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Processing of Nontelomeric 3′ Ends by Telomerase: Default Template Alignment and Endonucleolytic Cleavage

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Telomerase is a specialized reverse transcriptase that maintains telomeres at chromosome ends by extending preexisting tracts of telomeric DNA and forming telomeres de novo on broken chromosomes. Whereas the interaction of telomerase with telomeric DNA has been studied in some detail, relatively little is known about how this enzyme processes nontelomeric DNA. In this study we recruited the *Euplotes* telomerase to nontelomeric 3′ termini in vitro using chimeric DNA primers that carried one repeat of a telomeric sequence at various positions upstream of a nontelomeric 3′ end. Such primers were processed in two distinct pathways. First, nontelomeric 3′ ends could be elongated directly by positioning a primer terminus at a specific site on the RNA template. Delivery to this default site was precise, always resulting in the addition of 4 dG residues to the nontelomeric 3′ ends. These same residues initiate new telomeres formed in vivo. Alternatively, 3′ nontelomeric nucleotides were removed from primers prior to initiating the first elongation cycle. As with default positioning of nontelomeric 3′ ends, the cleavage event was extremely precise and was followed by the addition of dG residues to the primer 3′ ends. The specificity of the cleavage reaction was mediated by primer interaction with the RNA template and, remarkably, proceeded by an endonucleolytic mechanism. These observations suggest a mechanism for the precision of developmentally regulated de novo telomere formation and expand our understanding of the enzymatic properties of telomerase.

Telomerase is the specialized DNA polymerase responsible for maintaining tracts of telomeric DNA at chromosome ends (15) and for building new telomeres on broken chromosomes (33, 50). A ribonucleoprotein, telomerase is an unusual reverse transcriptase that directs the synthesis of telomere repeat arrays by copying a sequence within its RNA subunit (4, 12, 18, 29, 31, 43, 44). For example, the *Euplotes crassus* telomerase RNA carries the sequence 5′CAAAACCCCAAAACC3′. This domain specifies the addition of TTTTGGGG repeats onto chromosome ends (43).

Protein subunits as well as the telomerase RNA moiety are needed to carry out polymerization (15). Two telomerase proteins have recently been cloned from *Tetrahymena thermophila*. One protein, p95, contains a DNA binding domain and functions in primer recognition (10, 20). The second protein, p80, binds the telomerase RNA and displays sequence similarity to the eukaryotic transcription factor TFIIS (10), which stimulates transcript cleavage by RNA polymerase (22, 39). Like RNA polymerase, the *Tetrahymena* and *Saccharomyces cerevisiae* telomerases have associated cleavage activities that remove 3′ terminal nucleotides from telomeric primers (2, 7, 8). The mechanism and biological relevance of this activity in telomere synthesis are unknown.

The interaction of telomerase with telomeric DNA has been examined in some detail (14, 16, 17, 27, 42, 52, 53). In vitro, telomerase efficiently elongates single-stranded DNA primers resembling the natural G-rich 3′ overhangs on chromosome termini. Telomeric primers appear to interact with two sites in telomerase (8, 10, 19, 20, 27, 28, 32, 35): the anchor site which resides in a protein subunit, presumably the *Tetrahymena* telomerase p95 equivalent (10), and the template site in the RNA subunit. Analogous to the leading and lagging sites proposed for RNA polymerase (6), these telomerase domains are thought to act in concert to facilitate polymerization of long tracts of G-rich telomeric repeats. Telomere synthesis is initiated by Watson-Crick base-paired alignment of a DNA primer 3′ terminus on the RNA template, while upstream primer sequences bind in the anchor site. A short stretch of DNA is added onto the primer 3′ terminus by copying nucleotides in the RNA template. Once the end of the templating region is copied, the new primer 3′ terminus is repositioned back at the beginning of the RNA template, simultaneously feeding the primer 5′ terminus further into the anchor site. Primer association with the anchor site maintains DNA-enzyme contact during the translocation event, permitting successive rounds of polymerization and translocation instead of primer dissociation.

In addition to elongating preexisting telomere tracts, telomerase can synthesize telomeres de novo onto nontelomeric DNA termini (50). The process of de novo telomere formation has been well documented in the ciliated protozoa, in which chromosome fragmentation and telomere formation are temporally coupled (11, 48) and are part of a massive developmentally regulated program of genome reorganization (37). Nuclear reorganization involves fragmentation of a copy of the germ line micronuclear genome into hundreds to thousands of linear DNA molecules that will ultimately make up the macronuclear genome. Telomerase caps each nascent DNA terminus with telomeres. Chromosome break sites are typically A/T rich (3, 47, 49), displaying no resemblance to the G-rich telomeric sequences that will be added. Hence, Watson-Crick base-paired alignments of the new chromosome 3′ ends onto the RNA template are precluded. Despite this, de novo telomere formation is extremely precise. All new telomeres initiate with the same register of repeats. In *E. crassus* this sequence is 5′GGGGTTTT. . .3′ (24), only one of the eight possible permutations of the G_4T_4 telomere repeat.

Studies with the human and *Tetrahymena* telomerases showed that telomeric repeats could be added directly onto nontelomeric 3′ ends in vitro (19, 35). Using chimeric primers bearing a cassette of telomeric sequence at the 5′ terminus of

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an otherwise nontelomeric primer, Harrington and Greider demonstrated that the *Tetrahymena* telomerase can catalyze the addition of telomeric DNAs onto nontelomeric 3′ ends (19). A telomeric cassette positioned up to 36 residues away from the 3′ terminus was sufficient for primer elongation. This study and others (8, 27, 28, 32, 35) were instrumental in defining the anchor site in telomerase. Presumably, the interaction of the upstream telomeric cassette with the anchor site facilitates delivery of a primer 3′ terminus into the enzyme active site for polymerization.

To better understand the interaction of telomerase with nontelomeric DNA, we examined the fate of nontelomeric 3′ ends during the initial primer elongation cycle. Our approach utilized chimeric DNA primers with a telomeric cassette sequence located at various positions upstream of a primer 3′ terminus. Although all such primers were extended, telomerase employed two distinct strategies in preparing the nontelomeric 3′ ends for elongation. In the first pathway, the primers could be elongated directly by positioning the 3′ ends at a default site on the RNA template independent of Watson-Crick base pairing. In the second pathway, telomerase eliminated a large number of nucleotides of nontelomeric DNA from the primer 3′ terminus prior to the first cycle of elongation. This reaction was mediated by primer interactions with the RNA template site and, surprisingly, proceeded by an endonucleolytic mechanism. These findings suggest a mechanism for the precision of developmentally regulated de novo telomere formation in vivo and offer new insight into the functions of the telomerase enzyme active site.

MATERIALS AND METHODS

Isolation of *Euplotes* macronuclei. *E. crassus* was grown and mated as described previously (40). Macronuclei containing active telomerase were isolated from cells at approximately 64 h after mating and were purified on Percoll-sucrose gradients (42). Nuclei were resuspended to approximately 10^5/ml in TMG buffer (10 mM Tris-HCl [pH 7.5], 3 mM $MgCl_2$, 10% glycerol) and stored at −80°C until use.

