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Specific RNA Residue Interactions Required for Enzymatic Functions of *Tetrahymena* Telomerase

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The ribonucleoprotein enzyme telomerase is a specialized reverse transcriptase that synthesizes telomeric DNA by copying a template sequence within the telomerase RNA. Here we analyze the actions of telomerase from *Tetrahymena thermophila* assembled in vivo with mutated or wild-type telomerase RNA to define further the roles of particular telomerase RNA residues involved in essential enzymatic functions: templating, substrate alignment, and promotion of polymerization. Position 49 of the telomerase RNA defined the 3′ templating residue boundary, demonstrating that seven positions, residues 43 to 49, are capable of acting as templating residues. We demonstrate directly that positioning of the primer substrate involves Watson-Crick base pairing between the primer with telomerase RNA residues. Unexpectedly, formation of a Watson-Crick base pair specifically between the primer DNA and telomerase RNA residue 50 is critical in promoting primer elongation. In contrast, mutant telomerase with the cytosine at position 49 mutated to a G exhibited efficient 3′ mispair extension. This work provides new evidence for specific primer-telomerase interactions, as well as base-specific interactions involving the telomerase RNA, playing roles in essential active-site functions of telomerase.

The active sites of many nucleic acid polymerases are composed of a network of interactions among amino acid residues, creating a site not only for catalysis of phosphodiester bond formation but also for binding the template-primer and nucleoside triphosphate substrates to allow templated synthesis. The three-dimensional structures of three polymerases—*Escherichia coli* DNA-dependent DNA polymerase I, human immunodeficiency virus type 1 RNA-dependent reverse transcriptase, and T7 DNA-dependent RNA polymerase—all show remarkable similarities in their catalytic-site structures, involving a conserved set of catalytic amino acid residues (15, 24, 29). In contrast to these purely protein polymerases, telomerase, a specialized cellular reverse transcriptase which performs a critical role in chromosome maintenance by adding telomeric sequence to chromosome ends, is a ribonucleoprotein.

Telomerase activity has been detected in several diverse eukaryotes (6, 19, 21, 25; for a review, see reference 4). In the ciliate *Tetrahymena thermophila*, telomerase consists of an essential 159-nucleotide RNA moiety (12) and two polypeptide subunits, p95 and p80 (8). Ciliate telomerase RNAs have a highly conserved secondary structure, including a conserved pseudoknot and a bent helix (3, 20, 22, 26, 30) (Fig. 1A). The p95 polypeptide has limited similarity to a subset of sequence motifs found in DNA- and RNA-dependent RNA polymerases, although a complete set of conserved amino acids known to form the catalytic site of other nucleic acid polymerases has not been identified (8). Our recent finding that single base changes in telomerase RNA can cause dramatic impairment of active-site functions, including enzyme processivity and fidelity, suggests that specific RNA residues, as well as the protein components of telomerase, may play important roles in enzyme action (10).

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Telomeric DNA sequences are usually composed of short G-rich tandem repeats, running 5′ to 3′ toward the distal end of the chromosome, although significant variations of this simple telomeric sequence motif have been found (reviewed in reference 5). In the *T. thermophila* telomerase RNA, the sequence 3′-AACCCCAAC-5′, referred to as the template domain, is complementary to the TTGGGG telomeric repeats of *T. thermophila*. The template domain is located at positions 43 to 51 within the 159-base telomerase RNA (Fig. 1A) and is defined as those RNA residues that function in templating and positioning the DNA primer by complementary base pairing. Certain nucleotides within this domain have been found to function as templating positions for the synthesis of telomeric DNA (33). When introduced into *T. thermophila*, mutant telomerase RNA genes with certain residues altered within the template domain cause the synthesis of the specifically altered telomeric repeats (10, 32, 33). These studies, as well as in vitro analysis of product synthesis by reconstituted telomerase activity or mutant telomerases partially purified from cells, showed that residues 43 to 48 are used as templating residues (1, 10). A model for telomere repeat synthesis by the *Tetrahymena* telomerase is shown in Fig. 1B (12, 28). Initially, the 3′ end of the DNA primer base pairs with the template domain of the telomerase RNA (Fig. 1B, step 1). The primer is elongated, copying specific telomerase RNA templating residues, making first-round products (Fig. 1B, step 2). At the end of the telomerase RNA template domain (RNA residue 43), the primer translocates and repositions for second-round synthesis (Fig. 1B, step 3). Thus, according to this model, some of the nine RNA residues within the template domain of *T. thermophila* telomerase can be copied into the 6-base telomeric DNA repeat of *T. thermophila* (GGGGTT), while other bases allow alignment of a GGGGTT repeat primer for synthesis of tandem repeat units.

The catalytic rate constant of polymerization was shown previously to be altered by base-specific interactions of the DNA primer with telomerase, in a manner independent of

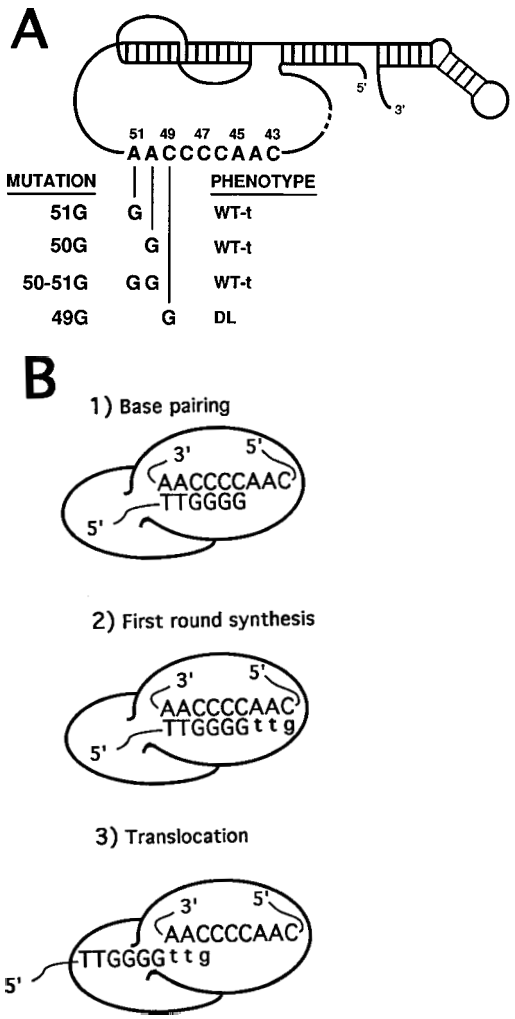

FIG. 1. (A) Telomerase RNA template domain nucleotides are shown with residue numbers above selected positions in relation to a schematic of the secondary structure of the telomerase RNA. The dashed region indicates the position of a helix present in some but not all ciliate telomerase RNA structures (20, 22, 26). Mutant telomerase RNA genes are designated by the telomerase RNA position followed by the altered nucleotide. Phenotypes are denoted by DL (delayed lethal) and WT-t (wild-type transformed). (B) Model for the addition of telomere repeats by telomerase (12, 28). The two ovals indicate schematically the two telomerase proteins (8). Step 1, 3′ nucleotides of the G-rich primer, $(T_2G_4)_n$, base pair with the template domain of the telomerase RNA; 3′-terminal input primer residues are shown in capital letters. Step 2, the G-rich primer is elongated, copying the telomerase RNA templating residues, making first-round products; elongation products are shown in lowercase letters. Step 3, the G-rich primer translocates, repositioning for second-round synthesis.

base pairing to the template domain (17). Recently, we have also found that specific mutations within RNA templating residues cause high misincorporation rates and premature product dissociation; neither type of malfunctioning was explainable by altered base pairing between the telomerase RNA and DNA substrate (10). These results revealed that as well as providing templating functions, specific telomerase RNA residues might contribute specific functional groups to the enzyme active site. In vivo chemical footprinting of the telomerase RNA has also recently shown that template domain residues are hyperreactive to dimethyl sulfate (34). This finding suggests that this domain is in a constrained conformation and is consistent with the involvement of this region in specific interactions in the telomerase ribonucleoprotein complex.

