Euplotes Telomerase: Evidence for Limited Base-Pairing during Primer Elongation and dGTP as an Effector of Translocation†

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ABSTRACT: The telomeric sequence repeats at the ends of eukaryotic chromosomes are maintained by the ribonucleoprotein enzyme telomerase. Telomeric DNA primers are bound by telomerase both at the active site, which includes base-pairing with the RNA template, and at a second anchor site. The stabilities of *Euplotes aediculatus* primer−telomerase complexes were determined by measuring their dissociation rates (k_{off}), using an assay involving photo-cross-linking at the anchor site. The primer length was varied, and mismatched substitutions were introduced in a systematic manner. We observed that k_{off} does not scale with primer length as expected for accumulated primer-template base-pairing. This suggests that telomerase maintains a more-or-less constant number of base pairs, similar to the transcription bubble maintained by RNA polymerase. An upper limit was estimated by comparing the experimental k_{off} for the primer−telomerase complex to that of a model DNA−RNA duplex. All the binding energy could be attributed to 10 or 11 base pairs; alternatively, there could be <10 base pairs, with the remaining energy contributed by other parts of telomerase. Most primers exhibited biphasic dissociation kinetics, with variations in both the amount in each phase and the rate for each phase. Since the cross-links monitored in the dissociation assay were all formed with the 5′ region of the primer, the two phases may arise from different base-pairing registers with the RNA template, possibly representing pre- and post-translocation complexes. A shift from slow phase to fast phase dissociation was observed in the presence of dGTP, which may implicate dGTP as a positive effector of translocation.

The termini of eukaryotic chromosomes are capped by a specialized structure called the telomere, composed of proteins associated with a region of simple repetitive DNA (for reviews, see references *1*, *2*). The repeat sequence varies between species (*3*), but is generally G- and T-rich in the strand running 5′ to 3′ toward the end of the chromosome. The GT-rich strands of ciliated protozoa, such as *Euplotes*, have been shown to have constitutive 3′ overhangs of 10−16 single-stranded nucleotides (*4*). Overhangs of different lengths have been found in diverse eukaryotes (*5*−*8*; W. Wright and J. Shay, personal communication), indicating that a 3′ overhang may be a general feature of telomeres. One role for the 3′ overhang is as a protein binding site on the end of the chromosome (*9*; for review, see *10*). The telomeric DNA and associated proteins form a cap that protects against exonuclease degradation and prevents chromosome fusion (*9, 11*).

Maintenance of a single-stranded telomeric overhang requires a special step during chromosomal replication (*12*; for the special case of hypotrichous ciliates, see *13*). Both leading and lagging strand syntheses are initiated using short RNA primers that are subsequently removed. The single-stranded overhang on the end produced by lagging strand replication can be regenerated by this RNA primer removal. However, at the terminus of the leading strand the overhang would be lost and must be restored by a different mechanism. This problem is solved by a specialized DNA polymerase that specifically synthesizes telomeric DNA repeats (*14*). This enzyme was dubbed telomerase, and has been shown to be responsible for the in vivo synthesis of telomeres during DNA replication (*15, 16*).

Telomerase is a ribonucleoprotein containing a single RNA moiety (*17*), and a sequence within the RNA serves as the template for the synthesis of telomeric repeats (*15, 16, 18*). The catalytic subunit of telomerase has been identified as a reverse transcriptase-related protein in *Euplotes* (*19*), *Saccharomyces cerevisiae* (*19, 20*), *Schizosaccharomyces pombe* (*21*), and human (*21, 22*). Additional telomerase-associated proteins have been found in *Tetrahymena* and mammals (*23*−*25*).

One special feature of telomerase is that it can processively add multiple repeats to a telomeric DNA primer through discontinuous synthesis. The template region of telomerase RNA codes for a single telomeric repeat, although additional nucleotides complementary to the telomere are present for substrate alignment (Figure 1A,B). To add subsequent repeats, the growing 3′ end of the primer must realign on the template after each round of addition (Figure 1C,D). To

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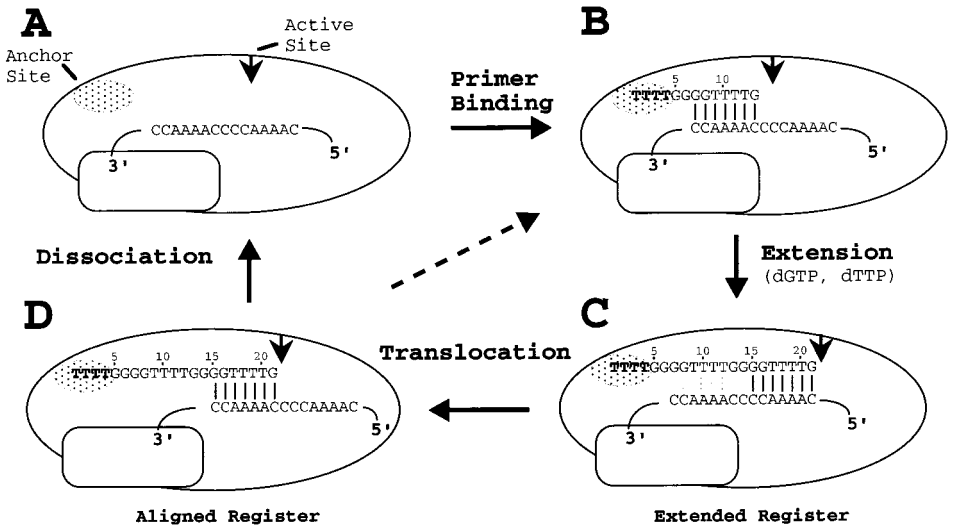

FIGURE 1: Schematic of the reaction cycle of telomerase. (A) Telomerase alone with nucleotides of the RNA template region specified. (B) A primer bound to the enzyme with its 5′ end in the anchor site and 3′ terminus aligned on the RNA template. (C) Deoxynucleotides are added to the primer until a full repeat is synthesized. (D) The primer is translocated on the RNA template and realigned for another repeat addition. Base pairs are indicated by solid lines for the transcription bubble model that is supported by the data presented herein; dashed lines represent the potential for additional Watson−Crick base-pairing. The boldface block of T's contains ^{I}U substitutions at the first and third positions to allow cross-linking.

explain this property of the enzyme, a two-site binding model was proposed (*26*−*28*). A primer being extended by telomerase has its 3′ terminus aligned on the RNA for repeat addition and its 5′ region bound in a separate anchor site (Figure 1). As long as the 5′ region remains bound in the anchor site, the extended primer 3′ end can be translocated back to the beginning of the template without dissociation of primer from the enzyme; dissociation only occurs when both sites are released simultaneously.