Telomerase assays. *Euplotes* macronuclei (5 μl) were assayed in 20-μl reaction mixtures containing 0.4 μM primer, 5 mM $MgCl_2$, 20 mM EGTA, 50 mM Tris-HCl (pH 8.0), 1 mM spermidine, 1 mM dithiothreitol, 0.1 mM dTTP, and 0.5 μM [^{32}P]dGTP (800 Ci/mmol) unless otherwise stated. Concentrations of nucleotides used are given in the figure legends. Reaction mixtures were incubated at 30°C for 1 h. DNA was precipitated, and products were resolved on sequencing gels and subjected to autoradiography. When indicated, products were quantified on a FUJIX BAS2000 PhosphorImager (National Science Foundation grant BIR9217251). All telomerase reactions were carried out at saturating primer concentrations.

Oligonucleotide preparation. DNA oligonucleotides were obtained from Gibco-BRL or GlaxoWellcome, Inc. Methylphosphonate oligonucleotides were synthesized and purified as previously described (21). All oligonucleotides were purified on 20% denaturing polyacrylamide gels before use. To generate the plus-one-product markers, gel-purified oligonucleotides were reacted with [^{32}P]dGTP and terminal deoxynucleotidyl transferase (Boehringer Mannheim) for 15 min at 37°C as per the manufacturer's instructions. Following labeling, the plus-one product was excised from a 20% denaturing gel and eluted overnight in TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). To generate primers carrying 3′-terminal dideoxynucleotides, gel-purified primers were incubated with terminal deoxynucleotidyl transferase and ddGTP for 2 h at 37°C and gel purified as described above.

RESULTS

***Euplotes* telomeres synthesized de novo initiate with dG residues in vitro.** To examine the synthesis of the first telomeric repeat added onto nontelomeric DNA 3′ ends, macronuclear extracts from *E. crassus* undergoing chromosome fragmentation and de novo telomere addition were reacted with standard telomeric oligonucleotide primers or chimeric oligonucleotides that carried stretches of telomeric and nontelomeric DNA. Chimeric primers consisted of various 3′ nontelomeric sequences and a distal or internal telomeric cassette of the 8-nucleotide sequence 5′GGGGTTTT3′. For example, pBRGT13T is a 21-mer oligonucleotide that bears 13 residues of the pBR plasmid sequence at its 3′ end and a cassette of 8 nucleotides (G_4T_4) its 5′ end. Similarly, EupGT13 is a 21-mer with a 5′ G_4T_4 cassette and 13 nucleotides at its 3′ end which are complementary to the *Euplotes* telomerase RNA outside the templating domain. As expected, a primer lacking any telomeric sequence failed to be extended into products typical of telomerase elongation (Fig. 1A, lane 4). However, inclusion of a telomeric cassette allowed for efficient extension of both EupGT13 and pBRGT13T (Fig. 1A, lanes 2 and 3), generating ladders of elongation products with an 8-nucleotide periodicity that are characteristic of *Euplotes* telomerase extension (see below). Since the products extended upward from the plus-one-nucleotide-product marker, we conclude that the 13-nucleotide nontelomeric 3′ terminus had not been eliminated from these primers prior to telomerase extension (see below).

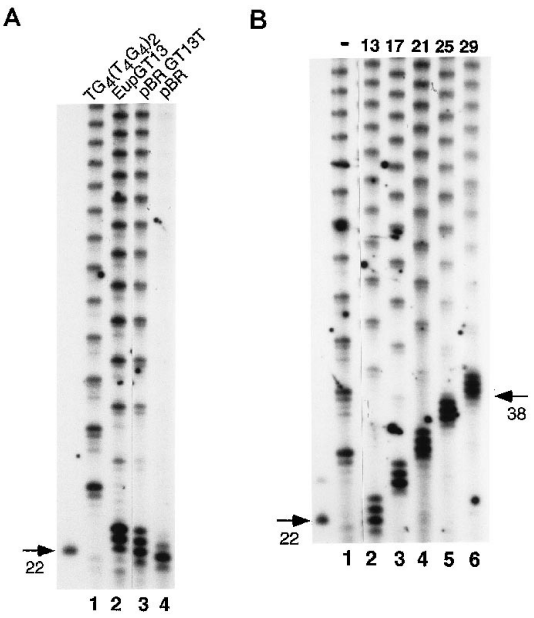

FIG. 1. Default extension of nontelomeric 3′ ends by telomerase. The products of *Euplotes* telomerase reactions were resolved on a 6% sequencing gel and subjected to autoradiography. (A) Nontelomeric 3′ ends are extended by the same register of telomeric repeats. Reactions are shown with $TG_4(T_4G_4)_2$ (lane 1); EupGT13, (G_4T_4)CGCACCTATTGAT (lane 2); pBRGT13T, (G_4T_4)ACTACGCGATCAT (lane 3); and pBR, CACTATCGACTACGCGATCAT (lane 4). The arrow indicates the migration of a 22-nucleotide marker. Some of the autoradiographs shown were exposed for long periods so that signals are not in the linear range of the film. Standard telomeric primers are utilized more efficiently than chimeric primers (see Fig. 3). The small products observed with pBR are generated by nonspecific activities in the nuclear extract (42). (B) Increasing the length of the 3′ nontelomeric sequence does not alter telomere synthesis by telomerase. Numbers above lanes 2 to 6 indicate the numbers of 3′ nontelomeric nucleotides that each primer carries. The minus sign over lane 1 indicates a completely telomeric primer. Primers $TG_4(T_4G_4)_2$ (lane 1); pBRGT13T (lane 2); pBRGT17, (G_4T_4)ATCGACTACGCGATCAT (lane 3); pBRGT21, (G_4T_4)CACTATCGACTACGCGATCAT (lane 4); pBRGT25, (G_4T_4)TAGCCACTATCGACTACGCGATCAT (lane 5); and pBRGT29, (G_4T_4)ATCATAGCCACTATCGACTACGCGATCAT (lane 6), were reacted with telomerase. A 22-nucleotide marker is shown in the far left lane; the migration of a 38-nucleotide primer is denoted by an arrow on the far right.

The strongest bands in the elongation ladders shown in Fig. 1A correspond to enzyme pausing or product dissociation (42, 43), with the periodicity of the pattern being set by the first nucleotides added by telomerase. Reactions with the two chimeric primers produced identical banding profiles, the most intense bands aligning across both lanes (Fig. 1A, lanes 2 and

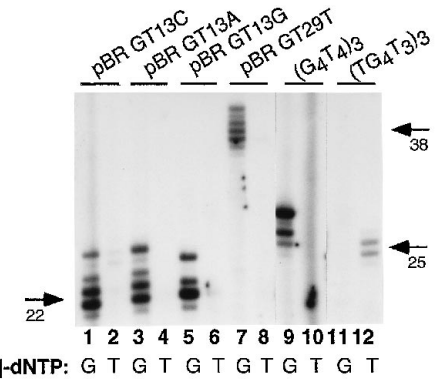

FIG. 2. Telomere synthesis onto nontelomeric DNA initiates with dG residues. Telomerase assays are shown with pBRGT13C, (G_4T_4)ACTACGCCAT CAC (lanes 1 and 2); pBRGT13A, (G_4T_4)ACTACGCCATCAA (lanes 3 and 4); pBRGT13G, (G_4T_4)ACTACGCCATCAG (lanes 5 and 6); pBRGT29 (lanes 7 and 8); $(G_4T_4)_3$ (lanes 9 and 10); and $(TG_4T_3)_3$ (lanes 11 and 12). Odd-numbered lanes contain 0.5 μM [^{32}P]dGTP (800 Ci/mmol) and 0.5 mM ddTTP, and even-numbered lanes contain 1.0 μM [^{32}P]dTTP (800 Ci/mmol) and 0.5 mM ddGTP. Reaction products were resolved on a 10% sequencing gel. The arrows mark the migrations of 22-, 25-, and 38-nucleotide markers.

