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The Anchor Site of Telomerase from *Euplotes aediculatus* Revealed by Photo-Cross-Linking to Single- and Double-Stranded DNA Primers

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Telomerase is a ribonucleoprotein enzyme that adds telomeric sequence repeats to the ends of linear chromosomes. In vitro, telomerase has been observed to add repeats to a DNA oligonucleotide primer in a processive manner, leading to the postulation of a DNA anchor site separate from the catalytic site of the enzyme. We have substituted photoreactive 5-iododeoxypyrimidines into the DNA oligonucleotide primer d($T_4G_4T_4G_4T_4G_2$) and, upon irradiation, obtained cross-links with the anchor site of telomerase from *Euplotes aediculatus* nuclear extract. No cross-linking occurred with a primer having the same 5′ end and a nontelomeric 3′ end. These cross-links were shown to be between the DNA primer and (i) a protein moiety of approximately 130 kDa and (ii) U51-U52 of the telomerase RNA. The cross-linked primer could be extended by telomerase in the presence of [α-^{32}P]dGTP, thus indicating that the 3′ end was bound in the enzyme active site. The locations of the cross-links within the single-stranded primers were 20 to 22 nucleotides upstream of the 3′ end, providing a measure of the length of DNA required to span the telomerase active and anchor sites. When the single-stranded primers are aligned with the G-rich strand of a *Euplotes* telomere, the cross-linked nucleotides correspond to the duplex region. Consistent with this finding, a cross-link to telomerase was obtained by substitution of 5-iododeoxycytidine into the CA strand of the duplex region of telomere analogs. We conclude that the anchor site in the ~130-kDa protein can bind duplex as well as single-stranded DNA, which may be critical for its function at chromosome ends. Quantitation of the processivity with single-stranded DNA primers and double-stranded primers with 3′ tails showed that only 60% of the primer remains bound after each repeat addition.

Telomeres are the terminal DNA-protein structures of eukaryotic chromosomes (for reviews see references 4 and 42). The DNA portion consists of simple sequence repeats, which in many organisms are rich in G and T nucleotides on the strand running 5′ to 3′ toward the end of the chromosome (3). In some ciliates, the GT strand has been shown to extend beyond the complementary CA strand, creating a single-stranded 3′ end (21). Telomere proteins that recognize and bind to this DNA structure have been isolated and characterized from the hypotrichous ciliates *Oxytricha nova* (9, 10, 18, 32) and *Euplotes crassus* (31, 33). The resulting DNA-protein telomere complex provides a protective cap on the end of the chromosome that is thought to prevent degradation and recombination of the chromosome ends.

Telomeric DNA sequence repeats are synthesized de novo at the chromosome ends by a unique enzyme called telomerase (12). Telomerase is a ribonucleoprotein (RNP) containing a single RNA strand (13). The sequence of this RNA has been determined for several ciliate species, and a comparison of the sequences has revealed a conserved RNA secondary structure (26, 28, 34). These RNAs all contain a CA-rich region, complementary to the GT-rich telomeric sequence, that functions as a template for synthesis of telomere DNA (14). An additional CA-rich sequence downstream of the template serves to properly align the 3′ end of a DNA primer for telomere repeat addition (36). In addition to the RNA moiety, telomerase protein components of 80 and 95 kDa for *Tetrahymena thermophila* (6) and proteins of ~43 and ~120 kDa for *Euplotes aediculatus* (24) have been reported. The RNA component of telomerase has also been identified in several fungi and mammals (5, 8, 29, 37), with protein subunits yet to be purified.

The telomeric DNA repeats also function in the faithful replication of the linear eukaryotic chromosome. DNA replication is initiated with RNA primers which are extended along a DNA template in the 5′-to-3′ direction and subsequently removed from the newly synthesized strand. Recently, a model for replication that takes into account the 3′ overhang on the GT-rich strand has been proposed. Lingner et al. (25) argue that during replication, the 3′ overhang would be lost on the leading strand and must somehow be reestablished; this is accomplished by the action of telomerase.

In in vitro assays, telomerase from *T. thermophila* has been shown to add repeats to a telomeric primer in a processive manner (11, 14). To function processively, the primer must translocate, without dissociation from the enzyme, and realign on the template for each additional repeat to be added. Other studies of primer sequence specificity have shown that a G-rich sequence is required for binding to telomerase but that this sequence can be at the 5′ end of a primer with a nontelomeric 3′ end (17, 30). These observations have led to the proposal of a two-site binding model for telomerase (11, 30) in which the 3′ end of the primer is bound on the template region of the RNA while the 5′ end is bound in a separate anchor site that has specificity for telomeric sequences. With the 5′ end of the primer held in the anchor site, the 3′ end can translocate on the template without complete dissociation of the primer from telomerase.

Primer binding and alignment on the telomerase RNA tem-

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plate have been extensively studied. The template region and surrounding nucleotides of telomerase RNA have been varied in vivo by expression of altered telomerase RNA sequences (37, 41) and in vitro by reconstitution of telomerase with transcribed RNA (2). Mutations in the templating nucleotides were shown to produce the predicted altered telomere sequence. Corresponding mutations in the alignment region, immediately downstream of the template, have demonstrated the role of these bases in properly positioning the 3′ end of the telomere. In vitro studies have confirmed the primer-template alignment by examination of extension products from primers with varied 3′ termini (1, 26, 36).

Much less is known about primer binding to the telomerase anchor site. Primer extension assays have indicated that the anchor site has some specificity for primers that contain G-rich sequences (17, 30). Evidence from processive primer extension in *Tetrahymena* is consistent with primers having to be 20 to 30 nucleotides long to bind in both the active and anchor sites (23).

In this study we have used primers containing substitutions of the photoreactive nucleotide analogs 5-iododeoxyuridine (^{I}U) and 5-iododeoxycytidine (^{I}C) to investigate the anchor site by UV-induced cross-linking. The 5-iododeoxypyrimidine nucleotides have cross-linking properties advantageous to this study: irradiation can be performed at 312 nm, thereby minimizing photodamage to proteins and nucleic acids, and the photoreactive moiety is an intimate part of the nucleotide (rather than one attached by a tether), such that cross-linking occurs at bond-length distances between the nucleotide and the target (19, 40).

Previous cross-linking studies with telomerase focused on the active site (16) and the region of a primer thought to align on the RNA template (6). Here we report that when the 3′ end of a primer is bound in the active site of the *Euplotes* enzyme, cross-links to telomerase are formed most efficiently with ^{I}U substitutions 20 to 22 nucleotides away. This distance between the active site and cross-links is consistent with cross-linking to the anchor site of telomerase. Cross-links to two subunits of telomerase, a protein with an apparent molecular weight of ~130 kDa and telomerase RNA nucleotides U51-U52, are observed. Furthermore, we use this cross-linking system to test the binding specificity of the anchor site by varying the location of photoreactive nucleotide analogs within single-stranded primers and partially duplex telomere analogs.

MATERIALS AND METHODS

Growth of *E. aediculatus* and preparation of nuclear extract. *E. aediculatus* was grown as described previously (38) under nonsterile conditions with *Chlorogonium* spp. as the food source. Cultures were grown in continuously aerated 5-gallon reactors. Nuclei were isolated from a 40- to 70-g cell pellet, and an extract (1 ml per 10 g of cells) was prepared by Dounce homogenization as previously described (26).

Nucleic acid sequences. The oligodeoxynucleotides used, with sequences written 5′ to 3′, were as follows: TELO22, TTTTGGGGTTTTGGGGTTTTGG; ssRNDM, ATTGAATGACTACGAGATGAA; and hpRNDM, GGGTTCCCT AGTTAGCCAGAGAGCTCCCAGGCTCAGATCTGGTCTAACCAGAGAG ACCCTATAGTGAGTCGTATTAATTTC. Other oligodeoxynucleotides not listed here are described in detail in the text.

Synthesis of 5-iododeoxypyrimidine-substituted oligodeoxynucleotides. ^{I}U and ^{I}C phosphoramidites (Glen Research) were used in standard deoxynucleotide synthesis. After synthesis, the DNA was deprotected with ethanolic ammonium hydroxide for approximately 24 h at room temperature. The DNA was dried down, redissolved, and then purified by denaturing gel electrophoresis in a 20% polyacrylamide gel (1× Tris-borate-EDTA [1× TBE], 7.3 M urea). The DNA was visualized by UV backshadowing, with care taken to minimize exposure time. Bands containing the DNA were excised from the gel, and the DNA was eluted in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The DNA was recovered by ethanol precipitation (35), redissolved in TE, and quantitated by UV absorption at 260 nm.

T7 RNA transcript. A T7 RNA polymerase transcript of *E. aediculatus* telomerase RNA (26) was prepared from a plasmid template provided by L. Hendrick and J. Lingner (24). The RNA 3′ terminus (. . .UCAUCUUUU-3′) comes from run-off transcription from a DNA template cleaved by the restriction enzyme *Ear*I. The 5′ terminus (5′-AGUUUCUC. . .) was produced by self-cleavage of a precursor RNA transcript containing a hammerhead ribozyme (15). The transcription reaction was incubated overnight at 37°C, and then the $MgCl_2$ concentration was adjusted to 20 mM and the reaction was incubated at 55°C for 1 h to facilitate cleavage by the ribozyme. The processed RNA product was gel purified as described above.

Oligonucleotide labeling. DNA primers and the T7 RNA polymerase transcript were radiolabeled with T4 polynucleotide kinase. Each reaction contained 20 pmol of 5′ ends and 165 nM [γ-^{32}P]ATP (6,000 Ci/mmol; NEN). Different protocols were used for single-stranded primers (and T7 polymerase RNA transcript) and hairpin primers and were performed according to the methods described previously (35). Labeling reactions were diluted to 50 μl with TE buffer and heat denatured 3 min at 85°C. Unincorporated nucleotides were removed with a Beckman G-25 TE spin column (DNA primers) or Beckman G-50 spin column (RNA transcript).

