Tetrahymena Telomerase Protein p65 Induces Conformational Changes throughout Telomerase RNA (TER) and Rescues Telomerase Reverse Transcriptase and TER Assembly Mutants†

Andrea J. Berman  
*University of Colorado Boulder*

Anne R. Gooding  
*University of Colorado Boulder*

Thomas R. Cech  
*University of Colorado Boulder, thomas.cech@colorado.edu*

Follow this and additional works at: [http://scholar.colorado.edu/chem_facpapers](http://scholar.colorado.edu/chem_facpapers)  
Part of the [Biochemistry Commons](http://scholar.colorado.edu/chem_facpapers), and the [Molecular Biology Commons](http://scholar.colorado.edu/chem_facpapers)

**Recommended Citation**  
[http://scholar.colorado.edu/chem_facpapers/22](http://scholar.colorado.edu/chem_facpapers/22)

This Article is brought to you for free and open access by Chemistry & Biochemistry at CU Scholar. It has been accepted for inclusion in Chemistry & Biochemistry Faculty Contributions by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
Transcriptase and TER Assembly Mutants Rescue Telomerase Reverse Transcriptase and TER Assembly Mutants

Andrea J. Berman, Anne R. Gooding, and Thomas R. Cech

Published Ahead of Print 16 August 2010.

Updated information and services can be found at:
http://mcb.asm.org/content/30/20/4965

These include:

SUPPLEMENTAL MATERIAL
Supplemental material

REFERENCES
This article cites 48 articles, 31 of which can be accessed free at:
http://mcb.asm.org/content/30/20/4965#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Tetrahymena Telomerase Protein p65 Induces Conformational Changes throughout Telomerase RNA (TER) and Rescues Telomerase Reverse Transcriptase and TER Assembly Mutants

Andrea J. Berman, Anne R. Gooding, and Thomas R. Cech*
Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received 16 July 2010/Accepted 1 August 2010

The biogenesis of the Tetrahymena telomerase ribonucleoprotein particle (RNP) is enhanced by p65, a La family protein. Single-molecule and biochemical studies have uncovered a hierarchical assembly of the RNP, wherein the binding of p65 to stems I and IV of telomerase RNA (TER) causes a conformational change that facilitates the subsequent binding of telomerase reverse transcriptase (TERT) to TER. We used purified p65 and variants of TERT and TER to investigate the conformational rearrangements that occur during RNP assembly. Nuclease protection assays and mutational analysis revealed that p65 interacts with and stimulates conformational changes in regions of TER beyond stem IV. Several TER mutants exhibited telomerase activity only in the presence of p65, revealing the importance of p65 in promoting the correct RNP assembly pathway. In addition, p65 rescued TERT assembly mutants but not TERT activity mutants. Taken together, these results suggest that p65 stimulates telomerase assembly and activity in two ways. First, by sequestering stems I and IV, p65 limits the ensemble of structural conformations of TER, thereby presenting TERT with the active conformation of TER. Second, p65 acts as a molecular buttress within the assembled RNP, mutually stabilizing TERT and TER in catalytically active conformations.

The catalytic core of the telomerase RNP is comprised of the protein reverse transcriptase TERT (for telomerase reverse transcriptase) and the RNA component TER (for telomerase RNA). TERT uses a region of TER that is complementary to the telomere DNA sequence to template the synthesis of telomeric repeats onto a single-stranded telomeric DNA primer (15). Like most reverse transcriptases, telomerase copies its template without dissociation, a phenomenon known as nucleotide addition processivity (NAP). In addition, telomerase is unique among reverse transcriptases in that it also undergoes repeat addition processivity (RAP), during which an already-extended primer is repositioned within its active site so the same intrinsic RNA sequence templates the addition of several telomeric repeats (14, 23).

Besides providing the template for telomere synthesis, TER primary, secondary, and tertiary structural elements play critical architectural and functional roles (34, 37, 39, 44, 45). In Tetrahymena thermophila TER (Fig. 1A), the base of stem II and part of the predicted single-stranded RNA on either side of stem II, called the template boundary element or the TERT-binding element (TBE), have been shown to be important for both TERT binding and delineating the 5′ boundary of the template sequence during synthesis (4, 18). The template recognition element (TRE), a single-stranded region of TER that is located 3′ of the template, has been shown to be involved in maintaining NAP and essential for RAP (4, 25). Just 3′ of the TRE is the pseudoknot, an interaction between stem-loop III and nucleotides preceding it, which contributes to NAP, RAP, RNA stability, and RNP assembly (12, 34, 39, 43, 44). The TERT secondary structure continues 3′ of the pseudoknot to complete stem I through long-range base pairing with the nucleotides at the 5′ end of TER. Stem I is connected through a linker to stem-loop IV, which enhances NAP and RAP, potentially through its interaction with TERT (17). Since TER in ciliated protozoa is an RNA polymerase III transcript, there is an oligo(U) tail at the 3′ end of the molecule that makes a small contribution to RNP assembly (3).

Telomerase holoenzymes contain other components besides TERT and TER, including both transiently and stably associated proteins (27, 31, 40). The initial purification of telomerase identified TER, TERT, and p43 in Euplotes aediculatus (22). p43 is a telomerase-specific protein containing a La motif and an RNA recognition motif (RRM); it binds primarily to stem-loop IV, which enhances NAP and RAP, potentially through its interaction with TERT (17). Since TER in ciliated protozoa is an RNA polymerase III transcript, there is an oligo(U) tail at the 3′ end of the molecule that makes a small contribution to RNP assembly (3).