The template and alignment regions of telomerase RNA together contain nucleotides complementary to 1.5−2 telomeric repeats (*29*, *30*). If base-pairing accumulated during primer extension, then in *Euplotes* there could be as many as 15 Watson−Crick base pairs between primer and template (Figure 1C). Disrupting all of these base pairs in order to translocate the primer would require substantial energy. It has been proposed (*28*, *31*) that addition of each nucleotide to the 3′ end of the primer may be accompanied by disruption of a single base pair at the other end of the primer-template duplex, in a manner analogous to the transcription bubble formed by RNA polymerase (*32*). This would maintain a constant amount of base-pairing spanning less than the entire template (indicated by bold base pair marks, Figure 1C) and reduce the energy required for translocation. It is not yet known how translocation occurs, whether it is a directed process, or what energy source might be required. Neither ATP nor GTP is needed for processive repeat addition (*14*), but it has been difficult to rule out participation of dGTP or dTTP, as both are substrates for repeat synthesis.

The repetitive nature of the telomerase primers, the reaction products, and the RNA template makes multiple primer alignments possible, thus creating difficulty in characterization of enzyme−primer complexes. When primer−telomerase interactions are assayed by activity, only the primer aligned as shown in Figure 1 panels B and D, but not panel C, can be observed, and primer binding cannot be assayed in the absence of dNTP's. These limitations can be circumvented through the use of a primer that can be cross-linked to telomerase. We have previously reported cross-links between the anchor site of telomerase from *E. aediculatus* and telomeric primers that contain the photoactive nucleotide analogue 5-iododeoxyuridine (^{I}U) (*33*). Because this substitution is isosteric with deoxythymidine, cross-linkable nucleotides may be embedded within simple telomeric repeat primers (*34*). The optimal ^{I}U location for cross-linking, within a 22 nucleotide telomeric primer, was determined to be in the 5′-most block of T's (shown in bold type in Figure 1). Upon irradiation of a primer preincubated with nuclear extract, cross-linked complexes with telomerase were formed with an ∼130 kDa protein (presumably the 123 kDa subunit described above) and with nucleotides U51−U52 of the RNA moiety. These cross-linked complexes became labeled by incubation with [α-^{32}P]dGTP either before or after irradiation, under conditions where rebinding of primer was inhibited, demonstrating that the 3′ end of the primer could align in the polymerase active site (*33*).

Several studies have demonstrated the ability of telomerase to utilize primers containing very few or no telomeric nucleotides at the 3′ end (*27*, *30*, *35*−*38*). However, only one of these reports includes quantitative data indicative of primer binding affinities (*35*). In the study reported herein, we have used cross-linking assays to compare quantitatively the binding affinity for primers with different 3′ sequences, thereby extending our understanding of primer interactions with telomerase. Specifically, we have measured the rate of primer dissociation (k_{off}) to investigate the extent of base-pairing interaction between primers and the RNA template. Determining the k_{off} in the presence and absence of dNTPs revealed that dGTP has a marked effect on the dissociation rate.

MATERIALS AND METHODS

Growth of Euplotes and Preparation of Nuclear Extract. *E. aediculatus* was grown as described (*39*) under nonsterile conditions, in aerated 15 gallon reactors, with *Chlorogonium* as the food source. Cells were collected on a 15 μm Nytex

filter and lysed in the presence of Nonidet P-40 nonionic detergent. Nuclei were isolated by sucrose cushion centrifugation, and a nuclear extract was prepared by Dounce homogenization as previously described (*30*).

Partial Purification of Telomerase. Nuclear extracts were partially purified by centrifugation in a 15−40% glycerol gradient as previously described (*33*). Fractions containing telomerase were identified by a gel-shift assay with a primer that hybridized to the telomerase RNA component. Peak fractions were pooled and dialyzed against telomerase reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM sodium glutamate, 1 mM dithiothreitol, 10% glycerol] using a Spectra/Por CE membrane with a 100 kDa molecular mass cutoff.

Primer Synthesis, Purification, and 5′ End-Labeling. Primers containing ^{I}U (Glen Research) were prepared as previously described (*33*) using standard phosphoramidite synthesis, and purified by polyacrylamide gel electrophoresis. The nontelomeric competitor primer had the sequence 5′-ATTGAATGACTACGAGATGAA; all other primer sequences are provided under the Results. Purified primers were 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol; NEN), and then purified using Beckman G-25 TE spin columns.

Preparation of 3′-Dideoxy Primers. Primers were synthesized and 5′ end-labeled as above; then a single dideoxynucleotide was added using terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim). Each reaction contained 20−60 pmol of primer and 1 mM ddNTP (ddTTP or ddGTP) in 1× TdT buffer (Boehringer Mannheim). Reactions were incubated for 1 h at 37 °C, and then quenched with 10 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA). The dideoxy-terminated primer was isolated from unreacted primer by denaturing polyacrylamide gel electrophoresis.

k_{off} Determination by Cross-Linking. Cross-linking reactions were performed as previously described (*33*). Partially purified telomerase was preincubated with a nontelomeric primer (6.5 mM) in telomerase reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM sodium glutamate, 1 mM dithiothreitol, 10% glycerol]; then 5′ end-labeled telomeric primer was added (0.015 mM) and allowed to bind for 10 min at 25 °C. Time zero samples were removed and transferred to prechilled tubes containing specific competitor primer (5 mM, or H_2O as a control). For each of the primers tested, the specific competitor was the same sequence as the labeled primer. To test for enzyme and primer stability, a portion of the reaction mixture was also removed, chased with H_2O, and returned to 25 °C for the duration of the time course (15−40 min). After controls were removed, the remainder of the reaction was transferred to a chase containing specific competitor primer (5 mM). Individual time points were removed, and primer dissociation was halted by transfer to a prechilled tube. To form the cross-links, samples were spotted on Parafilm stretched over an iced metal block and irradiated for 10 min in a Stratalinker equipped with 312 nm bulbs, and then transferred to a tube containing 3× gel loading buffer and heat denatured 3 min at 85 °C. The cross-linked complexes were separated by SDS−PAGE using Novex 4−20% polyacrylamide gels. After electrophoresis, gels were dried and visualized using a Molecular Dynamics Phosphorimager system. To quantitate each species, a box was drawn to encompass the cross-link band in its entirety, and the counts within the box were recorded. An equivalent area was quantitated in the prechased lane and that value subtracted from the cross-link band to correct for background.