SDS-PAGE gel system. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Novex 4 to 20% polyacrylamide gradient gels (1 mm thick; 15 well) were run in 1× Laemmli gel buffer (22) (90 V for gel shift of DNA primer by telomerase RNA; 120 to 150 V for all others). Prior to electrophoresis, samples were adjusted to 1× Laemmli gel loading buffer with a 3× stock solution (190 mM Tris-HCl [pH 6.8], 30% glycerol, 6% SDS, 0.08% bromophenol blue). Kaleidoscope prestained protein standards were obtained from Bio-Rad. Molecular masses are indicated on individual gels for which these protein standards were used.

Partial purification of telomerase. Crude nuclear extracts were partially purified by glycerol gradient fractionation as described previously (24). Glycerol gradients were poured from the bottom; 2.5 ml each of top (15% glycerol) and bottom (40% glycerol) buffers containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 300 mM sodium glutamate, 1 mM EDTA, and 1 mM dithiothreitol were used. Approximately 200 to 250 μl of nuclear extract was layered onto the gradient and centrifuged for 14 to 16 h at 287,000 × *g* (average) in a Beckman SW55 rotor. Fractions (200 μl) were removed manually from the top of the gradient and assayed by the standard cross-linking protocol with the following changes: (i) no specific competitor DNA primer was added prior to denaturation and (ii) after denaturation, the reactions were incubated 10 min at 45°C to allow annealing of DNA primer with telomerase RNA. Reaction products were separated by SDS-PAGE at 90 V. Peak fractions containing telomerase were pooled and dialyzed against telomerase reaction buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM sodium glutamate, 1 mM dithiothreitol, 10% glycerol). Aliquots were frozen with liquid N_2 and stored at −70°C. The concentration of telomerase was determined by comparison to standards of telomerase RNA transcript in a Northern analysis (35).

Standard protocol for cross-linking assay. Partially purified telomerase (1 to 6 nM) was preincubated with nontelomeric DNA oligonucleotide (7.2 μM ssRNDM) for 10 min at 25°C. [5′-^{32}P]DNA primer was added (final concentration, 16 nM) and incubated 10 min at 25°C. Reactions (10 μl) were spotted on Parafilm stretched over an iced metal block and irradiated for 10 min in a Stratalinker equipped with bulbs with a light wavelength of 312 nm. The reactions were then transferred to tubes containing 5 μl of 3× Laemmli gel loading buffer and specific competitor DNA primer (final concentration, 3 μM) where indicated. Reaction products were heat denatured for 3 min at 85°C and separated by SDS-PAGE.

Proteinase K and RNase T_2 digests of cross-linked complexes. Cross-linking reactions were performed by the standard protocol except that unlabeled competitor DNA primer [0.5 μl of 100 μM $^{I}U_{1/3}$TELO22] was added to iced 10-μl samples prior to irradiation. Enzyme digestions were performed following irradiation: for the RNase T_2 digest, Triton X-100 was added (final concentration, 0.5%) along with 1 U of RNase T_2; for the proteinase K digest, SDS was added (final concentration, 0.5%) along with proteinase K (final concentration, 0.02 mg/ml). The reactions were incubated for 1 h at 45°C. For the double digestion, the RNase T_2 digestion was performed first and was followed by the proteinase K digestion. The products were denatured and separated by SDS-PAGE as described above.

DNase digest and denaturing gel electrophoresis of DNA-RNA cross-linked complex. Telomerase (300 μl), partially purified by glycerol gradient, and the primer [5′-^{32}P]$^{I}U_1$TELO20 were cross-linked as described above. After irradiation, the reaction was diluted with an equal volume of proteinase K digestion buffer (20 mM Tris-HCl [pH 7.9], 10 mM EDTA, 1% SDS) and heat denatured for 3 min at 85°C. Proteinase K was added to 50 μg/ml and incubated 1 h at 45°C. The nucleic acid component was recovered by ethanol precipitation as described previously (35) and resuspended in 100 μl of TE buffer. An aliquot of the recovered nucleic acid fraction (8 μl) was combined with 1 μl of a 10× DNase buffer (400 mM Tris-HCl [pH 7.9], 100 mM NaCl, 60 mM $MgCl_2$, 100 mM $CaCl_2$) and 3 U of RQI DNase (Promega), or water as a control, and then incubated for 20 min at 37°C. After heat denaturation (3 min at 85°C) the reaction mixture was again treated with proteinase K and ethanol precipitated. The nucleic acid pellet was redissolved in 8 μl of TE–5 μl of denaturing gel loading buffer (95% formamide, 30 mM EDTA [pH 8.0], 0.02% xylene cyanol,

0.02% bromophenol blue), and the products were separated by electrophoresis in an 8% polyacrylamide gel (acrylamide-bisacrylamide [19:1], 1× TBE, 7 M urea).

Primer extension analysis of the DNA-RNA cross-linked complex. A large-scale cross-linking reaction was done using 1.2 ml of glycerol gradient-purified telomerase and 1.6 μM $^{I}U_{1}$TELO20. The reaction was incubated for 10 min at 25°C and then irradiated 15 min at 312 nm. Following cross-linking, the cross-linked complex was treated with proteinase K as described above and the nucleic acid component was recovered by ethanol precipitation. The reaction products were separated by electrophoresis in a denaturing 8% polyacrylamide gel. A cross-linking reaction with [5′-^{32}P]$^{I}U_{1}$TELO20 was performed as described above to produce a radiolabeled marker for the cross-linked complex. Radiolabeled T7 RNA polymerase transcript was used as a marker for un-cross-linked RNA. The cross-linked DNA-RNA complex and un-cross-linked RNA from the large-scale reaction were excised from the gel and eluted into TE. The cross-linked DNA-RNA complex was digested with DNase I as described above, and the RNA portion was recovered by ethanol precipitation. Primer extension reactions were performed on the un-cross-linked RNA and the DNase I-treated cross-linked complex as described previously (43) with a [5′-^{32}P]DNA primer complementary to nucleotides 88 to 113 of telomerase. Dideoxy sequencing reactions were performed on a T7 transcript of the telomerase RNA by using the same primer. The extension products were separated on a denaturing 8% polyacrylamide gel.

Telomerase extension of a cross-linked primer-telomerase complex after irradiation. Peak fractions of glycerol gradient-purified telomerase were pooled and concentrated sixfold in a Centricon-30 device. Partially purified telomerase (15 μl) was mixed with unlabeled primer $^{I}U_{odd}$TELO22 or $^{I}U_{3}$TELO22 (0.75 μl of a 0.4 μM stock solution) and irradiated as described above. The reactions were transferred to tubes containing competitor oligonucleotide where indicated (1 μl of 100 μM stock solution), ddTTP (0.75 μl of 100 μM stock solution), and radionucleotide [α-^{32}P]dGTP or [α-^{32}P]dATP (3.5 μl; 3,000 Ci/mmol). After incubation for 60 min at 30°C, unincorporated nucleotides were removed by ultrafiltration (once each with 2, 1, and 2 ml of TE with 0.1% SDS) in a Centricon-30 device via centrifugation in a Beckman JA-20 rotor at 5,000 × *g*. The retentate was recovered, concentrated, resuspended in 1× Laemmli gel loading buffer (15 μl) and denatured 3 min at 85°C. The reaction products were separated by SDS-PAGE.

Telomerase extension of a cross-linked primer-telomerase complex before irradiation. Partially purified telomerase (15 μl) was incubated with unlabeled DNA primer $^{I}U_{1/3}$TELO22 (1 μl of 100 μM stock solution) for 10 min at 25°C. Nucleotides—dideoxynucleoside triphosphate (ddNTP) as indicated (1 μl of 50 μM stock solution or H_2O) and [α-^{32}P]dGTP (3.5 μl; 3,000 Ci/mmol; NEN)—were then added to the reaction mixture. The reactions were incubated for 4 min at 25°C, and labeling with [α-^{32}P]dGTP was competed by addition of unlabeled dGTP (1 μl of 10 mM stock solution). The tubes were iced and the reactions were irradiated as described in the standard cross-linking protocol. Unincorporated nucleotides were removed by ultrafiltration (twice with 1 ml and once with 0.8 ml of TE with 0.02% SDS) in a Centricon-30 device via centrifugation in a Beckman JA-20 rotor at 5,000 × *g*. The retentate was recovered and concentrated. The pellet was resuspended in 20% glycerol (12 μl) and denatured for 3 min at 85°C, and the reaction products were separated by SDS-PAGE.

Quantitation of cross-links to telomerase. Quantitation of species observed on gels was done with a Molecular Dynamics PhosphorImager and Image Quant software. The amount of telomerase was taken to equal the amount of [5′-^{32}P]$^{I}U_{1/3}$TELO22 oligodeoxynucleotide that was gel shifted due to annealing to the template region of telomerase RNA. In order to verify complete annealing of the oligodeoxynucleotide to the RNA, three concentrations of oligodeoxynucleotide were used in the annealing reaction, 10, 30, and 80 nM. All gave the same signal, indicating that the gel shift was quantitative and that all of the telomerase RNA was saturated with oligodeoxynucleotide (data not shown). Cross-links to telomerase RNA were quantitated in reactions that had unlabeled DNA competitor (5 μM TELO22) added after cross-linking but before denaturation and electrophoresis. Cross-linking efficiency was determined by the following formula: [(cross-link intensity)/(RNA gel shift intensity)] × 100.