Although certain template domain residues have been proposed to be involved in primer substrate positioning (12, 28), the specific actions of these proposed alignment residues have not been reported and the exact 3′ templating-residue boundary has not been defined. Here we show that, unexpectedly, residue 49 can be copied with high efficiency by telomerases containing either mutated or wild-type RNA. Furthermore, mutating the C residue normally at position 49 to a G causes efficient 3′ mispair extension at this position, revealing another unanticipated alteration of telomerase action caused by a telomerase RNA base change. In contrast, under all conditions used and with all telomerase RNA mutants tested, we found no evidence for copying of position 50. Instead, we show that formation of a Watson-Crick base pair between the primer and specifically position 50 of the RNA template is important for processive action of telomerase, even during copying of a distant part of the template. Together with previous work (10, 17), these results suggest that an optimal active site for telomerase action is built by a collaboration between structural components of the DNA primer, telomerase RNA, and telomerase proteins.

MATERIALS AND METHODS

Strains and electroporation. *T. thermophila* strains used in these experiments were CU427, ChxA/ChxA (cy-sens, VI) and CU428, and Mpr/Mpr (6-mp sens, VII), kindly provided by P. J. Bruns, Cornell University. Both strains are paromomycin sensitive and contain B rDNA. Cells were grown at 30°C to late logarithmic phase on 2% PPYS (2% Proteose Peptone, 0.2% yeast extract, 0.003% Sequestrene) supplemented with penicillin (100 μg/ml), amphotericin B (250 pg/ml), and streptomycin (100 μg/ml) with shaking at 100 rpm. The cells were starved for ~24 h, and mating types were mixed. At 10 to 11 h after mixing, electroporation was performed essentially as described by Gaertig and Gorovsky (9). The electroporation apparatus used was a Bio-Rad Gene Pulser set at 440 V, 200 Ω, and 25 μF with 0.4-cm electrode gap cuvettes. The vector used for electroporation was prD4-1, which contains a recombinant rDNA allele carrying the two direct-repeat replication origins from C3 rDNA (the first origin contains an additional G residue within the rDNA promoter) and the Pmr marker and downstream region from B rDNA (31). Transformants were challenged with 100 μg of paromomycin per ml approximately 24 h after electroporation. Transformants were evident about 4 days after introduction of paromomycin.

Mutagenesis. Mutagenesis was performed by overlap extension via PCR as previously described (14) except for the following modifications. PCR was performed with 20 cycles of denaturation (30 s at 94°C), annealing (30 s at 50°C), and extension (30 s at 72°C) under conditions specified by the manufacturer of the DNA thermal cycler (Perkin-Elmer Cetus). Mutant telomerase RNA genes were inserted into the vector prD4-1 (31) at the *Xho*I site within the vector polylinker. Potential mutant genes were sequenced to detect possible unwanted errors by *Taq* polymerase.

Telomerase preparation. Partially purified mutant and wild-type telomerases were prepared as previously described with the following modifications (11, 17). Populations of mutant and wild-type telomerase RNA gene transformants were cultured at 30°C in 50 ml with shaking at 100 rpm to late logarithmic phase or, in the case of lethal mutants, to maximal population density before complete phenotypic expression. Cells were washed once with Dryl's solution (1.7 mM sodium citrate, 2.4 mM sodium phosphate, 2 mM $CaCl_2$), and incubated in Dryl's solution at 30°C with shaking at 100 rpm for ~24 h. S-100 extract was loaded onto a DEAE-agarose (Bio-Rad) column and eluted with 300 mM sodium acetate after being washed with 6 column volumes of 200 mM sodium acetate. Preparations from the previous column were brought to 500 mM sodium acetate and desalted by being loaded onto an octyl-Sepharose (Pharmacia) column and washed with 6 column volumes of TMG buffer (10 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride). Telomerase was eluted with TMG buffer containing 1% Triton X-100, and samples were kept frozen at −80°C until needed.

Telomerase assays. The primer was purified on 20% polyacrylamide–8 M urea gels, eluted passively with shaking at 100 rpm overnight at 37°C, and passed over a Sep-Pak C-18 column (Waters). Telomerase reactions were performed essentially as previously described (11, 17). Final reaction mixtures contained 50 mM Tris (pH 8.5 at 25°C), 1 mM spermidine, 1 mM dithiothreitol, 1 μM primer, 1.5 μM [α-^{32}P]deoxynucleoside triphosphate ([α-^{32}P]dNTP) (either dGTP, dATP, or TTP as indicated), and 100 μM other cold dNTPs. Reaction mixtures were incubated at 30°C for 45 min unless otherwise specified. ddNTPs were added to a final concentration of 100 μM. Products from in vitro reactions were run on 10% polyacrylamide–8 M urea gels. Gels were dried, exposed to X-ray film, and generally incubated for 1 to 2 days at −80°C before development.

RESULTS

Since the internal templating-domain sequence 3′-AAC CCCAAC-5′ within the *Tetrahymena* telomerase RNA is complementary to 1.5 telomeric T_2G_4 repeats, not all these RNA residues are theoretically required for templating. The terminally redundant trinucleotide sequence AAC, residues 51 to 49, has been proposed to participate in alignment by base pairing with the primer substrate to position it for elongation (Fig. 1B) (1, 12, 28). To test this proposal directly, the effects of mutating these residues on telomeric repeat synthesis were analyzed. Mutations in telomerase RNA genes are designated by the telomerase RNA position followed by the mutant nucleotide (Fig. 1A). To substitute telomerase in vivo with the mutated RNA, each altered telomerase RNA gene was inserted into the high-copy-number vector prD4-1 (31) and introduced into cells by electroporation (10). We have shown previously that in these experiments, the mutant telomerase RNA gene is fully dominant over the endogenous wild-type gene and that the extracted telomerase is completely substituted with the mutated telomerase RNA (10). For each mutation in the telomerase RNA gene, the cellular phenotypes were analyzed by monitoring several independent transformants. The effects of the mutations on telomerase activity were determined by assaying partially purified telomerase preparations in vitro under various conditions and with different DNA primers. The enzymatic reactions of the mutant and wild-type telomerases were compared by analyzing the patterns of reaction products fractionated on DNA sequencing gels, as described previously (10).

Cellular phenotypes of telomerase RNA mutants. Telomerase RNA mutations 50G, 51G, and 50-51G each produced transformants with cellular morphology and fission rates similar to those of cells transformed with a wild-type telomerase RNA gene on the same high-copy-number vector (WT-t phenotype), which was essentially the same as wild-type untransformed cells. Telomere lengths in the macronucleus were indistinguishable from those in wild-type or WT-t control transformants. Transformants with the 49G telomerase RNA gene showed a delayed lethal (DL) phenotype similar to that described previously for the 48U mutant (10): wild-type transformed morphology and fission rates were seen for up to ~25 fissions after the introduction of the particular mutant telomerase RNA gene, but then fission rates progressively decreased, and within the next ~5 to 10 fissions, divisions stopped completely. During the slowing and termination of fission, these transformed cells also progressively displayed a variable "monster" morphology (33). In contrast to other template mutants (10, 32, 33), the predicted mutant repeat sequences were not detected in the telomeres of either the 48U or 49G transformants by Southern blotting (9a, 33). However, one difference between these two mutants was that the telomeres were slightly lengthened in some 49G transformant lines and shortened in other lines whereas the telomeres in all the 48U mutant transformant lines were invariably shortened, as described previously (33).

Thus, the various aspects of the phenotypes of the 49G, 50G, 51G, and 50-51G mutants differed from those of the previously described 44G, 43A, and 49+C mutants (10, 27, 32). Therefore, to understand the basis of the WT-t and DL phenotypes of the 49G, 50G, 51G, and 50-51G transformants, in vitro analysis was performed on telomerase extracted from each mutant type.