The effectiveness of the chase was verified by premixing the unlabeled chase oligonucleotide with the labeled primer; in this reaction, no cross-link was observed (data not shown). The stability of the complex after the temperature-drop quench, but prior to irradiation, could be determined from the relative signal obtained for the zero time samples with and without chase oligo. Although an ∼5−15% loss of signal was observed due to addition of the chase oligo, the majority remained stable under the chase conditions. This loss was principally due to a minor component that completely dissociated under the quench conditions. This species may be due either to a mode of binding not seen in the canonical reaction cycle or possibly to enzyme that was damaged during purification. Similarly low stability was seen for nontelomerase cross-links.

The telomerase used for these experiments was only semipurified from a nuclear extract, so the following additional controls were performed. Cross-linking was done with primers of various lengths (12−30 nucleotides), and discrete complexes with appropriately different mobility were observed for each primer (data not shown). The electrophoretic homogeneity of these complexes indicated that primers bound to telomerase were unaffected by any nuclease activity in the extracts. Other possible complications were enzyme and primer degradation, or additional binding occurring during the time course. To test these possibilities, samples without chase oligonucleotide were collected at time zero and at the end of the time course. These two measurements were essentially the same, showing that the overall cross-link signal did not substantially decrease or increase during the incubation. Together, these results indicate that lack of enzyme purity did not adversely affect the measurement of cross-linking extent or dissociation rate.

k_{off} Determination by Pulse-Label Method. Unlabeled primer (5 mM, ^{I}UT^{I}UTGGGGTTTTGGGGTTTTGG) was bound to partially purified telomerase for 10 min at 25 °C in telomerase reaction buffer. Pulse-labeling was done by addition of [α-^{32}P]dGTP (0.35 mM) and ddTTP (130 mM), followed by 4 min incubation at 25 °C. Prior to the chase reaction, a zero time aliquot was transferred to an iced tube containing dGTP (62.5 mM). The remainder of the sample was then transferred to the dGTP chase (62.5 mM) and incubated at 25 °C. Time points were removed and dissociation halted by transfer to an iced tube. Samples were irradiated and analyzed as described above. Excess [α-^{32}P]-dGTP was removed by ultrafiltration in a Centricon-30 device prior to SDS−PAGE.

Primer Extension Assay of Telomerase Activity. Partially purified telomerase was preincubated with a nontelomeric primer in telomerase reaction buffer, and then 5′ end-labeled telomeric primer was added (0.015 mM) and allowed to bind for 10 min at 25 °C. A 10 μL aliquot of the reaction was then mixed with an equal volume of telomerase reaction buffer containing dGTP and ddTTP (125 mM each), and incubated 10 min at 25 °C. Extension reactions were stopped by addition of 100 μL of proteinase K buffer [20 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1% sodium dodecyl sulfate]

and incubated 3 min at 85 °C. The nucleic acid component was isolated by proteinase K digestion (80 mg/mL) for 45 min at 45 °C, followed by ethanol precipitation. Primer extension products were separated by denaturing 20% polyacrylamide gel electrophoresis. Reaction products were visualized and quantitated by phosphorimager analysis.

The correct template alignment of primers of different lengths (and therefore different 3′ ends) was verified by telomerase activity assay in the presence of dGTP and ddTTP, or dTTP and ddGTP (data not shown). For each of the primers tested (12−30 nucleotides), termination occurred at the expected template location due to addition of the specific ddNTP. Some apparent slippage (<50% of bound primer) occurred with primers that terminated with the 3′ ends GTT or GTTT; in each case, slippage allowed addition of five nucleotides, GGGGddT. This may be due in part to poor utilization of ddTTP as reported for *Tetrahymena* telomerase (*40*).

k_{off} Determination by Primer Extension Method. Telomerase was preincubated with nontelomeric primer; then 5′ end-labeled primer was added (0.03 mM, ^{I}UT^{I}UTGGGGTTTTGGGGTTTTG) and incubated for 10 min at 25 °C in telomerase reaction buffer. Time zero samples were transferred to extension reactions, and then the reaction was chased by addition of unlabeled specific competitor primer (5 mM) and time points were removed. Time points were transferred to extension reactions and treated as described above.

Primer Dissociation Data Analysis and Curve Fitting. The intensity of the cross-linked species at each time point corresponds to the amount of primer that remains bound to telomerase. Each individual time point was normalized to the intensity of the time zero sample. The data were generally fitted to a biphasic dissociation using the equation $Y = A_1 \exp(k_1 t) + A_2 \exp(k_2 t)$. In cases where the rates of the two phases were essentially the same, the data were instead fit to a single exponential $Y = A \exp(kt)$. For the data that were fit with a double exponential, the average correlation coefficient (R value) was 0.99, whereas when these same data were fit with a single exponential the average R value was 0.94. Fitting the data with an additional variable to allow the end point to float gave no substantial difference in the relative rates or populations of either phase. The end points obtained using this alternative fit were generally less than 10% (data not shown), which is consistent with a visual inspection of Figures 2 and 3A,C. In four independent determinations of the dissociation kinetics of the 22 nucleotide reference primer, performed over a period of several months, the following means and standard deviations were obtained: a fast phase of 31 ± 19%, $k_{off} = 0.43 \pm 0.21$ min^{-1}; and a slow phase of 70 ± 18%, $k_{off} = 0.035 \pm 0.006$ min^{-1}. Curve fits were done using the computer program Kaleidagraph 3.0.

Thermal Melts. Telomeric DNA primer (3−5 mM) was mixed with an equimolar amount of RNA corresponding to the telomerase RNA template (sequences are given under Results) in telomerase reaction buffer. Samples were preheated to 90 °C, and then rapidly cooled to the starting temperature (15 and 5 °C for extended and aligned register models, respectively), to allow annealing. Duplex melting was monitored at 260 nm in a CARY spectrophotometer at a heating rate of 1 °C per min (data not shown). Reverse melts were also performed as a control and gave the same results. The data were analyzed as described by Marky and Breslauer (1987).

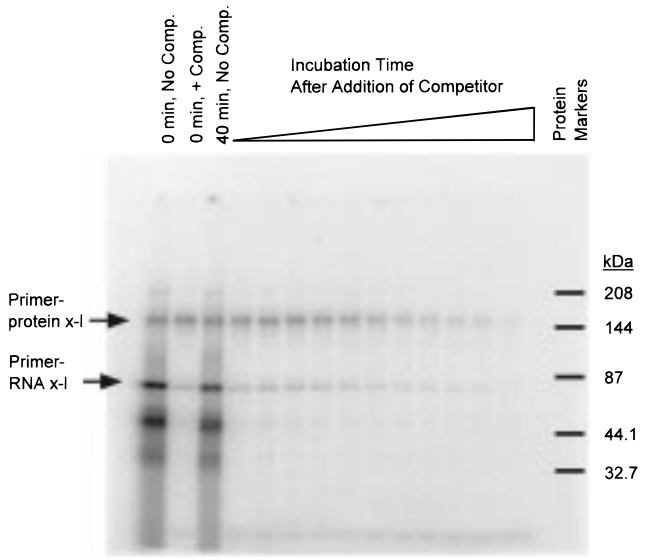

FIGURE 2: Dissociation of the 21 nucleotide primer ^{I}UT^{I}-UTGGGGTTTTGGGGTTTTG from telomerase measured by cross-linking. (A) Phosphorimager printout of an SDS−PAGE gel showing the time-dependent dissociation of 5′-labeled primer from telomerase during a 40 min chase reaction with an excess of unlabeled primer. Controls were included for the 0 and 40 min time points without chase addition. Primer that remained bound to telomerase was measured by quantitation of the ~150 kDa primer−protein cross-link (Primer-protein x-l). The molecular mass of prestained protein markers is indicated at the right.