Characterization of telomere analog constructs. Two types of telomere analog were prepared: (i) hairpins (HPTEL) and (ii) bimolecular duplexes utilizing three different GT strands (dsTEL). The extent of duplex formation with the dsTEL oligonucleotides was determined by gel shift assay. The CA strand (^{32}P labeled) was diluted in telomerase reaction buffer (see above), and the GT strand was added in 10, 20, or 100% excess. The primers were heat denatured for 3 min at 85°C, annealed for 15 min at 25°C, diluted 1:1 with native gel loading buffer (34 mM Tris-HCl, 66 mM HEPES, 1 mM EDTA, 20% glycerol), and then run on a nondenaturing 8% polyacrylamide gel in 1× THE buffer (34 mM Tris-HCl, 66 mM HEPES, 1 mM EDTA [pH 8]). The gel shift was >99% complete at 10% excess and complete at 100% excess GT strand, indicating that virtually all of the CA strand was in a duplex (data not shown).

The dsTEL constructs contain a *Hae*III restriction endonuclease recognition site at one end. This was included to allow verification of the register in which the two strands were annealing. Duplexes were formed as described above, with NEBuffer 2 (New England Biolabs) used in place of telomerase assay buffer. The reactions were incubated overnight at 37°C with 10 U of *Hae*III, diluted 1:1 with denaturing gel loading buffer, and then run on a denaturing 20% polyacrylamide gel (1× TBE, 7 M urea). Each of the constructs was cut to greater than 90%, consistent with the desired annealing register (data not shown).

The HPTEL constructs are hairpin oligonucleotides and require no annealing step to produce a duplex telomere analog. In order to verify that HPTEL formed the desired hairpin and not a dimer, [5′-^{32}P]^{I}C-HPTEL-1 was diluted in 1× NEBuffer 2, diluted 1:1 with native gel loading buffer, and then run on a nondenaturing polyacrylamide gel. The HPTEL primer was compared with the duplexed dsTEL construct, and a single band with a mobility similar to that of the latter was observed for the former (data not shown).

Cross-linking with dsTEL primers. Primer duplexes were preformed as follows. [5′-^{32}P]^{I}C-dsCA-3 and the indicated GT strand were mixed (75 nM each strand) in telomerase assay buffer, heat denatured for 3 min at 85°C, and annealed for 10 min at 25°C. After duplex formation, a threefold excess of unlabeled GT or CA strand was added as indicated. The preformed duplex (1 μl) was incubated with partially purified telomerase for 10 min at 25°C and then cross-linked as described above.

RESULTS

Identification of cross-links to telomerase. The nuclear extract from *E. aediculatus* used for most of the following experiments contained a mixture of proteins, any number of which could form cross-links to the DNA primers upon irradiation. To identify cross-links specific to telomerase, we assayed glycerol gradient fractions for the presence of telomerase RNA and for the ability to cross-link to a telomeric primer containing photoreactive nucleotide substitutions. Extract samples were incubated with an unlabeled nontelomeric oligodeoxynucleotide (7 μM ssRNDM) prior to addition of the telomeric primer in order to saturate any nonspecific DNA binding proteins. Initial screening for a cross-link to telomerase was done with a [5′-^{32}P]DNA primer with ^{I}U substituted at 6 of the 12 thymidines: d(^{I}UT^{I}UTGGGG^{I}UT^{I}UTGGGG^{I}UT^{I}UTGG) or $^{I}U_{odd}$TELO22. Subsequent assays were performed with substitutions at a subset of these positions (see below).

After UV irradiation at 312 nm, the reaction products were separated by SDS-PAGE to reveal cross-linked primer-protein complexes (Fig. 1A). The SDS denatures proteins but has little effect on DNA-RNA hybrids, allowing the gel shift of the [5′-^{32}P]DNA primer by telomerase RNA to be monitored on the same gel. This DNA-RNA hybrid gives a band at 60 kDa, identified by comparison to a control reaction with an in vitro transcript of telomerase RNA (first lane). Repeating the gel shift at higher primer concentrations showed that hybridization was quantitative with respect to the telomerase RNA (Materials and Methods). The band at 60 kDa also contains a minor component of primer covalently cross-linked to telomerase RNA (see below). The strongly cross-linked complex with an apparent molecular mass of ~150 kDa cosedimented with the telomerase RNA (Fig. 1B) as it exists in the extract, where it is known to be a ribonucleoprotein (24). Additional evidence that this is a specific cross-link to a protein subunit of telomerase is presented below. The peak fractions containing telomerase and a cross-link were numbers 14 through 18. For subsequent experiments, the peak fractions of such glycerol gradients were pooled, dialyzed against a telomerase reaction buffer, and stored in aliquots (Materials and Methods); this material is referred to hereafter in this work as partially purified telomerase.

The 150-kDa cross-linked complex contains a protein. In a reaction which was not irradiated, one major band, which resulted from a gel shift of [^{32}P]DNA primer by telomerase RNA, was seen at 60 kDa (Fig. 2, lane 1). After irradiation, an additional band was observed at 150 kDa (lane 2). When unlabeled competitor primer [$^{I}U_{1/3}$TELO22] was added prior to addition of the [^{32}P]DNA primer, no 150-kDa band was observed (lane 3). Alternatively, the competitor primer was added to iced samples after the [^{32}P]DNA primer was bound (10-min incubation at 25°C) but prior to irradiation. This elim-

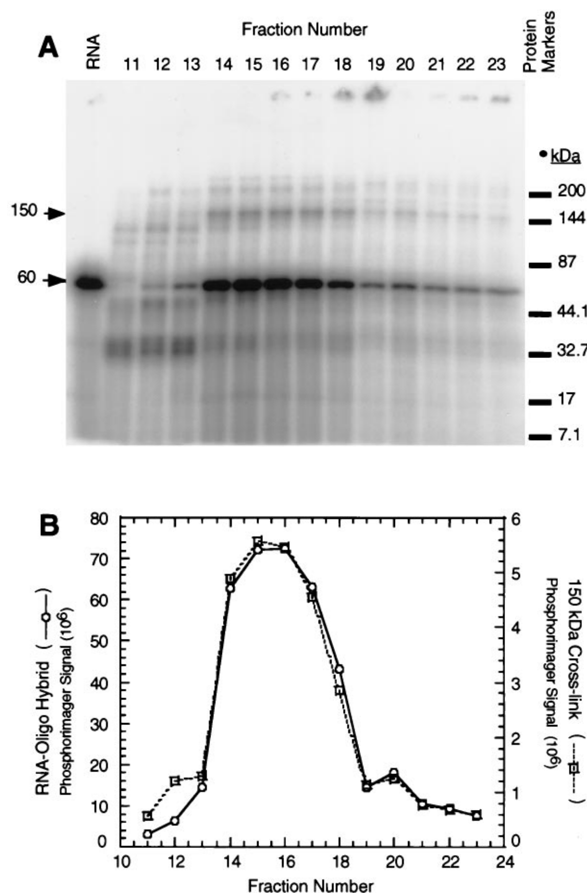


FIG. 1. Glycerol gradient assayed for telomerase-specific cross-links. (A) Nuclear extract from *E. aediculatus* was sedimented in a 15 to 40% glycerol gradient; sedimentation was from left to right. An aliquot of each fraction (fractions 11 to 23 are shown here) was assayed for cross-linking to the primer [5′-^{32}P]$^{I}U_{1/3}$ TELO22 using the standard protocol. The specific telomerase protein-DNA cross-linked complex has an apparent molecular mass of 150 kDa by SDS-PAGE. Under the conditions used for SDS-PAGE, the telomerase RNA hybridizes to the DNA primer and gives a gel-shifted complex at 60 kDa. Lane RNA, a synthetic telomerase RNA (prepared by in vitro transcription, not irradiated) run as a marker. The positions and sizes of prestained protein markers are indicated. Unlabeled cross-links have not been characterized. (B) The intensities of the 60-kDa hybridization band and the 150-kDa cross-linked complex were quantitated and plotted against the fraction number.

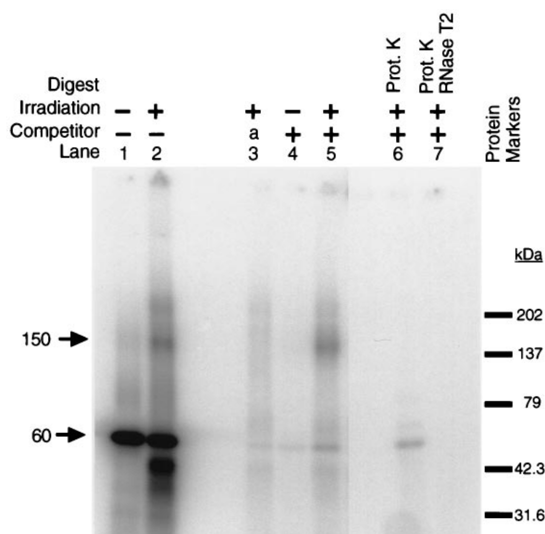


FIG. 2. The 150-kDa cross-linked complex is sensitive to proteinase, and the 60-kDa complex is sensitive to RNase. Peak fractions of telomerase from a glycerol gradient were pooled and aliquots were cross-linked to [5′-^{32}P]$^{I}U_{1/3}$ TELO22 by the standard protocol. The samples were irradiated 0 min (lanes 1 and 4) or 30 min (lanes 2, 3, and 5 to 7). After cross-linking, the reaction mixtures were treated with proteinase K (lane 6) or proteinase K and RNase T_2 (lane 7). An excess of unlabeled competitor primer was added where indicated (+) to iced samples prior to irradiation; this competes out low-stability interactions and primer gel shift by telomerase RNA. In lane 3, competitor primer was added prior to addition of [5′-^{32}P]$^{I}U_{1/3}$TELO22 (a). The samples were then denatured and analyzed by SDS-PAGE.

inated the majority of the [^{32}P]DNA primer gel shift by telomerase RNA (compare lanes 1 and 2 to lanes 4 and 5) and also eliminated low-stability cross-linked species, as observed in the decrease of the 44-kDa band (compare lane 2 to lane 5). The 150-kDa cross-linked complex was not significantly affected by competitor added at this point.