Position 49 of the telomerase RNA acts as a templating residue. It was shown previously that position 43 of the template is copied in both in vitro and in vivo assembled telomerases (1, 10). Therefore, it was anticipated that position 49 would not act as a templating residue, since the synthesis of hexameric GGGGTT repeats would require only copying residues 43 to 48 (Fig. 1B). To test this, telomerase with the C residue at position 49 of the telomerase RNA mutated to a G (mutant 49G [Fig. 1A]) was tested for its ability to incorporate dCTP at this position in vitro. This 49G mutant enzyme was supplied with the primer $(G_4T_2)_3$, [α-^{32}P]dGTP, and ddTTP in the presence or absence of dCTP. In the control reaction with the wild-type enzyme, the pattern of elongation products synthesized was unaffected by dCTP, as expected (Fig. 2A, lanes 2 and 4). The major stop was at the fifth addition position, as predicted for addition of gggg(ddt) to the primer (Fig. 2A, lanes 2 and 4). In contrast, with the mutant 49G enzyme, the position of the final major elongation stop depended on the presence or absence of dCTP. Replacing a dG or T residue in an oligonucleotide with a dC residue causes faster migration of a product under the sequencing-gel conditions used here. With dCTP present (Fig. 2A, lane 1), the final stop band migrated slightly faster than the wild-type final stop band (Fig. 2A, lane 2), consistent with addition of an initial (unlabeled) dC residue to the -TT ending primer $(G_4T_2)_3$, followed by three dG residues and then the chain-terminating ddT (schematic in Fig. 2B). Without dCTP, with the 49G enzyme the major stop was shifted one nucleotide down from the wild-type stop (Fig. 2A, lanes 3 and 4). To account for this pattern of elongation, we propose that in the absence of dCTP, the first nucleotide added by the 49G enzyme to the -TT 3′ end is [α-^{32}P]dG (*g), copying position 43, and after translocation, this *g 3′-end base pairs with the C residue at position 48 and is extended by addition of gg(ddt) (Fig. 2B). Additional direct evidence for position 49G acting as a template residue came from showing that a labeled dC residue was added at the +1 position when the 49G mutant enzyme was supplied with the same primer used in Fig. 2A, $(G_4T_2)_3$, [α-^{32}P]dCTP, dGTP, and TTP. As expected, this +1 product band was slightly shifted down compared with the +1 product made by the wild-type enzyme with labeled dGTP and the same primer (data not shown).

The scheme in Fig. 2B implies the formation of a non-Watson-Crick rG-T base mismatch between the penultimate residue of the primer and position 49 of telomerase RNA as shown in Fig. 2B. An alternative model is that the -TT ending primer copies position 43, as proposed above, but then after translocation, the residue at position 49 bulges out so that a mispair is not required. This mode of elongation would be similar to the dislocation-mediated mutagenesis that has been proposed for retroviral reverse transcriptases and DNA polymerases, although for these polymerases this proposed type of distortion would occur at much lower frequencies (2, 16) than the in vitro events we observe here.

With the primer $(G_4T_2)_3$ and all dNTPs present, the mutant 49G enzyme synthesized primarily one to two elongation rounds, with only small amounts of longer products (Fig. 2C, lane 4). When dCTP was omitted from the reaction, a faint 5-nucleotide repeat pattern was produced (Fig. 2C, lane 6). We propose that these 5-nucleotide repeats were synthesized by the minor pathway shown in Fig. 2D, with again an rG-T mispair at position 49. Further confirmation of these two pathways came from using the primers $(T_2G_4)_3$ and $(G_3T_2G)_3$, which yielded comparable results (data not shown).

Combined with the results of the ddTTP reactions (Fig. 2A), these experiments showed that when all dNTPs were present, the mutant 49G enzyme reaction consisted of the major and minor pathways shown in Fig. 2D and that the mutated position 49 is used as a templating residue. As described previously, with the 43A mutant enzyme, the wild-type rC at posi-

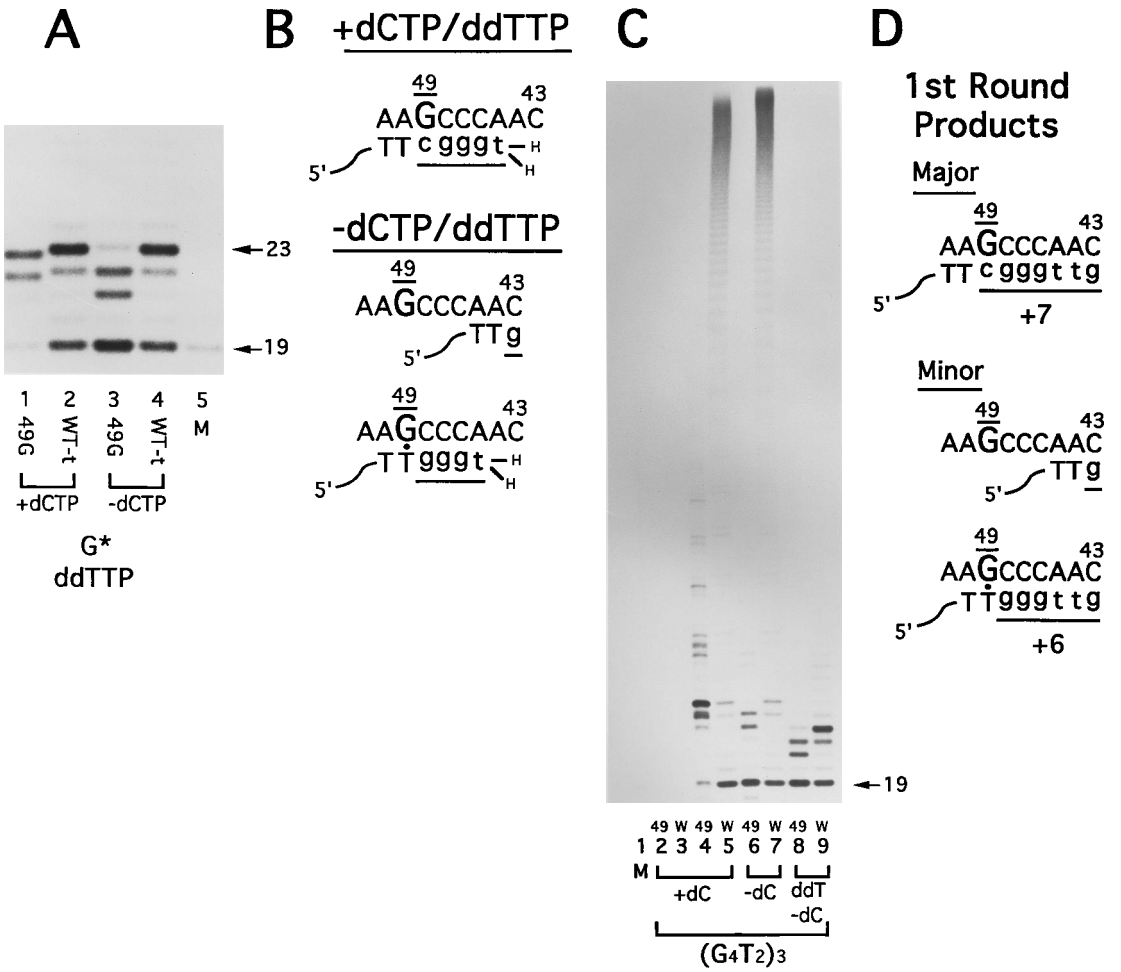

FIG. 2. The 49G telomerase uses residue 49 as a templating position. (A) Telomerase reaction products were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP, 100 μM ddTTP, and primer $(G_4T_2)_3$. Lanes: 1 and 3, mutant 49G enzyme; 2 and 4, wild-type transformed enzyme (WT-t); 5, marker. For lanes 1 and 2, reaction mixtures contained 100 μM dCTP; for lanes 3 and 4, reaction mixtures had no dCTP. (B) Schematic of products produced in reactions with and without dCTP that contained ddTTP by the mutant 49G enzyme in panel A. Elongation products are shown in underlined lowercase letters. The large dot indicates a G-T mispair when dCTP is absent. The two H's attached to the 3′ T residue of the product represent a ddT. (C) Telomerase reaction mixtures separated on a 10% sequencing gel. All reactions contained 1.5 μM [α-^{32}P]dGTP with primer $(G_4T_2)_3$. Lanes: 1, marker, light band at 19 nucleotides; 2, 4, 6, and 8, reactions with mutant 49G enzyme (lanes 49); 3, 5, 7, and 9, reactions with wild-type-transformed enzyme (lanes W). For lanes 2 to 5, reaction mixtures contained 100 μM dCTP and 100 μM TTP; for lanes 6 and 7, reaction mixtures contained 100 μM TTP and had no dCTP; for lanes 8 and 9, reaction mixtures contained 100 μM ddTTP and had no dCTP; for lanes 2 and 3, enzymes were preincubated with RNase A. (D) Schematic of major and minor products produced in reactions by mutant 49G enzyme from panel C during the first round of synthesis. The large dot indicates a G-T mispair when dCTP is absent. Elongation products are shown in underlined lowercase letters.

