THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 15, Issue of April 14, pp. 8893–8901, 1995
Printed in U.S.A.

Gel Shift and UV Cross-linking Analysis of *Tetrahymena* Telomerase*

(Received for publication, November 8, 1994, and in revised form, January 5, 1995)

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Telomerase is an unusual ribonucleoprotein that synthesizes new telomeres onto chromosome ends. The enzyme has been most extensively characterized in ciliates, where the RNA component has been cloned from several species, and its elongation properties have been characterized in detail. To understand the substrate specificity and protein composition of telomerase, we have used gel shift and UV cross-linking to characterize the enzyme from the ciliate *Tetrahymena thermophila*. In a mobility shift assay, a complex was identified that contained telomerase RNA, co-purified with telomerase activity, and was sensitive to nuclease treatment. The mobility shift complexes specifically formed using several different single-stranded, telomeric sequences but not non-telomeric primers. These results suggest that the specificity of telomerase for G-rich primer sequences occurs at least in part at the level of primer binding. UV cross-linking analysis identified a 100-kDa cross-linked protein that may be a telomerase component.

Chromosome ends are maintained by the unusual DNA polymerase, telomerase. This polymerase, first discovered in *Tetrahymena*, adds telomeric sequences *de novo* to chromosome ends to make up for sequence loss during replication (Greider and Blackburn, 1985; Yu *et al.*, 1990). Telomerase represents a unique class of DNA polymerase in that it contains an essential RNA component, which serves as an internal template for the addition of telomeric repeat sequences. In *Tetrahymena*, telomerase processively adds hundreds of telomeric $d(TTGGGG)_n$ repeats, one nucleotide at a time, using an internal RNA sequence CAACCCCAA as a template (Greider and Blackburn, 1989). The role of the telomerase RNA was demonstrated in two ways. First, inactivation of *Tetrahymena* telomerase using RNA-complementary oligonucleotides and RNase H cleavage abolished telomerase activity *in vitro* (Greider and Blackburn, 1989). Second, when a telomerase RNA gene mutation in the CAACCCCAA template region was introduced into *Tetrahymena*, telomeres corresponding to the mutant sequence were synthesized (Yu *et al.*, 1990). These results demonstrated that not only is the CAACCCCAA sequence used as a template but also that telomerase is the enzyme which synthesizes telomere sequences *in vivo*. Telomerase activities with similar properties to the *Tetrahymena* enzyme have been identified in several different organisms, including hypotrichous ciliates, human, mouse, and *Xenopus laevis* cells (Lingner *et al.*, 1994; Mantell and Greider, 1994; Morin, 1989; Prowse *et al.*, 1993; Shippen-Lentz and Blackburn, 1989; Zahler and Prescott, 1988). The RNA component of telomerase has so far been cloned only in ciliates (Greider and Blackburn, 1989; Lingner *et al.*, 1994; Romero and Blackburn, 1991; Shippen-Lentz and Blackburn, 1990).

Unlike the binding specificity of many DNA-binding proteins, telomerase primer specificity is not precise. Telomerase can elongate G-rich single-stranded DNA oligonucleotides regardless of the exact sequence of the primer. However, non-telomeric sequence primers are not efficiently elongated (Blackburn *et al.*, 1989; Greider and Blackburn, 1987). For a given primer 3′ end, telomerase will correctly add the next base in the telomeric sequence. The ability to correctly "fill out" any permutation of the telomeric sequence d(TTGGGG) is likely due to primer alignment with the telomerase RNA sequence CAACCCCAA (Greider and Blackburn, 1989).

The *Tetrahymena* telomerase enzyme can be reconstituted *in vitro* using synthetic telomerase RNA and partially purified telomerase proteins (Autexier and Greider, 1994). The reconstituted enzyme has similar elongation specificities as the wild type telomerase. Mutations in the 5′-CAACCCCAA-3′ sequence showed that the 5′ six nucleotides provide template information while the 3′ most CAA sequence is required for aligning primer 3′ ends with the template region. Thus the template region helps determine the specificity for both primer 3′ end sequence and the nucleotides incorporated by telomerase.

The "hybridization-directed" model of telomerase elongation predicts that alignment of the substrate 3′ end with telomerase RNA is important for elongation. However, *in vivo* chromosome healing by telomere addition has been observed in ciliates, yeast, and humans at broken ends that have little or no G-rich sequence (reviewed in Greider (1991a)). *In vitro*, telomerase will elongate "chimeric" oligonucleotides containing two d(TTGGGG) repeats at the 5′ end followed by up to 36 bases of non-telomeric DNA at the 3′ end (Harrington and Greider, 1991). A similar analysis of human telomerase primer specificity *in vitro* showed that the 3′ end of a primer does not have to be strictly complementary to the RNA template to be elongated (Morin, 1991). These results suggest that the sequences necessary for primer recognition can be spatially separated from the site of telomere addition, and led to the hypothesis that there may be a site of primer recognition, or binding, that is distinct from the site of telomere synthesis (Harrington and Greider, 1991; Morin, 1991). Kinetic analysis using primers with a fixed 3′ but different 5′ end sequences also suggests that there must be a contribution of a site distinct from the template region in primer binding (Lee and Blackburn, 1993). Finally, the non-

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processive elongation of short primers implies that there must be a second site, or "anchor site" for primer binding (Collins and Greider, 1993; Lee and Blackburn, 1993). By analogy to RNA polymerases, a primer 10 nucleotides or longer can always be bound at one site or the other during processive elongation, while shorter primers may only be bound at the template site (Collins and Greider, 1993).

To date all experiments on primer specificity have assayed elongation as the end point. The relative contribution of primer binding and elongation have not been separately determined. We have used a mobility shift assay to directly examine whether the telomerase primer recognition step, like primer elongation, is specific for G-rich sequences. In addition, we have identified a 100-kDa protein which cross-links to telomerase products. The UV cross-linking and gel shift analysis may be useful for the identification of telomerase proteins in other organisms.

EXPERIMENTAL PROCEDURES

Purification of Tetrahymena Telomerase—At all steps in the purification, telomerase elongation activity was assayed as described previously (Harrington and Greider, 1991). Briefly, 20 μl of extract was incubated with 20 μl of a 2 × reaction buffer, such that the final concentration of the added components was 100 μM TTP (Pharmacia), 1 μM [^{32}P]dGTP, 0.1 μg of d(GGGGTT)$_3$ in 1 × telomerase buffer (1 mM spermidine, 60 mM K-acetate, 5 mM β-mercaptoethanol, and 50 mM Tris acetate, pH 8.5). Incubation at 30 °C for 1 h was followed by phenol extraction and ethanol precipitation of the radiolabeled products. The dried pellets were resuspended in 2.5 μl of sequencing gel loading buffer (37%, v/v, formamide, and approximately 0.25%, w/v, bromphenol blue, 0.25%, w/v, xylene cyanol), and resolved on a 10%, w/v, denaturing acrylamide gel as described previously (Greider and Blackburn, 1987).

Tetrahymena cells were grown and harvested after 12–18 h of starvation, without subsequent mating (Avilion *et al.*, 1992). Typically, 36 liters of *Tetrahymena* cells were grown to a density of 3.0–3.5 × 10^5 cells/ml in PPYS (2% w/v proteose peptone (Difco), 0.2%, w/v, yeast extract (Difco), 10 mM $FeCl_2$) at 30 °C, pelleted, and starved by resuspension in 8–12 liters of Dryls medium (1.3 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 1.7 mM Na-citrate, 2 mM $CaCl_2$) at 30 °C for 12–18 h. Cells were harvested and the cell pellet was lysed at 4 °C in 3 volumes of TMG (10 mM Tris, pH 8.0, 1 mM $MgCl_2$, 10%, v/v, glycerol, 5 mM β-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride, and 0.25 μg/ml each of leupeptin, pepstatin, chymostatin, and antipain) and 0.2%, v/v, Nonidet P-40, and centrifuged at 100,000 × *g* in a SW41 Beckman rotor at 4 °C for 1 h. All subsequent purification was carried out at 4 °C, and telomerase fractions were quick-frozen in liquid nitrogen and stored at −70 °C. Heparin-agarose-purified telomerase was obtained as described previously (Greider and Blackburn, 1987) or as follows. The S100 was loaded onto a heparin-agarose (Bio-Rad) column equilibrated with TMG, washed with TMG, and eluted with TMG containing 0.2 M potassium glutamate. The eluate was loaded onto DEAE-agarose (Bio-Rad) column equilibrated with TMG, washed with TMG, 0.15 M potassium glutamate, and eluted with a linear salt gradient of 0.15–0.6 M potassium glutamate in TMG.