3). The $TG_4(T_4G_4)_2$ control was extended by the addition of $(TTTTGGGG)_n$ (Fig. 1A, lane 1). Since the banding profile in the chimeric primer reactions was offset by 4 nucleotides from this control (Fig. 1A, lanes 1 and 2), we surmised that the chimeric primers were initially extended by dGGGGTTTT. Addition of this permutation of telomeric repeats was not altered when the cassette was moved further away from the primer 3′ end in 4-nucleotide increments (Fig. 1B). The periodicity of the profile shifted by exactly 4 nucleotides (Fig. 1B, lanes 2 to 6), corresponding to the increased length of the input primer. Despite the variable nontelomeric 3′ ends, telomerase appeared to initiate synthesis by adding the same set of nucleotides to all the chimeric primers.

We determined which nucleotides were first added to nontelomeric 3′ ends by conducting telomerase assays in the presence of [^{32}P]dGTP and ddTTP or [^{32}P]dTTP and ddGTP. Under these conditions, elongation will be terminated by dideoxynucleotide incorporation. For instance, the telomeric primer $(TG_4T_3)_3$ acquired dTddG (Fig. 2, lanes 11 and 12) while the primer $(G_4T_4)_3$ was extended by dGGGGddT (lanes 9 and 10). Similar assays were conducted with chimeric primers bearing diverse 3′ sequences. No radiolabeled extension products were obtained in reactions with [^{32}P]dTTP and ddGTP for any of the chimeric primers (Fig. 2, lanes 2, 4, 6, and 8). However, in the presence of [^{32}P]dGTP and ddTTP, chimeric primers bearing 3′ dT, dA, or dC residues were extended by the addition of 5 nucleotides corresponding to the incorporation of dGGGG ddT (Fig. 2, lanes 1, 3, and 7). When the primer terminated in one or more dG residues, a different result was obtained. A primer terminating in a single 3′ dG residue was extended by dGGGddT (Fig. 2, lane 5), while a primer bearing 3 3′ dG residues was extended by dGddT (data not shown). These data indicate that a terminal dG is sufficient to align a nontelomeric 3′ end on the RNA template. This observation agrees with results obtained with human telomerase showing that as few as 2 bp of complementarity to the RNA template can correctly align a primer for elongation (35).

Interestingly, a single 3′ dT residue on a chimeric primer was not able to align a primer 3′ terminus to a complementary rA in the *Euplotes* telomerase RNA template (Fig. 2, lanes 7 and 8). These data are consistent with delivery of nontelomeric 3′ ends to the vicinity of rC nucleotides on the RNA such that a terminal dG can base pair directly with the RNA template for extension while polymerization onto other 3′ terminal residues proceeds without prior Watson-Crick paired alignment. Thus, the *Euplotes* telomerase can direct nontelomeric 3′ ends to a default position on the RNA template, which always results in a telomere sequence initiating with dGGGGTTTT, the same sequence found at sites of de novo telomere formation in vivo.

Recruitment of telomerase to nontelomeric DNA. Sequences distal to a primer 3′ terminus appear to bind outside the RNA template, presumably in the anchor site of a protein subunit. To further investigate the interaction between primers and the telomerase anchor site, we examined the efficiency with which telomerase utilizes primers carrying different 5′ telomeric sequences. Specifically, we explored the minimal sequence requirements for telomerase recognition by assaying primers with telomeric cassettes of varying dG and dT content. A sampling of telomerase products from such reactions is shown in Fig. 3A. Only a subset of the chimeric primers tested were efficiently extended into long reaction products. A completely nontelomeric primer (Fig. 3A, lane 11) and primers containing 5′ cassettes consisting of nonconsecutive dG residues or dT residues alone were very poorly utilized by telomerase (Fig. 3A, lanes 5 to 7 and 10). Under our conditions, the *Euplotes* telomerase synthesized predominantly short products with such primers; longer products having the characteristic 8-nucleotide repeated banding pattern were extremely faint.

In contrast, chimeric primers carrying as few as three consecutive dG residues at their 5′ termini and no adjacent dT residues extended were much more efficiently and only slightly more poorly than primers carrying the complete G_4T_4 cassette (Fig. 3A, lanes 2 and 9 and see Fig. 3B). All primers carrying dG residues at their 5′ termini that were extended by telomerase acquired the default permutation of telomere repeats (Fig. 3A). Since altering the sequence composition of the cassette had no effect on primer positioning within the RNA template site, we conclude that the upstream telomeric cassette must interact at the anchor site rather than at the RNA template.

To quantitate the amount of product synthesized with chimeric primers, telomerase reactions were carried out in the presence of [^{32}P]dGTP and ddTTP. Because the primers tested were extended by the addition of the same residues, dGGGG ddT (Fig. 2 and data not shown), we could make a direct comparison of the amounts of radioactivity incorporated among the samples by PhosphorImaging (Fig. 3B). The results obtained under these assay conditions were congruent with the results of the qualitative analysis displayed in Fig. 3A. Elongation of primers is reported relative to a 21-nucleotide chimeric primer carrying a perfect 5′ G_4T_4 telomeric repeat at its 5′ terminus and 13 nucleotides of the 3′ nontelomeric sequence (Fig. 3B, second bar). As with the *Tetrahymena* telomerase (18), the specificity of the *Euplotes* enzyme for chimeric primers was substantially lower than those of standard telomeric primers. Chimeric primers incorporated only about one-third the amount of radioactivity (Fig. 3B, compare the first two bars). Interestingly, increasing the length of nontelomeric DNA on the primer 3′ terminus did not substantially affect product abundance. Chimeric primers with up to 29 3′ nontelomeric nucleotides were elongated approximately as efficiently as primers carrying only 13 3′ nontelomeric residues. Furthermore, the level of incorporation did not substantially increase when a second telomeric repeat was added to the chimeric primer 5′ terminus (Fig. 3B, compare the bars labeled G_4T_4, G_4T_4-29, and $(G_4T_4)_2$-29). In agreement with the data shown in Fig. 3A, primers bearing as few as three consecutive