tion 49 is also used as a templating nucleotide, in this case producing a 7-nucleotide G_4T_3 repeat during each round by copying positions 49 to 43 (10). However, it was possible that the use of position 49 as a templating position represented aberrant behavior caused by the 49G and 43A mutations. To determine whether wild-type telomerase can use position 49 as a templating nucleotide, the wild-type enzyme was assayed with telomeric primers ending in $-T_2$, $-T_3$, or $-T_4$. Previous work has shown that with complementary DNA oligonucleotides, telomerase RNA residues immediately 3′ to the template region are cleavable by RNase H, indicating that they are capable of forming a DNA-RNA hybrid (12). With the $-T_2$ ending primers $(G_4T_2)_2$ (designated P12) or $(G_4T_2)_3$ (designated P18), a strong labeled band (multiple turnover +*g product) corresponding to the addition of one dG residue was produced [Fig. 3A, lanes 3 and 4, P12+1, and lanes 5 and 6, P18+1; see also Fig. 5A, lanes 2 and 4, for primer $(G_4T_2)_2$]. As expected, this first turnover band was unaffected by the presence of ddTTP (Fig. 3A). The strong turnover product suggested that the $-T_2$ end of these primers annealed with positions 45 and 44 and that the first added nucleotide (*g) was templated by position 43, after which dissociation occurred with high frequency (Fig. 3B). In contrast, when a primer with a $-T_3$ 3′ end, the 14-nucleotide $(G_4T_3)_2$ (designated P14), was used to prime wild-type telomerase, formation of the initial strong +*g turnover product was greatly reduced (Fig. 3A, lanes 1 and 2, P14+1). We propose that the three terminal T residues of $(G_4T_3)_2$ anneal to telomerase RNA positions 50 to 52 (Fig. 3B) and that elongation then proceeds without a strong turnover of the initial +*g product, by copying residues 49 through 43 of the RNA, producing the +7 band. As described below, similar results were obtained with a primer with a $-T_4$ 3′ end, $(G_4T_4)_2$, which can potentially base pair with residues 50 to 53 (see Fig. 5A, lanes 10 and 12). Hence, we

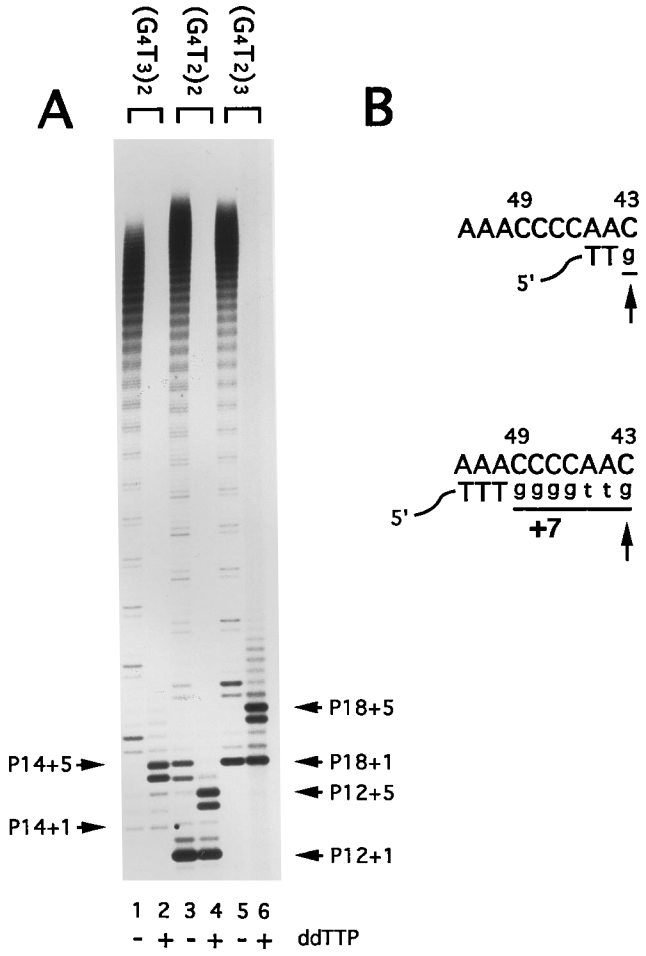

FIG. 3. (A) Wild-type enzyme uses residue 49 as a templating position. Telomerase reaction mixtures were separated on a 10% sequencing gel. All reaction mixtures contained wild-type transformed telomerase with 1.5 μM [α-^{32}P]dGTP. Lanes: 1, 3, and 5, reaction mixtures contained 100 μM TTP; 2, 4, and 6, reaction mixtures contained 100 μM ddTTP. Lanes 1 and 2, primer $(G_4T_3)_2$, designated P14; lanes 3 and 4, primer $(G_4T_2)_2$, designated P12; lanes 5 and 6, primer $(G_4T_2)_3$, designated P18. (B) Schematic of wild-type template region with either the -TT-ending primer or the -TTT-ending primer. Elongation products are shown in underlined lowercase letters. The arrow denotes the position of the first major turnover product. +7 denotes the 7-nucleotide repeat produced during first-round synthesis with the -TTT-ending primer before the first major turnover product was produced.

conclude that with these primers, wild-type telomerase can use RNA position 49 as a templating residue.

Efficient elongation of a 3′ rG-T mispair by 49G mutant telomerase. When the mutant 49G enzyme was assayed with the -T_3-ending primer $(G_4T_3)_2$, [α-^{32}P]dGTP, and ddTTP, in the presence or absence of dCTP, the final stop position was again determined by the presence of dCTP. With dCTP present (Fig. 4A, lane 1), the final stop bands at +4 and +5 were shifted down slightly from the positions of the two corresponding stop bands produced by wild-type telomerase (Fig. 4A, lane 2). This pattern was consistent with incorporation of an initial dC residue to the -T_3-ending primer by the 49G enzyme during first-round synthesis (schematic in Fig. 4B) and was similar to the result described above for the -T_2-ending primer (Fig. 2). However, in contrast to the -T_2 primer, with the -T_3 primer, only negligible amounts of label were incorporated into the first addition product. Hence, even though the two T residues at the 3′ end of this primer could pair with positions 44 and 45 of the template, or the penultimate 6 bases of this -GGGGTTT-ending primer could pair with positions 49 to 44 of the mutant 49G RNA, there was no evidence that either of these occurred. These alternatives would be expected to lead either to incorporation of a [^{32}P]dG by copying position 43 (producing a high turnover +1 band) or to removal of a mispaired 3′ T residue of the primer by the cleavage reaction of telomerase at position 43 and addition of *g, which would produce a labeled product the same size as the 14-nucleotide input primer. This cleavage reaction has been described previously for the wild-type enzyme (7). Without dCTP, the final stop band produced by the 49G enzyme was shifted 1 nucleotide down from the major, final stop band produced by the wild-type enzyme (Fig. 4A, lanes 3 and 4). These results were confirmed in experiments with the primers $(T_2G_4)_3$ and $(G_3T_2G)_3$ (data not shown) and are also consistent with the results shown in Fig. 2A, lane 3, and Fig. 2C, lane 8. Hence, the available data suggest that the mutated rG residue at telomerase RNA position 49 forms a non-Watson-Crick base mismatch with the 3′-terminal dT residue of the -T_3-ending primer, which, in the absence of dCTP, is extended by addition of the first nucleotide [α-^{32}P]dG (*g) by copying position 48 (Fig. 4B).