Some purifications were carried out using a similar procedure as above, except the order of columns was changed to DEAE-agarose, phenyl-Sepharose, and heparin-agarose, followed by spermine-agarose, concentration on a DEAE-agarose column, and sucrose gradient sedimentation (Harrington, 1993). Briefly, S100 extract prepared from approximately 10^{10}-10^{11} cells was loaded onto DEAE-agarose (Bio-Rad) equilibrated in TMG, 0.15 M potassium glutamate (KGlu), and eluted with a linear salt gradient of 0.15 M KGlu to 0.6 M KGlu in TMG. Active fractions were pooled and loaded onto phenyl-Sepharose equilibrated in TMG, 0.4 M KGlu, and eluted with TM, 50%, v/v, ethylene glycol. The pooled active fraction was adjusted to approximately 0.05 M KGlu, loaded onto heparin-agarose (Pharmacia) equilibrated in TMG, and eluted with TMG, 0.2 M KGlu. Active fractions were pooled and loaded onto spermine-agarose (Sigma) equilibrated in TMG, 0.2 M KGlu, and eluted with TMG, 0.6 M KGlu. To concentrate the enzyme, active fractions were diluted to 0.15 M KGlu with TMG, loaded onto a small DEAE-agarose column, and eluted with TMG, 0.4 M KGlu. A portion of the most active eluate (100–150 μl) was loaded onto a 3.6–3.8-ml 7–30%, w/v, sucrose gradient in TMG, 0.4 M KGlu, and centrifuged in a Beckman SW50.1 rotor for 17 h, 150,000 × *g* at 4 °C. Fractions were collected from the bottom to the top of the gradient using a glass capillary tube and a fraction collector, and the samples were immediately assayed for telomerase activity before storage in liquid nitrogen. In this purification scheme, telomerase was purified approximately 100-1000-fold based on measuring the enrichment for the telomerase RNA component (Harrington, 1993). The fold purification relative to activity was not possible to determine accurately because activity increased over the first few columns suggesting that inhibitors were being removed.

Affinity Purification—Affinity purification of telomerase extracts was carried out by a modification of the method of Franza *et al.* (1987). Approximately 1 ml of partially purified extract (sizing column, heparin-agarose, DEAE-agarose) was incubated with 75 μl of streptavidin-agarose beads (Bethesda Research Laboratories), and rocked at 4 °C for 30 min. The agarose beads were pelleted in a microcentrifuge, and the supernatant was removed to a new tube. To this supernatant was added 30–100 μg of the nonspecific competitor, pBR oligonucleotide (Harrington and Greider, 1991), and the sample was rocked at 4 °C for another 30 min. Fifty to 100 μg of biotinylated (TTGGGG)$_8$ was added, and the sample was rocked for 1 h at 4 °C (Photoprobe Biotin obtained from Vector Laboratories and DNA prepared according to manufacturer's instructions). The supernatant was then divided into three, and serially precipitated with approximately 200 μl of streptavidin-agarose for 20 min at 4 °C. After each incubation, the beads were pelleted, the supernatant was removed, and another one-third supernatant was added. After all supernatant had been exposed to the agarose beads, the beads were extensively washed in TMG. A small amount of TMG remained over the washed pellet. The beads were assayed directly for telomerase activity and UV cross-linking.

Oligonucleotides and Mobility Shift Probes—All oligonucleotides were obtained from Operon Technologies, Inc. and were gel-purified as described previously (Harrington and Greider, 1991). For generation of probes for the mobility shift assay, 0.1 μg of gel-purified oligonucleotide was added to 1 × kinase buffer (Sambrook *et al.*, 1989), 100 μCi of [γ-^{32}P]ATP (DuPont NEN), and 5 units of T4 polynucleotide kinase (BRL) in a final volume of 20 μl, and incubated at 37 °C for 15–30 min. The reaction was stopped by addition of 80 μl of 1 × TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at −20 °C. The probe was diluted to a final concentration of 1 ng/μl and 1 μl was used for each reaction in the mobility shift assay.

Telomerase Mobility Shift Assay—For the standard mobility shift, 10 μl of extract was incubated with 10 μl of a mixture containing 1 μl of ^{32}P-d(TTGGGG)$_3$ (or ^{32}P-d(GGGGTT)$_3$ in some experiments), 3.0 μl of mobility shift buffer (33 mM Tris acetate, pH 8.0, 3.3 mM $MgCl_2$, 33%, v/v, glycerol), 1.5 μl of 100 ng/ml d(T)$_{18}$, and 4.5 μl of sterile water. The sample was incubated on ice for 10–30 min, and was loaded directly onto a pre-run 6%, w/v, nondenaturing acrylamide gel (20:1 acrylamide: bis-acrylamide) in 1 × TBE (Sambrook *et al.*, 1989), electrophoresed for 4 h at 240 V, dried, and exposed to XAR-5 film. For the competition assays, the standard mobility shift assay was performed as described above, except that 1 μl of the appropriate concentration of unlabeled competitor oligonucleotide was added to the probe mixture. For the nuclease experiments, 10 μl of telomerase extract was incubated with 1–2 units of micrococcal nuclease and 1 mM $CaCl_2$ (final concentration) at 30 °C for 10 min. The sample was returned to ice, and brought to 10 mM EGTA and 10 mM $MgCl_2$, followed by addition of 10 μl of probe mixture as described above. Control samples were treated identically, except micrococcal nuclease was omitted. For treatment with ribonuclease ONE (Promega) or ribonuclease CL3 (Pharmacia Biotech Inc.), the appropriate units as indicated in Fig. 3 were added to 10 μl of partially purified telomerase extract, and incubated on ice for 10 min prior to the addition of 10 μl of probe mixture.

Reverse Transcriptase-PCR[1] Analysis of Telomerase RNA—Telomerase RNA was isolated from telomerase preparations with one phenol extraction, and ethanol precipitated with 5 μg of yeast tRNA (Sigma) as a carrier. *In vitro* transcribed telomerase RNA was used as a positive control for the PCR, and was prepared using T7 polymerase as described previously (Avilion *et al.*, 1992). For isolation of RNA from excised gel slices as indicated in Fig. 4, the gel slices were crushed into 0.3 M sodium acetate, 0.2%, w/v, SDS and eluted overnight at 30 °C, and the RNA was precipitated from the supernatant with 600 μl of absolute ethanol. The sequence of the primers used in the PCR reaction are: "oligo 10'," 5'-AAAAATAAGACATCCATTGATAAATAGTGTATCAAATG-3', and "oligo 9'," 5'-ATACCCGCTTAATTCATTCAGA-3'.

[1] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); N_3RdUTP, 5-[*N*-(*p*-azidobenzoyl)-allyl]-deoxyuridine monophosphate.