The telomerase holoenzyme from T. thermophila contains a 65-kDa protein (p65) that is the ortholog of Euplotes p43 (47). p65, encoded by an essential gene, copurifies with TER, TERT, and telomerase activity, stimulates holoenzyme biogenesis, and stabilizes TER levels (33, 47). Homologs of p43/p65 have not been found outside the ciliates, but the complex of dyskerin, NHP2, NOP10, and GAR1 (11, 28, 32, 46) may be the functional analog in mammalian telomerase. These proteins are not telomerase specific but are components of small nucleolar RNPs (snoRNPs) (16). Despite the presence of the La motif in p65 and an oligo(U)
with TER beyond the established binding site of stems I and IV. Through a combination of electrophoretic mobility shift assays, nucleic acid structure probing experiments, and telomerase assays, we have determined that p65 stimulates holoenzyme formation by directly and indirectly promoting the proper folding of the entire TER structure, suggesting that p65 is a TER-specific RNA folding protein. We also have shown that the previously observed modest stimulation of telomerase activity by p65 becomes much more substantial in the context of TER and TERT mutations; several highly deleterious TER and TERT assembly mutants can be rescued to near-wild-type activity with p65, and in fact, p65 has emerged as a tool for sorting assembly and activity mutants of TER and TERT. Finally, these results suggest that p65 stabilizes the functional interactions between TERT and TER that are required for the telomerase reaction cycle.

**MATERIALS AND METHODS**

**Protein purification.** The gene expressing p65 used in these studies was re-cloned from pET28-HisTEV-p65(1-542) into pET28, giving the full-length p65 protein an N-terminal His6 tag followed by a thrombin cleavage sequence. E. coli BL21(DE3) cells transformed with HisThrombin-p65(1-542) were grown in LB supplemented with 30 μg/ml kanamycin at 37°C until an optical density at 600 nm of 0.4 to 0.6 was reached. The temperature was then decreased to 18°C for 30 min prior to induction with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 to 6 h. Cells were harvested and stored at −80°C.

Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 10% glycerol) with Complete EDTA-free protease inhibitor tablets (Roche). Prior to lysis by sonication, cells were incubated with lysosome at a final concentration of 5 mg/ml for 40 to 60 min. Lysate then was clarified and passed over a 5-ml HiTrap column (GE). Protein was dialyzed overnight to remove first the imidazole and then the NaCl concentration to 100 mM, loaded onto a HiTrap Q column (GE), and eluted with a 125-ml gradient from 100 mM to 1 M NaCl. The p65-containing fractions were pooled; concentrated; buffer exchanged into 20 mM HEPES, pH 7.5, 250 mM NaCl, 2 mM DTT; and loaded onto a gel filtration column (Sephadex 200; GE). The peak then was concentrated and stored in the presence of 5% glycerol at −80°C. The concentration of active p65 (typically 50 to 70%) was determined by the titration of 50 nM 32P-labeled TER RNA and analyzed by electrophoretic mobility shift assay (EMSA).

**TERT-N (amino acids 1 to 516)** was subcloned from mp190 (8) and inserted into the NcoI and BamHI sites of vector pET28a (Novagen), resulting in pET28-TERT516, which has a His6 tag followed by the first residue of TERT. This construct used for the RNA binding domain of TERT, RBD (amino acids 216 to 516), was a slightly smaller domain than that used in previous studies (18, 19, 29, 30) but had comparable TER and p65-TER binding affinity (C. O'Connor and K. Collins, unpublished results). This construct also was subcloned from mp190 (8) and inserted into the NdeI and BamHI sites of vector pET28a (Novagen), resulting in a His6 tag followed by a thrombin site and amino acids 216 to 516 of TERT.

An overnight culture of BL21(DE3) cells transformed with pET28-TERT516 or RBD was grown at 37°C and used to inoculate a 100-ml LB culture. This starter culture then was used to inoculate 1-liter cultures of LB (1:100) supplemented with 30 μg/ml kanamycin. These cultures then were grown until an optical density at 600 nm of 0.4 was reached. The temperature then was decreased to 18°C for 30 min prior to induction with a final concentration of 0.1 mM IPTG for 16 h. Cells were harvested and stored at −80°C.

We set out to determine what other roles p65 plays in RNP biogenesis besides inducing the conformational change within stem IV. Specifically, we asked whether p65 interacts...
Cells expressing TERT-N were resuspended in lysis buffer with Complete EDTA-free protease inhibitor cocktail tablets (Roche). The purification proceeded in the same manner as the purification of p65 through the first nickel column. The pooled fractions of TERT-N from the nickel column then were diluted with 50 mM MES, pH 6.5, 2 mM DTT to bring the concentration of NaCl to 250 mM. After pelleting any protein that precipitated, the sample was loaded onto tandem HiTrapS/HITrapS (GE) columns. The protein then was eluted off the HiTrapS column with a gradient from 250 mM to 1 M NaCl over 100 ml. Fractions containing TERT-N were concentrated; buffer exchanged into 20 mM HEPES, pH 7.5, 250 mM NaCl, 2 mM DTT; and loaded onto a gel filtration column (Superdex 200; GE). The peak then was concentrated and stored in the presence of 5% glycerol at −80°C. The concentration of active TERT (typically 30%) was determined by a titration of 50 nM 5′-end-labeled TER RNA and analyzed by EMSA.