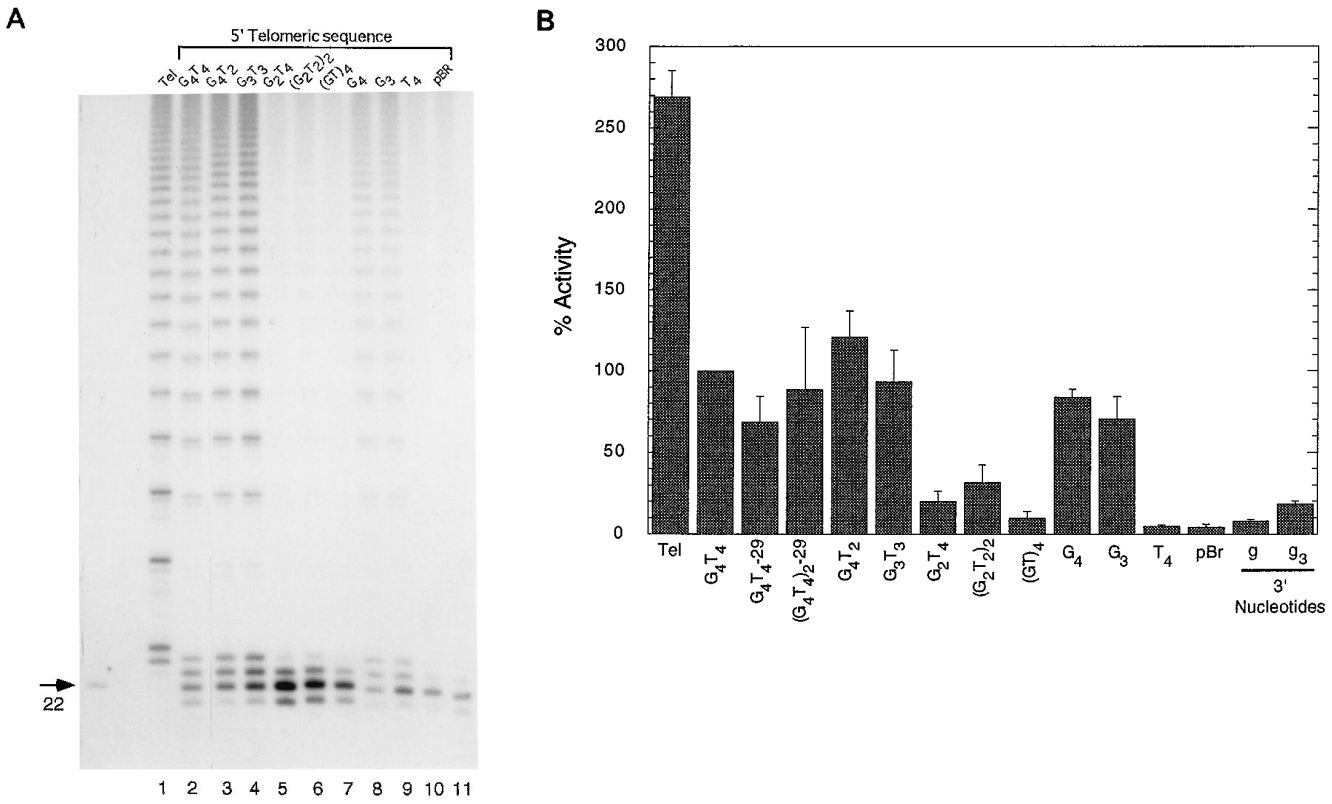

FIG. 3. Minimal sequence requirements of DNA primer-anchor site recognition. (A) As few as three consecutive 5′ dG residues are adequate for telomerase recruitment. Standard telomerase reactions were performed, and the elongation products were resolved on a 10% sequencing gel. Reactions are shown with (telomeric sequences are underlined) Tel, TGGGGTTTTGGGGTTTTGGGG (lane 1); G_4T_4, GGGGTTTTACTACGCGATCAT (lane 2); G_4T_2, GGGGTTCGACTACGCGATCAT (lane 3); G_3T_3, GGGTTTCGACTACGCGATCAT (lane 4); G_2T_4, GGTTTTCGACTACGCGATCAT (lane 5); $(G_2T_2)_2$, GGTTGGTTACTACGCGATCAT (lane 6); $(GT)_4$, GTGTGTGTACTACGCGATCAT (lane 7); G_4, GGGGATCGACTACGCGATCAT (lane 8); G_3, GGGTATCGACTACGCGATCAT (lane 9); T_4, TTTTATCGACTACGCGATCAT (lane 10); and pBR (lane 11). The arrow indicates the migration of a 22-nucleotide marker. (B) Telomerase reactions with [^{32}P]dGTP and ddTTP were carried out. The amount of dGGGGddT synthesized by telomerase is reported relative to the chimeric primer carrying a complete 5′ G_4T_4 repeat, (G_4T_4). The data represent three independent experiments. Primers tested, from left to right (5′ telomeric sequences are underlined), are Tel; G_4T_4, GGGGTTTTACTACGCGATCAT; G_4T_4-29, GGGGTTTTATCATAGCCACTATCGACTACGCGATCAT; $(G_4T_4)_2$-29, GGGGTTTTGGGGTTTTATCATAGCCACTATCGACTACGCGATCAT; G_4T_2; G_3T_3; $(G_2T_2)_2$; $(GT)_4$; G_4; G_3; T_4; and pBR. Results with primers containing 3′-terminal dG residues are shown in the two right bars and represent the averages of two experiments. g, CACTATCGACTACGCGATCAG; g_3, CACTATCGACTACGCGATGGG.

dG residues were utilized nearly as efficiently as those containing a complete G_4T_4 cassette. Finally, primers with 3 dG residues at their 3′ termini incorporated much less radioactivity than primers with 3 5′ dG residues (Fig. 3B), suggesting that DNA-anchor site interactions may be more important for primer recruitment and elongation than the DNA interaction at the RNA template.

The *Euplotes* telomerase can eliminate nontelomeric DNA before initiating telomere synthesis. The default mode for initiating telomere synthesis was observed only with primers carrying telomeric cassettes at their 5′ termini. Primers with internal telomeric cassettes were processed differently. The most striking finding was the generation of reaction products that migrated below the full-length input primer (Fig. 4A). This result is in stark contrast to that observed in reactions with primers bearing a 5′ telomeric cassette, in which all elongation products migrate above the input primer. Primers carrying internal telomeric cassettes generated banding profiles offset from each other (Fig. 4A, lanes 2 to 5) and from pBRGT13T (lane 1), a primer elongated in the default mode. The offset in banding patterns and the migration of products below the input primer corresponded exactly to the amount of nontelomeric sequence at the primer 3′ end (Fig. 4B). For example, a reaction with pBRGT6, which carries 7 nucleotides of nontelomeric sequence at its 5′ end and 6 nontelomeric nucleotides at its 3′ end, generated products that migrated a distance 6 residues shorter than the 21-nucleotide input primer. The overall banding profile from this reaction was offset by 6 nucleotides compared with that of pBRGT13T (Fig. 4A, lane 5).