RESULTS

Dissociation Rate (k_{off}) of Primer Bound to Telomerase. We have developed a photo-cross-linking assay to study DNA primer binding to telomerase from *E. aediculatus* (*33*). As a starting point for the following experiments, we chose a 21 nucleotide primer that corresponded to a telomerase primer extension product (Figure 1, panels C and D). This primer had the 3′ terminus -TTTTG that would allow binding in either the aligned or the extended register, and contained ^{I}U substitutions in the 5′-most block of T's (shown in bold type in Figure 1) to allow cross-linking to the anchor site. The stability of the primer−telomerase complex was determined by measuring the rate constant for primer dissociation (k_{off}). Primer labeled at the 5′ end was incubated with partially purified telomerase to allow binding, and then the reaction was chased with a 300-fold excess of unlabeled primer. Time points were removed after chase addition, and primer dissociation was halted by rapidly lowering the temperature. Primer−telomerase cross-links were induced by irradiation at 312 nm and the products analyzed by SDS−PAGE (Figure 2). The cross-link intensity at each time point corresponds to the amount of primer that remains bound to telomerase. Primer cross-linked with the telomerase protein (~150 kDa complex) was quantitated, and then normalized to the zero time signal and plotted versus time after chase (filled circles, Figure 3A−C).

The 60 kDa cross-linked complex of primer and telomerase RNA seen in Figure 2 was previously described (*33*). This cross-link was not used for quantitation of primer dissociation for several reasons: the covalently cross-linked complex is

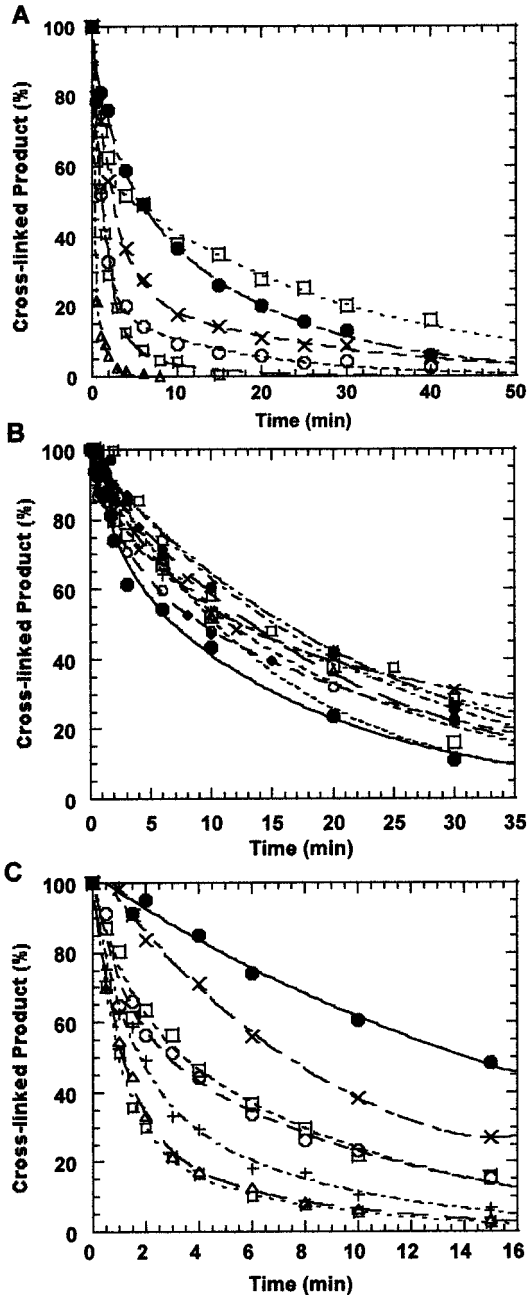

FIGURE 3: Effect of primer length and sequence on dissociation rate. (A) Primers that were 21 nucleotides (●) and shorter: 20 (×), 19 (○), 16 (□), 14 (small open square), 12 (△). (B) Primers that were 21 nucleotides (●) and longer: 22 (×), 23 (○), 24 (big open box), 25 (+), 26 (△), 27 (small filled circle), 28 (small open square), 30 (♦). (C) Primers containing point mutations relative to the 22 nucleotide primer (●): A21 (×), C20 (○), C18 (big open box), A16 (small open square), A14 (△), C12 (+). Mismatched primers were prepared in the 22 nucleotide background and are shown with the corresponding reference curve.

formed less efficiently than that involving telomerase protein; the complex comigrates in SDS−PAGE with a noncovalent primer−RNA duplex; and the complex forms even with the deproteinized RNA subunit, and therefore may not accurately reflect active telomerase.

Effects of Primer Length and Mismatches on the Dissociation Rates. Dissociation rates were measured for a variety of primers using the cross-linking method (Figure 3). The data were grouped into three categories: primers that were shorter (Figure 3A) or longer (Figure 3B) than the 21 nucleotide reference, or that contained mismatched bases (Figure 3C). The parent primer for the mismatched variants is 22 nucleotides long, but the similarity of the 21-mer and 22-mer allows comparisons to be made with either one. The efficiency of cross-linking was similar for all of the primers, generally within ±10−50% of the reference. Some variation in the efficiency is expected due to the possibility (especially with long primers) of primer binding in registers that do not place an ^{I}U residue at a cross-linkable position in the anchor site. This alternative register binding was observed with a 30-mer containing ^{I}U residues in the most 5′ or second most 5′ block of T's (*33*). However, such binding does not appear to constitute more than 50% of the total bound primer, and, as no cross-link is observed, this alternative binding does not affect the dissociation data measured by cross-linking. The observed similar efficiencies of cross-linking of different primers may result from a single location within the anchor site constituting the majority of cross-links.