The telomerase enzyme is composed of both RNA and protein subunits, either of which can cross-link to the DNA primer. The 150-kDa cross-linked complex was eliminated by proteinase K treatment (Fig. 2; compare lanes 5 and 6). The 60-kDa complex was not affected by proteinase K but was eliminated by RNase T_2 digestion (lane 7). Thus, the 150-kDa species consists of DNA primer cross-linked to a protein. Additional evidence that this protein is a telomerase component is presented below. The intensity of the 60-kDa band after irradiation (lanes 5 and 6) seemed to increase compared to that of an unirradiated sample (lane 4). This observation (and additional data not shown) raised the possibility that a cross-link was being formed between the DNA primer and telomerase RNA. This possibility was investigated further by denaturing gel electrophoresis, and those results are shown below.

The mobility of the 150-kDa complex reflects both the size of the protein component and retardation due to the attached 22-nucleotide DNA primer. To estimate the protein size in the absence of the primer, the gel mobility was determined for cross-linked complexes containing primers that ranged in size from 21 to 28 nucleotides (data not shown). The mobilities of these complexes were plotted against primer length and extrapolated to a zero-length primer, resulting in a size estimate of 130 kDa for the protein component. The geometry of a complex also has a strong effect on its mobility through a gel matrix (27), so the protein molecular mass estimate is only approximate.

Cross-linking with highly purified telomerase and quantitation of cross-links. Highly purified telomerase, in which the major polypeptides correspond to the two protein subunits (24), was also tested for cross-linking (Fig. 3). The same cross-linked complexes of 150 and 60 kDa were observed with the highly purified telomerase as with partially purified extract, confirming that both the 150- and 60-kDa products contain telomerase subunits. All of the other cross-linked complexes seen with partially purified telomerase were eliminated by purification and are therefore presumably due to primer cross-linked to nontelomerase proteins in the extract.

Assuming a stoichiometry of one telomerase RNA molecule and one bound primer per holoenzyme complex, the efficiency of cross-linking can be estimated by comparison of the intensity of the signal from the cross-link to that resulting from gel-shift of primer by telomerase RNA (Materials and Methods). To determine the maximum extent of cross-linking, an irradiation time course up to 1 h was performed with partially purified extract. In three trials, the cross-link to protein (150 kDa) was 7 to 13% efficient and the cross-link to RNA (60

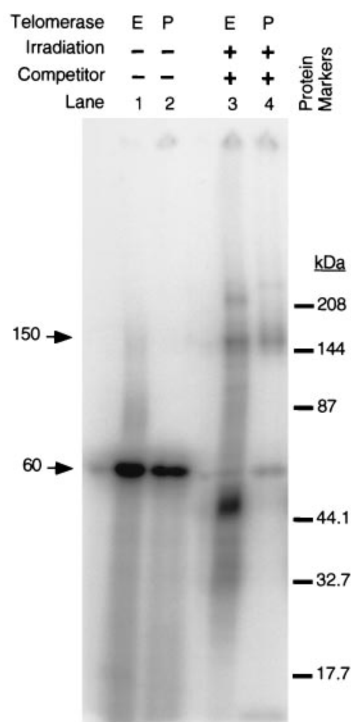


FIG. 3. Highly purified telomerase gives the same cross-linked complexes. The patterns and efficiencies of cross-linking of partially purified telomerase extract (E) and highly purified telomerase (P) were compared by using the primer $^{I}U_{1/3}$TELO22 in the standard protocol (lanes 3 and 4, respectively). The efficiency of cross-linking was determined by comparison to the quantity of telomerase in each extract, as determined by gel shift of the primer by telomerase RNA (lanes 1 and 2). The apparent molecular weights (in thousands) of the cross-linked complexes, indicated by arrows (left), were determined by comparison to protein markers (right).

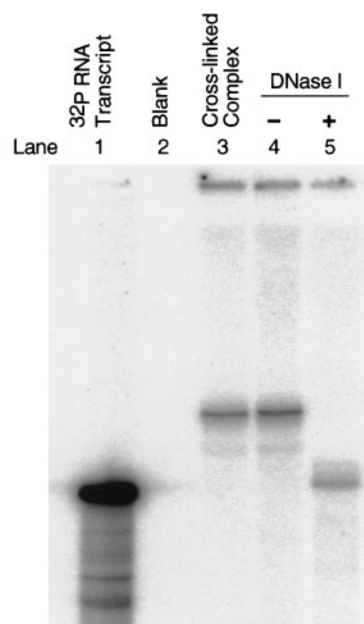


FIG. 4. The DNA primer forms a cross-link to telomerase RNA. A large-scale cross-linking reaction was performed with partially purified telomerase. After cross-linking was performed, the proteins were denatured with SDS and digested with proteinase K. The nucleic acid component was recovered (lane 3), and aliquots were incubated in a DNase I buffer without (lane 4) or with (lane 5) DNase I. Products were analyzed by electrophoresis in an 8% polyacrylamide gel (1× TBE, 7 M urea). A 189-nucleotide T7 RNA polymerase transcript of *E. aediculatus* telomerase RNA was 5′ ^{32}P labeled as a marker (lane 1).

kDa) was 2 to 4% efficient (data not shown). In a single time point assay with 20 min of irradiation, the cross-linking efficiencies for highly purified and partially purified telomerase preparations were the same (Fig. 3).

Cross-link to the RNA moiety of telomerase. Experiments with the DNA-RNA cross-link (DNase I treatment and cross-link mapping) were performed with a 20-nucleotide primer, $d(T_2G_4T_4G_4T_4G_2)$, with a single ^{I}U substitution at its extreme 5′ nucleotide. When this primer is 5′ end labeled with ^{32}P, the radiolabel and photoreactive moiety are on the same nucleotide and are unlikely to be removed when the cross-linked complex is treated with DNase I.

In a nondenaturing gel, a cross-linked complex between a DNA primer and the RNA moiety of telomerase should have the same electrophoretic mobility as the DNA-RNA hybrid. To test whether the labeled 60-kDa band really contained a covalent DNA-RNA cross-link, we utilized an electrophoretic gel system designed to be denaturing to noncovalent nucleic acid hybrids (Fig. 4). The cross-linked complex was observed (lane 3); as expected, its mobility was reduced relative to that of a telomerase RNA marker (lane 1) due to the attached DNA primer. Treatment of this complex with DNase I removed most of the DNA primer, leaving a labeled RNA (lane 5) with a mobility similar to that of the RNA marker (lane 1). Incubation in DNase I buffer without enzyme had no effect (lane 4). The identity of this band as telomerase RNA has been confirmed by RNase H digestion in the presence of oligonucleotides complementary to the RNA (data not shown) and by primer extension (see below). Consistent with the digestions shown in Fig. 2, the cross-linked complexes in Fig. 4 (lanes 3 to 5) were all resistant to treatment with proteinase K-SDS that was part of the postirradiation workup.

We tested whether telomerase proteins were required for formation of the DNA-RNA cross-link by treating the extract with proteinase K prior to irradiation (data not shown). A 1-h incubation of extract at 45°C with SDS and proteinase K gave no apparent diminution of the DNA-RNA cross-link. When the extract was heated to 85°C in SDS prior to proteinase K treatment at 45°C, the cross-link/RNA ratio was reduced severalfold, possibly due to misfolding of the RNA after denaturation. The location within the RNA of the cross-link formed in the presence of protein has been mapped (see below), but the cross-link formed after deproteinization has not. It is therefore not certain if the same or a different cross-link is being observed.

The location of the cross-link to telomerase RNA, formed in the intact telomerase nucleoprotein particle, was determined by primer extension analysis (Fig. 5A). First, the cross-linked complex and the un-cross-linked RNA were separated by denaturing gel electrophoresis and isolated from the gel. After treatment of the cross-linked complex with DNase I to remove the primer, these RNAs were used as templates for extension of a [^{32}P]DNA primer by reverse transcription (lanes 6 and 7). The region of sequence shown spans nucleotides U27 through G69 of telomerase RNA. Some stops are seen in both lane 6 and lane 7, for example, at positions A64 and A68; these are common in primer extension reactions and could correspond to modified nucleotides, damage due to irradiation, or regions with strong secondary structure. A stop unique to the cross-linked complex (lane 6) is seen at position U52, and a lesser stop is seen at position U51, with no primer extension beyond

FIG. 5. Mapping the location of the DNA-RNA cross-link within the telomerase RNA. (A) After cross-linking was performed, the DNA-RNA cross-linked complex (lane 6) and un-cross-linked RNA (lane 7) were separated by denaturing PAGE and isolated by elution from the gel. The cross-linked complex was treated with DNase I, and then primer extension reactions were performed on both RNAs with the [5′-^{32}P]DNA primer shown in panel B. A sequence ladder was prepared by dideoxy sequencing of an in vitro-transcribed telomerase RNA (lanes 1 to 5). The RNA sequence is shown at the left, and the locations of some strong stops found in both cross-linked and un-cross-linked RNA are indicated by arrows at the right. Photo-cross-linking at U51-U52 was determined by lack of primer extension beyond this point. (B) The location of the primer cross-links to telomerase RNA (nucleotides U51 and U52) are shown on a schematic of the secondary structure of *E. aediculatus* RNA (26). The sequencing primer spans nucleotides 88 to 113 as indicated.

these two positions (compare lane 6 to lane 7). Therefore, U51-U52 of the telomerase RNA has been identified as a site of cross-linking to a DNA primer (Fig. 5B). Evidence that this binding represents the anchor site is presented below.

Cross-linked primers reside in the active site and can be extended in *cis* by telomerase. Telomerase adds nucleotides to the 3′ end of a DNA oligonucleotide primer, using the RNA moiety as a template (14). If the cross-linked primers were bound to an active enzyme in a manner competent for extension, then the enzyme's own polymerase activity should be able to label the primer with [α-^{32}P]dGTP. This was tested in two ways which can be summarized as "cross-link then extend" and "extend then cross-link."

