Interaction of Human Telomerase with Its Primer Substrate

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ABSTRACT: Telomerase is a ribonucleoprotein responsible for maintaining the ends of linear chromosomes in nearly all eukaryotic cells. In humans, expression of the enzyme is limited primarily to the germ line and progenitor cell populations. In the absence of telomerase activity, telomeres shorten with each cell division until a critical length is reached, which can result in the cessation of cell division. The enzyme is required for cell immortality, and its activity has been detected in the vast majority of human tumors. Because of this, telomerase is an attractive target for inhibition in anticancer therapy. To learn more about the biochemistry of the human enzyme and its substrate recognition, we have examined the binding properties of single-stranded oligonucleotide primers that serve as telomerase substrates in vitro. We have used highly purified human enzyme and employed a two-primer method for determining the dissociation rates of these primers. Primers having sequence permutations of $(TTAGGG)_3$ were found to have considerably different affinities. They had $t_{1/2}$ values that ranged from 14 min to greater than 1200 min at room temperature. A primer ending in the GGG register formed the most stable complex with the enzyme. This particular register imparted stability to a nontelomeric primer resulting in a nearly 100-fold decrease in the k_{off}. We have found that interactions of telomerase with primer substrates are stabilized mainly by contacts with the protein subunit of the enzyme (hTERT). Base-pairing between the primer and the template region of telomerase contributes minimally to its stabilization.

Human somatic cells have a finite proliferative capacity that is limited by their telomere length (*1*). In the absence of the enzyme telomerase, chromosome ends may be shortened by dozens of nucleotides with each cell division resulting from the inability of DNA polymerases to replicate the ends of linear DNA (*2*). The consequences of telomere shortening may be either a cessation of cell division or chromosome instability and apoptosis when telomeres reach a critically short length (*3*−*6*). Because of this, the activation of telomerase can be linked to its ability to confer immortality to cells, which has indeed been demonstrated for fibroblasts (*7*, *8*), retinal pigment epithelial cells (*7*), endothelial cells (*9*), and other cell types (reviewed in ref *10*).

Cells in the vast majority of human cancers express telomerase whereas most normal somatic tissues lack expression. Cells that undergo malignant transformation necessitate several independent genetic or epigenetic changes that contribute to growth deregulation. Activation of telomerase is required for cell immortality and is one of several events that must occur for conversion of primary human cells into tumor cells (*11*). These studies provided the rationale for selecting telomerase as an attractive target for anticancer therapy. The support for this approach was further buttressed by the finding that overexpression of a dominant-negative form of the enzyme could result in growth arrest or death of tumor cell lines. The amount of time or number of cell divisions necessary to reach crisis in a particular cell line was related to the telomere length of that cell line (*12*). Additional experiments with antisense oligonucleotides directed against the template region of telomerase have yielded similar results and support the key role of telomerase in tumor cell maintenance (*13*−*17*).

Telomerase is a ribonucleoprotein that utilizes a short sequence within its RNA subunit as the template for reverse transcription, synthesizing d(TG)-rich repeats onto the ends of eukaryotic telomeres, which in vertebrates comprises the hexanucleotide d(TTAGGG) (*18*). In vitro, telomerase catalyzes the addition of d(TTAGGG) repeats onto a single-stranded DNA primer. For the human enzyme, two telomerase components are necessary and sufficient for this activity in vitro: the previously mentioned RNA (hTR)[1] and a protein that constitutes the catalytic subunit (hTERT). We have been interested in a rational approach to finding telomerase inhibitors as potential therapeutics and recently described several oligonucleotide phosphoramidates and thio-phosphoramidates addressed to the RNA component of the enzyme (*17*, *19*, *20*). These oligonucleotides have picomolar to low nanomolar potency in inhibiting telomerase activity in cell free assays, suggesting very efficient template antagonism. Further knowledge of human telomerase, especially its substrate recognition, should allow us to better select reagents as potential competitive inhibitors of the enzyme.

In cells, the single-stranded DNA 3′-overhang at the end of the telomere, d(TTAGGG)n, is the natural substrate for the enzyme. Short single-stranded oligonucleotides that have minimal complementary to the template region of telomerase can serve as primers in cell free assays (*21*). In this work,

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[1] Abbreviations: hTR, human telomerase RNA subunit; hTERT, human telomerase protein subunit; k_{off}, dissociation rate constant; $t_{1/2}$, ln $2/k_{off}$.

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we have begun to investigate the interactions of human telomerase with its DNA primer substrate and have measured the relative affinities of several primers for the enzyme. We found that while enzyme−primer complexes can be stabilized by base-pairing with the hTR template as well as by interactions between hTERT and the 5′-end of the primer, an additional strong interaction occurs between hTERT and the 3′-end nucleotides of the primer.