Failure to copy position 50 of mutant telomerase RNA. Since position 49 unexpectedly acted as a templating position, we also tested whether the same was true for position 50, by using the 50G mutant telomerase (Fig. 1B) and primers ending in -T_2, -T_3, or -T_4. The rationale was to determine whether elongation could proceed through position 50G, copying it into a dC, by forcing the 3′ end of the primer to pair just upstream of

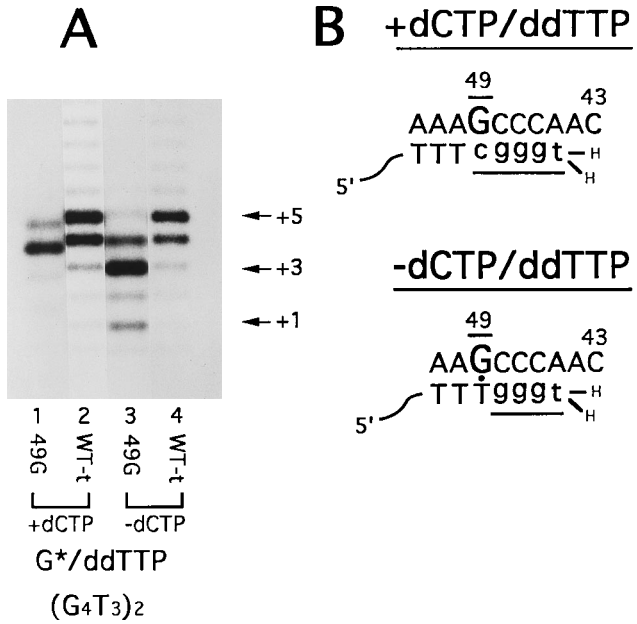

FIG. 4. Efficient 3′ G-T mispair extension by the 49G mutant enzyme. (A) Telomerase reaction mixtures were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP, 100 μM ddTTP, and primer $(G_4T_3)_2$. Lanes: 1 and 3, mutant 49G enzyme; 2 and 4, wild-type-transformed enzyme (WT-t). For lanes 1 and 2, reaction mixtures contained 100 μM dCTP; for lanes 3 and 4, reaction mixtures had no dCTP. Markers; +1, primer plus 1 nucleotide; +3, primer plus 3 nucleotides; +5, primer plus 5 nucleotides. (B) Schematic of products produced in reactions with and without dCTP that contained ddTTP by mutant 49G enzyme in Fig. 2C with primer $(G_4T_3)_2$. The large dot indicates a G-T mispair when dCTP is absent. The two H's attached to the 3′ T residue of the product represents a ddT. Elongation products are shown in underlined lowercase letters.

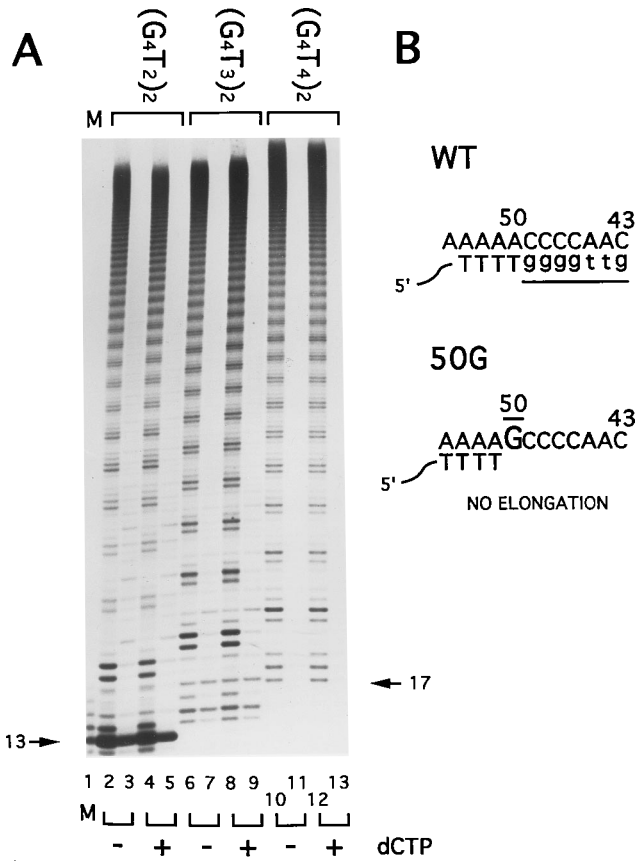

FIG. 5. Residue 50 is not used as a templating residue. (A) Telomerase reaction products were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP and 100 μM TTP. Lanes: 1, marker; the major band corresponds to 13 nucleotides; 2, 4, 6, 8, 10, and 12, reactions with wild-type-transformed enzyme; 3, 5, 7, 9, 11, and 13 reactions with mutant 50G enzyme; 4, 5, 8, 9, 12, and 13, reaction mixtures contained 100 μM dCTP; 2, 3, 6, 7, 10, and 11, reaction mixtures did not contain dCTP; 2 to 5, primer $(G_4T_2)_2$; 6 to 9, primer $(G_4T_3)_2$; 10 to 13, primer $(G_4T_4)_2$. (B) Schematic of mutant 50G template region and wild-type template region interaction with the -T_4-ending primer $(G_4T_4)_2$. Elongation products are shown in underlined lowercase letters.

position 50. However, with or without dCTP present, the 50G mutant enzyme synthesized essentially only abortive products (+*g) with the primer $(G_4T_2)_2$, consistent with copying position 43 (Fig. 5A, lanes 3 and 5). With the $(G_4T_3)_2$ or $(G_4T_4)_2$ primers, only small amounts of short products were made (Fig. 5A, lanes 7, 9, 11, and 13). We propose that although the $(G_4T_4)_2$ primer can potentially anneal with the A residues at positions 51 to 54, residue 50 cannot be copied, thereby blocking elongation (Fig. 5B).

In contrast to the 50G enzyme, wild-type enzyme efficiently synthesized long products with the $(G_4T_3)_2$ or $(G_4T_4)_2$ primers as well as with primer $(G_4T_2)_2$ (Fig. 5A, lanes 2, 4, 6, 8, 10, and 12). However, the intensity of the initial +*g product seen with $(G_4T_3)_2$ and $(G_4T_4)_2$ was lower than with $(G_4T_2)_2$, consistent with $(G_4T_3)_2$ and $(G_4T_4)_2$ annealing preferentially with positions 50 to 52 and 50 to 53, respectively, and elongation proceeding through positions 49 to 43 to produce the primer +7 product (Fig. 3B and 5B). Therefore, we conclude that position 50 of the telomerase RNA did not serve as a templating residue under these conditions.

A specific role for Watson-Crick base pairing of the primer with telomerase RNA position 50. With the -T_2-ending primers $(G_4T_2)_2$ and $(G_4T_2)_3$, both the 50G mutant telomerase (Fig. 6A, lanes 5 and 9) and the double-mutant 50-51G enzyme (lanes 7 and 11) were capable of adding primarily only one G residue, as shown by the incorporation of labeled dG at the +1 position. The second (and subsequent)-round elongation products synthesized by these two mutant telomerases were drastically reduced in amount compared with the products of 51G mutant telomerase (lanes 6 and 10) or control WT-t telomerase (lanes 8 and 12).