In the first approach, an unlabeled primer ($^{I}U_{odd}$TELO22 or $^{I}U_{3}$TELO22) was cross-linked to the enzyme and then the complex was incubated with [α-^{32}P]dGTP. SDS-PAGE revealed a labeled cross-linked complex at the expected position of 150 kDa (Fig. 6A, lanes 2 and 3). In addition, we observed a strong 60-kDa band due to annealing of un-cross-linked, extended primer to the telomerase RNA (lanes 1 to 3 and 8). The cross-linked complex became labeled upon incubation with [α-^{32}P]dGTP but not [α-^{32}P]dATP (lanes 4 and 5), consistent with the nucleotide specificity of telomerase. The addition of excess unlabeled primer after irradiation but prior to labeling did not weaken the signal at 150 kDa (lanes 6 and 7), indicating that the labeling of cross-linked complex occurred in *cis*. The addition of this unlabeled specific competitor primer prior to denaturation for SDS-PAGE eliminates the gel shift of [^{32}P]DNA primer (Fig. 2); therefore, the remaining signal at 60 kDa (lanes 6 and 7) is due to label incorporated into the primer in the DNA-RNA cross-linked complex. The singly substituted $^{I}U_{3}$TELO22 primer primarily forms the 60-kDa cross-linked complex and not the 150-kDa complex. This selectivity is seen in the reduced signal at 150 kDa relative to that at 60 kDa (lane 7). The relationship between ^{I}U position and cross-link efficiency was investigated in detail (see below).

A second approach was also taken to demonstrate that the cross-link is to an active enzyme, with the primer 3′ end in a position where it can be extended. In this approach, an unlabeled primer ($^{I}U_{1/3}$TELO22) (Fig. 6B, lanes 2 to 7) was bound to telomerase and then [α-^{32}P]dGTP was added for a 4-min pulse-labeling. The primer was present at 5 μM such that rebinding of ^{32}P-labeled primer which dissociated from telomerase (ca. 4 nM) would be negligible. The sample was placed in an ice bath after pulse-labeling to slow the dissociation of bound primer, and the labeling by [α-^{32}P]dGTP (0.33 μM) was chased with an excess of unlabeled dGTP (475 μM). The reaction mixtures were then irradiated, and the reaction products were separated by SDS-PAGE. The expected cross-linked complexes were seen at 150 and 60 kDa (Fig. 6B, lane 3). No cross-link was seen in the absence of added primer (lane 1) or when the sample was not irradiated (lane 2).

In order to confirm that labeling was due to telomerase, reactions in which the [α-^{32}P]dGTP pulse-labeling was done in the presence of 2.5 μM of each dideoxynucleoside triphosphate were included (lanes 4 to 7). Incorporation of a dideoxynucleotide would prevent further extension of the primer. Only the presence of ddGTP caused a diminution of the label incorporated into the cross-linked complexes (lane 4). This indicates the specific addition of a G nucleotide as predicted from primer alignment on the telomerase RNA template (Fig. 6C).

These results demonstrate that the 150-kDa cross-link to protein and the 60-kDa cross-link to telomerase RNA are both due to complexes between the primer and active telomerase. The 3′ end of the primer is positioned for polymerase extension both prior and subsequent to cross-link formation. The alignment of the cross-linked primer on the telomerase template was verified by a telomerase activity assay in the presence of dGTP and ddTTP (see last section of Results).

Location within the DNA primer of the cross-links to telomerase protein and RNA. Initial cross-linking experiments were performed with the primer $^{I}U_{odd}$TELO22 (Fig. 7A), containing ^{I}U substituted for every other thymidine in the parent oligodeoxynucleotide primer $d(T_4G_4T_4G_4T_4G_2)$. To determine which of these nucleotides was responsible for the observed cross-links, we prepared six singly substituted primers and tested them individually for their ability to form the 150- and 60-kDa cross-linked complexes (Fig. 7B, lanes 3 to 8; results summarized in Fig. 7A). An excess of unlabeled primer was added to each cross-linking reaction after irradiation but before denaturation (except where indicated) in order to compete away the gel shift of the [^{32}P]DNA primer by telomerase RNA. This approach allowed the cross-link to telomerase RNA to be observed on the same gel as the cross-link to the protein described above. Cross-link efficiency was quantitated as described earlier, by comparison to a control with no com-

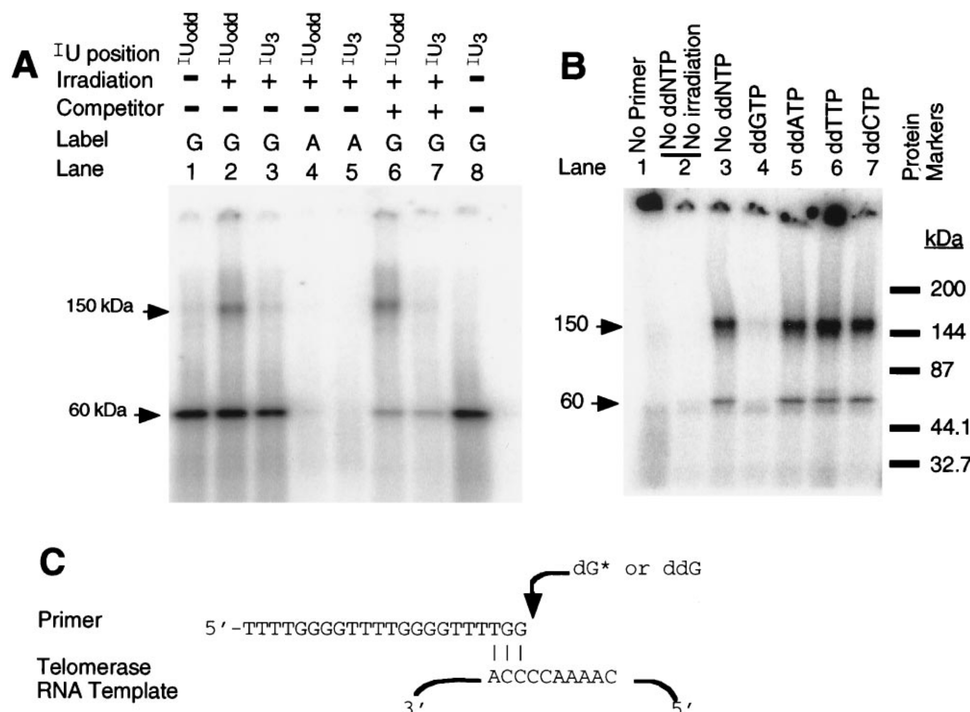


FIG. 6. Primers cross-linked to telomerase can be extended in *cis* by the enzyme. (A) DNA primers containing either six ᴵU substitutions, $^{I}U_{odd}$TELO22 (lanes 1, 2, 4, and 6), or a single substitution, $^{I}U_{3}$TELO22 (lanes 3, 5, 7, and 8), were cross-linked to partially purified telomerase. After irradiation, an excess of unlabeled TELO22 competitor was added to reactions in lanes 6 and 7. Telomerase activity was initiated by addition of ddTTP and radionucleotides [α-^{32}P]dGTP (lanes 1 to 3 and 6 to 8) or [α-^{32}P]dATP (lanes 4 and 5). Unincorporated nucleotides were removed by ultrafiltration, and then the retentate was concentrated and analyzed by SDS-PAGE. (B) A DNA primer containing two ᴵU substitutions, $^{I}U_{1/3}$TELO22 (lanes 2 to 7), was incubated with partially purified telomerase for 10 min; a control with no primer was included (lane 1). Aliquots were then transferred to pulse-labeling reactions containing [α-^{32}P]dGTP (0.33 μM) and the indicated ddNTP (2.5 μM) and incubated for 4 min. Reactions were then chased with dGTP (475 μM), placed on ice, and irradiated. Unincorporated nucleotides were removed by ultrafiltration; the retentate was concentrated and analyzed by SDS-PAGE. (C) A telomeric primer is shown aligned on the telomerase RNA template. The next nucleotide encoded by the template is dG (or ddG).

petitor in which [^{32}P]DNA primer was gel shifted by telomerase RNA (lane 12). The six-substitution (all odd positions) primer was included for reference (lane 2). A primer that contained six ᴵU substitutions at all of the even numbered positions also formed the 150-kDa cross-linked complex (data not shown); the formation of the 60-kDa cross-linked complex was not tested.

The 150-kDa cross-linked primer-protein complex was formed primarily with ᴵU nucleotides at positions 1 and 3 (Fig. 7B, lanes 7 and 8) located at the 5′ end of the primer. Very little cross-linking was seen with ᴵU substitutions closer to the 3′ end (lanes 3 to 6). A primer that contained ᴵU substitutions at both positions 1 and 3 gave a cross-linking efficiency equal to the sum of the individual substitutions (lane 9; quantitation shown in Fig. 7A). An ᴵU nucleotide at position 3 was responsible for the majority of the 60-kDa cross-link to telomerase RNA (lane 7). To test whether the 5′ telomeric region alone was sufficient for cross-linking, a primer which contained ᴵU substitutions at the 1 and 3 positions but which had a nontelomeric 3′ sequence was prepared ($^{I}U_{1/3}$CON) (Fig. 7A); no cross-linked complexes were observed with this control (Fig. 7B, lane 1).

The observation that position 3 gives cross-links to both protein and RNA may indicate a single binding site in which both a telomerase amino acid and a telomerase nucleotide are in a position to form cross-links with this primer nucleotide, or it may indicate the existence of two different binding sites. The equilibrium constant for primer binding to telomerase was measured by using these two cross-links as reporter groups (15a), and the same affinity was seen for both. It is unlikely that telomerase has two different anchor sites with the same affinity for binding single-stranded primer, supporting the former explanation. While cross-linking provides a good indication of physical proximity, it remains to be determined whether both protein and RNA moieties make energetically significant contacts with the primer.