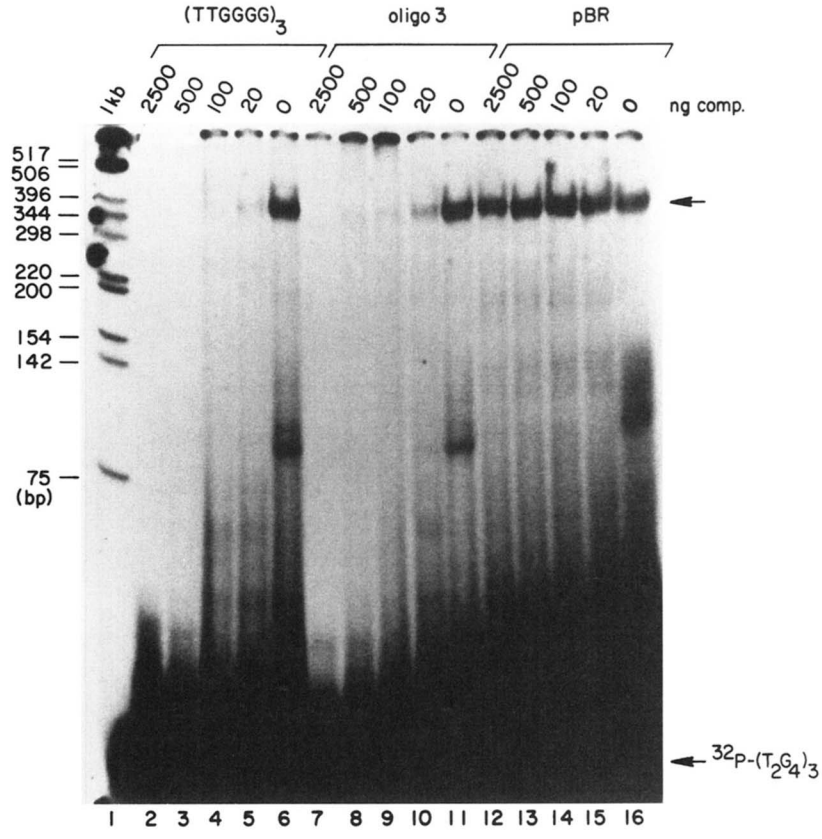

FIG. 1. **Gel mobility shift of d(TTGGGG) by telomerase.** Partially purified telomerase was incubated with 1 ng of ^{32}P-d(TTGGGG)$_3$ and resolved on a nondenaturing gel. *Lane 1*, ^{32}P-labeled 1-kbp marker, with base pairs indicated at the left. *Lanes 2–16*, the indicated amount of unlabled competitor oligonucleotide was added to the mobility shift mixture; for d(TTGGGG)$_3$, from 2500 ng (*lane 2*) to no competitor (*lane 6*), and similarly, competition with oligo 3 and pBR, *lanes 7–11* and *12–16*, respectively. The position of the d(TTGGGG)$_3$-specific mobility shift is indicated with an *arrow* at *right*, and the *arrow* at *bottom right* marks the approximate position of unbound ^{32}P-d(TTGGGG)$_3$ probe. The sequences of oligonucleotides used in this experiment are: pBR, 5′-AGCCACTATCGACTACGCGATCAT-3′; oligo 3, 5′-GCACTAGATTTTTGGGGTTG-3′.

Telomerase RNA samples (ranging from 50 pg to 1 ng) were brought to 4.5 μl with diethyl pyrocarbonate-treated sterile water, and added to a 20 μl of mixture containing 1 × reaction buffer (50 mM Tris-Cl, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl), 100 pmol of primer (oligo 10), 1.0 mM each of dATP, dCTP, dGTP, and TTP, 1.0 mM dithiothreitol, 20 units of RNasin (Promega), and 2–5 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). The sample was incubated at room temperature for 10 min, and subsequently at 52 °C for 60 min, and 95 °C for 5 min. To this sample was added a 80-μl mixture containing 1 × PCR buffer (20 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.2 mM $MgCl_2$), 200 μM each of dATP, dCTP, dGTP, and TTP, 1.0 μM of each primer (oligo 10, oligo 9), and 2.5 to 5 units of AmpliTaq polymerase (Perkin-Elmer). The sample was overlaid with 100 μl of light white mineral oil (Sigma), and amplified in a Perkin-Elmer PCR machine for 30 cycles of 1 min at 94 °C, 1 min at 46 °C, and 1 min at 72 °C. To visualize the products, 10–30 μl of each reaction were resolved on a 1%, w/v, agarose gel and the gel was stained in 1 μg/ml ethidium bromide.

UV Cross-linking of Telomerase—For the N_3RdUTP elongation assay, all reactions were carried out in subdued light. Ten μl of telomerase extract was incubated with a 4-μl reaction mixture, consisting of 1 μl of 10 × telomerase buffer, 1 μl of [^{32}P]dGTP (400 Ci/mmol; NEN), 1.0 μl of 1 mM N_3RdUTP (synthesized according to Bartholomew *et al.* (1990, 1991)), and 0.5 μg of gel-purified d(G_4T_2)$_3$ primer. Elongation reactions proceeded for 6–10 min at 30 °C. Samples to be cross-linked were placed in a 96-well microtiter plate pre-blocked with 1 mg/ml bovine serum albumin or approximately 50 μl of Sigmacote (Sigma), covered in plastic wrap, and placed at room temperature directly underneath a hand-held UV monitor at 254 nm for 5–10 min. For unirradiated controls, the samples remained at room temperature in the dark during the course of cross-linking. The sample was removed, and approximately 5 units each of RNase ONE, DNase, and MNase were added to the sample, and incubated at 30 °C for 10 min. The sample was boiled in protein gel loading buffer and resolved on a 7–16%, w/v, acrylamide linear gradient protein gel, with a 3%, w/v, acrylamide stack.

RESULTS

Telomerase Primer Recognition—The specificity of *Tetrahymena* telomerase primer elongation has been extensively characterized (Blackburn *et al.*, 1989; Collins and Greider, 1993; Greider, 1991b; Greider and Blackburn, 1987; Harrington and Greider, 1991; Lee and Blackburn, 1993). However, since it is not possible to distinguish between primer binding and nucleotide addition in the previously used elongation reactions, we used a mobility shift assay to directly study the primer binding specificity of highly purified telomerase. Radiolabeled telomeric d(TTGGGG)$_3$ oligonucleotide was incubated with fractions containing telomerase and resolved on a nondenaturing gel. Using partially purified extracts, we identified a protein-DNA complex that was specific for telomeric DNA and that migrated with the 344-bp DNA marker (Fig. 1). This complex was efficiently competed by a 20-fold excess of unlabeled competitor telomeric oligonucleotide, d(TTGGGG)$_3$, but was not competed by up to a 2500-fold excess of non-telomeric oligonucleotide, pBR (Fig. 1). Other telomeric or telomere-like oligonucleotides that are substrates for telomerase elongation, such as d(TTGGGG), d(GGGGTT)$_3$, d(TTAGGG)$_3$, d(TG)$_9$, d(TGTGTGGG)$_2$TG, and d(TTGGGG)$_2$pBR (Blackburn *et al.*, 1989; Greider and Blackburn, 1987; Harrington and Greider, 1991), also competed for formation of this complex (data not shown). An oligonucleotide complementary to the telomerase RNA template region, oligo 3, also competed for ^{32}P-d(TTGGGG)$_3$-binding (Fig. 1). This oligonucleotide contains 21 nucleotides complementary to the RNA, including the nine nucleotides, TTGGGGTTG, covering the RNA template (Greider and Blackburn, 1989). A second RNA complementary oligonucleotide, oligo 8, which hybridizes adjacent to but does not cover the CAACCCCAA RNA template (Greider and Blackburn, 1989) did not efficiently compete for ^{32}P-d(TTGGGG)$_3$ binding (data not shown). The telomere-specific mobility shift did not require Mg^{2+}, TTP, or dGTP for complex formation (data not shown). This complex was not formed when telomerase extracts were incubated with the ^{32}P-labeled non-telomeric oligonucleotide, pBR, or when telomerase extracts were treated with Proteinase K (data not shown).