We verified that nucleotides were being removed from the primer 3′ terminus and not the 5′ terminus. Several primers were reacted with terminal deoxynucleotidyl transferase and a dideoxynucleotide to yield a 22-nucleotide primer terminating in a ddNTP residue. DNA molecules carrying the 3′ ddNTP are extended by telomerase only upon removal of the terminal dideoxynucleotide. Telomerase failed to elongate both the telomeric primer $(G_4T_4)_3$ (Fig. 4C, lane 2) and a primer carrying a 5′ telomeric cassette which is extended in the default mode (lane 4) when they terminated in 3′ ddGTP. In contrast, pBRGT4, a primer bearing an internal telomeric cassette and 4 nontelomeric residues at its 3′ end, was extended whether it terminated in 3′ ddGTP or not, generating initial products that migrated below the input primer (Fig. 4C, lanes 5 and 6). Extension occurred not simply because the 3′ ddGTP was removed; a total of 5 nucleotides corresponding to 4 residues of 3′ nontelomeric nucleotides plus the terminal ddGTP were eliminated prior to elongation by telomerase. The plus-one

product from this reaction migrated as 18 nucleotides (Fig. 4C, lane 6).

The specificity of the cleavage reaction indicated that primer shortening was directed by telomerase and not a nonspecific nuclease in the nuclear extract. First, although a faint 1-nucleotide ladder of products migrating below the input primer was observed upon long exposures of the autoradiographs, addition of excess nonspecific DNA eliminated these products but had no effect on the cleavage products obtained with the chimeric primers (data not shown). Second, the number of nucleotides eliminated from a primer 3′ terminus was strictly dependent upon the alignment of the primer on the RNA template (see below). Finally, cleavage activity copurified with telomerase activity over five consecutive purification steps (13).

The cleavage reaction is specified by primer alignment on the telomerase RNA template. DNA cleavage in our reactions was extremely precise. 3′ nontelomeric DNA was cleanly removed from all of the chimeric primers bearing internal G_4T_4 cassettes to expose the telomeric sequence for elongation. In all cases, a homogeneous population of cleaved products was generated. Imprecise cleavage followed by extension was expected to alter the precise 8-nucleotide periodicity of the banding profile. This was not observed. There are two routes that telomerase could follow to remove a nontelomeric sequence. First, nucleotides could be eliminated until a telomere sequence was exposed for elongation. Alternatively, cleavage could occur at a fixed site relative to the *Euplotes* telomerase RNA templating domain, as has been proposed for the *Tetrahymena* enzyme (2, 8).

We examined these possibilities by varying the permutation of the telomeric cassette sequence within the chimeric primers. The modified telomeric cassettes are predicted to align at two different positions along the RNA template (Fig. 5A). If telomerase eliminates nontelomeric DNA until the telomeric sequence is reached, we predict 3 nucleotides would be removed from a chimeric primer containing a dGGGGTTTT internal cassette and 3 nucleotides of 3′ nontelomeric DNA [pBR(G_4T_4)-3]. Similarly, 3 nucleotides should be removed from a primer containing the cassette G_3T_4G and 3 3′ nontelomeric residues [pBR(G_3T_4G)-3]. As predicted, 3 nucleotides were removed from pBR(G_4T_4)-3, as determined from the migration positions of products running below the 22-nucleotide marker (Fig. 5B, lane 1). However, 4 nucleotides were removed from pBR(G_3T_4G)-3 prior to telomerase elongation (Fig. 5B, lane 3). Although the intensity of the plus-one product is reduced, the 1-nucleotide offset in banding patterns of the longer products with pBR(G_4T_4)-3 versus those with pBR(G_3T_4G)-$_3$ supports this conclusion. Thus, cleavage does not appear to fulfill a classical proofreading function, since nucleotides that can form base pairs with a final rC residue in the RNA template can be removed as well as those that cannot. This interpretation is further supported by results obtained with pBRG_4T_5-3, a primer that coincidentally contains an additional dT residue adjacent to the telomere cassette, GGGGTTTTT, as well as 3 nontelomeric 3′ nucleotides. Four nucleotides were removed from this primer prior to elongation (Fig. 5B, lane 2 and see also Fig. 4C, lane 6). We conclude from these data that the cleavage reaction was not operating specifically to expose telomeric DNA. Rather, cleavage occurred at a fixed site in the *Euplotes* telomerase and resulted in the elimination of any DNA residues that extended 5′ of position 35 in the telomerase RNA (Fig. 5A).

Cleavage by telomerase is endonucleolytic. Since cleavage by telomerase did not appear to serve a classical proofreading function, it was of interest to determine whether the reaction proceeded by an endo- or exonucleolytic mechanism. To an-

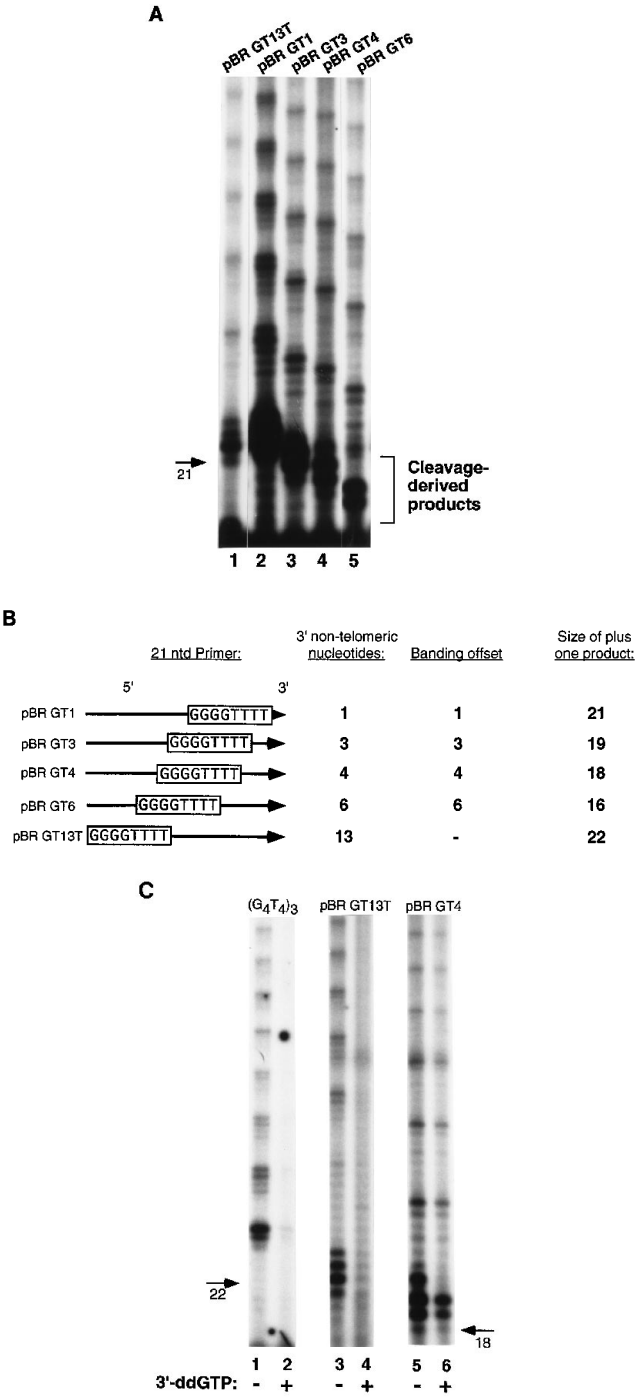

FIG. 4. Cleavage of nontelomeric 3′ ends. (A) Cleavage precedes telomerase elongation. Telomerase reactions are shown with pBRGT13T (lane 1); pBRGT1, CACTATCGACTA(G_4T_4)T (lane 2); pBRGT3, CACTATCGAC(G_4T_4)CAT (lane 3); pBRGT4, CACTATCGA(G_4T_4)TCAT (lane 4); and pBRGT6, CACTATC(G_4T_4)GATCAT (lane 5). Reaction mixtures were resolved on a 6% sequencing gel. The arrow indicates the migration of a 21-nucleotide input primer. (B) Diagram of primers tested in panel A. The G_4T_4 telomeric cassettes are boxed, and lines represent nontelomeric sequences. Banding profiles are offset relative to that of the pBRGT13T primer and extended in the default mode, as indicated. ntd, nucleotide. (C) DNA cleavage eliminates nucleotides from the primer 3′ end. Telomerase reactions were conducted with primers as indicated. Reactions shown in lanes 2, 4, and 6 were conducted with primers carrying a 3′-terminal ddGTP. The arrows denote the migrations of 18- and 22-nucleotide markers.