To determine the effect of an additional G_4T_4 repeat preceding (5′ of) the cross-linkable nucleotides, such that they were no longer in the 5′-terminal block of T's, two primers were synthesized. These primers were 30 nucleotides long, d($T_4G_4T_4G_4T_4G_4T_4G_2$), and contained two ᴵU substitutions in either the 5′-most block of T's (28 and 30 nucleotides from the 3′ end) or in the second block (20 and 22 nucleotides from the 3′ end). Both of these primers gave a cross-link to telomerase when used in the standard assay (data not shown), indicating that the anchor site recognizes the telomeric repeat and not simply the 5′-terminal nucleotides of a bound primer.

A natural telomere in *E. aediculatus* has a duplexed region of 28 bp and a 14-nucleotide 3′ overhang, as shown in Fig. 8A. Alignment of the single-stranded primers with this structure reveals that the cross-linkable nucleotides correspond to a double-stranded region of the telomere (arrows in Fig. 8A). This raises the following question: does the anchor site recognize and bind to duplex DNA?

Cross-linking with hairpin primers (HPTEL). Hairpin primers (Fig. 8B) which mimic the structure of an *E. aediculatus* macronuclear telomere (21) (Fig. 8A), except that the duplexed repeat regions have been truncated, were designed.

A

Oligo	Position of ^{I}U	Protein % X-link	RNA % X-link
$^{I}U_{odd}$	UTUTGGGGUTUTGGGGUTUTGG	3.8	3.4
$^{I}U_{1}$	UTTTGGGGTTTTGGGGTTTTGG	1.8	0.3
$^{I}U_{3}$	TTUTGGGGTTTTGGGGTTTTGG	0.8	2.0
$^{I}U_{9}$	TTTTGGGGUTTTGGGGTTTTGG	0.2	0.2
$^{I}U_{11}$	TTTTGGGGTTUTGGGGTTTTGG	0.2	0.4
$^{I}U_{17}$	TTTTGGGGTTTTGGGGUTTTGG	0.2	0.5
$^{I}U_{19}$	TTTTGGGGTTTTGGGGTTUTGG	0.3	0.2
$^{I}U_{1/3}$	UTUTGGGGTTTTGGGGTTTTGG	2.9	2.0
$^{I}U_{1/3CON}$	UTUTGAATGACTACGAGATGAA	0.0	0.0

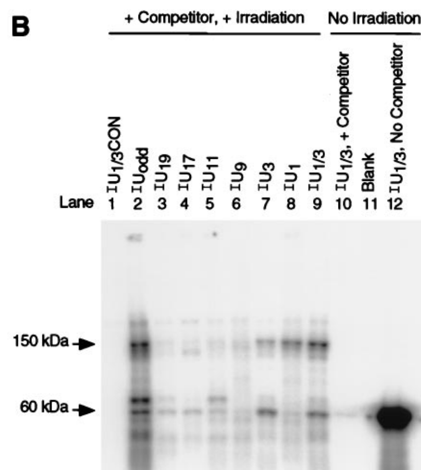
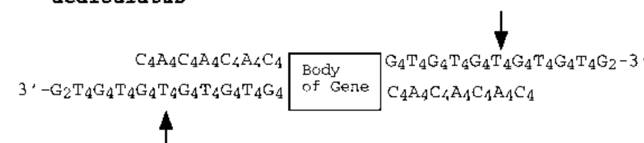


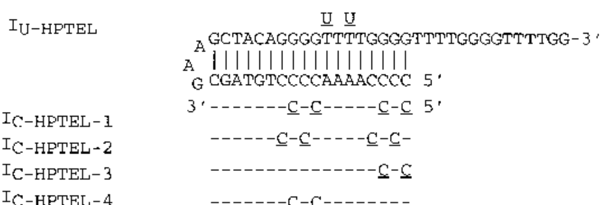
FIG. 7. The positions in the DNA primer responsible for efficient protein and RNA cross-linking are far from its 3′ end. (A) Each position of ^{I}U substitution in the single-stranded DNA primers is indicated (U). The percent efficiency of cross-linking to protein (150 kDa) and RNA (60 kDa) with each primer is indicated in the columns on the right. Efficiency is determined by dividing the intensity of the cross-links (panel B, lanes 1 to 10) by the RNA gel shift signal (panel B, lane 12) and multiplying by 100 (Materials and Methods). (B) Primers shown in panel A were assayed for cross-linking by the standard protocol (lanes 1 to 9). Unlabeled DNA primer (0.5 μM TELO22) was added after cross-linking but before denaturation (lanes 1 to 10) to compete for the gel shift of [^{32}P]DNA primer by telomerase RNA (lane 12).

A Schematic of a macronuclear gene from *E. aediculatus*



B HPTEL Constructs

Primer Substitutions



C dsTEL constructs

FIG. 8. Telomere analog primer constructs. (A) A natural telomere in *E. aediculatus* consists of a 28-bp duplex region and a 14-nucleotide single-stranded 3′ overhang (21). The locations of ^{I}U nucleotides, within a single-stranded primer, which form cross-links to telomerase are indicated by arrows. (B) Hairpin constructs, all 53 nucleotides long, were synthesized with ^{I}C (C) and ^{I}U (U) substitutions. Nucleotides identical to those in the HPTEL primer are indicated (-). (C) Duplex telomere analogs (dsTEL) formed by hybridization of separate GT and CA strands. Three different GT strands were synthesized; when annealed to the CA strand (^{I}C-dsCA-3), they give duplexes with a 12-nucleotide telomeric overhang (dsTEL), with no tail (nt), or with a random-sequence tail (rt).

Photoreactive nucleotide analogs were incorporated in the duplex region of these primers in either the GT or CA strand. The hairpin sequence was chosen to provide maximum stability (20), and a region of nontelomeric sequence was used to ensure annealing in the desired register. A single-stranded telomeric tail of 14 nucleotides was included in all of the HPTEL constructs. Proper formation of the hairpin structure was confirmed as described in Materials and Methods.

An initial screening experiment to determine which ^{I}C or ^{I}U substitutions would give a cross-link was performed (data not shown). The primer ^{I}C-HPTEL-1 gave the strongest cross-link, and a comparison of the derivative primers ^{I}C-HPTEL-3 and ^{I}C-HPTEL-4 showed that the ^{I}C substitutions in the 5′-most block of C's were responsible for most of the cross-link. A much weaker cross-link was seen with ^{I}U-HPTEL and ^{I}C-HPTEL-2 primers. The DNA-protein complex formed with the HPTEL primers (53 nucleotides) has an apparent molecular mass of 175 kDa. This is consistent with the difference in size between the hairpin primer (53 nucleotides) and the single-stranded primer (22 nucleotides). The band due to gel shift by telomerase RNA is similarly shifted to approximately 75 kDa. The methods described below were used to confirm that the HPTEL primers were cross-linked with telomerase.

A glycerol gradient was assayed for cross-linking as described above with the ^{I}C-HPTEL-1 primer, and a cross-link at 175 kDa that cofractionated with the telomerase RNA was observed (Fig. 9). The ^{I}C-HPTEL-1 cross-link was 5- to 10-fold less efficient than the cross-links observed with the single-stranded primers when compared side by side (data not shown). The efficiency of cross-linking derived by direct comparison to the RNA gel-shift in Fig. 9 is not accurate, because the HPTEL primer does not give as efficient a gel shift as the single-stranded primer.

To determine if the cross-linking of telomerase with the ^{I}C-HPTEL-1 primer resulted in a functional complex, [α-^{32}P]dGTP labeling experiments were performed. Cross-linking to primer ^{I}C-HPTEL-1 followed by labeling gave a cross-linked complex at 175 kDa (data not shown). In a pulse-labeling experiment analogous to those shown in Fig. 6B, the signal from the ^{I}C-HPTEL-1 primer was much weaker than that of the single-stranded primer, but the same pattern of incorporation and ddNTP competition was evident (data not shown). These experiments demonstrate that the HPTEL-telomerase complex is aligned in a manner competent for nucleotide addition.

Cross-linking with double-stranded bimolecular primers (dsTEL). The dsTEL primers (Fig. 8C) were designed to answer two specific questions. (i) Can the apparent cross-linking with the duplex region in the HPTEL experiments be confirmed with a double-stranded primer in which the labeled

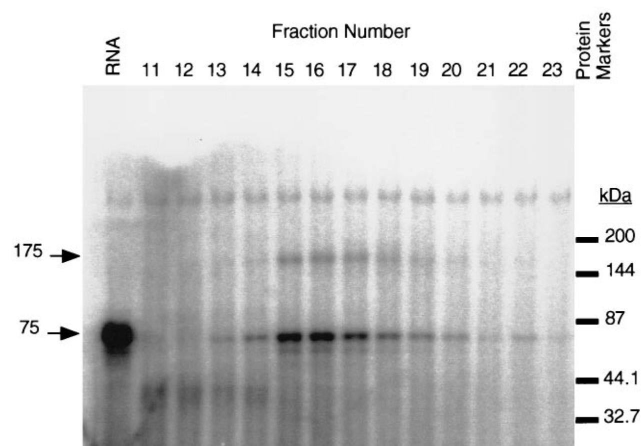


FIG. 9. A telomere analog primer forms a cross-link with an entity that cofractionates with telomerase in a glycerol gradient. Nuclear extract from *E. aediculatus* was sedimented in a 15 to 40% glycerol gradient. A portion of each fraction (fractions 11 to 23 are shown here) was assayed for cross-linking by the standard protocol and with the primer [5′-^{32}P]^{I}C-HPTEL-3 (Fig. 8B). Positions and sizes of prestained protein markers are indicated. RNA, in vitro-transcribed telomerase RNA assayed without irradiation and run as a marker. Unlabeled cross-links have not been characterized.