As primers became successively shorter, the general trend was a steady increase in the overall dissociation rate (Figure 3A). A break in the trend was observed with the 16-mer, which may reflect its 3′ terminus being stabilized by 4 G-C base pairs with the RNA template. As primer length decreased, the incremental changes in the net dissociation rate were relatively small, far less than expected for the loss of corresponding base-pairing interactions in a DNA−RNA duplex. Loss of a single base pair from a simple duplex results in an ∼1−2 kcal mol^{-1} loss of binding energy (*41*), which would give an ∼5−40-fold increase in dissociation rate.

Lengthening the primer gave no substantial decrease in the net dissociation rate (Figure 3B). Similar dissociation rates were observed for primers ranging in length from 21 to 28 nucleotides, even though the sequence at the 3′ end varied greatly.

The effects of introducing single-base mismatches depended on the location of the mismatch (Figure 3C). The overall dissociation rate steadily increased as the mismatch was moved toward the 5′ end, with the greatest effect at A16. As the mutation was moved even further, the overall dissociation rate started to decrease again; no mutations preceding C12 were tested. Again, these changes were less than expected for introduction of a mismatch into a simple duplex, where the corresponding RNA−RNA mismatches are destabilizing by ∼2 kcal mol^{-1} (*42*). A change of this magnitude would give a corresponding increase in the dissociation rate of >10−40-fold.

The effects of double mismatches at the 3′ terminus and of replacement of the entire center of the primer were also determined and gave results qualitatively similar to the single mismatches (Table 1). A primer containing telomeric sequence only at the 5′ end gave no detectable cross-link.

Determination of Dissociation Rate Constants (k_{off}). The majority of the primers that were tested exhibited markedly biphasic dissociation kinetics (Figure 3). These data were therefore fit with a double exponential curve as described (Materials and Methods). In cases where the two rates obtained with a double exponential were essentially the same, a single exponential was used. The rates and amplitudes derived from these curve fits are given in Table 1.

As the length and sequence of the primers were varied, two types of changes in the dissociation kinetics were

Table 1: Summary of Primer Dissociation Rate Data[a]

primer		fast phase %	fast phase k_{off} (min^{-1})	slow phase %	slow phase k_{off} (min^{-1})
	Reference				
21	UTUTGGGGTTTTGGGGTTTTG	31	0.34	64	0.06
	Shorter				
20	UTUTGGGGTTTTGGGGTTTT	74	0.41	26	0.04
19	UTUTGGGGTTTTGGGGTTT	79	0.87	21	0.07
16	UTUTGGGGTTTTGGGG	41	0.97	58	0.04
14	UTUTGGGGTTTTGG	92	0.69	11	0.11
12	UTUTGGGGTTTT	77	5.23	23	0.67
	Longer				
22	UTUTGGGGTTTTGGGGTTTTGG	31	0.37	75	0.04
23	UTUTGGGGTTTTGGGGTTTTGGG	31	0.35	68	0.04
24	UTUTGGGGTTTTGGGGTTTTGGGG	22	0.79	87	0.05
25	UTUTGGGGTTTTGGGGTTTTGGGGT	26[b]	0.24	74	0.04
26	UTUTGGGGTTTTGGGGTTTTGGGGTT	–[c]	–	97	0.05
27	UTUTGGGGTTTTGGGGTTTTGGGGTTT	–	–	94	0.04
28	UTUTGGGGTTTTGGGGTTTTGGGGTTTT	–	–	100	0.05
30	UTUTGGGGTTTTGGGGTTTTGGGGTTTTGG	–	–	93	0.02
	Mismatched				
C12	UTUTGGGGTTTCGGGGTTTTGG	54	0.71	44	0.14
A14	UTUTGGGGTTTTGAGGTTTTGG	72	0.84	26	0.14
A16	UTUTGGGGTTTTGGGATTTTGG	74	0.99	27	0.16
C18	UTUTGGGGTTTTGGGGTCTTGG	31	0.82	70	0.11
C20	UTUTGGGGTTTTGGGGTTTCGG	39	0.91	62	0.10
A21	UTUTGGGGTTTTGGGGTTTTAG	–	–	95	0.11
A22	UTUTGGGGTTTTGGGGTTTTGA	22	0.61	78	0.04
AA	UTUTGGGGTTTTGGGGTTTAA	71	0.21	29	0.04
CC	UTUTGGGGTTTTGGGGTTTTCC	89	0.21	14	0.03
CHM	UTUTGAATGACTACGATTTTGG	72	2.52	27	0.37
CON	UTUTGAATGACTACGAGATGAA	*d*			

[a] The data were obtained by a double exponential curve fit, so the total percentage may not equal 100%. Error values were derived from the curve fits: average error in percent was ±8; individual error values ranged from 2 to 17%. In the oligonucleotide sequences, U represents 5-iodouridine. [b] The data for the 25-mer are a compilation of two data sets. [c] The symbol - in place of a value indicates that the data were best fit by a single exponential. [d] No detectable cross-link.

observed: a change in partitioning between the fast and slow phases, and a change in k_{off} of the individual phases (Table 1). The appearance of a faster net dissociation rate (Figure 3) can result from changes in either or both of these. What is most striking upon examination of the data in Table 1 is the relatively minor changes in rate constant (k_{off}) that accompany significant changes in primer length and 3′-terminal sequence. The majority of the increases observed in the net dissociation rate were due to a population shift from the slow to the fast phase.

Because the cross-link to the 5′ end of the primer is believed to arise from a single orientation within the anchor site, the observation of two phases indicated that the 3′ end of the primer was partitioned between two distinct states. A simple explanation for this result was that the primer was bound in two different base-pairing registers, such as the extended and aligned (Figure 1, panels C and D) (for more detail regarding this model, see ref *43*).

An alternative possibility was that the biphasic curve represented a mixed population of telomerase enzyme, for example, damaged and undamaged. This does not provide a consistent explanation, however, because modifications of the primer produced different ratios of fast/slow rates with a single telomerase preparation (see below). Furthermore, controls showed that the telomerase and the bound primer were not degraded during the time course (see Materials and Methods).

Free Energy Contribution of Primer−RNA Interaction. Primers are aligned on the telomerase RNA template through base-pairing interactions with the primer 3′ terminus (*29*). Additional contributions to primer binding may come from interactions with nontemplate regions of the RNA, as well as with the protein subunits of telomerase. A lower limit on base-pairing may be estimated from the minimum number required to give a unique alignment on the RNA template. For the G_4T_4 repeat sequence in *Euplotes*, this would be 4 base pairs. An upper limit for the extent of primer-template base-pairing was established by comparing the dissociation rate determined for the primer−telomerase complex with values for a simple DNA−RNA duplex (Table 2).