The protein purification protocol for RBD was similar to that for p65, except the peak fractions from the second nickel column were diluted with 50 mM MES, pH 6.5, 2 mM DTT to decrease the concentration of NaCl to 100 mM. This diluted solution was loaded onto a HiTrapS column and eluted with a gradient of 100 mM to 1 M NaCl. The sample then was concentrated and buffer exchanged into 20 mM HEPES, pH 7.0, 250 mM NaCl, 2 mM DTT prior to gel filtration (Superdex 75; GE). The peak then was concentrated and stored in the presence of 5% glycerol at −80°C. The concentration of active RBD (typically 30%) was determined by the titration of 50 nM 5′-end-labeled TER RNA and analyzed by EMSA.

RNA preparation. RNA was synthesized in vitro using T7 RNA polymerase for the runoff transcription of EarI-digested plasmids (pTET ELO) containing the template for TER (48). EarI digestion results in a DNA template encoding four Us at the 3′ end of the TER. All TER mutants were generated using the OpenBase site-specific mutagenesis kit QuikChange (Strategen) with pTET ELO as the template. The construct for the circularly permuted RNA (cpRNA) was generated by two rounds of PCR using ptetTELO as the template and then inserted into the runoff transcription of EarI-digested plasmids (ptetTELO) containing the template. The construct for the circularly permuted RNA (cpRNA) was generated by two rounds of PCR using ptetTELO as the template and then inserted into the runoff transcription of EarI-digested plasmids (ptetTELO) containing the template. The construct for the circularly permuted RNA (cpRNA) was generated by two rounds of PCR using ptetTELO as the template and then inserted into the runoff transcription of EarI-digested plasmids (ptetTELO) containing the template. The construct for the circularly permuted RNA (cpRNA) was generated by two rounds of PCR using ptetTELO as the template and then inserted into the runoff transcription of EarI-digested plasmids (ptetTELO) containing the template.

RNA was 5′ end labeled with [α-32P]ATP using T4-poly nucleotide kinase (NEB). After labeling, unincorporated nucleotide was removed with Illustra G-25 spin columns (GE) prior to 6% polyacrylamide–7 M urea–100 mM to 1 M NaCl. The sample then was concentrated and buffer exchanged into 20 mM HEPES, pH 7.0, 250 mM NaCl, 2 mM DTT prior to gel filtration (Superdex 75; GE). The peak then was concentrated and stored in the presence of 5% glycerol at −80°C. The concentration of active RBD (typically 30%) was determined by the titration of 50 nM 5′-end-labeled TER RNA and analyzed by EMSA.

EMSA was performed as previously described (33). Briefly, reactions were carried out in 20 μl containing 0.33 mM [α-32P]dUTP, 0.1 mM dTTP, 9.67 mM dGTP, 1 mM oligonucleotide Tel3a ([dGdGd]$_n$); and 10 μl of diluted telomerase beads. Reaction mixtures were incubated at 30°C for 1 h and then stopped with stop buffer containing 3.6 M sodium acetate, pH 5.2, 1 μl glycerol, and 5,000 counts of a recovery control (a 5′-end-labeled and gel-purified 63-mer DNA oligonucleotide of random sequence). Samples then were ethanol precipitated, resuspended in 20 μl 1× formamide loading buffer, and heated to 95°C for 5 min. Eight microliters of each sample was loaded onto 10% polyacrylamide (40:1)–7 M urea–1× Tris-borate-EDTA sequencing gels. Gels were run at 90 W for 1.75 h, dried, and exposed to storage phosphor screens. Screens were scanned using a Typhoon Trio (GE) and analyzed using ImageQuant TL (GE).

The telomerase assays shown in Fig. 2D were performed with a modified protocol. In these assays, STEV was added at 20 times the indicated final concentrations. In the samples that received p65-stem IV, 10 μM p65 was prebound to stem IV at a 20-fold level of the indicated final concentration.

Structure-probing assays. Nuclease digestion was performed in several buffer conditions (see below). While buffer conditions affected the intensities of the nucleic digestion patterns, the reported cleavage and protection patterns generally were consistent among multiple conditions.

RNase T1 assays. RNase T1 (Ambion) digestions were performed on either trace amounts of 5′-end-labeled TER RNA or with 100 nM cold TER RNA spiked with a trace amount of 5′-end-labeled TER RNA. Digests typically were performed in 10 mM Tris-HCl, pH 7.0, 100 mM KCl, 10 mM MgCl$_2$, 100 ng/ml yeast tRNA, and either 1× binding buffer, 50 to 500 nM p65 (active concentration), 50 to 250 mM TERT-N (active concentration), or both proteins. Other buffer conditions tested include the buffer used by Bhattacharyya and Blackburn (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl$_2$) (6), selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) buffer (100 mM HEPES, pH 8.0, 100 mM NaCl, 6.7 mM MgCl$_2$), and high-salt buffer (25 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM MgCl$_2$, 5% glycerol). Proteins and RNA were incubated at room temperature for 5 to 30 min before the addition of T1 nuclease. T1 nuclease was added at 0.05, 0.025, or 0.013 U/μl and allowed to digest for 10 min at room temperature. Digests then were ethanol precipitated, resuspended, and counted in a scintillation counter. Equal counts for each were loaded onto a 12% polyacrylamide sequencing gel. T1 ladders were prepared by the T1 nuclease digestion of RNA in a denaturing buffer (Ambion).