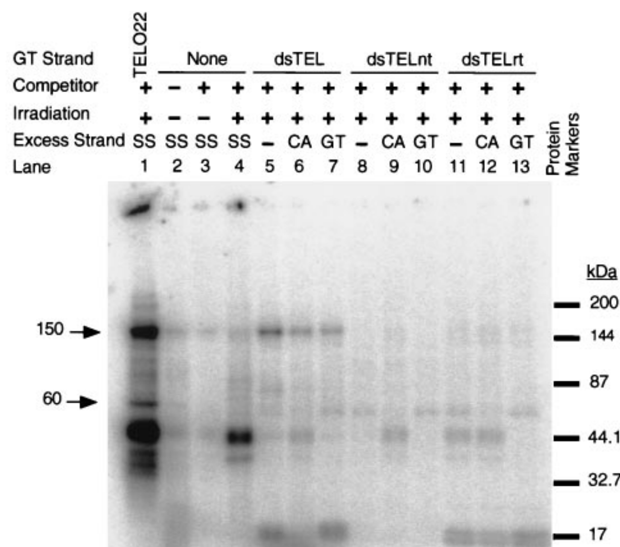


FIG. 10. Bimolecular (dsTEL) telomere analogs form cross-links to telomerase. dsTEL primer constructs (Fig. 8C) were tested for cross-linking by the standard protocol. The duplexes were formed with equimolar amounts of CA strand and GT strand (lanes 5, 8, and 11); in some cases a threefold excess of unlabeled CA strand (lanes 6, 9, and 12) or GT strand (lanes 7, 10, and 13) was added prior to incubation with extract. Single-stranded $^{I}U_{1/3}$TELO22 was also cross-linked and run as a marker (lane 1). In all the dsTEL constructs the same [5′-^{32}P]^{I}C-dsCA-3 oligonucleotide was used (lanes 2 to 13), and the GT strand was varied as indicated above the lanes. With no GT strand present (single-stranded ^{I}C-dsCA-3 [SS]) three assays were performed: with no irradiation and no competitor (lane 2); with no irradiation and with competitor (lane 3); and with irradiation and with competitor (lane 4). After irradiation and prior to denaturation, an excess of unlabeled CA strand competitor was added to all other dsTEL reaction mixtures (lanes 5 to 13). The bands with molecular masses less than 60 kDa are not believed to be due to telomerase and have not been characterized further. The positions of prestained protein markers are shown at the right.

strand has no T_4G_4 sequence? (ii) Is the single-stranded tail required for binding and cross-linking of duplex primers? For these experiments, the CA strand was always 5′ end labeled and contained the photoreactive nucleotide analog ^{I}C. As with the HPTEL primers, control experiments were performed to verify that the duplexes formed to a high extent and in the expected register (Materials and Methods). Labeling experiments with [α-^{32}P]dGTP were not performed with these constructs, because the ^{I}C nucleotides are in the strand opposite to that extended by telomerase. The ^{I}C substitutions in the CA strand primer had the same relative positions in the duplexed construct as those in the primer ^{I}C-HPTEL-3.

Four sets of cross-linking reactions were performed (Fig. 10), each differing in the sequence of the GT strand used to form the duplex (Fig. 8C). The same ^{32}P-labeled ^{I}C-dsCA-3 primer was used in all the reactions. For each reaction, a duplex was preformed with equimolar amounts of GT and CA strand (Fig. 10, lanes 5, 8, and 11), and this was followed by the addition of a threefold excess of either the unlabeled CA strand (lanes 6, 9, and 12) or GT strand (lanes 7, 10, and 13). The excess CA or GT strand was added to exaggerate the effect of either strand being in slight excess in the attempt to achieve an equimolar duplex. A parallel reaction using the $^{I}U_{1/3}$TELO22 oligonucleotide was included (lane 1) to provide a comparison for the mobility and efficiency of the cross-links to the dsTEL duplexes. $^{I}U_{1/3}$TELO22 and ^{I}C-dsCA-3 are 22 and 21 nucleotides long, respectively, and are expected to give cross-linked complexes with similar mobilities. After cross-linking but prior to denaturation, an excess of unlabeled ^{I}C-dsCA-3 was added to compete for noncovalent mobility shifts of the [^{32}P]DNA primer (lanes 3 to 13). A cross-linking reaction with the ^{I}C-dsCA-3 primer alone showed a weak band in the region of the expected cross-link (lane 4); however, a similar reaction without irradiation gave a band of the same intensity (lane 3).

The construct in which the GT strand formed a 14-nucleotide single-stranded overhang of telomeric sequence (dsTEL) gave a cross-linked complex with the same mobility as that formed with the $^{I}U_{1/3}$TELO22 primer (Fig. 10 [compare lanes 5 to 7 with lane 1]). Comparing the intensity of these cross-links revealed that the dsTEL cross-link was 5 to 10-fold less efficient; however, cross-linking requires both proximity and correct alignment of the photoreactive moiety with the cross-linking target. In a duplex, ^{I}C is located deep in the major groove and may have a lower accessibility than ^{I}U in the single-stranded primers. The intensity of the dsTEL cross-link was only partially diminished by the addition of a threefold excess of either unlabeled CA or GT strands (lanes 6 and 7). The unlabeled GT strand would be expected to directly compete for binding of the labeled duplex to the telomerase, while the CA strand could anneal to the 3′ overhang region of dsTEL and convert it to a complete duplex that binds poorly. Thus, the signal in lane 5 cannot be ascribed to a failure to form a stoichiometric complex; an excess of either strand does not eliminate cross-linking, nor does it enhance the efficiency. No significant 150-kDa cross-linked complex was formed with duplexes lacking a single-stranded tail (lanes 8 to 10) or containing a random tail (lanes 11 to 13). No cross-link to telomerase RNA was seen at 60 kDa with any of these complexes.

Processivity of primer extension. Since the cross-links observed with both single-stranded and duplex primers indicated that they were bound in the anchor site of telomerase, we tested whether these primers would be processively extended. Labeled primers (5′ end labeled) were bound to telomerase in the absence of dNTPs and then were chased with unlabeled primer and dNTPs (Fig. 11A). In the presence of excess unlabeled primer, the 8-nucleotide repeat pattern characteristic of extension by *Euplotes* telomerase (26, 36) was observed (lanes 1 and 7). The strong bands in the ladder have been attributed

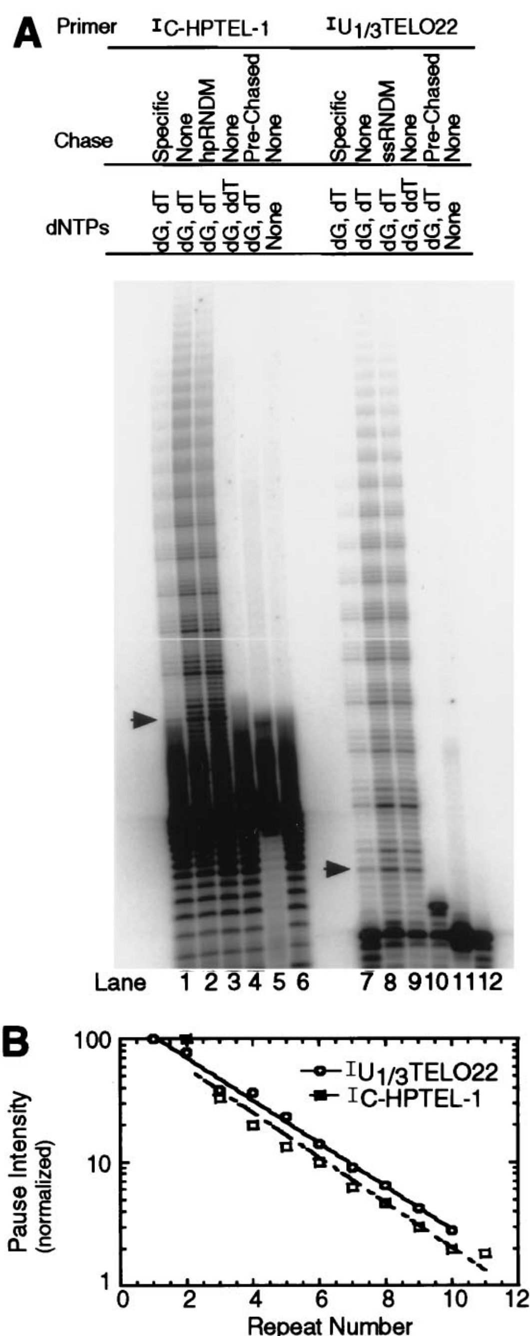


FIG. 11. Processivity of telomerase. (A) Partially purified telomerase was incubated with [5′-^{32}P]DNA primers (30 nM) $^{I}C_{1}$HPTEL (lanes 1 to 6) and $^{I}U_{1/3}$TELO22 (lanes 7 to 12). Prechased reactions (lanes 5 and 11) had unlabeled specific competitor added along with the [5′-^{32}P]DNA primer. Aliquots were transferred to telomerase extension reactions containing 125 μM nucleotides and an excess (5 μM) of unlabeled competitor primer and incubated for 10 min at 25°C (specific competitor, lanes 1 and 7; no competitor (water added), lanes 2 and 8; nonspecific competitor, lanes 3 and 9). The nucleic acid components were isolated by proteinase K-SDS treatment and ethanol precipitation. Telomerase reaction products were separated on a denaturing 7% polyacrylamide gel. (B) The intensity of each band that corresponds to a complete repeat addition was quantitated and divided by the intensity of the first quantitated band (indicated by arrows in panel A). The results were normalized and plotted against the number of repeats. The exponential decrease is fit by straight lines on the semilog plot.

to either a pause in extension or dissociation of primer upon extension to the end of the template (14). In this bind-and-chase experiment, any [^{32}P]DNA primer that remains bound to telomerase can be extended and any primer that dissociates is replaced by an unlabeled primer. Under these conditions, any extension of the primer, beyond a single repeat addition, is indicative of processivity (lanes 1 and 7). In the absence of specific chase primer (lanes 2 and 8) or with a non-telomeric primer (lanes 3 and 9), telomerase was able to rebind [^{32}P]DNA primer. In these reactions, each molecule of telomerase can bind and extend more than one [^{32}P]DNA primer during the course of the reaction; therefore, more-intense signals are seen (compare lane 1 with lanes 2 and 3 and lane 7 with lanes 8 and 9). To establish that the concentration of chase oligonucleotide was sufficient to completely eliminate extension of [^{32}P]DNA primer, the unlabeled chase primer was added along with [^{32}P]DNA primer (lanes 5 and 11); no primer extension was seen. To verify that the primer was bound to telomerase and aligned in the predicted register (26), a reaction in which ddTTP was used in place of dTTP was included (lanes 4 and 10). In this case, the primers were extended by 3 nucleotides (GGddT) as expected (lane 10); the 3-nucleotide extension product of the HPTEL primer was visible in a shorter exposure.