To determine whether the ^{32}P-d(TTGGGG)$_3$-binding activity corresponds to telomerase or to some other telomere-binding

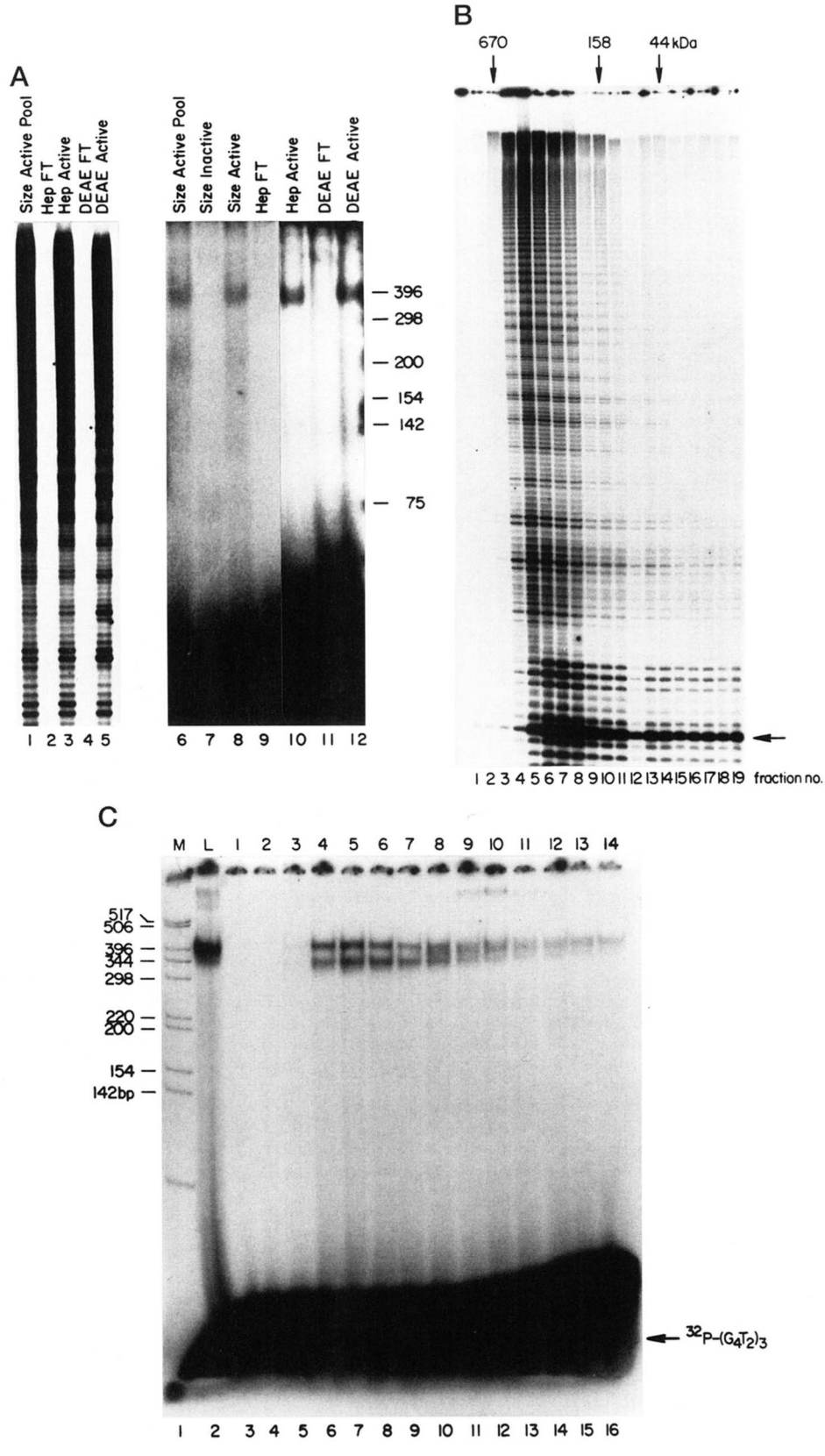

FIG. 2. **Mobility shift co-purifies with telomerase activity.** *A*: *lanes 1–5*, telomerase elongation activity through a partial purification. *Lanes 1, 3,* and *5,* pooled fractions containing telomerase activity after gel filtration, heparin-agarose, and DEAE-agarose, respectively. *Lanes 2* and *4*, flow-through fractions from the heparin-agarose and DEAE-agarose columns, respectively. *Lanes 6–12*, ^{32}P-d(TTGGGG)$_3$ mobility shift assay on respective fractions throughout the purification at left: *lane 6*, the same pooled, active fractions after gel filtration as in *lane 1*; *lane 7*, a fraction from the gel filtration column which did not contain telomerase activity; *lane 8*, a gel filtration fraction containing telomerase activity. *Lanes 9–12*, mobility shift assay using the same fractions as in *lanes 2–5*. Markers are indicated at *right* in base pairs. *B*, telomerase activity fractionated over DEAE-agarose, phenyl-Sepharose, heparin-agarose, spermine-agarose, and concentrated on a DEAE-agarose column was sedimented on sucrose gradient. Each fraction was assayed for telomerase elongation activity, and the DNA products were resolved on a 10%, w/v, denaturing acrylamide gel. *Lanes 1–19*, sucrose gradient fractions from the bottom to the top of the gradient, indicated at bottom. The sedimentation of native protein markers in the gradient are indicated with *arrows* above lanes 3, 10, and *14* in kDa. The position of the first nucleotide added to the d(GGGGTT)$_3$ primer is shown with an *arrow* at *bottom right*. *C,* fractions 1–14 of the sucrase gradient shown in *part B* were tested for the ability to bind the ^{32}P-d(TTGGGG)$_3$ probe. *Lane 1*, ^{32}P-labeled 1-kbp marker, with the molecular weight of each band as indicated to the *left*. *Lane 2,* the purified fraction used to load the sucrose gradient. *Lanes 2–16*, sucrose fractions 1–14 from the bottom to the top of the gradient.

protein, we followed telomerase elongation activity and the mobility shift complex throughout telomerase purification (Fig. 2). *Tetrahymena* S100 extracts were purified over gel filtration, heparin-agarose, and DEAE-agarose (Fig. 2*A*). The ^{32}P-d(TTGGGG)$_3$-binding activity further co-purified with telomerase over the series: DEAE-agarose, phenyl-Sepharose, heparin-agarose, spermine-agarose, a DEAE concentrating column, and finally sucrose gradient sedimentation (Fig. 2*B*). Co-purification of the band shift with telomerase on the final gradient is shown in Fig. 2*C*. In this experiment the band shift appeared as a doublet. This doublet, seen in several highly purified preparations, has identical specificity as the single shift and may be due to limited telomerase degradation in these fractions (data not shown).

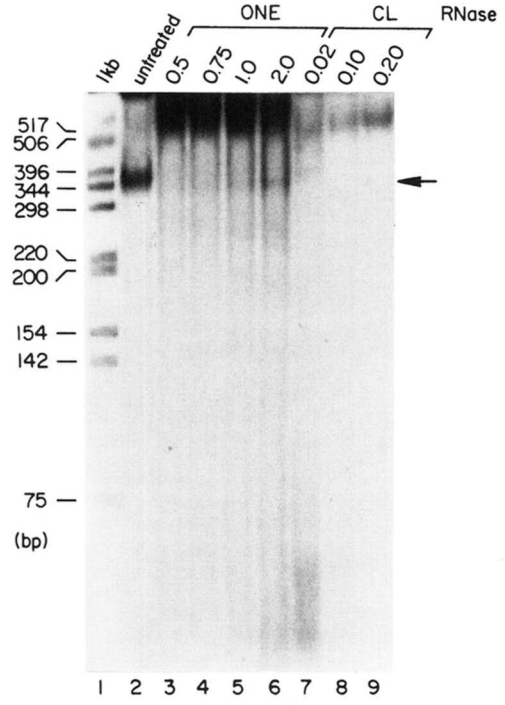

FIG. 3. **RNase sensitivity of the mobility shift complex.** *Lane 1*, ^{32}P-labeled 1-kbp marker, with base pairs as shown at the *left*. *Lane 2*, untreated telomerase extracts assayed for ^{32}P-d(TTGGGG)$_3$ complex formation; *lanes 3–6*, telomerase extracts preincubated on ice with increasing units of RNase ONE prior to probe addition. *Lanes 7–9*, preincubation with increasing units of RNase CL3. The position of the telomerase mobility shift is indicated with an *arrow*. The labeled input probe was run off the gel.