RNase V1 assays. RNase V1 (Ambion) digestions were performed as for T1, except the buffer contained 0.008, or 0.002, or 0.001 U/μl and the reaction was allowed to proceed for 10 to 15 min at room temperature. RNase V1 assays were performed in 10 mM Tris-HCl, pH 7.0, 100 mM KCl, 10 mM MgCl$_2$, 100 ng/ml yeast tRNA, or the buffer used by Bhattacharyya and Blackburn (6).

RNase ONE assays. RNase ONE (Promega) digestions were performed as for T1, except the buffer contained 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 200 mM sodium acetate, 15 mM magnesium acetate. Another buffer condition was tested in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate. Samples were not precipitated but stopped with 1% SDS and formamide gel-loading dye, and equal volumes were loaded directly onto the 12% sequencing gel.

RESULTS

p65 improves the activity of telomerase by facilitating functional interactions between TERT and TER. To assess the effect of p65 on telomerase RNP formation, we added 5′-end 32P-labeled TER either by itself or prebound to highly purified full-length p65 (Fig. 1A and B) to translations of TERT in rabbit reticulocyte lysates (RRLs). The use of radiolabeled TER facilitated the direct assessment of how much RNA was bound per molecule of TERT synthesized during the transla-
The addition of p65 increased the activity of telomerase 3-fold (3.0 ± 0.3 [mean ± standard error of the mean (SEM)]) (Fig. 1C), which could be partially explained by more efficient complex formation (RNA pulldown was increased 1.4-fold ± 0.3-fold). In the absence of TERT, only trace amounts of TER were recovered from the antibody-coated beads used for the immunopurification of the complex (Fig. 1D). Consistent with an effect on the amount of complex, RAP was not affected by p65 (the \( R_{1/2} \) value, which describes the number of repeats at which half of the primers have dissociated from telomerase [20], was 0.40 ± 0.02 repeats for telomerase and 0.44 ± 0.02 repeats for telomerase assembled in the presence of p65).

It has been proposed that p65 bridges stems I and IV of TER (33). Telomerase activity was drastically reduced (Fig. 2A) when the RNA was broken between stems I and IV (nucleotides 1 to 107 and stem IV) (Fig. 2B). However, the addition of p65 to the translation system resulted in the recovery of robust telomerase activity (Fig. 2A). The p65-mediated restoration of activity was, at least in part, due to the retention in the RNP of stem IV (Fig. 2C), which has been shown to stimulate telomerase activity (5, 24, 41). The ability of p65 to bridge these 5' and 3' half molecules of TER, resulting in the recovery of stem IV during the pulldown of telomerase, was not seen in previously published experiments performed with similar RNA constructs (33).

To test the effect of stem IV on the stimulation of p65-assisted assembly and activity, we synthesized telomerase in the absence of stem IV. RNA containing nucleotides 1 to 107 was tested for telomerase activity in the presence or absence of p65 and with increasing amounts of stem IV. Without p65, the addition of a 500-fold excess of stem IV was required to obtain a modest recovery of activity and repeat addition processivity (Fig. 2D, lane set 1). However, when p65 was present in the translation, activity and RAP were recovered even at very low concentrations of stem IV. While robust activity and RAP were recovered in the presence of p65 at low concentrations of stem IV, an increase in telomerase activity was apparent at higher concentrations of stem IV when p65 was added during the activity assay (compare lane sets 2 and 3 in Fig. 2D), confirming the importance of the order of the addition of the components to the reaction (29). Furthermore, adding excess p65 during the telomerase assay to samples that contained p65 from the translation did not increase telomerase activity, suggesting that the system can be saturated by p65 (compare lane sets 3 and 4 in Fig. 2D).
recovered by coimmunoprecipitation. (C) Telomerase activity as-
ing stems I and IV bound to p65 with high affinity (cpRNA31-25) (25) comprising the 5' terminal cleft of the TERT RT domain.

Surprisingly, stem I was not required for telomerase activity in the presence of p65 (Fig. 3). A circularly permuted RNA (cpRNA31-25) (25) comprising the 5' wheel of TER but missing stems I and IV bound to p65 with high affinity (Kd = 38 nM; Kd stands for dissociation constant) but limited activity; the recovery of processive telomerase activity occurred only when stem IV and p65 were added in trans, presumably because p65 was necessary for binding stem IV in telomerase molecules containing cpRNA (Fig. 3B and C). These results suggest that p65 can bridge stem IV and the central wheel of TER but miss-
ing stems I and IV. (B) Duplicate SDS-PAGE analysis of TERT immunoprecipitates following translations in RRLs in the presence or absence of p65 and the indicated RNA components. The top panel shows the [35S]methionine incorporated into TERT, while the bottom panel shows only the 32P-end-labeled TER components recovered by coimmunoprecipitation. (C) Telomerase activity assays using the indicated components. RC, recovery control.