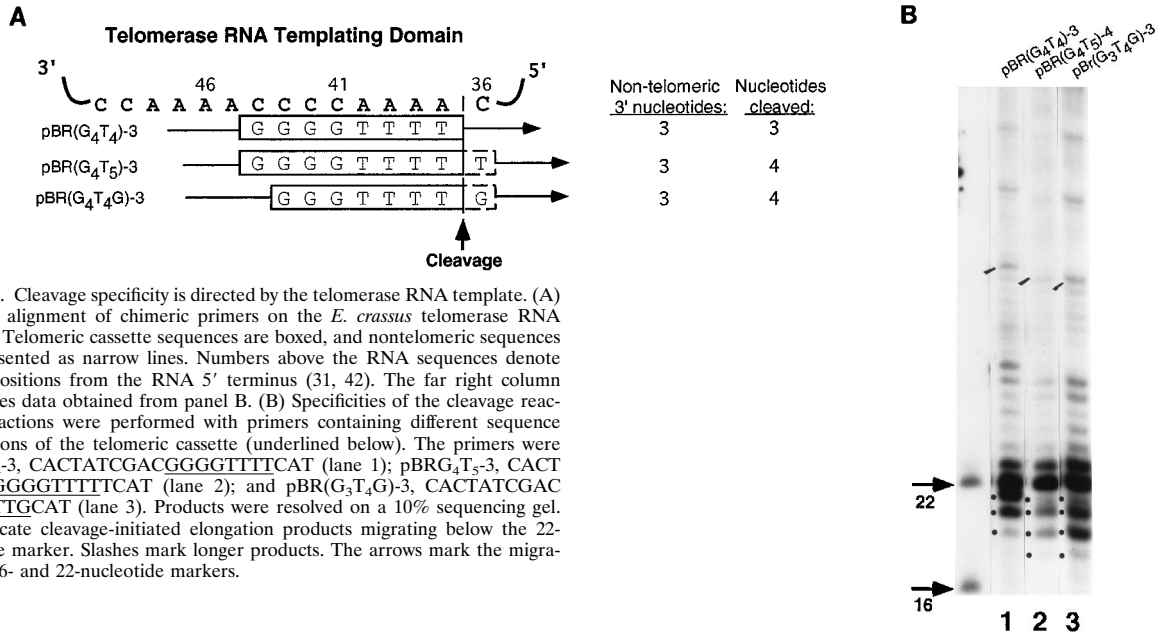

FIG. 5. Cleavage specificity is directed by the telomerase RNA template. (A) Predicted alignment of chimeric primers on the *E. crassus* telomerase RNA template. Telomeric cassette sequences are boxed, and nontelomeric sequences are represented as narrow lines. Numbers above the RNA sequences denote relative positions from the RNA 5′ terminus (31, 42). The far right column summarizes data obtained from panel B. (B) Specificities of the cleavage reactions. Reactions were performed with primers containing different sequence permutations of the telomeric cassette (underlined below). The primers were $pBRG_4T_4$-3, CACTATCGACGGGGTTTTCAT (lane 1); $pBRG_4T_5$-3, CACT ATCGAGGGGTTTTTCAT (lane 2); and $pBR(G_3T_4G)$-3, CACTATCGAC GGGTTTTGCAT (lane 3). Products were resolved on a 10% sequencing gel. Dots indicate cleavage-initiated elongation products migrating below the 22-nucleotide marker. Slashes mark longer products. The arrows mark the migrations of 16- and 22-nucleotide markers.

swer this question, we examined the cleavage reaction using primers containing a single nuclease-resistant phosphodiester substitution. Substituting the nonbridging oxygen of a phosphodiester bond for a methyl group renders the linkage resistant to nuclease attack (45). A telomeric primer containing a single methylphosphonate linkage was extended as efficiently as unsubstituted primers, indicating that the substitution did not alter primer binding and extension (data not shown).

We next examined cleavage and elongation of a chimeric primer, pBRGT6, which carries 6 nucleotides of a 3′ nontelomeric sequence. Telomerase reactions with unmodified pBRGT6 resulted in the elimination of 6 3′ nucleotides prior to elongation (Fig. 6, lane 1). Therefore, a substitution of the phosphodiester bond 3 nucleotides from the 3′ terminus should not affect an endonucleolytic cleavage reaction but would be expected to alter an exonucleolytic reaction. Products of the same size and abundance were generated with the methylphosphonate-substituted primer (Fig. 6, lane 2). To confirm that the reaction was endonucleolytic, the phosphodiester substitution was placed 6 residues in from the primer 3′ end, at the predicted cleavage site. In this case, cleavage and extension were inhibited by 90% (Fig. 6, lane 3). We conclude that primer cleavage is predominately mediated by an endonucleolytic mechanism.

DISCUSSION

A minimal sequence requirement for telomerase recognition of DNA. The interaction of telomerase with telomere DNA has been the subject of several in vitro studies. DNA specificity for telomerase is thought to depend upon a variety of factors, including primer length (8, 27, 36), the ability to base pair with the RNA template (1, 18, 32, 35, 43), and sequences at the primer 5′ terminus (references 19, 27, 32, 35, and this study). Together these observations provide the framework for a two-primer binding-site model for telomerase in which the protein-associated anchor site binds and maintains contact with sequences distal to the primer 3′ terminus while the RNA template site aligns the primer 3′ terminus at the catalytic center and directs the polymerization reaction. Whereas the individual contributions of the anchor versus the template site in DNA recognition are still largely unknown, DNA interactions within the anchor site are particularly enigmatic. A detailed kinetic analysis of the *Tetrahymena* telomerase revealed that sequences at the 5′ terminus of a primer alter the reaction rate. Moreover, even when the primer had been extended by the addition of a few telomeric repeats, sequences at the 5′ end continued to exert an effect on enzyme processivity (27). In this study, we exploited the concept of a telomere cassette sequence embedded within nontelomeric DNA to further explore DNA-anchor site interactions and to examine how the *Euplotes* telomerase processes nontelomeric DNA.