Since telomerase contains an essential RNA component, we tested whether the complex was sensitive to RNase treatment. Partially purified telomerase extracts were preincubated with different ribonucleases, and then assayed for complex formation. Treatment with either RNase ONE, which cleaves after any nucleotide, or RNase CL3, which cleaves primarily at C residues, eliminated the complexes migrating with the 344-bp DNA marker (Fig. 3). Incubation with each RNase resulted in the formation of other complexes at lower mobilities than the telomerase complex (Fig. 3). The reason for this effect is not known. Pre-treatment with active MNase also eliminated the 344-bp complex, whereas the complex was unaffected by incubation of telomerase extracts with MNase that had been inactivated with EGTA (data not shown). These results indicate that the telomeric primer binding complex contains an RNA component necessary for complex stability.

We next assayed directly for telomerase RNA in the mobility shift complex. Telomerase fractions were incubated either with or without radiolabeled telomeric primer and electrophoresed on adjacent lanes on a nondenaturing gel (Fig. 4*A*). The two lanes were subsequently excised, cut into 10 slices and RNA eluted from each gel slice. Reverse transcription and PCR using telomerase RNA specific primers showed that telomerase RNA was present in gel slice number 3 that contained the mobility shift complex in the presence of primer (Fig. 4*B*). Telomerase RNA was not detected at this position in the gel when telomerase extracts were not incubated with the ^{32}P-d(TTGGGG)$_3$ probe. Telomerase RNA was also detected near the wells of the mobility shift gel, possibly as a result of enzyme that did not enter the acrylamide gel (Fig. 4*B*). Thus, the presence of telomerase RNA in the gel section containing the (TTGGGG) binding complex suggests that this complex represents telomerase.

UV Cross-linking of Telomerase Products to Potential Protein Components—To identify the protein components present in the telomerase complex, we generated a photoreactive telomeric oligonucleotide probe containing the TTP analog, N_3RdUTP (Bartholomew *et al.*, 1990, 1991). We first attempted to directly bind telomerase to the ^{32}P-labeled N_3RdUTP-containing DNA, and then cross-link the complex. However, using the mobility shift assay it was apparent that telomerase would not bind a probe that contained N_3RdUTP (data not shown). We therefore took advantage of the ability of telomerase to incorporate the photoreactive TTP analog, N_3RdUTP, during the elongation of telomeric primers. In this assay, the generation of a radioactive, cross-linkable primer depends upon the action of telomerase to incorporate the N_3RdUTP and [^{32}P]dGTP onto unlabeled d(TTGGGG)$_3$ primer. Upon irradiation of the telomerase reaction with UV light, three bands at approximately 100, 50, and 25 kDa were apparent (Fig. 5). Only the 100-kDa cross-linked protein was specific to the presence of telomerase elongation products. Telomerase extracts that were inactive, either through ribonuclease treatment or omission of telomeric primer, did not generate a 100-kDa band. The cross-linked protein at 100 kDa was seen only upon exposure to UV, whereas the two smaller proteins were labeled even in the absence of UV irradiation (Fig. 5). Proteinase K treatment either before primer elongation or just prior to UV irradiation abolished the signal at 100 kDa indicating that this band is a protein (data not shown).

Using telomerase purified over 4 steps, DEAE, phenyl-Sepharose, heparin-agarose, spermine-agarose, and concentrated on a DEAE column, we compared the elongation of different telomeric and non-telomeric primers in the UV cross-linking assay. Oligonucleotides that showed significant incorporation of N_3RdUTP and [^{32}P]dGTP generated a labeled 100-kDa protein, for example, d(GGGGTT)$_3$ and d(TTGGGG)$_3$ (Fig. 6). Oligonucleotides which competed for elongation activity, such as oligo 3 and to a lesser extent oligo 8, also competed for labeling of the 100-kDa cross-linked protein. In contrast, oligonucleotides which were not efficiently elongated in the presence of N_3RdUTP, such as pBR and pBRG$_4$, showed little or no detectable cross-linked protein at 100 kDa (Fig. 6*B*). Preincubation of extracts with the non-telomeric oligonucleotides, pBR and an 18-base oligo(dT), d(T)$_{18}$, prior to d(G$_4$T$_2$)$_3$ addition did not compete for cross-linking to the 100-kDa protein (Fig. 6*B*). In extracts preincubated with the pBR oligonucleotide, both the elongation products and the 100-kDa cross-link were enhanced, suggesting that pBR is competing for nonspecific single-stranded DNA binding activity in the extract (Fig. 6*B*).

In this assay the generation of radiolabeled product for cross-linking depends on the presence of telomerase activity, thus the 100-kDa protein was not labeled when fractions were preincubated with RNase (Fig. 6). To determine whether the cross-linking of the 100-kDa protein to the N_3RdUTP probe is RNA-dependent, we carried out a telomerase reaction and then subsequently treated with RNase before UV cross-linking. Incubation with ribonuclease after the telomerase elongation reaction had no effect on the synthesized products yet labeling of the 100-kDa cross-linked protein was reduced (Fig. 6, *A* and *B*). Thus primer binding may require the integrity of the telomerase complex.

Co-purification of the 100-kDa Species with Telomerase—To examine the co-localization of the 100-kDa cross-linkable protein with telomerase, enzyme purified over DEAE-agarose, phenyl-Sepharose, spermine-agarose, DEAE concentration, and sucrose gradient sedimentation was assayed by UV cross-linking (Fig. 7). Upon sucrose gradient sedimentation, long telomerase elongation products with N_3RdUTP incorporated peaked in fractions 4 and 5 and the 100-kDa cross-linked species was also present in fractions 4 and 5 (Fig. 7, *A* and *B*).