The slow phase for the 22 nucleotide primer had $k_{off} = 0.04\ min^{-1}$ (Table 1); how many base-paired nucleotides in a DNA−RNA duplex would give the same dissociation rate? The sequence of the template region of telomerase RNA is shown vertically in Table 2, along with a 22 nucleotide primer in the extended register. The cumulative free energy of duplex formation between primer and RNA was estimated from theoretical values (*41*, *44*). As each successive base pair is added to the duplex (reading down the table), the corresponding free energy is given as a running sum.

To verify the calculated values, the oligonucleotides shown in Table 2 were synthesized, and the thermal melting profile of the full length duplex was measured. The experimental free energy (*45*) of $-20.7\ kcal\ mol^{-1}$ (bracketed in Table 2) matched the theoretical value for the 16 base pair duplex (15 Watson−Crick pairs plus 1 G−U).

The duplex free energies were then used to calculate the theoretical k_{off} values for incrementally longer duplexes. The

Table 2: Free Energy of Duplexes between DNA Primer and RNA Template

RNA 5′		DNA 3′	$\Delta G°_{calcd}$(sum) (kcal mol^{-1})	k_{off}(calcd)[a] (min^{-1})
A				
G		G	+3.1[b]	
C	-	**G**	+1.2	
A	-	**T**	+0.3	
A	-	**T**	−0.7	
A	-	**T**	−1.7	
A	-	**T**	−2.7	
C	-	**G**	−4.8	nd
C	-	**G**	−6.9	522
C	-	**G**	−9.0	15.0
C	-	**G**	−11.1	0.43
A	-	**T**	−12.0	0.095
A	-	**T**	−13.0	0.018
A	-	T	−14.0	0.003
A	-	T	−15.0	0.0006
C	-	G	−17.1	nd
C	-	G	−19.2	
U	∘	G	−20.7[c]	
U	∘	G	−21.3[c]	
A	-	T	−22.0[c]	
C		T	−22.7[c] [−20.7]	[3.9 × 10^{-8}]
A	-	T		
C		T		

[a] $k_{off} = K_d k_{on}$; $k_{on} = 6 \times 10^7$ min^{-1} (*53*). [b] Free energy of helix initiation = +3.1 kcal. [c] Values from (*44*); all others from (*41*).

experimental free energy for the 22 nucleotide primer fully base-paired to the RNA template (−20.7 kcal mol^{-1}) gave a predicted k_{off} value of 3.9×10^{-8} min^{-1}. Comparison to the actual k_{off} for the primer−telomerase complex, 0.04 min^{-1} (Table 1), revealed that substantially less base-pairing must be involved. If 10 or 11 base pairs were formed between the telomerase RNA template and a bound primer (boldface lettering in Table 2), all of the binding energy could be attributed to base-pairing. If less than 10 base pairs were formed, the remaining binding energy could be contributed by interactions with other regions of telomerase (RNA or protein).

The difference in dissociation rates for the fast and slow phases of the 22-mer was ∼10-fold, corresponding to an energetic difference of ∼1.3 kcal mol^{-1}. This amount of energy could mean a difference between these two species of a single base pair, different types of base pairs, or different protein−nucleic acid interactions.

Pulse-Label Method for Primer Dissociation Rate Measurement. To verify that the rates measured using 5′-labeled primers reflected binding to active telomerase, an alternative assay format was used. In this approach, an unlabeled 22 nucleotide primer was bound to telomerase and pulse-labeled by a 4 min incubation with [α-^{32}P]dGTP and ddTTP. The label was thereby incorporated into a dideoxy-terminated 25-mer (ending in -GGGddT), but only when primer was bound to active enzyme. The unlabeled primer was present in sufficient concentration to prevent rebinding of any labeled primer that dissociated. To measure k_{off}, the labeling reaction was chased with an excess of dGTP that halted radiolabel incorporation; time points were removed after dGTP chase addition, irradiated, and then analyzed by SDS−PAGE.

Using this pulse-labeling technique, primer dissociation again exhibited biphasic kinetics (Figure 4). The fast phase (77%) had $k_{off} = 0.26$ min^{-1}, and the slow phase (22%) had

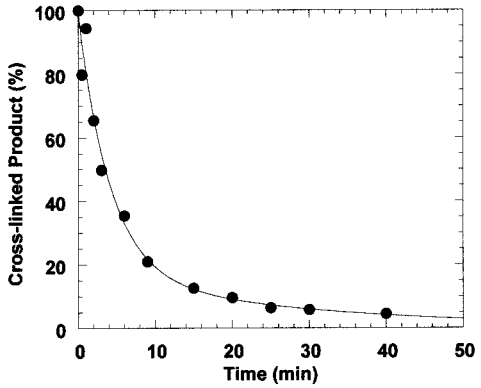

FIGURE 4: Primer dissociation measured in a pulse−chase reaction. The 22 nucleotide primer ^{I}UT^{I}UTGGGGTTTTGGGGTTTTGG was bound to telomerase in vast primer excess and labeled by incorporation of [α-^{32}P]dGTP and ddTTP. The reaction was then chased with dGTP and incubated for various times prior to irradiation. Formation of the cross-linked complex was monitored by SDS−PAGE; the amount of complex at each time point was normalized to the zero time and plotted versus time of incubation. The curve shown is the result of a double exponential fit.

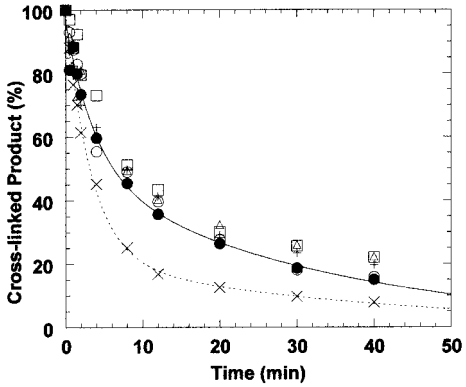

FIGURE 5: Primer dissociation in the presence of nucleotides. The dissociation rate for the 3′-dideoxy terminated primer ^{I}UT^{I}UTGGGGTTTTGGGGTTTTddG was determined by cross-linking. The nucleotides indicated (125 μM) were included during the primer binding step: no nucleotides (●), dGTP (×), dTTP (○), dATP (□), dCTP (+), dGMP (△). The no dNTP and dGTP data were fitted with a double exponential, and the curves are shown.

$k_{off} = 0.05$ min^{-1}, rates that were approximately the same as previously determined for the 5′-labeled 25-mer (Table 1). The primary difference between the two measurements was that the relative amounts of fast and slow phase were reversed. Two major differences between the techniques used for 5′-labeled and pulse-labeled primers were the dideoxy primer terminus and the presence of dGTP and ddTTP. These differences were next tested individually for their effects on k_{off}.

