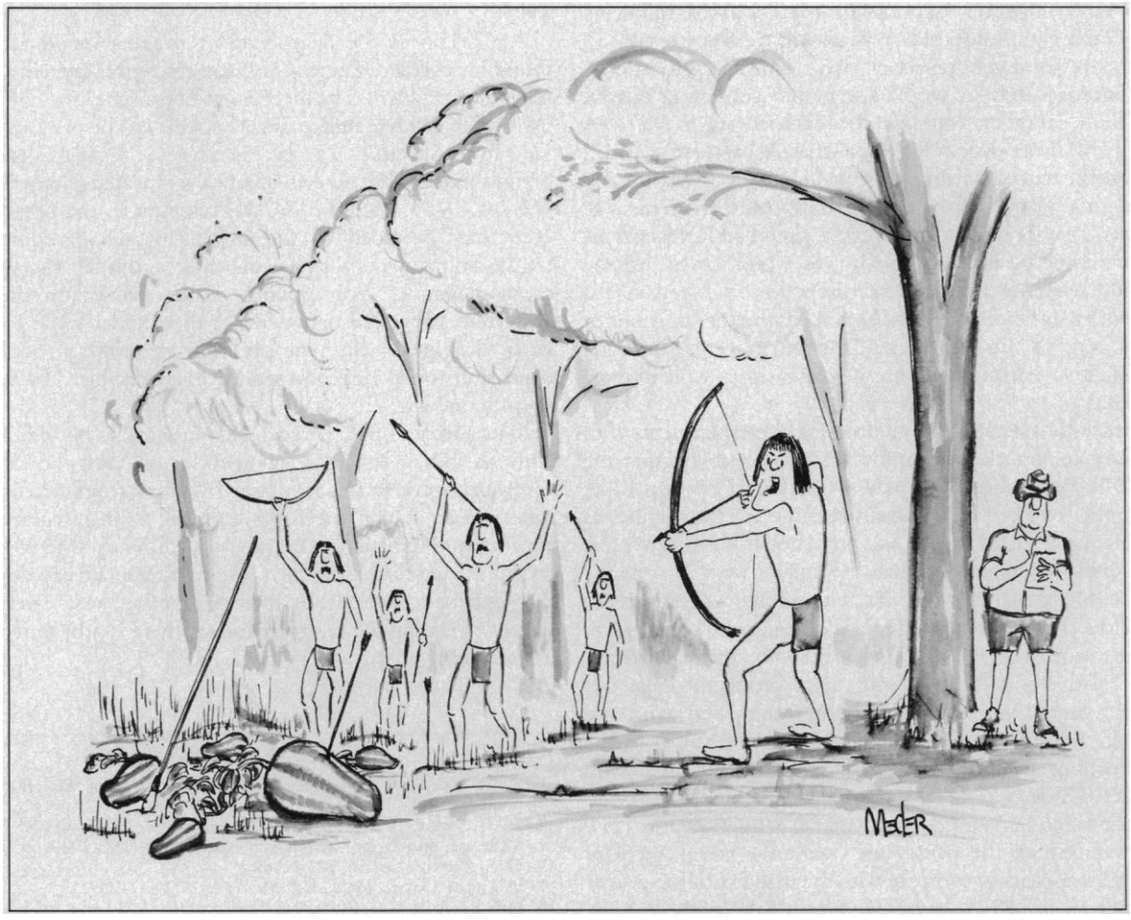

" Poulson found that the Naciremas were simple hunter/gatherers, although they sometimes confused the two concepts."