To test whether the 50G and 50-51G mutant telomerases failed to carry out post-first-round synthesis because the elongated products were unable to position their 3′ ends correctly by base pairing (Fig. 6C), we tested primer DNAs containing base changes that restored Watson-Crick base pairing with mutant telomerase RNA positions 50 and 51. The primer $G_4T_2G_4\mathbf{C_2}$ was elongated by the 50-51G mutant telomerase with a prominent stop after the addition of 7 nucleotides (Fig. 6A, lane 3). A first-round product of this length is predicted if Watson-Crick dG · rC base pairing dictates the position of primer annealing; this result also provided additional evidence that position 49 can be copied (Fig. 6B). A +7-nucleotide product from the 50G enzyme was visible after longer autoradiographic exposures (not shown). However, the 51G and WT-t telomerases did not detectably elongate the -C_2-ending primer to make round 1 products (Fig. 6A, lanes 2 and 4). As expected, neither mutant nor wild-type enzyme showed any evidence of pairing of the -C_2-ending primer with positions 44 and 45, which would have been predicted to produce the characteristic strong +*g dissociation product from copying position 43 (Fig. 6A, lane 3). These results suggested that efficient positioning for post-first-round synthesis requires Watson-Crick pairing of the primer with residues 50 and 51 (Fig. 6B).

Surprisingly, telomerase still showed a requirement for telomerase RNA residue 50 and, to a lesser extent, 51 to be in a Watson-Crick base pair even when the 3′ end of the primer was paired with other residues in the telomerase RNA template. As shown in Fig. 6A, lane 3, although the +7 product produced by the 50-51G mutant enzyme ends in -tt*g and thus could potentially Watson-Crick base pair its 3′ *g to position 49 and form wobble pairs between the tt and positions 50 and 51, second-round synthesis was greatly reduced. The need for a Watson-Crick base pair at position 50 was even more strikingly demonstrated by using primers with -G_3 at the 3′ end, allowing three dG · rC Watson-Crick base pairs to form at the 3′ end of the primer with template positions 49 to 47. These primers consisted of either the wild-type telomeric sequence $T_2G_4T_2G_3$ or the sequence $T_2G_4\mathbf{C_2}G_3$, containing internal compensatory nucleotide changes restoring complementary Watson-Crick base pairing to positions 50 and 51 in the 50-51G mutant and to position 50 in the 50G mutant. Each primer was tested with the wild-type, 51G, 50G, and 50-51G telomerases in the presence of [α-^{32}P]dGTP and the other unlabeled triphosphates. The degree of dissociation of the elongation products as synthesis proceeded along the template, copying positions 46 to 43, was determined by comparing the relative intensities of the +1 to +4 product bands (Fig. 6D). The 50G and 50-51G mutant telomerases barely elongated the wild-type $T_2G_4T_2G_3$ primer beyond 1 or 2 nucleotides (Fig. 6D, lanes 5 and 7). In contrast, the TT-to-CC change in the primer $T_2G_4\mathbf{C_2}G_3$ relieved this early product dissociation, with elongation by 50G and 50-51G proceeding efficiently to complete first-round synthesis before product dissociation at position 43 (addition of *gtt*g [Fig. 6D, lanes 1 and 3]). Conversely, with the wild-type primer, first-round synthesis by the 51G and WT-t telomerases proceeded to completion of the first round (Fig. 6D, lanes 6 and 8), whereas these telomerases mostly failed to complete first-round synthesis with the $T_2G_4\mathbf{C_2}G_3$ primer (Fig. 6D, lanes

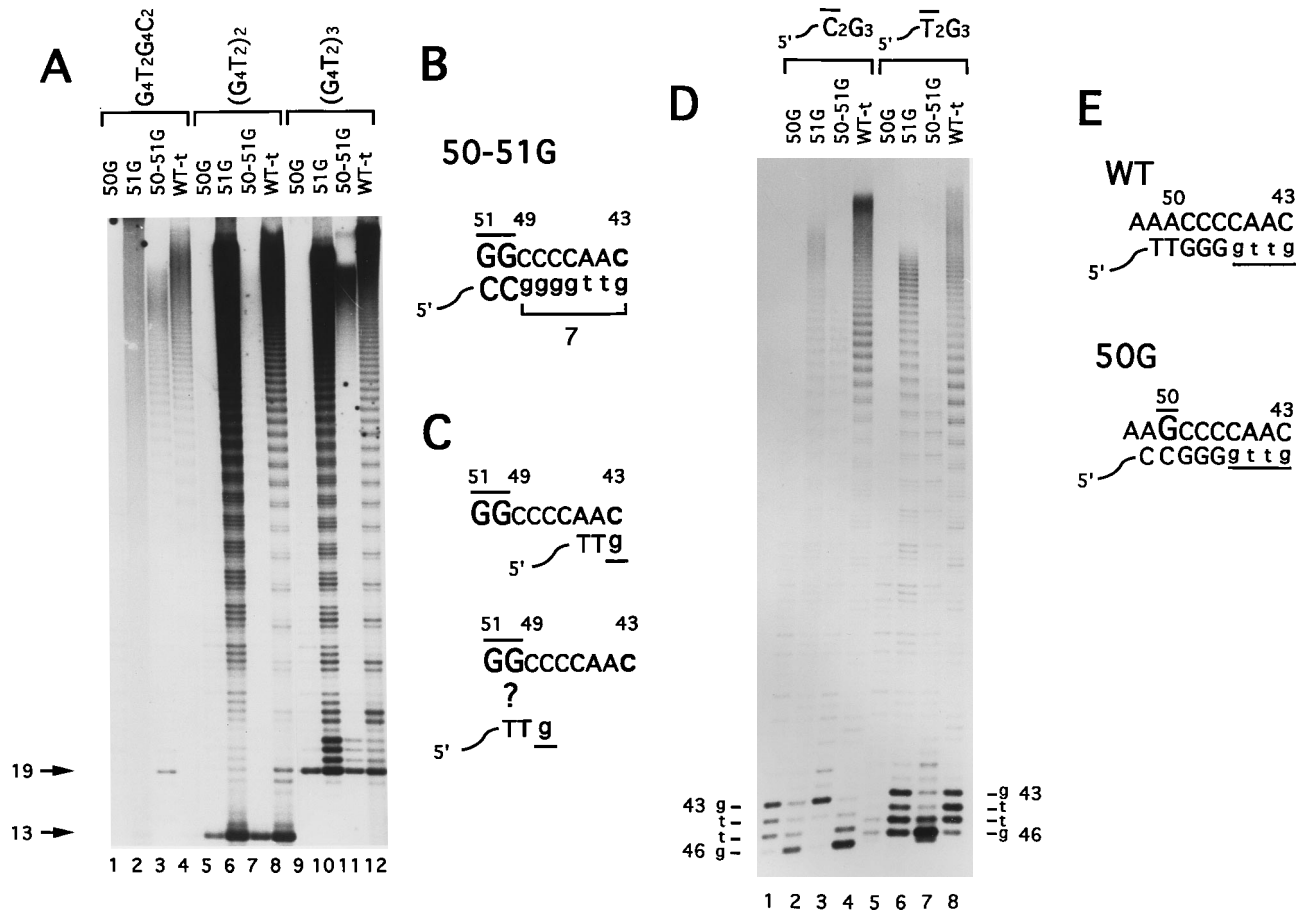

FIG. 6. Base pairing at residues 50 is essential for primer positioning and elongation. (A) Telomerase reaction products were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP and 100 μM dCTP, TTP, and dATP. Lanes: 1, 5, and 9, mutant 50G enzyme; 2, 6, and 10, mutant 51G enzyme; 3, 7, and 11, mutant 50-51G enzyme; 4, 8, and 12, wild-type-transformed enzyme. Lanes 1 to 4 contained primer $G_4T_2G_4C_2$; lanes 5 to 8 contained primer $(G_4T_2)_2$; lanes 9 to 12 contained primer $(G_4T_2)_3$. (B) Schematic of the mutant 50-51G template region. First-round synthesis of the 7-nucleotide product with the -CC-ending primer $G_4T_2G_4C_2$. Elongation products are shown in underlined lowercase letters. (C) Schematic of the mutant 50-51G template region. First-round product with TT 3′-ending primers $(G_4T_2)_2$ or $(G_4T_2)_3$ and the inability of first-round products to position for second-round synthesis. (D) Telomerase reaction products were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP and 100 μM dCTP, TTP, and dATP. Lanes: 1 and 5, mutant 50G enzyme; 2 and 6, mutant 51G enzyme; 3 and 7, mutant 50-51G enzyme; 4 and 8, wild-type-transformed enzyme. Lanes 1 to 4 contained primer $T_2G_4\mathbf{C_2}G_3$; lanes 5 to 8 contained primer $T_2G_4T_2G_3$. (E) Schematic of the wild-type (wt) template domain base paired with a wild-type primer and the 50G mutant template domain base paired with a primer with a compensatory base change forming a rG · dC base pair at position 50 with the primer.