Effects of a Dideoxy Terminus and of dNTP's on Primer Dissociation. Terminal deoxynucleotidyl transferase (TdT) was used to prepare primers with 3′-dideoxy termini (see Materials and Methods). The dissociation rates of these primers were determined using the standard cross-linking assay in the presence and absence of 125 mM dNTPs. The results of measurements made with the blocked 21 nucleotide primer are plotted in Figure 5, and the data for all oligos tested are given in Table 3. For comparison, the values for the all-deoxy primers are given in brackets next to the values for the corresponding dideoxy-blocked primers.

Table 3: Summary of Primer Dissociation Rate Data[a] for Dideoxy-Blocked Primers

primer	3′ end		relative signal[b]	fast phase %	fast phase k_{off} (min^{-1})	slow phase %	slow phase k_{off} (min^{-1})
17	-GGGGT	no dNTP	1.00	67	0.77	30	0.04
		dGTP	0.13	47	0.86	50	0.04
		dTTP	1.15	62	0.46	39	0.04
		dGTP + dTTP	0.25	58	0.49	41	0.03
21	-TTTTG	no dNTP	1.00	47[31][c]	0.26[0.34]	49[64]	0.03[0.06]
		dGTP	1.24	77	0.30	21	0.03
		dTTP	0.97	44	0.32	57	0.03
22	-TTTGG	no dNTP	1.00	29[31]	0.31[0.37]	72[75]	0.03[0.04]
		dGTP	1.43	61	0.23	37	0.04
		dTTP	0.99	22	0.27	77	0.04
25	-GGGGT	no dNTP	1.00	17[26]	0.97[0.24]	83[74]	0.04[0.04]
		dGTP	0.48	33	0.54	67	0.04
		dTTP	1.11	30	0.21	70	0.03

[a] Data fit with two independent exponentials. Error for percentages was ±8; individual errors ranged from 3 to 16. [b] Relative yield of cross-linked telomerase−primer complex at zero time after chase. [c] Data in brackets are for all-deoxy primers of the same length and sequence from Table 1.

No substantial difference was observed upon blocking the primer 3′ end, indicating that the 3′-hydroxyl group was not contributing to the overall binding energy or to the relative populations of fast and slow phases. Primers have been shown to be able to align on the telomerase of *Euplotes crassus* with the 3′ end overhanging the template (*36*), also indicating no substantial preference for 3′-OH binding. However, a significant difference was observed for the 21 nucleotide primer in the presence of dGTP (Figure 5). The dissociation rate constants were similar to those observed without nucleotides, but a shift occurred to mostly fast phase. This effect was not observed with dTTP, dCTP, dATP, or dGMP. The 22 nucleotide primer also showed a shift from slow phase to fast phase when dGTP was included in the assay (Table 3). The different partitioning that was observed in the pulse−chase assay (above) may therefore be due to the presence of dGTP.

One explanation for the dGTP-induced shift could be cooperative binding with the next cognate nucleotide. To test this possibility, binding was assayed with 3′-dideoxy-blocked 17 and 25 nucleotide primers for which the next cognate nucleotide is dTTP (Table 3). For these primers, dTTP did not have a substantial effect. dGTP still had an effect, but it was qualitatively different; the ratio of fast/slow phase remained unchanged, but the relative cross-link intensity was decreased 2−7-fold. The reason for this difference in dGTP effect remains unclear. One possibility is that both the 21 and 22 nucleotide primers have 3′ termini that are native substrates of telomerase, whereas the primers with a T terminus correspond to partially extended products, and may therefore be discriminated against by the translocation and alignment processes. Alternatively, the presence of dGTP may bias the binding of the 5′ end in the anchor site, favoring a non-cross-linkable orientation.

Primer Dissociation Rate Measured Using a Telomerase Activity Assay. A third method of determining k_{off} was also used. As in the cross-linking protocol, a 5′-labeled primer (22-mer) was bound to telomerase and chased with an excess of unlabeled competitor. However, rather than cooling the sample and then cross-linking, nucleotides were added (dGTP and ddTTP) and the reactions incubated an additional 10 min to allow translocation and primer extension. The extended primer was then separated by PAGE (Figure 6A), and the +3 product was quantitated, normalized to the zero time reaction, and plotted versus time (Figure 6B). Dissociation was again biphasic, with approximately the same fast phase (19%, $k_{off} = 1.23 \pm 0.74$ min^{-1}) and slow phase (82%, $k_{off} = 0.05 \pm 0.005$ min^{-1}) as measured by cross-linking. In

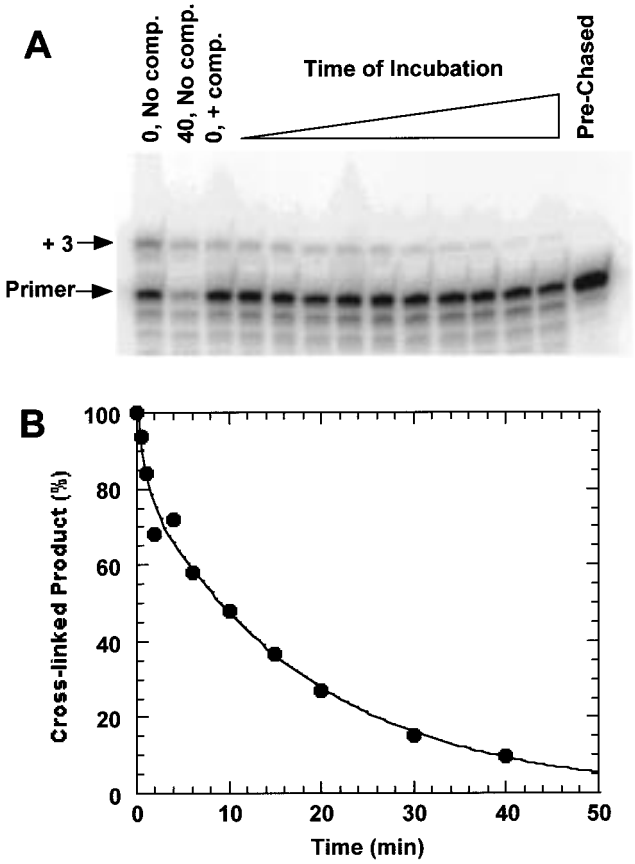

FIGURE 6: Primer dissociation measured using primer extension by telomerase. (A) Phosphorimager printout of a polyacrylamide gel showing the time-dependent dissociation of a 5′-^{32}P-labeled 22 nucleotide primer from telomerase after the reaction was chased with an excess of unlabeled primer. Time points were added to a reaction containing dGTP and ddTTP and incubated to allow telomerase to extend bound primer. (B) A plot of the amount of extended primer (+3 product), quantitated from (A). The data were fit with a double exponential curve as shown.

this assay, there was no dNTP present during the chase step, so the majority of primer dissociated in the slow phase.