FIG. 3. p65 interacts with regions of TER outside stems I and IV. (A) Cartoon of the two-piece TER system consisting of stem IV and cpRNA31-25, a circularly permuted region of TER that is missing stems I and IV. (B) Duplicate SDS-PAGE analysis of TERT immunoprecipitates following translations in RRLs in the presence or absence of p65 and the indicated RNA components. The top panel shows the [35S]methionine incorporated into TERT, while the bottom panel shows only the 32P-end-labeled TER components recovered by coimmunoprecipitation. (C) Telomerase activity assays using the indicated components. RC, recovery control.

p65 binding to TER causes conformational rearrangements in the telomerase RNP. We then set out to define the specific p65-TER interactions and conformational rearrangements re-
quired for the functional reconstitution of the telomerase RNP. We used RNase T1 and RNase ONE to probe the single-stranded regions of TER and RNase V1 to probe its double-stranded and stacked regions (Fig. 4). In addition, several sites of nuclease-independent cleavage were observed. To understand the contribution of p65 to complex formation, we tested TER alone, the binary complexes of TER plus p65 and TER plus TERT*, and the ternary complex TER plus p65 plus TERT*. TERT* describes either the RBD of TERT (amino acids 216 to 516) or the N-terminal half of TERT containing the TEN and RBD domains, also known as TERT(1-516) (see Fig. S3 in the supplemental material); these regions account for most of the RNA-binding affinity of full-length TERT (9, 19). Both of these truncated proteins were tested as binary and ternary complexes in the nuclease protection assays and yielded equivalent results.

The nuclease protection data are consistent with a direct footprint for p65 in stems I and IV of TER (Fig. 4A to E), as summarized by the large arrowheads in Fig. 4E. The single-
stranded RNA probes revealed protections of the GA bulge (G121) and the linker between stems I and IV (Fig. 4D and E and data not shown). Also, proximal stem IV (G114) was susceptible to single-strand-specific RNase T1 in the absence of p65 but was protected from cleavage in the presence of p65 (Fig. 4C and E). Comparative sequence analysis indicates that this region is double stranded (37), suggesting that p65 is necessary to facilitate proper base pair formation within TER stems I and IV.

Notably, the 5' half of hairpin loop IV became protected from the single-stranded RNA probe when p65 was bound (Fig. 4B, inset, and D and E). Cleavage by RNase ONE was reduced at nucleotides C132 to C134; selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) experiments also support this conclusion (data not shown). These protections by p65 might be direct or indirect, since the conforma-
tional change induced in stem IV by p65 brings loop IV into proximity with other regions of TER (35, 42).

The protection data reveal that TERT* binds at the TBE and the base of stem II. In the presence of TERT*, nucleotides at the base of stem II, such as A20 and G21, were protected from V1 digestion, while nucleotides closer to loop II, such as A23 to U25, had increased V1 reactivity. In addition, nucleotides U38 to U41 were protected from the single-strand-specific RNase ONE upon TERT* binding (Fig. 4B). TERT* binding to the TBE and stem II is consistent with published data (6, 19, 21, 48). An unexpected finding was that TERT* binding causes structural rearrangements in positions within the central wheel of the RNA that are not directly bound by protein, such as nucleotides in the TRE.

The sequential binding of p65 to TER stems I and IV and TERT* to stem II induces conformational rearrangements that increase the single-stranded and stacked nature of the nucleotides in the region spanning the TBE, the template, and the TRE. For example, when both p65 and TERT* were bound to TER, G59, G61, and G64 had increased susceptibility to RNase T1, and TRE nucleotides A52 to U60 and template alignment nucleotides C49 to A51 exhibited enhanced cleavage by RNase ONE. Under similar conditions, U55, C56, and G61 to U63 had increased digestion by V1 nuclease (Fig. 4A and G), presumably indicative of base stacking (6). The observed conformational changes induced by protein binding may disrupt nonproductive base-pairing interactions within the central wheel of TER, which is consistent with in vivo DMS (dimethylsulfate) probing data (48). In addition, the binding of the proteins may stabilize the RNA into a more rigid structure that limits the sampling of various conformations by the nucleotides. Such rearrange-
ments would facilitate template binding in the active-
site cleft of the TERT RT domain.

FIG. 3. p65 interacts with regions of TER outside stems I and IV. (A) Cartoon of the two-piece TER system consisting of stem IV and cpRNA31-25, a circularly permuted region of TER that is missing stems I and IV. (B) Duplicate SDS-PAGE analysis of TERT immunoprecipitates following translations in RRLs in the presence or absence of p65 and the indicated RNA components. The top panel shows the [35S]methionine incorporated into TERT, while the bottom panel shows only the 32P-end-labeled TER components recovered by coimmunoprecipitation. (C) Telomerase activity assays using the indicated components. RC, recovery control.
p65 helps distinguish between assembly and activity mutants of TER. The reorganization of the RNA between TER stems II and III upon p65 binding led us to ask how p65 affects the activity of telomerase containing different TER mutations. We generated a series of point, deletion, and sequence mutants that scan the secondary structural elements of TER (Fig. 5A). Conveniently, some of these mutants had been characterized previously in the absence of p65 (4, 5, 17, 18, 21). Our studies revealed three classes of TER mutants: (i) those that lose activity in the absence of p65 but are restored to near-wild-type activity levels or higher by p65, (ii) those that are rescued but not fully resuscitated, and (iii) those that are unresponsive to p65.