2 and 4). The fact that only the wild-type and 51G mutant enzymes synthesized intermediate and long products relatively efficiently (Fig. 6D, lanes 2, 4, 6, and 8) further suggests that a Watson-Crick base pair is especially critical at residue 50 of the telomerase RNA. Moreover, with primer $T_2G_4\mathbf{C_2}G_3$, the mutant 50G and 50-51G telomerases showed less early dissociation before complete first-round synthesis than did the wild-type telomerase primed by the wild-type primer $T_2G_4T_2G_3$ (Fig. 6D, compare lanes 1 and 3 with lane 8). Thus, creating telomerase RNA-primer rG · dC base pairs, as opposed to the wild-type rA · T pairs, at position 50 in particular greatly reduced premature dissociation during the first round. Because rG · dC base pairs are expected to be more stable than rA · T base pairs, this observation further reinforced the importance of Watson-Crick base pairing to residues 50 and, to a lesser degree, 51 of the telomerase RNA.

The 51G mutation increases product dissociation at position 47. In Fig. 6A, lanes 6 and 10, an altered pattern of bands after first-round synthesis was particularly apparent for the 51G mutant enzyme product profile compared with that of the wild type, with two strong bands appearing above the strongest band in the wild-type repeat pattern (Fig. 6A, lanes 8 and 12), considered to be the position 43 translocation band (12, 17). For GGGGTT repeat synthesis copying positions 48 to 43, these stronger bands apparent in the 51G patterns would correspond to pausing and/or dissociation at positions 48 and 47. Similarly, altered product profiles were also seen when 51G enzyme was primed by $(T_2G_4)_2$, $(T_2G_4)_3$, and $(G_3T_2G)_3$ (data not shown). Pulse-chase experiments were performed with primer $(GT_2G_3)_3$ to determine whether the altered pattern was caused by pausing or product dissociation (Fig. 7). Comparison of Fig. 7, lanes 5 and 6, showed that for products ~50 nucleotides in length or shorter (arrowhead), the strong bands corresponded to product dissociation, as they were not chased into longer products; products longer than ~50 nucleotides could be chased into high-molecular-weight products, and hence pausing accounted for their increased strength compared with the wild type. Unexpectedly, the dissociation and pausing were especially marked after copying position 47 rather than position 48 (Fig. 6A, lane 6, and Fig. 7, lanes 4 and 5). If weakened pairing caused by the mispair at 51G were the major reason for premature product dissociation, it would have

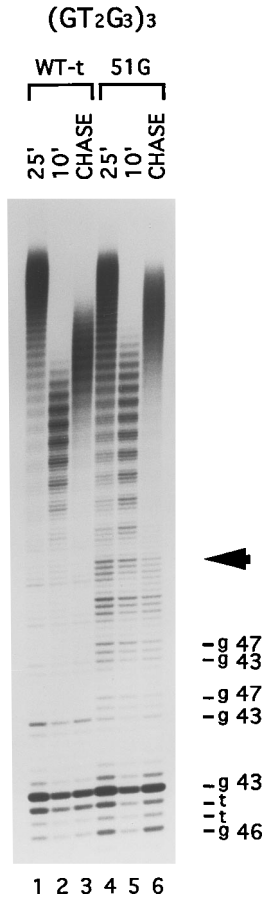

FIG. 7. The 51G mutation causes product dissociation at position 47. Telomerase reaction products were separated on a 10% sequencing gel. All reaction mixtures contained 1.5 μM [α-^{32}P]dGTP and 100 μM TTP. Lanes: 1 to 3, reaction mixtures contained wild-type-transformed enzyme; 4 to 6, reaction mixtures contained mutant 51G enzyme. For lanes 1 and 4, reactions were carried out for a total of 25 min; for lanes 2 and 5, reactions were stopped after 10 min; and for lanes 3 and 6, reactions were chased with the addition 10 μM unlabeled dGTP after 10 min and the reactions were stopped after a total reaction time of 25 min. The large arrow indicates the point of transition between products that are dissociated or complex with mutant and wild-type telomerases.

been predicted that dissociation would be most affected after copying position 48, since after position 47 one more dG-rC pair has been created between the product and template RNA. Therefore, this result is more reminiscent of previous findings that mutating position 43 to an A residue or position 48 to a U residue each caused premature product dissociation specifically at position 45, neither of which could be attributed to weaker base pairing (10).

DISCUSSION

An extended template in telomerase RNA. Unexpectedly, we have found that 7 nucleotides, from telomerase RNA positions 49 to 43 (Fig. 8), can be copied directly into DNA rather than the 6 nucleotides predicted to be required for copying the hexanucleotide telomeric DNA repeat of *T. thermophila* (GGGG TT). The templating nucleotides were delineated in several ways, using both wild-type and mutant telomerases. First, direct evidence that positions 49 through 43 are templating residues has come from showing that mutated residues at both these positions are copied into the complementary base, in this and previous work (10). Second, elongation products produced by mutant enzymes revealed the use of residues 49 to 43 to template the first round of product synthesis in the case of the 49G enzyme and 50-51G enzyme, when primed by the -CC ending primer $G_4T_2G_4C_2$, and subsequent rounds of copying by the 43A enzyme (10). Third, wild-type telomerase used positions 49 to 43 as templating residues during first-round synthesis from primers with a -T_3 or -T_4 3′ end. Hence, position 49 can act as either a templating or an alignment residue (Fig. 8). Using a different approach (in vitro reconstituted versus in vivo assembled telomerase), Autexier and Greider concluded that residue 49 was not used as a templating residue (1). In contrast, our results suggest the possibility that the template utilized by the wild-type enzyme to synthesize GGGGTT repeats is either 48 through 43 or 49 through 44. Consistent with the latter possibility, we have shown previously with wild-type telomerase that under certain in vitro conditions, the elongating primer dissociates at a high rate at position 44 but still synthesizes 6-base GGGGTT repeats (18).

Use of the seven possible templating nucleotides from positions 49 to 43 permits certain primers to be elongated from two annealing positions: primers with -T_2 at the 3′ end annealed either at positions 44 and 45 or at positions 50 and 51, while -T_3- and especially -T_4-ending primers annealed predominantly at positions 50 and 51, followed by copying of residues 49 to 43. This result also suggests that the A residues at positions 52 and 53, considered to be outside the templating domain because of their inability to be copied into TTGGGG repeats, can act as alignment residues to augment the alignment function of 50 and 51.

Positions 50 and 51 function in primer alignment. In contrast to residue 49, position 50 of the telomerase RNA did not serve as a templating residue to any detectable level under the conditions tested here. We showed by several direct tests that

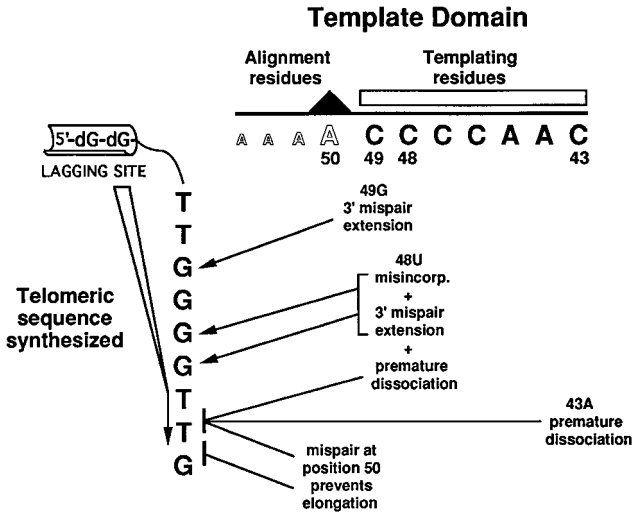

FIG. 8. Model for the active site of *T. thermophila* telomerase. The RNA template domain (residues 43 to 51) is shown with the two neighboring 3′ A residues which help align -T_3- and -T_4-ending primers by base pairing. Templating residues are designated by the open box and black letters (residues 43 to 49). Alignment residues are designated by the solid line and triangle (residues 43 to 53). Residues that function only to align are denoted by open letters (residues 50 to 53). The lagging product site as described previously is indicated by a half-cylinder (18). The telomeric DNA primer can bind its 5′ portion in the lagging product site, promoting k_{cat} for elongation at positions 45 and 44 (16) (open arrow). The Watson-Crick base pair at position 50 (solid triangle) with the corresponding primer position stimulates elongation to position 43. Mutations at positions 48 and 49 affect correct base recognition (10). Mutations at positions 48 and 43 cause premature product dissociation after position 45 is copied (10).