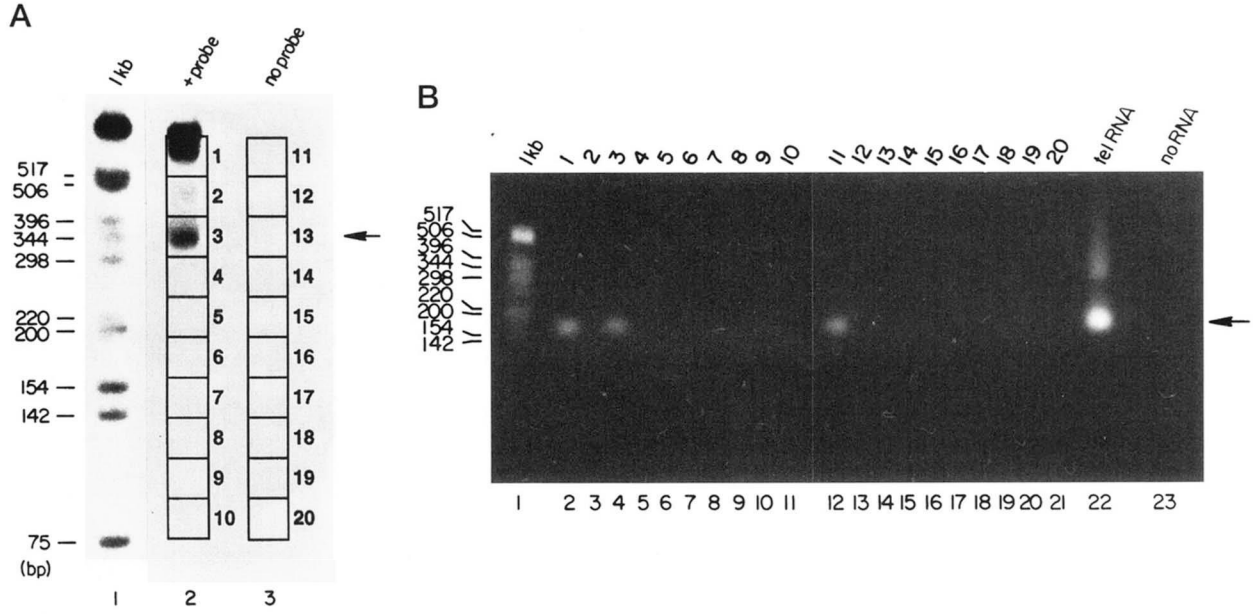

FIG. 4. **Telomerase RNA is present in the shifted complex.** *A*, a mobility shift gel was run and each lane was cut into 10 sections to determine the position of telomerase RNA in the gel. *Lane 1*, ^{32}P-labeled 1-bp marker, with base pairs indicated at the *left*. *Lane 2*, partially purified telomerase (200-fold) was incubated under standard conditions with ^{32}P-d(TTGGGG)$_3$, and electrophoresed on a 6%, w/v, nondenaturing acrylamide gel. *Lane 3*, telomerase extract was incubated with all components of the mobility shift mixture except the probe. The wet gel was wrapped and briefly exposed on a PhosphorImager imaging plate to visualize the telomerase complex, and both lanes were divided into several gel fragments, *1–10* for *lane 2* (gel slice number 3 contained the telomerase complex and is indicated at *right* with an *arrow*), and *11–20* for *lane 3*. *B*, the products of reverse transcriptase-PCR using telomerase RNA-specific primers for each gel slice are shown after electrophoresis on a 1%, w/v, agarose gel and stained with ethidium bromide. *Lane 1*, 1-bp marker, with base pairs as indicated at the *left*. *Lanes 2–11*, gel slices 1–10, corresponding to *lane 2* in *A*. *Lanes 12–21*, gel slices 11–20, corresponding to *lane 3* in *A*. *Lane 22*, 100 pg of *in vitro* transcribed telomerase RNA used as a positive control in the PCR reaction. The position of the expected product is indicated to the *right* with an *arrow*. *Lane 23*, as a negative control, no RNA added in the reverse transcriptase-PCR reaction.

SDS gel analysis of these sucrose gradient fractions showed that a band of approximately 100 kDa was present in the fraction that contained the 100-kDa cross-linked protein. Because it is not possible to quantitate the amount of protein that is cross-linking with the reagents currently available, we cannot determine if the 100-kDa protein seen by silver staining is the same protein that cross-links to the telomerase products. A number of other polypeptides were also present in the active gradient fractions. Quantitative estimates of the fold purification indicate that telomerase is not purified to homogeneity in the fractions (data not shown). Thus, from the available data, we cannot yet conclude that the 100-kDa band seen by silver staining is a telomerase component. Further purification of telomerase is currently underway.[2]

To examine whether the 100-kDa cross-link co-purified with telomerase using a different purification, we developed an affinity purification for telomerase. Biotinylated telomeric oligonucleotides were tested for their ability to precipitate telomerase activity onto streptavidin-agarose. Telomerase fractions purified over a sizing column, heparin-agarose, and DEAE-agarose were incubated with a biotinylated 48-base telomeric oligonucleotide, d(TTGGGG)$_8$, followed by precipitation of the extract onto streptavidin-agarose. Using this procedure up to 25% of telomerase activity was recovered on the agarose beads (Harrington, 1993). The agarose beads containing the affinity-purified telomerase activity were incubated with telomeric primer, N_3RdUTP, [^{32}P]dGTP, and subjected to UV cross-linking. SDS-PAGE and autoradiography of the affinity-purified, cross-linked extracts also showed radiolabeling of a 100-kDa protein (Fig. 8). Similar to the extracts purified using standard chromatography, cross-linking of the 100-kDa protein was dependent on UV treatment and the generation of telomerase reaction products (Fig. 8). The 100-kDa protein was also labeled in the absence of added d(GGGGTT)$_3$ primer (Fig. 8, *lane 2*), since telomerase could elongate the primer bound to streptavidin-agarose, d(TTGGGG)$_8$ (data not shown).

DISCUSSION

Telomerase Primer Binding Specificity—Telomerase is an unusual DNA polymerase in that it carries the template sequence for telomere repeat synthesis as an essential RNA component. Although the enzyme copies RNA into DNA, like a reverse transcriptase, the telomerase mechanism is unique since only six nucleotides of the template are copied and telomeric primers are specifically elongated. Telomerase elongates telomeric sequences, whereas non-telomeric primers are very poor substrates (Blackburn *et al.*, 1989; Greider and Blackburn, 1987; Harrington and Greider, 1991). The primer elongation specificity may lie in the initial binding of telomerase to primer substrates or in the ability to elongate primers once they are bound, or both. To distinguish between these two steps, binding and elongation, we sought an assay which would directly measure binding of primer oligonucleotides. We identified a telomeric primer d(TTGGGG)$_3$-specific complex using a mobility shift assay that was competed by several G-rich oligonucleotides. The specificity of primer binding reflects that of primer elongation, suggesting that elongation specificity is in part at the level of binding. The ability of telomerase to specifically bind telomeric primers was distinct from elongation, since binding occurred in the absence of nucleotides and Mg^{2+}. This property is similar to those of other RNPs, such as RNase P, which require Mg^{2+}, and nucleotides only during catalysis, and not for substrate recognition (Smith and Pace, 1993). It is not yet clear whether the complex we have identified is a true "preinitiation" complex that is competent for elongation upon addition of nucleotides.

Specificity at Both Anchor and Template Sites—Current

[2] K. Collins and C. Greider, unpublished results.

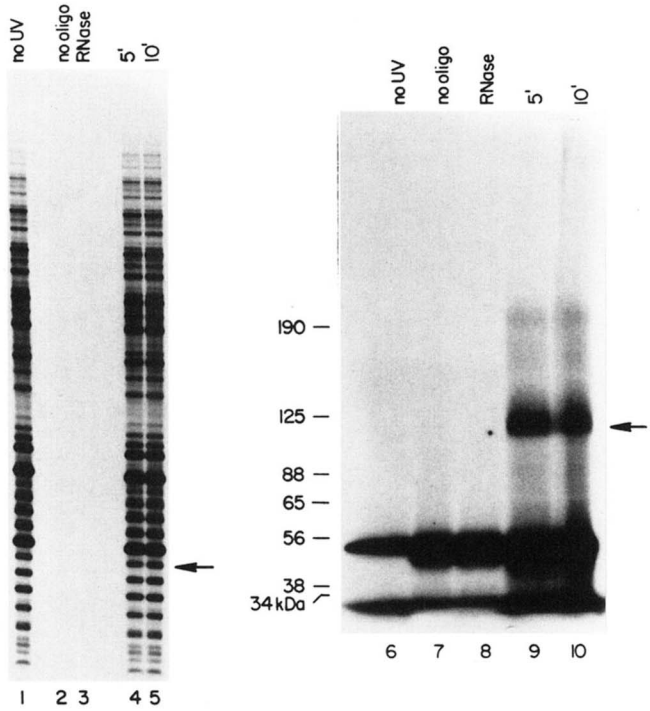

FIG. 5. **Cross-linking of a 100-kDa protein to telomerase products.** Telomerase elongation activity was assayed in the presence of [^{32}P]dGTP and N_3RdUTP. *Lane 1*, standard elongation assay; *lane 2*, telomeric primer was omitted from the reaction; *lane 3*, the telomerase extract was pretreated with 10 units of ribonuclease ONE for 10 min on ice prior to the elongation assay; *lanes 4* and *5*, standard assay conditions as in *lane 1*. The position of the input primer $d(G_4T_2)_3$ is indicated at the *bottom right* by an *arrow*. Aliquots of the same samples in *lanes 1–5* were exposed to UV light and resolved by SDS-PAGE. *Lanes 7, 8,* and *10*, 10-min UV treatment. *Lane 9*, 5-min UV treatment. The 100-kDa cross-linked protein is indicated at the *right* by an *arrow*; protein standards are at the *left* in kDa.