The addition of p65 to several extremely debilitated mutants restored telomerase activity to within 50% of wild-type levels. Comparing the telomerase activity of these class 1 mutants in the absence of p65 (Fig. 5D, top panel, white bars) to their activity in the presence of p65 (gray bars) indicates a recovery of approximately 30-fold (i.e., 3000%), but the accuracy of these estimates is limited by the weak signals for telomerase activity in the absence of p65. Included in this class are several mutations in the sites of direct TERT binding, such as CA15-40AGU, CAUU15-18GUAA, CAUUC15-19GUAAG, UCA38-40AGU, and the stem IV mutants C62G, which display recovery to near-wild-type activity with p65. Resuscitation in activity was much greater than can be explained by the increase in RNA association between TERT and the mutant TER in the presence of p65 (Fig. 5C and D, bottom). The pseudoknot mutants PKA and PKB also had minimal activity in the absence of p65 but near-wild-type levels of activity in the presence of p65. The rescue of the activity of these mutants cannot be correlated with an increase in RNA association (Fig. 5D, bottom). The compensatory mutations in the pseudoknot (PKC) were included as a control and demonstrate wild-type levels in the presence of p65 (Fig. 5D, bottom). The compensatory mutations in the pseudoknot (PKC) were included as a control and demonstrate wild-type levels in the presence of p65 (Fig. 5D, bottom). The compensatory mutations in the pseudoknot (PKC) were included as a control and demonstrate wild-type levels in the presence of p65 (Fig. 5D, bottom).

In our system, p65 rescued the TER mutant containing a deletion of the GA bulge in stem IV to ~50% of the level of wild-type activity (1.7-fold ± 0.2-fold for A4GA and 3.0-fold ± 0.3-fold for wild-type TER) by p65. The presence of p65 resulted in a modest increase (2-fold) in RNA association with TERT and an ~5-fold increase in telomerase activity (Fig. 5) for this mutant. In contrast, other studies have indicated that the deletion of the GA bulge prevents p65 from affecting telomerase RNP assembly (1, 33, 42).

In class 2, p65 increased the activity of several TER assembly mutants and stem IV mutants to a more modest extent. For example, CAUUC15-18GUAA, CAUUC15-19GUAAG, UCA38-40AGU, CAUUC15-19GUAAG/UCA38-40AGU, and the stem II deletion mutant (ΔII) were recovered to ~15 to 40% of wild-type levels in the presence of p65 (Fig. 5D). While the presence of p65 resulted in an increase in RNA pulldown in some of these cases, the levels of RNA recovery were too little to explain the activity recovery (Fig. 5D). The mutation of loop IV to a GAAA tetraloop resulted in the incomplete recovery of activity in the presence of p65 despite comparable levels of RNA association with TERT; although stem IV of this construct is a binding substrate for p65, these results confirm that the sequence of loop IV is important for telomerase function (36).

In class 3, several TER mutants were not suppressed by the presence of p65. Although the mutants combining altered TRE and the TBE regions (such as CAUUC15-18GUAA/C62G) had an increase in RNA pulldown in the presence of p65 (Fig. 5D), activity was not recovered. p65 also was unable to affect TER that lacked stem IV (ΔIV); since stem IV is a major site of p65 interaction (3, 29, 33), the presence of p65 in the translation and assembly reactions was not able to rescue RNA recovery or activity. Only one round of replication occurred with ΔIV (Fig. 5B), which is consistent with the role loop IV has been shown to play in RAP (17). Finally, another subgroup of class 3 included the template mutation C48U that was unaffected by p65. This was expected, as the template is not involved in assembly. This mutant gave a single round of addition because the extended primer cannot base pair properly with the mutated template.

p65 rescues TERT assembly mutants but not activity mutants. We then asked if the assembly and activity of telomerases containing TERT mutants could be rescued by p65. The panel of mutants that we tested spanned the CP and T motifs of the RBD, which are involved in RNP assembly, and motifs 1, A, B, and E of the RT domain (Fig. 6) (9). We predicted that the RT motif mutants previously characterized as activity mutants would not be rescued by p65, while some of the RBD mutants would be aided by p65 because they are defective in TER binding and, therefore, RNP assembly.

The point mutants in the T motif of the RBD showed notable p65 stimulation of telomerase activity. While the recovery can be explained partially by an increase in RNA binding (typically a ~2-fold increase), this does not completely account for the magnitude of activity stimulation observed for these mutants, which is typically greater than 10-fold (Fig. 6D). As before, the magnitude of recovery for some mutant pairs is difficult to measure accurately because of the extremely weak activity in the absence of p65 (Fig. 6B). CP point mutants (L327A and P334A) were increased by p65 by a factor similar to the activity of wild-type TER in the presence of p65 (Fig. 5D).
to that for wild-type TERT, correlating with similar moderate increases in RNA binding.