Watson-Crick base pairing with residues 50 and 51 is responsible for positioning the primer for elongation.

This in vitro analysis revealed that the 50G and 50-51G mutant telomerases were essentially competent only at first-round synthesis, displaying a severe decrease in long-product formation. However, strikingly, *Tetrahymena* cells containing 50G or 50-51G mutant telomerase had a completely wild-type phenotype, with no apparent decrease in fission rate or changes in cell morphology or telomere length over several hundred generations. These results suggest that first-round product formation may be sufficient for telomere maintenance in vivo. Consistent with this interpretation, Yu and Blackburn (32) concluded, from experiments in which two different telomerase RNAs with different template sequences were present in the same cell, that synthesis of telomeric repeats in vivo is nonprocessive, with on average one to two repeats being added at a time by a given telomerase ribonucleoprotein. Thus, we have shown that mutations within the telomerase RNA can have large effects on the enzymatic capacity of the mutant telomerases with minimal or nonexistent cellular consequences. These results also suggest that telomerase activity in cells may be present in considerable excess over the amount required for telomere maintenance, so that certain types of mutations resulting in decreased telomerase activity are tolerated.

Efficient elongation specifically requires position 50 to be in a Watson-Crick base pair with the primer. Surprisingly, pairing the three dG residues at the 3′ end of a -TTGGG primer to positions 49 to 46 of the 50G or 50-51G template was insufficient to allow its efficient elongation along the template in vitro, but efficient elongation was restored by using a $T_2G_4\mathbf{C_2}G_3$ primer, containing internal compensatory nucleotide changes complementary to the mutant enzyme position 50 (and 51 in the case of the 50-51G mutant). We conclude that the major requirement for a Watson-Crick base pair is at position 50.

Independent confirmation of the importance of a Watson-Crick base pair at position 50 also comes from the previous analysis of the 44G mutant telomerase (10). The 44G and 50G mutations are located at corresponding positions (underlined) in the terminally redundant AAC sequence at each end of the 3′-A<u>A</u>CCCCA<u>A</u>C-5′ templating domain. By using the primer ($T_2G_4T_2G_3$), the 44G mutant enzyme incorporates a C residue at position 44 and produces first-round elongation products ending with -G_3gt**c**g (10), while the mutant 50G enzyme produces a product ending in -G_3gt**t**g. After translocation following the first round, each enzyme might be expected to position its newly elongated 3′ end at residues 51 to 49, with Watson-Crick base pairs at 51 and 49 flanking either the dC-rA mismatch (44G mutant) or the dT-rG wobble pair (50G mutant) at position 50. However, both enzymes produced essentially only one round of synthesis. Thus, altering the primer or template sequence at corresponding nucleotide positions (44 and 50) to create a mispair at position 50 gives similar results.

We propose that the correct local base pair geometry of Watson-Crick base pairing is especially critical at residue 50 of the telomerase RNA. Specifically, the altered geometry of a wobble pair or mispair appears to be a significant factor in the inability of the mutant 50G, 50-51G, and 44G enzymes to synthesize beyond the first round. Especially when there are three Watson-Crick base pairs at the 3′ end of the primer, the difference in stability between a mutant rG-T mispair (wobble pair) and a wild-type rA · T pair at position 50 is unlikely to be sufficient to explain the dramatic decrease in post-first-round products by these mutant enzymes.

The specificity of the requirement for base pairing at position 50 is highlighted by the contrasting ability of the 49G mutant telomerase to extend a primer with mismatches at position 49. In the absence of dCTP, the 49G enzyme extended primers with an rG · T wobble pair between the 3′ nucleotide of the primer and position 49G. In these cases, copying proceeded efficiently all the way to the end of the template. In addition, in previous work, we have shown that 48U mutant telomerase, containing a C-to-U transition in position 48 of telomerase RNA, efficiently elongates rC-dA mispairs at the 3′ end of the primer at positions 47 and 46 (10).

Interactions between DNA, RNA, and protein at the telomerase active site. We propose a model (Fig. 8), incorporating these new results and previous findings (10, 17, 18), in which a collaboration between the DNA primer, the telomerase RNA, and the telomerase protein(s) is required for optimal active-site function. We have now identified several specific components of this set of interactions. The present work has identified a Watson-Crick pair, formed specifically with the primer and position 50 of the telomerase (Fig. 8), that is required for synthesis to proceed efficiently to the 5′ end (positions 44 and 43) of the template, even when multiple additional Watson-Crick base pairs can form between the downstream region of the primer and the template. This primer-telomerase interaction at position 50 (Fig. 8) is clearly distinguished from the Watson-Crick pairing that occurs at other positions in the templating domain by its high degree of intolerance of a wobble or other mispair. The base pairing at position 50 is also distinct from the base-specific interactions of the 5′ end of the primer shown previously to cause changes in k_{cat} of polymerization at distant positions on the template (18) (Fig. 8). These interactions did not involve the RNA template residues but, instead, either protein and/or other regions of the RNA. The region of telomerase responsible for these 5′ interactions (Fig. 8) has been called the second site (23), anchor site (7), or lagging-product site (18), the last by analogy with RNA polymerase of *E. coli*. Positive effects on k_{cat} (18) and on primer binding (13) were always found when dG residues were present in this portion of telomerase. Thus, the stimulatory effects on elongation of the position 50 primer pairing and the 5′ dG residue interactions identify two specific components of the telomeric DNA substrate specificity of telomerase.

In addition to primer DNA-telomerase interactions, we have shown previously that altering specific template residues affects active-site function in ways not explainable by altered base pairing (10). On the basis of these findings, we proposed that certain template residues may play critical roles in active-site function. Mutating position 48 or 43 led to premature product dissociation at position 45 (Fig. 8). We also showed that the 48U mutant telomerase, containing a C-to-U mutation at position 48, synthesizes and extends rC-dA mispairs by incorporating dA where dG should be incorporated at positions 47 and 46 (10). Here we have shown that mutating another residue, to create the 49G mutant telomerase, also causes a specific loss in fidelity, by allowing a rG-T mispair at the 3′ end of the primer to be extended. The specific losses of fidelity caused by the 48U and 49G mutations (Fig. 8) could result from a distortion of the active site or failure of the mutated RNA residue to contribute a critical functional group to the active site, which prevents a mispair from being distinguished from the correct local geometry of a Watson-Crick base pair.

In summary, we have shown directly that the *T. thermophila* telomerase RNA templating residues extend from positions 43 to 49 and that residues 3′ to the 9-nucleotide templating domain can augment alignment of the primer for elongation. Most unexpectedly, a highly specific contribution of the primer to efficient elongation on distant positions on the template involves formation of a Watson-Crick base pair with position

50 in the RNA. From the combined results of this and previous work, we propose a model in which a three-way interaction exists between the DNA product, the telomerase RNA template region, and the telomerase proteins to promote active-site function. The emerging picture of the active site of this unusual reverse transcriptase is one in which base-specific RNA-protein interactions are required for correct elongation, which leads in turn to base-specific interactions with the telomeric DNA sequence that is synthesized, further promoting polymerization.

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