models for telomerase primer binding suggest that there are two distinct sites on telomerase for binding primer oligonucleotides (Collins and Greider, 1993; Harrington and Greider, 1991; Lee and Blackburn, 1993; Morin, 1991). The template region CAACCCCAA plays a role in aligning the 3′ end of primer oligonucleotides (Autexier and Greider, 1994). A second site for primer binding, the anchor site, possibly on a protein component, is required for processive elongation of bound primers (Collins and Greider, 1993). How each of these sites determines primer specificity has not yet been determined. Telomerase is not restricted to elongating $d(TTGGGG)_n$ primers. Oligonucleotides containing the telomere sequence of other eukaryotes such as d(TTAGGG), d(TTTTGGGG), d(TGGGTGTG), and even the sequence $(TG)_9$, not found at telomeres in nature, are elongated (Blackburn *et al.*, 1989; Greider and Blackburn, 1987). These data suggest that the role of the RNA CAACCCCAA sequence in primer binding is not strictly through base pairing. The primer $d(TG)_9$ will only form two base pairs with the template region. In addition, the anchor site, which may play an important role in primer recognition, must not have strict specificity for d(TTGGGG) in the way that many DNA-binding proteins do. The results presented here suggest that telomerase binding primer specificity matches primer elongation specificity. Thus binding may play a key step in determining what substrates are elongated.

The competition for primer binding in the gel shift and cross-linking assays using oligonucleotides complementary to telomerase RNA also suggests two possible modes for primer binding. The oligonucleotides oligo 3 and oligo 8 are both elongated by telomerase. When oligo 3, which binds adjacent to and covers the template region, is preincubated with telomerase it inhibits subsequent elongation of d(TTGGGG), while preincubation with oligo 8 which hybridizes adjacent to the template does not (Greider and Blackburn, 1989). Consistent with this, in the gel shift and cross-linking studies oligo 3 competed for $d(TTGGGG)_3$ binding, but oligo 8 did not compete efficiently. The ability of oligo 3 to compete for telomeric primer binding could occur at either the anchor site or RNA template since oligo 3 contains the sequence TTGGGGTTG at its 3′ end. Oligo 8, however, is probably elongated solely by hybridizing adjacent to the CAACCCCAA template.

In vivo, telomerase will add $(TTGGGG)_n$ repeats onto AT-rich DNA with no pre-existing telomeric sequence (Yu and Blackburn, 1991). This specificity is clearly different than that seen *in vitro*. Binding to nontelomeric sequences was not detectable in the gel shift assay. There may be other factors in the cell which associate with telomerase to facilitate the recognition of new or broken chromosome ends, which do not co-purify with telomerase activity *in vitro*, or which are expressed only at specific times in the *Tetrahymena* life cycle.

Telomerase Has Properties Similar to Other Polymerases—The proposal for two distinct binding sites for the elongating chain in telomerase is based on the two-site model for RNA binding during elongation by RNA polymerase ((Collins and Greider, 1993) reviewed in Chamberlin (1993)). The polymerase moves in saltatory steps along the template and at each translocation the growing chain is fed from the template site (site 1) to the anchor site (site 2). The crystal structure has been solved for T7 RNA polymerase, Klenow fragment, human immunodeficiency virus reverse transcriptase, and DNA polymerase β (Kohlstaedt *et al.*, 1992; Pelletier *et al.*, 1994). Each of these polymerases has a separate site for polymerization and binding of the elongating chain. The overall arrangement of these sites is remarkably conserved (reviewed in Joyce and Steitz (1994) and Pelletier *et al.* (1994)). The identification of a primer specific cleavage activity further supports this analogy between telomerase and RNA polymerase (Collins and Greider, 1993). The band shift complex identified in this study is analogous to the ternary complexes of RNA polymerase, RNA and DNA template isolated on native gels (Hagler and Shuman, 1993). Using this assay it may be possible to physically isolate intermediates in the telomerase elongation reaction.

Polymerase interactions with primer and template have been studied extensively using gel mobility shift assays. Kinetic parameters of primer and template binding (Hsieh *et al.*, 1993), analysis of DNA binding specificities (Ng *et al.*, 1993), identification of auxiliary factors (Flores *et al.*, 1992), and mutational analysis of amino acids involved in primer and template binding (Date *et al.*, 1991; Joung and Engler, 1992) have been defined using this assay. Having extensively characterized the telomerase primer complex, we can now use this assay to address the mechanism by which telomerase recognizes primer substrates. In addition, the initial binding affinity and the initial rate of nucleotide addition can be separately compared for a wide variety of telomeric and non-telomeric primers.

Identification of a Potential Telomerase Subunit by UV Cross-linking—Several observations indicate that a protein of approximately 100 kDa may be a subunit of the telomerase enzyme. First, cross-linking of this protein in our assays depended on UV treatment, telomerase activity, $d(GGGGTT)_3$ primer, N_3RdUTP, and [^{32}P]dGTP. Second, preincubation with the nonspecific oligonucleotide pBR prior to telomeric primer addition enhanced the 100-kDa cross-link, whereas a nonspecific protein might be competed by excess oligonucleotide. Third, labeling of the 100-kDa protein was sensitive to ribonuclease treatment of the telomerase complex after generation of