The processivity of telomerase on these different substrates was measured. The intensities of the strong bands that mark addition of a telomeric repeat were quantitated; the first such band is marked by an arrow in Fig. 11A. The intensity of each band was then divided by the value obtained for the first (or the second for ^{I}C-HPTEL-1, as the first is obscured by smearing seen in all lanes including the unextended primer), and the resulting ratio was normalized as a percentage (Fig. 11B). Each band is approximately 60% as intense as the previous band for both single-stranded and hairpin substrates; this result did not change significantly when the chase time was varied from 2 to 10 min (data not shown). Based on these results, the translocation efficiency of telomerase from *E. aediculatus* under these conditions is 60% and the processivity (number of additions before 50% of the primer dissociates) is approximately two repeats (16 nucleotides).

DISCUSSION

Telomerase adds repeated GT-rich sequences to the ends of eukaryotic chromosomes. After the addition of each repeat, the 3′ end of the primer must reposition on the RNA template to prime the synthesis of the next repeat. This translocation can occur without release of the DNA primer from telomerase, giving processive extension. According to the two-site model for telomerase processivity, this can occur because the primer is bound not only at the RNA template but also at a separate anchor site (11, 30). The primer can only dissociate from telomerase when it is simultaneously released from both sites. Our studies of the *Euplotes* enzyme now reveal two new properties of the anchor site: (i) it consists of, or is in physical proximity to, both a specific protein and a portion of the RNA subunit and (ii) it can bind either duplex or single-stranded regions of telomeric DNA primers. A schematic for the binding of primer to both the anchor and active sites of telomerase based on our cross-linking and telomerase activity data is shown in Fig. 12; detailed aspects are discussed below.

The anchor site of telomerase is composed of both protein and RNA components. We have demonstrated cross-links between the telomerase from *E. aediculatus* and telomeric DNA primers containing photoreactive ^{I}U substitutions (40). Incubation of these primers with nuclear extract followed by irra-

FIG. 12. Model for primer bound to telomerase at both the template and anchor sites. The telomerase RNA secondary structure of *E. aediculatus* (26) is depicted with two potential protein subunits (24), represented by ovals. The ~130-kDa protein observed here is assumed to correspond to the ~120 kDa protein reported in purified telomerase by Lingner and Cech (24). A DNA primer is shown with its 3′ end (G_{OH}) aligned on the RNA template as indicated by telomerase activity experiments. Potential base pairing between the DNA primer and RNA template is indicated by a question mark because the extent of base pairing is not currently known. Cross-links to the protein subunit can occur when the 5′ end of the primer is single-stranded (sequence shown), or in a base-paired duplex, indicated schematically. Cross-links to U51-U52 of telomerase RNA and to the ~130-kDa protein subunit of telomerase are indicated (×). The location of a second protein subunit relative to the RNA is arbitrary and is included only for completeness.

diation gave an ~150-kDa cross-linked complex with a telomerase protein (un-cross-linked molecular mass, ~130 kDa) and an ~60-kDa cross-linked complex with telomerase RNA. Several lines of evidence indicated that these cross-links were to the telomerase enzyme. The protein which formed the 150-kDa cross-linked complex cosedimented with the telomerase RNA in a glycerol gradient. The cross-linked complexes were formed with purified telomerase, which contains an ~120-kDa protein (24). The cross-linked primer was extended by telomerase in the presence of dGTP, not dATP; extension was inhibited by only one of the ddNTPs, ddGTP. The cross-links were specific to telomeric primers; cross-linking was not inhibited by the presence of 7 μM nontelomeric oligonucleotide competitor, and an oligonucleotide with the same 5′ end but a nontelomeric 3′ end did not cross-link.

The identity of the RNA component was confirmed by comparing the size, after removal of the DNA primer by DNase I, to that of an in vitro transcript of telomerase RNA (Fig. 4), by oligonucleotide-directed RNase H digestion (data not shown) and by primer extension. The location of this cross-link within the telomerase RNA was mapped to nucleotides U51-U52 by primer extension (Fig. 5). The telomerase RNA alone, after removal of the protein components by proteinase K-SDS treatment, was sufficient for forming a DNA-RNA cross-link (data not shown). The cross-link formed in the absence of telomerase proteins was not mapped to a specific site within the RNA; therefore, it is possible that different cross-links were formed in the presence and absence of protein. Alternatively, even in the absence of protein, the telomerase RNA alone may have sufficient secondary and tertiary structure to position the primer for cross-linking. When the telomerase RNA was heat denatured as well as deproteinized prior to cross-linking, the efficiency of the cross-link was reduced severalfold. This reduction may be due to partial misfolding of the RNA after denaturation, as has been observed with telomerase RNA from *Tetrahymena* (43).

The most efficient cross-linking to telomerase protein occurred with 22-nucleotide telomeric primers containing ^{I}U substitutions in the 5′-most T_4 block. Positions 1 and 3 form two independent cross-links to the telomerase protein, while the cross-link to telomerase RNA was formed primarily with the ^{I}U substitution at position 3. The position of each cross-link is indicated in Fig. 12. The fact that cross-links were observed with substitutions 20 and 22 nucleotides upstream of the 3′ end of the primer, in preference to substitutions 4, 6, 12, or 14 nucleotides upstream, supports the idea of an anchor site which is separate and distinct from the active site of telomerase. It is unlikely that the active site of telomerase recognizes 20 nucleotides of DNA, since it has been demonstrated that a very few telomeric nucleotides are sufficient to prime telomerase activity (17, 26, 30).

Primer bound to telomerase at both active and anchor sites can be looped out from the enzyme. The cross-link between primer and U51-U52 of telomerase RNA defines a point of contact between the 5′ end of the primer and a site immediately adjacent to the alignment region of the RNA. Telomerase activity experiments (described above) indicate that the primer that forms this cross-link has its 3′ end aligned at position C43 in the template region of the RNA. These two points of contact are only 8 nucleotides apart on the telomerase RNA but are 20 nucleotides apart on the primer. In order to make these two contacts simultaneously, the middle region of the primer must be looped out from the RNA as shown in Fig. 12. While it seems clear that a primer can be looped out from telomerase RNA between the active and anchor sites, it remains to be determined whether contacts to telomerase are made with the middle region of the primer. Although these data indicate that primer bound to telomerase can be looped out, they do not distinguish whether this is the most stable or most prevalent conformation.

The anchor site of telomerase recognizes a natural telomere analog. Cross-links between a telomerase protein and a telo-

mere analog containing ^{I}C nucleotides in the CA strand of the duplex region were also observed. These cross-links showed the same specificity for telomerase as those obtained with single-stranded primers. If any cross-link between telomere analogs and telomerase RNA was formed, the signal was too weak to be seen. The finding that telomerase can recognize a natural telomere through binding at both the active and anchor sites may give some insight into its in vivo substrate. Two models have been proposed for the timing of telomerase function during DNA replication (25). In one model, telomerase would add repeats to the 3′ end of a natural telomere prior to replication. It has been shown that telomerase is present in the replication band of *O. nova* prior to DNA replication (7). In the other model, the blunt-ended DNA produced by replication would have to be opened up or resected by a 5′-to-3′ nuclease prior to telomerase action (25, 39). The cross-linking data presented here demonstrate the feasibility of telomerase's recognizing duplex DNA with a 3′ single-stranded tail as required by either model.

Processivity of telomerase on single-stranded and telomere analog substrates. One prediction that follows from cross-linking to natural telomere analogs is that they would be processively extended by telomerase. This was demonstrated in a bind-and-chase assay for telomerase activity (Fig. 11). The processivity was measured by comparing the relative intensities of the bands that correspond to each complete repeat addition. It was determined that approximately 60% of the primer remains bound to telomerase after each translocation. Approximately half of the bound primer is therefore either released by the enzyme or is paused in an nonextendable conformation after the addition of just two repeats (16 nucleotides). This limited processivity for the *E. aediculatus* telomerase can be compared to the in vitro processivity of 520 nucleotides determined for the *T. thermophila* enzyme (11). It is tempting to compare the relative processivities of these enzymes to the telomere lengths observed in vivo. The telomeres of *E. aediculatus* are relatively short and well defined (42 nucleotides on the GT strand), while those of *T. thermophila* are much longer (up to several hundred nucleotides) and vary in length. However, this comparison could be misleading, because the in vivo processivity of telomerase from *T. thermophila* could be different from that observed in vitro. Cell lines that express two different telomerase RNA template sequences have a random mix of telomere repeats added by these two enzymes, rather than long stretches added by either one (41). The processivity reported here for telomerase from *E. aediculatus* is consistent with this in vivo result, although the data are from two different organisms.

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