Several point mutants of amino acids in the RT domain showed near-wild-type telomerase activity and RNA binding (K538A and S880A). The catalytic carboxylate mutant (D618A) and the 5-amino-acid deletion in the B motif (Δ763-767) were not activated by p65, which is consistent with severe disruptions of the enzyme active site (9), despite RNA binding that was comparable to that of wild-type TERT (Fig. 6C and D). Taken together, these data demonstrate that p65 can com-

FIG. 5. p65 rescue distinguishes TER assembly mutants from TER activity mutants. (A) Secondary-structure diagram of TER indicating the various mutations tested. (B) Telomerase assays for each of these mutants with equal amounts of 35S-labeled TERT. The activity for each mutant is shown in the absence and presence of p65. RC, recovery control. (C) SDS-PAGE analysis of a selection of telomerases containing the indicated TER mutations assembled in the absence and presence of p65. The samples are shown in duplicate, and the 35S-labeled TERT and coimmunoprecipitation of 32P-labeled mutant TER are indicated. (D) Quantitation of telomerase activity assays and RNA pulldowns. Mutants are listed at the bottom. The top panel shows relative telomerase activity for each mutant in the absence (white bars) and presence (gray bars) of p65. All activities were normalized to the activity of telomerase containing wild-type TER in the absence of p65. The error bars indicate SEM (n ≥ 3). The bottom panel shows the RNA recovered by the coimmunoprecipitation of TERT normalized to the amount of TERT and setting the amount of wild-type TER recovered in the absence of p65 to 1.0. White bars, without p65; gray bars, with p65. The error bars indicate the SEM from three independent measurements.
DISCUSSION

Previous work established p65 as a telomerase-specific protein that enhances telomerase RNP assembly by binding to the TER RNA and inducing a structural change in the RNA, thereby enhancing the binding of TERT; furthermore, the site of p65 action was localized to the proximal half of stem IV, a conserved GA bulge, and stem I of TER (3, 29, 33, 42, 47). In agreement, the most-straightforward interpretation of our nuclease protection data is that stem I, proximal stem IV, and the GA bulge in stem IV constitute the binding site of p65 (Fig. 4E). Moreover, our binding data show p65 binding beyond stems I and IV, perhaps most convincingly demonstrated by Fig. 3C. In addition, we find that p65 reorganizes the RNA structure outside the regions of its binding, which helps explain why p65 enhances telomerase activity above and beyond its increasing the amount of complex formed. Finally, we show that the normally modest effect of p65 on telomerase activity is greatly amplified in the case of TER and TERT assembly mutants to the extent that many severe mutants are fully resuscitated. Thus, p65 is a tool for distinguishing between assembly and activity mutants of *Tetrahymena* telomerase.

p65-assembled telomerase RNPs are of higher quality. The addition of p65 to *in vitro* translation and assembly systems of *T. thermophila* telomerase results in telomerase RNPs...
with higher telomerase activity. This can be explained partially by the enrichment of TER-bound TERT molecules that occurs with p65. However, the 1.4-fold ± 0.3-fold increase in RNA association with TERT does not completely account for the 3.0-fold ± 0.3-fold (means ± SEM) increase in telomerase activity. As described below, some mutant telomerasases show much more dramatic effects of p65 on activity above and beyond assembly; that is, they have greater activity per RNP molecule. Thus, p65 contributes not only to the quantity of RNP formed but also to the quality. This conclusion is consistent with published FRET data indicating that the presence of p65 increases the homogeneity of telomerase RNPs (42), thereby improving the quality of the complexes overall.

Nuclease probing experiments identified RNA structural changes that may explain the improvement of activity of telomerase assembled in the presence of p65. Nucleotides 3′ of the template become more single stranded and yet more organized in nature, as evidenced by RNase ONE and RNase V1 cleavage, respectively. These changes are expected to facilitate template accessibility and primer binding. Perhaps these nucleotides participate in nonproductive base-pairing interactions that are disrupted when p65 binds to their illegitimate pairing partners.

In addition, several loop IV nucleotides are protected from nuclease digestion by p65 and remain protected in the p65-TERT-TER ternary complex. In combination with the telomerase activity assay for the loop IV_GAAA TER mutant (Fig. 5D), these results suggest that loop IV is poised for proper holoenzyme formation upon the complexation of p65 and TER, with the sequence of loop IV contributing to the assembly of TERT, as suggested by Robart et al. (36). It also agrees with the single-molecule studies that suggest loop IV is brought into the core of the telomerase RNP during assembly (42).

p65 is thought to serve as a bridge between stems I and IV (33); in agreement, we find greater activity of p65-assembled telomerase with nucleotides 1 to 107 (which contains stem I) plus stem IV than with cpRNA (no stem I) plus stem IV (Fig. 2A and 3C). In addition, the cpRNA plus stem IV TER system revealed that p65 may have direct interactions with other regions of TER. The binding experiments of Fig. S1 in the supplemental material indicate a direct interaction between p65 and the central wheel of TER that is relatively high affinity and low specificity. The presence of TERT may enhance these interactions. Such interactions may contribute to the quality of the RNP; if p65 promotes proper secondary and tertiary structure formation for all of TER, TERT would bind TER molecules that were folded in their active conformation more often.

p65 is a tool for distinguishing between assembly and activity mutants. Since p65 participates in telomerase RNP assembly, we predicted that p65 would be able to rescue mutations in regions of TER that have been shown to be responsible for the TER-TERT interaction. Indeed, altering nucleotides in the TBE, stem II, and/or the TRE abrogated assembly and therefore activity in the absence of p65, which is consistent with previously published results (4, 21), but many of these mutants were rescued by p65.