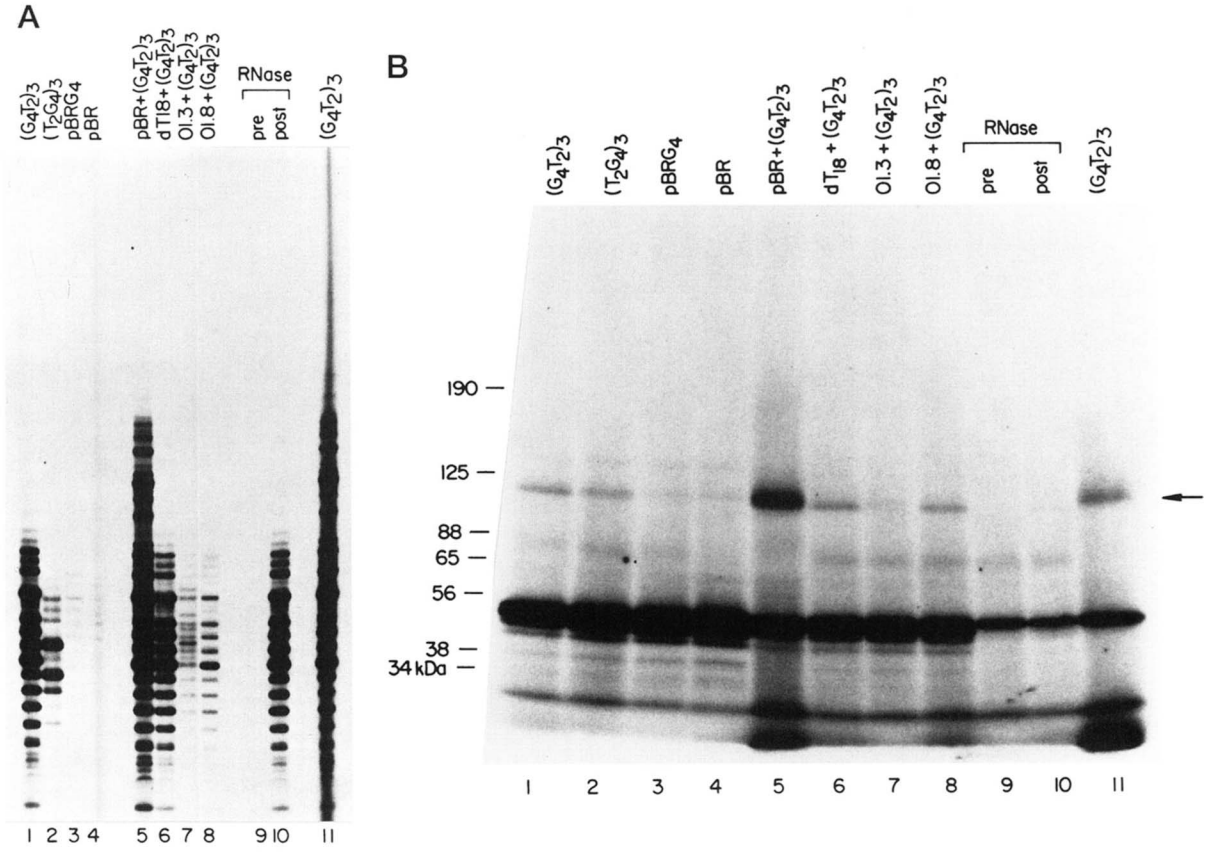

FIG. 6. **Specificity of the 100-kDa cross-link is similar to telomerase primer specificity.** *A: lanes 1–4,* telomerase was reacted with 0.5 μg of the primer indicated above each lane. *Lanes 1* and *2*, telomerase elongation products using the primers $d(G_4T_2)_3$ and $d(T_2G_4)_3$, respectively. *Lanes 3* and *4*, elongation of $pBRG_4$ and pBR oligonucleotides, respectively. *Lanes 5–8*, samples were preincubated with 1.0 μg of the oligonucleotides pBR, oligo $d(T)_{18}$, oligo 3, and oligo 8, respectively, prior to the addition of telomeric primer $d(G_4T_2)_3$ (0.5 μg) and reaction mixture. *Lane 9*, telomerase was preincubated with 2 units of RNase ONE (Promega) prior to the elongation assay. *Lane 10*, telomerase was incubated with 2 units of RNase ONE immediately following the elongation assay, prior to UV cross-linking. *Lane 11*, elongation of $d(G_4T_2)_3$ in the presence of 5 mM EDTA and 2 mM $MgCl_2$. *B,* aliquots of the same samples as in *A* were exposed to UV light for 5 min and analyzed by SDS-PAGE. The position of the 100-kDa cross-linked protein is indicated with an *arrow* at the *right*. Protein markers are indicated in kDa at the *left*.

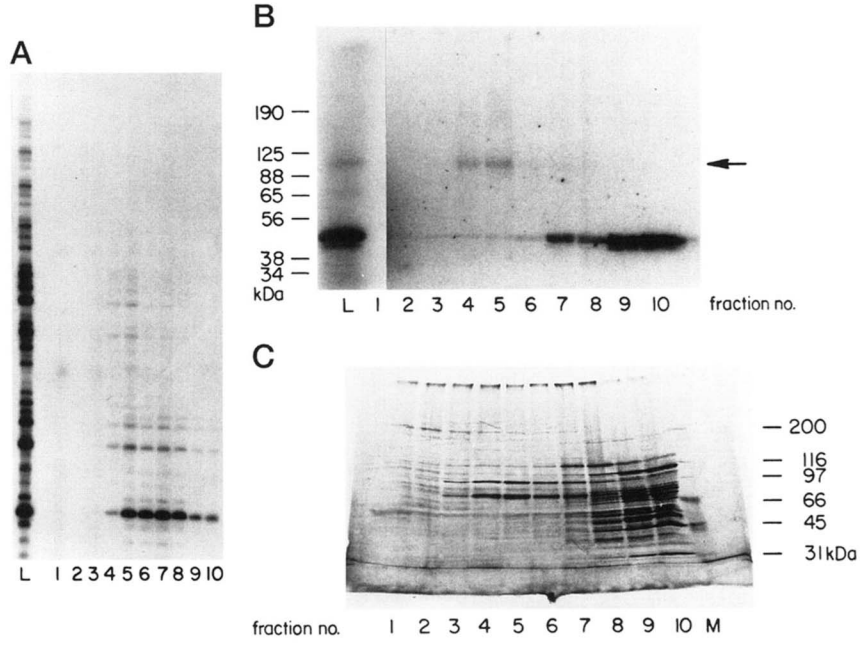

FIG. 7. **Sedimentation of 100-kDa cross-linked protein with telomerase.** *A*, as the last step in the large scale telomerase purification (see "Experimental Procedures"), telomerase extract was sedimented on a 7–30%, w/v, sucrose gradient, and fractions were collected from the bottom to the top of the gradient. This gradient is the same as shown in Fig. 2*B*. For this experiment, each fraction was assayed in the presence of N_3RdUTP, and the DNA products were resolved on a 10%, w/v, denaturing acrylamide gel. *B,* the same sucrose gradient samples as shown in *A* were cross-linked, and resolved by SDS-PAGE. The position of the 100-kDa cross-linked protein is indicated at the *right* with an *arrow*, and molecular weight standards are shown at the *left* in kDa. *C*, approximately one-twentieth of each fraction from the sucrose gradient was loaded onto a SDS-PAGE gel. The fraction numbers are indicated at the *bottom*. *M* indicated the protein size standards.

the ^{32}P-labeled N_3RdUTP-containing DNA, suggesting this protein is part of an RNP complex. Fourth, the 100-kDa cross-linked protein was present in sucrose gradient fractions and affinity purified fractions that contained extensively purified telomerase activity. One limitation of the cross-linking approach taken here is that telomerase activity itself is required to generate the products that cross-link to the 100-kDa protein. The cross-link is present in fractions with telomerase activity

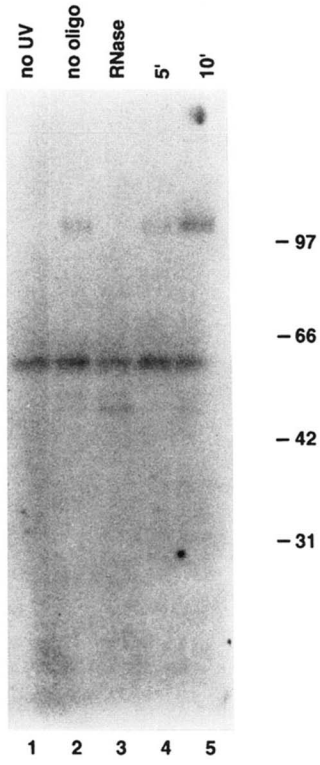

FIG. 8. **Affinity purification and cross-linking of telomerase.** Telomerase extracts were purified to approximately 1000-fold by affinity chromatography, and samples were assayed for elongation and cross-linking. *Lane 1*, no UV treatment. *Lane 2*, no added $d(G_4T_2)_3$ primer. *Lane 3*, affinity-purified extracts were incubated with approximately 10 μg of ribonuclease A prior to the telomerase elongation assay. *Lanes 2* and *3* were exposed to UV light for 10 min. *Lanes 4* and *5*, telomerase elongation products were exposed to UV light for 5 and 10 min, respectively.

simply because only those fractions that contain telomerase activity can synthesize the cross-linkable products. To avoid this problem we attempted to first generate ^{32}P-labeled N_3RdUTP-containing DNA and then to bind it to telomerase for cross-linking. However, gel shift experiments using d(TTGGGG) probes substituted with N_3RdUTP indicated that telomerase would not bind these probes. Although the polymerase activity of telomerase will incorporate the analog, the initial binding of the primer is inhibited. The anchor site on telomerase may be unable to accommodate the large bulky arylazide group present in the N_3RdUTP.

If the 100-kDa protein is a telomerase component, the sedimentation of telomerase between 200 and 400 kDa in sucrose gradients suggests that other polypeptides in these fractions may also be telomerase components, but are not cross-linked during elongation (Harrington, 1993). Further purification is underway to unambiguously define the polypeptides that make up the telomerase RNP and to clone the genes for these subunits.[2] Once the telomerase protein component(s) are cloned we can use the band shift assay to define the contribution of the protein to the primer binding specificity.

Acknowledgments—We thank Winship Herr and Bruce Stillman for helpful advice and the following people for critical reading of the manuscript: Winship Herr, Bruce Stillman, Erich Grotewold, Brenda Andrews, Mike Tyers, Kathleen Collins, Stephanie Smith, and Chantal Autexier. We also thank Jim Duffy, Phillip Renna, and Mike Ockler for photography and artwork.

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