DISCUSSION

Telomerase binds and extends single-stranded DNA primers with a preference for those with telomeric sequence (*35*). Primer binding at the telomerase active site occurs at least partially through base-pairing to the RNA template, since completely double-stranded primers do not bind (*33, 37*). This serves to align primers so that correct repeats are consistently synthesized (*29*). The stability of the interaction between telomerase and synthetic oligodeoxynucleotide primers has now been studied by measuring rate constants for dissociation (k_{off}). Our analysis was restricted to primers bound in a productive mode by two criteria. First, the photocross-linking method only monitored primers with their 5′ end in the anchor site (*33*). The observation that primers with different lengths gave a similar efficiency of cross-linking is most consistent with their having a single mode of binding within the anchor site. Second, when the analysis was restricted to primers that had been pulse-labeled by telomerase and were still associated with the same enzyme responsible for their extension, similar k_{off} values were measured.

One striking observation is that the dissociation rates are remarkably insensitive to changes in primer length which, according to the rules of RNA−DNA duplex stability (*41*), should have a very large effect. The difference in potential Watson−Crick interactions between a primer bound in the aligned register (7 base pairs) and extended register (15 base pairs) is predicted to give a difference in k_{off} of $>10^{10}$ (Table 2). In contrast to that expectation, increasing the primer length from 12 or 14 nucleotides to 21 nucleotides decreased k_{off} from telomerase by only 10^1-10^2 (Table 1). The k_{off} values are also markedly insensitive to base substitutions designed to give mismatches in the DNA−RNA template duplex. We conclude that a protein component or components of telomerase act to equalize the binding energy of different telomeric DNA primers.

The Transcription Bubble Model for Telomerase−Primer Interactions. The easiest way to understand the insensitivity of binding to primer length is if the protein maintains a more-or-less fixed amount of primer-template base-pairing during initial primer binding, and throughout the primer extension reaction (*28*). This situation is reminiscent of RNA polymerase, which maintains a constant amount of base-pairing in a transcription bubble during elongation. In contrast, DNA polymerases and retroviral reverse transcriptases create ever-longer duplexes during elongation. The amount of duplex maintained during primer extension by *Euplotes* telomerase has not been determined, but it is inferred to be between 4 and 10 nucleotides. The lower limit is based on the minimum number of base pairs required to give a unique alignment on the template, and the upper limit on a comparison of the calculated and measured k_{off} values.

Biphasic Dissociation Kinetics. Many of the primers dissociated with biphasic kinetics, particularly those shorter than 21 nucleotides (Figure 3A) and those containing mismatches (Figure 3C). As all of the measurements were based on cross-linking at the 5′ end, we propose that the two phases are due to different base-pairing registers of the 3′ end with the telomerase RNA template. For example, one plausible model (*43*) attributes the fast phase dissociation to the aligned register (Figure 1D) and the slow phase dissociation to the extended register (Figure 1C). Future studies to establish more definitively the source of biphasic dissociation rates may require different experimental approaches. One possibility is the testing of telomerase RNA template mutants with complementary and noncomplementary primers; however, such mutants are not yet available in *Euplotes*. Even this may not provide an absolute answer, as template mutations in *Tetrahymena* have significant and variable effects depending on the type and location of the mutation, attributed to disruption of active site interactions (*46*).

Primer-Template Duplex Migration. During both macronuclear development and chromosome healing (reviewed in *47, 48*), it is likely that nontelomeric sequences have to be aligned with the telomerase template. This is a situation analogous to the binding of mismatched primers tested in this study. The effect of these mismatches on primer dissociation was lower than predicted by base-pairing energetics (Table 1). Telomerase is therefore compensating for the effect of the mismatch in some way. One possibility is that protein interactions serve to stabilize mismatched base pairs. Alternatively, the region of base-pairing may be able to migrate, such that at any given time there is a mixed population of duplexes. In this way, the effect of a mismatch would be damped because it would not always be present in the duplexed region. Recall that 10−11 or fewer base pairs out of a possible 15 are formed between the primer and the template. Mismatched nucleotides in the center of the template would be the most destabilizing (as was observed, Table 1), because they would be present in a larger number of the possible duplexes. However, migration of the duplex in these complexes could result in transient species with fewer base pairs and correspondingly lower stability. Preferential binding in one base-pairing register may align the mismatch so as to minimize its effect, resulting in the observed shift from mostly slow phase to mostly fast phase dissociation.

Translocation and dGTP. The 3′ end of an extended primer can be released from the RNA template and rebound in proper alignment for another repeat addition while the 5′ end of the primer remains bound in the anchor site. The presence of this translocation step in the telomerase reaction cycle has long been evident from processive repeat addition (*31*), yet little is known about how it occurs.

In one model, base-pairing between primer and template would be opened in an energy-requiring process, perhaps by a helicase-like activity. Neither ATP nor GTP is required for repeat addition, but it had been impossible to rule out dGTP or dTTP because both are required for primer extension. Determination of k_{off} for dideoxy-blocked primers has now indicated that dGTP may stimulate the translocation process (Table 3). The observed shift from slow phase to fast phase dissociation may accompany the changes in base-pairing which occur upon translocation. We have also observed an effect of dGTP on translocation by measuring its effect on the processivity of telomerase (*49*).

One possibility for this effect is that dGTP binding decreases the stability of the extended register, thereby favoring translocation. However, the same dissociation rates

were seen with and without dGTP; only the amount in each phase was changed. Alternatively, binding and perhaps hydrolysis of dGTP may provide energy for translocation. Future testing of nonhydrolyzable dGTP analogues may provide a definitive answer. Recent efforts have been directed at using nucleotide analogues to inhibit the activity of telomerase for a therapeutic effect in cancer (*50*−*52*). If dGTP is indeed the energy source for translocation, then analogues that inhibit the translocation step may prove pharmaceutically interesting.

Source of the Telomerase Pausing Pattern. Telomerase primer extension assays produce a distinctive banding pattern, the major bands corresponding to synthesis of complete telomeric repeats. It was shown using a bind and chase assay (*33*) that, for the telomerase of *Euplotes aediculatus*, the band intensity decreased exponentially as products became longer. Each major band was ∼60−70% as intense as the previous one. Two primary models have been proposed to explain this banding pattern: (i) translocation is relatively inefficient, such that primer has a significant probability of dissociating during each translocation event; or (ii) translocation is slow, and primer therefore accumulates as a paused species waiting to translocate. However, neither model is exclusive of the other.

The data presented herein lend support to the latter model. The half-life for dissociation of the slow phase species (k_{off} = 0.04 min^{-1}) is ∼17 min, even in the presence of dGTP. This reveals that primer can be bound to telomerase, yet remain unextended for a substantial time.

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