Like the *Tetrahymena* enzyme, *Euplotes* telomerase elongated DNA primers lacking 3′ complementarity to the RNA template. The levels of efficiency of such reactions versus those conducted with completely telomeric primers were significantly lower, consistent with previous studies indicating that primer binding in both the anchor and template sites is optimal for elongation. Surprisingly, the specificities for upstream telomeric sequences were markedly different for the *Tetrahymena* and *Euplotes* enzymes. Efficient elongation of nontelomeric 3′ ends by the *Tetrahymena* telomerase occurs only when a primer contains two full TTGGGG repeats (19). In contrast, the *Euplotes* telomerase extended primers bearing a single cluster of 3 or 4 dG residues at the 5′ terminus almost as well as a primer carrying two full GGGGTTTT repeats. Adjacent dT residues did not substantially increase recognition. Primers that lacked dG clusters or contained fewer than three consecutive dG residues were very poorly elongated by the *Euplotes* enzyme. The *Tetrahymena* telomerase has been shown to efficiently elongate a primer consisting of repeated dGdT sequences, $(GT)_9$ (17). Whether an extended tract of this sequence would be sufficient for *Euplotes* telomerase recognition and elongation is unknown.

Since the sequence alterations made to the 5′ telomeric cassette had no effect on the nucleotides added to the primer 3′ terminus, we are confident our experiments probed DNA interactions within the telomerase anchor site. This conclusion is supported by the observation that a primer bearing three consecutive dG residues at its 3′ terminus, which must neces-

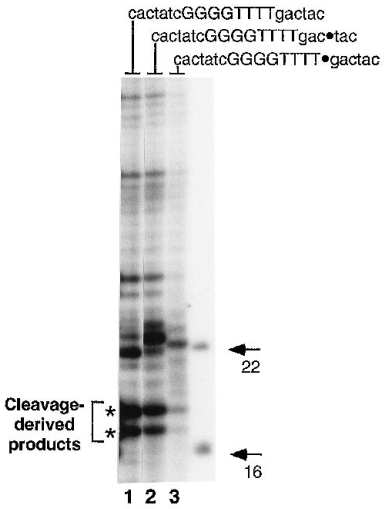

FIG. 6. Telomerase-mediated cleavage is endonucleolytic. Telomerase was reacted with an unmodified primer (pBRGT6) or a primer containing a single methylphosphonate linkage located at the position indicated in its sequence by a ●. The telomeric sequences within the primers are capitalized. Asterisks indicate bands that were quantified by PhosphorImaging. The arrows mark the migrations of 16- and 22-nucleotide markers.

sarily bind in the template site, was used much less efficiently than a primer carrying 3 5′ dG residues. This finding argues that primer contacts at the anchor site may be more important for establishing a productive elongation complex than primer interactions with the RNA template.

The extremely lax sequence specificity displayed by the *Euplotes* telomerase cannot fully account for the ability of this enzyme to form new telomeres in vivo. Internal tracts of telomeric sequence or even short clusters of dG residues are not found adjacent to telomere addition sites (3). Moreover, sequences corresponding to the site of de novo telomere formation in vivo do not prime elongation by the *Euplotes* telomerase in vitro (34). Efficient recruitment of telomerase to nontelomeric 3′ ends in vivo is likely to require the participation of *trans*-acting factors. Since chromosome breakage and de novo telomere formation appear to be temporally linked in ciliates (11, 48), it has been suggested that telomerase is recruited to a site of de novo telomere formation as part of a multisubunit complex, one component of which binds the chromosome breakage sequence element and cleaves the DNA. As part of this complex, telomerase would be localized to the new chromosome terminus (see below). Telomerase's lax sequence specificity and ability to precisely position nontelomeric DNA within the enzyme active site could be sufficient to initiate telomere synthesis.

Default positioning of nontelomeric DNA at the telomerase template. Once telomerase has been recruited to a nontelomeric 3′ terminus, it is not obvious how the polymerization reaction initiates. Watson-Crick base-paired alignment of the primer 3′ terminus onto the RNA template is precluded. Evidence that this reaction might be regulated came from experiments with human, *Tetrahymena*, and *Oxytricha* telomerases which showed that elongation of nontelomeric 3′ ends generated discrete banding profiles (19, 32, 35). Thus, primer termini appeared to be specifically positioned and not randomly delivered into the enzyme active site for the first round of elongation. Our data provide an explanation for these observations by demonstrating that nontelomeric DNA 3′ ends are positioned at a default alignment site on the RNA template for the initial elongation cycle. Delivery to this site is extremely precise and, in the case of the *Euplotes* telomerase, always results in the addition of 4 dG residues to the nontelomeric 3′ end. Remarkably, default positioning is maintained even when a primer carries 29 residues of 3′ nontelomeric DNA, the longest nontelomeric 3′ end we tested (Fig. 2). We postulate that the nontelomeric DNA segment of the primer loops out of the enzyme while the 5′ telomeric cassette maintains contact with the anchor site (see Fig. 7). If this model is accurate, then telomerase is able to capture a primer 3′ end that lies a long distance from the point of primer binding in the anchor site and to direct that terminus into the catalytic center. Perhaps default positioning provides a "landing pad" for primers to increase the efficiency level of polymerization initiation when telomerase extends nontelomeric 3′ ends.

The default setting of the *Euplotes* telomerase provides a plausible explanation for the precision of developmentally regulated telomere formation in vivo. In both milieus, the *Euplotes* telomerase initiates new telomeres by adding dG residues onto nontelomeric DNA. It is likely that default positioning will be a conserved mechanism, extending beyond ciliate telomerases. New telomeres formed in *S. cerevisiae* also initiate with a precisely defined register of telomeric repeats (26) which corresponds to an 11-nucleotide core within the telomerase RNA (44).

Telomerase-mediated endonuclease activity. Our studies with chimeric primers revealed that telomerase uses two pathways for processing nontelomeric 3′ ends. One pathway results in the direct addition of telomeric repeats onto a nontelomeric 3′ terminus. Alternatively, 3′ terminal nucleotides can be eliminated prior to telomerase elongation (Fig. 7). The cleavage reaction is most striking with chimeric primers that carry an internal cassette of telomeric DNA, although we and others have observed 3′ cleavage with completely telomeric primers (2, 7, 8, 34). The mechanism of primer partitioning between the default and cleavage modes is unclear. Our data suggest that nontelomeric 3′ ends are directed to the default position when an upstream cluster of dG residues binds in the anchor site. Interaction of telomeric sequences with the template site, by contrast, leads to cleavage of the nontelomeric 3′ end (Fig. 7). This model of primer interaction with telomerase is strongly supported by the observation that changing the permutation of the telomeric cassette sequence affected the number of nucleotides removed from a primer 3′ terminus, while varying the 5′ telomeric sequence altered the efficiency of primer utilization but had no effect on positioning the 3′ terminus on the RNA template.

The cleavage reaction is almost certainly catalyzed by telomerase. Not only is cleavage activity associated with telomerase through extensive enzyme purification, but the specificity of the reaction is strikingly similar to *Tetrahymena* telomerase cleavage activity (2, 8). Both enzymes have a fixed cleavage site that corresponds to the same position on the two RNA templates; both activities remove any nucleotides that extend beyond the 5′ boundary of the templating domain. In contrast to previous studies, our work was conducted with oligonucleotides bearing nontelomeric 3′ ends. This was a fortuitous choice that permitted a more detailed examination of the cleavage mechanism. The 3′ nontelomeric ends of some oligonucleotides we tested are predicted to extend far beyond the 5′ boundary of the *Euplotes* templating domain. In a reaction with 1 24-mer oligonucleotide, 13 nontelomeric nucleotides were cleaved from the primer 3′ terminus (33). In this case, about 50% of the input primer was eliminated prior to elongation by telomerase. We have yet to define an upper limit for nucleotide elimination in the cleavage reaction.