The pseudoknot mutants were rescued by p65, but this increase in telomerase activity was not due to an increase in RNA association (Fig. 7). In this case, it is likely that the quality rather than the quantity of the complex is enhanced, and in fact for yeast and human telomerasases it has been proposed that the pseudoknot triple-helix region of the RNA is involved in the alignment of the primer-template relative to the active site (34). Previously, Autexier and Greider reported that pseudoknot mutations were not deleterious to telomerase activity in vitro (5). Their starting preparations of telomerase were assembled in vivo, so they can be assumed to include the p65 subunit; thus, it is not surprising that their activity data agree with our data for PKA and PKB in the presence of p65.

Finally, the mutation in the template region, a segment of TER that is not involved in assembly but in the activity of the holoenzyme, was not rescued by p65. Thus, the RNA mutants studied can be sorted into three classes: those aided by p65 because they are defective in RNP assembly, mutants helped by p65 not in assembly per se but because of the quality of the assembly, and mutants unaffected by p65 because they have a major defect in RNA activity (Fig. 7).

All of the TERT mutants studied followed the trends in RNA association and telomerase activity reported by Bryan et al. (9). In general, the mutation of amino acids responsible for binding TER (in the RBD) decreased telomerase activity because these mutations destroyed the TERT-TER association, while the mutation of several conserved amino acids in the active site of the RT domain decreased or eliminated telomerase activity without decreasing RNA binding. In the presence of p65, mutations in the RBD, especially those in the T motif, were suppressed; telomerase activity was significantly stimulated (4- to 30-fold), and this was partially due to stimulation in the TERT-TER association (less than or equal to 3-fold) (Fig. 6D). Thus, our data suggest that the T motif has a function beyond simple RNA binding, a conclusion that is consistent with the recent protein crystal structure showing that the T motif may structurally support part of the RT domain; the β-hairpin of the T motif extends toward the active site of the RT domain and may form functionally relevant interactions with the fingers and the presumed thumb subdomains, which typically stabilize the incoming nucleotide and the primer-template duplex, respectively (13, 38). Additionally, the T motif may form a hinge between the RBD and the RT domain, possibly playing a role in coordinating the movements of these subdomains during polymerization and translocation. The TERT mutants in several conserved amino acids of the RT domain were not rescued by p65, as expected. Thus, p65 rescued mutants that were deficient in assembly (7, 19, 30) but could not rescue mutants deficient in polymerization activity (Fig. 7).

Model for the mechanism of p65-induced stimulation of telomerase activity. The binding of p65 to stems I and IV [and the 3′-oligo(U) tail] sequesters these nucleotides from the rest of the TER molecule. This may help the template and surrounding regions of TER assume the correct conformations required for telomerase activity. Indeed, the structural effects of p65 seen far from its primary binding site in stem IV are consistent with the idea that improving the single-stranded and stacked nature of the template and the surrounding regions of TER are important for increasing the efficiency of complex
formation. In short, p65 may limit the ensemble of structural possibilities to those that are catalytically active.

Beyond interacting with TER, several observations suggest that p65 also participates in interactions with TERT. The TRE and template become more accessible only in the presence of both proteins, while stem II assumes its predicted secondary structure only in the presence of both proteins. Even more compelling, the TER stem II deletion mutant data suggest that p65 directly contacts TERT. In this example, the main site in TER of TERT binding is missing, yet p65 stimulates a robust recovery of telomerase activity; here, the simplest explanation is a direct contact between the two proteins. Previous studies have tested for the direct interaction between p65 and TERT, and none was found in the absence of TER (33). However, it is possible that the binding of p65 to TER induces a conformational change in p65 that allows it to interact with TERT.

Several of the TERT mutants also hint at a direct interaction between the two proteins. For example, point mutations in the conserved T motif demonstrate a substantial rescue in the presence of p65, yet point mutations in another RNA-binding element, the CP motif (10, 26), do not. These data support the hypothesis that p65 directly interacts with the T motif of the RBD. Alternatively, by helping to organize the RNA structures necessary for productive RBD binding, p65 may indirectly suppress T motif mutants.

Taken together, these data suggest that p65 acts as a molecular buttress within the telomerase RNP. In the context of a wild-type system, p65 enhances the number of TERT molecules that are stably associated with TER, thereby increasing the activity of the bulk telomerase population. When TER is broken into two pieces, p65 is required for the association of stem IV with TERT, and activity is recovered only in the presence of p65. In the context of many TERT and TER assembly mutants, p65 is not simply a useful accessory factor but is essentially required for stable RNA association. Thus, we conclude not only that p65 is a telomerase-specific assembly protein but also that its presence in the telomerase RNP maintains active TERT conformations.

FIG. 7. Summary of the effect of p65 on mutants of TER and TERT.

ACKNOWLEDGMENTS

We are grateful to the members of the Cech laboratory for helpful discussions. We also thank Kathleen Collins (University of California, Berkeley) for the plasmid encoding p65 and for helpful discussions in the initial phases of the project. We also thank Catherine O’Connor and Kathleen Collins for sharing unpublished studies in which they designed RBD 216-516 and determined that it gave robust soluble expression and retained full interaction with TER and TER-p65. A.J.B. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

REFERENCES