This study is the first to demonstrate that DNA elimination by telomerase is catalyzed by an endonucleolytic mechanism. Three observations led us to suspect that telomerase-mediated cleavage is endonucleolytic. First, the event was extremely precise, generating a homogeneous population of elongation products. Second, both telomeric and nontelomeric nucleotides could be eliminated (Fig. 5B). This finding strongly argues that cleavage does not serve a classical proofreading function, as was demonstrated for many DNA polymerase-associated exonucleolytic activities (25). Finally, we considered the striking parallels noted between the telomerase elongation mechanism and those of RNA polymerases (8–10, 28, 32). In response to transcription arrest, RNA polymerase on its own (41, 46) or in conjunction with *trans*-acting factors (5, 38) can endonucleolytically cleave phosphodiester bonds 17 nucleotides from a transcript 3′ terminus (23, 41). Current elongation models for RNA polymerase propose that arrest occurs when the polymerase loses contact with the RNA transcript 3′ terminus. Endonucleolytic cleavage generates a new transcript 3′ terminus positioned within the catalytic center. Contact with the transcript terminus is now reestablished and elongation can proceed.

Some chimeric primers used in this study appear to form a complex with telomerase that resembles an arrested RNA polymerase. The nontelomeric 3′ termini of such primers extend outside the telomerase enzyme active site (Fig. 7). Under these circumstances, polymerization cannot be initiated. Endonucleolytic cleavage can solve this problem by eliminating nucleotides that extend outside the templating domain. This model is strongly supported by the observation that telomerase is extremely inefficient at elongating a primer that carries a nonhydrolyzable methylphosphonate linkage at the junction between telomeric and nontelomeric DNA.

The primary function of the telomerase endonuclease is not likely to be involved in enhancing enzyme processivity, as is hypothesized for RNA polymerase. The available data indicate that telomerase is not highly processive in vivo (50). Instead, we postulate that telomerase endonuclease, while not having a classical proofreading activity, serves to ensure that nucleotides beyond the templating domain are not copied into DNA. In vivo and in vitro, the *Euplotes* telomerase synthesizes perfect 8-nucleotide repeats of TTTTGGGG in a mechanism involving successive rounds of primer elongation, translocation, and realignment of the newly synthesized 3′ terminus on the RNA template (24, 42, 43). The fidelity of these three events is essential, as altering the telomere repeat sequence dramatically changes telomere length and may lead to cellular senescence and death (31, 44, 51). One way in which variant telomere sequences could be produced is if the primer failed to translocate at the proper place and nucleotides in the telomerase RNA beyond the templating domain were copied into DNA. Telomerase's capacity to endonucleolytically cleave DNA that extends beyond the 5′ boundary of the template ensures that only those residues within the functional templating domain are copied during telomere synthesis.

Another more speculative model for the telomerase endonuclease of ciliates proposes that its activity may be directly involved in developmentally regulated chromosome fragmentation. Once recruited to the site of DNA cleavage, it is conceivable that telomerase catalyzes endonucleolytic cleavage of one DNA strand and then initiates new telomere synthesis on the exposed 3′ terminus. The yeast mitochondrial group II intron aI2 (54, 55) and the *Bombyx mori* R2 element (30) have been shown to catalyze site-specific endonucleolytic cleavage and then reverse transcription of a closely associated RNA template. The aI2 intron encodes a protein with endonuclease and reverse transcriptase activities. To initiate insertion of the intron into an intronless allele, the aI2 ribonucleoprotein complex, consisting of the aI2-encoded reverse transcriptase protein and the excised aI2 RNA intron, cuts the recipient DNA at a specific target site. The RNA component catalyzes the cleavage of the sense strand first, with the resulting 3′ OH serving as a primer for reverse transcription of the RNA template. The antisense strand is then cleaved by the protein component of the ribonucleoprotein. The ranges of telomerase cleavage substrates have not yet been defined. Therefore, it is unknown whether telomerase is capable of cleaving both strands of duplex DNA or if other factors are needed. Further studies are under way to determine if telomerase endonuclease plays a role in de novo telomere formation.

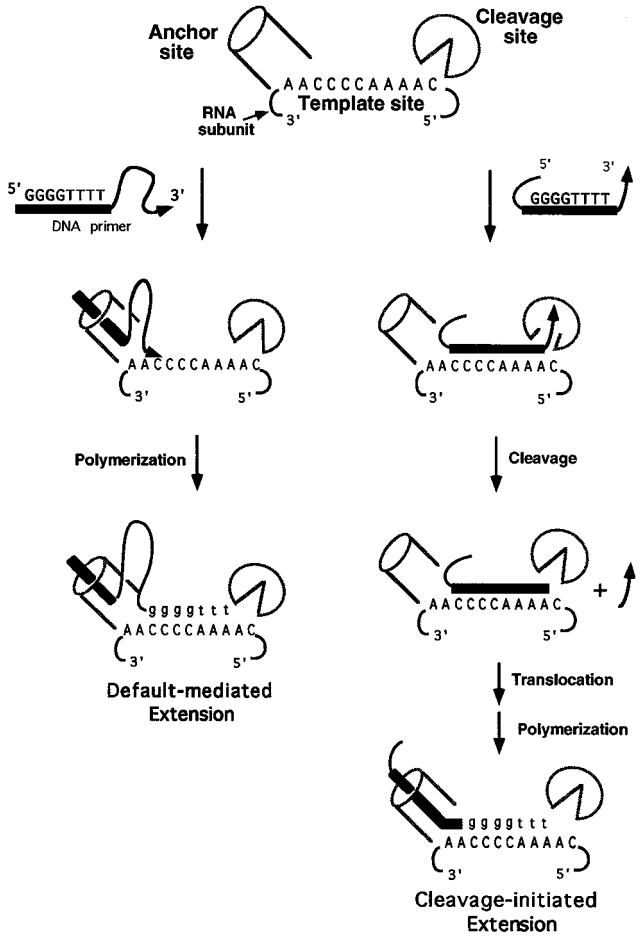

FIG. 7. Processing and extension of nontelomeric 3′ termini by the *Euplotes* telomerase. Telomerase is proposed to contain three functional domains: the RNA template site, a protein anchor site, and a cleavage site. The distal telomeric sequence (thick black line) in an otherwise nontelomeric (thin black line) primer binds the anchor site or the template site. This initial interaction dictates the pathway by which a chimeric primer will be processed. Anchor site binding facilitates delivery of the primer 3′ terminus to a default position on the RNA template to initiate telomere synthesis with the dG addition. Alternatively, binding of the telomeric sequence to the RNA template pushes the nontelomeric 3′ terminus outside the polymerization site and into the cleavage site. This results in an endonucleolytic reaction that removes DNA that aligns with or beyond residue 36 on the RNA template. Polymerization commences with the addition of dG residues.

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