MATERIALS AND METHODS

Purification of Human Telomerase. Telomerase was prepared from 293 suspension cells that overexpressed the hTERT gene, using a myeloproliferative sarcoma virus promoter. Whole cell extracts were prepared from frozen cell pellets. The cell pellets were resuspended in one packed cell volume of H buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-KOH (pH 7.9), 10 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl floride, and 0.5 μg/mL Leupeptin) and then lysed with a dounce homogenizer. The concentration of salt in the lysate was adjusted to 0.3 M NaCl, which was stirred for 15 min and then centrifuged at 100 000*g*. Solid ammonium sulfate was added to the supernatant to achieve 42% saturation, and insoluble proteins were pelleted. The pellets were resuspended in A buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-KOH (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethyleneglycoltetraacetic acid, 10% glycerol, and 0.5 μg/mL Leupeptin) containing 0.1 M NaCl (1/5 of their original volume) and dialyzed against this buffer. Following dialysis, the extract was spun at 25 000*g* to remove insoluble material. Telomerase was purified by antisense affinity chromatography similar to previously described methods, using a 2′-O-Me RNA complementary to the template region of hTR (*22*, *23*). Affinity purified extracts were further purified by size-exclusion chromatography (G5000PW, Tosoh Biosep LLC). The amount of telomerase in the extracts was determined by quantitative northern blot analysis of hTR and by quantitative measurement of the telomerase activity. The activity measurement was conducted by pulse-labeling a saturating amount of primer (100 nM 18AGG, Table 1) with 20 μCi [α-^{32}P] dGTP (3000 Ci/mmol) for 30 min at 22 °C, which permitted the addition of a single nucleotide. Under the conditions used, there was no substrate turnover during the pulse-labeling, and thus, we were able to measure the moles of active enzyme in the preparation. The number of active enzyme molecules found agreed with the number of hTR molecules determined by northern analysis in the preparation, which was between 0.1 and 0.2 pmol of telomerase per milliliter of extract, and we thus inferred an equal stiochiometry between hTR and hTERT.

Determination of Dissociation Rates of Primers. Extracts containing telomerase (G5000PW pool, 0.5 fmol/20 μL reaction) were incubated with stated primers (100 nM) at 22 °C for 30 min in telomerase reaction buffer that contained 50 mM (*N*-2-hydroxyethylpiperazine-*N*′-3 propanesulfonic acid)-NaOH (pH 8.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, and 0.5 mM ethyleneglycoltetraacetic acid. Primer−telomerase complexes were then challenged by the addition of 20 μM 24GGG or 18GGG (Table 1). At times indicated in the figures, aliquots (18 μL) were removed and added to a nucleotide mix (2 μL) containing 2 mM dTTP and 10 μCi [α-^{32}P] dATP (3000 Ci/mmol) and labeled for 5 min. The reactions were terminated with sodium dodecyl sulfate (0.1%), NH_4 acetate (750 mM), and pellet paint (2 μL, Novagen) in a final volume of 200 μL. The terminated reactions were extracted with an equal volume of phenol-chloroform (1:1) and precipitated with 2.5 volumes of ethanol. Following centrifugation, the pellets were washed with 80% ethanol and resuspended in 90% formamide (10 μL) containing bromophenol blue and xylene cylanole. The samples were applied to a 20% polyacrylamide gel containing 7 M urea, 115 mM tris(hydroxymethyl) aminomethane, 15 mM boric acid, and 0.6 mM ethylenediaminetetraacetic acid and electrophoresed at 21 W until the bromophenol blue migrated off the gel. Following electrophoresis, the gel was fixed in 5% trichloroacetic acid (w/v), rinsed in water, dried, and analyzed with a PhosphorImager (Molecular Dynamics).

Table 1: Primers Used for Binding Studies

Name	Sequence
24GGG	TTAGGGTTAGGGTTAGGGTTAGGG
18TAG	GGTTAGGGTTAGGGTTAG
18AGG	GTTAGGGTTAGGGTTAGG
18GGG	TTAGGGTTAGGGTTAGGG
18GGT	TAGGGTTAGGGTTAGGGT
18GTT	AGGGTTAGGGTTAGGGTT
18TTA	GGGTTAGGGTTAGGGTTA
19TTA	AGGGTTAGGGTTAGGGTTA
20TTA	TAGGGTTAGGGTTAGGGTTA
NT18GTT	AATCCGTCGAGCAGAGTT
NT18GGG	AATCCGTCGAGCAGTGGG
CH18GGG	AATCCGTCGAGGTTAGGG
18GGG	TTAGGGTTAGGGTTAGGG
GRN163	TAGGGTTAGACAA[a]

[a] Contains 3′-NHP(O)(S-)O-5′ thio-phosphoramidate (NPS) linkages and a 3′-terminal amino group.

For primers ending in GGG, GGT, or GTT, the rate constants were determined by following the disappearance of the labeled primer. Each primer is expressed as percent bound relative to the zero time point. Primers ending in TTA, TAG, or AGG are not extended with the dTTP and [α-^{32}P] dATP nucleotide mix, so rate constants were determined by following the appearance of the labeled 24GGG challenge primer. For this set of primers, percent bound is determined by first taking the difference from the maximum at each time point. In either case, the rate constants were determined by fitting the data to the equation $y = A \exp(-kt)$ (where A represents the amount of primer in complex at time zero, k is the rate constant, and t is the time in minutes), using the Prism software package (GraphPad Software Inc.).

Dissociation Rates of Primers Having a 3′-Terminal Guanosine Analogue. All modified primers are derivatives of the 18GGG (Table 1) sequence that terminate with a guanosine analogue. Primers ending in a 2′-deoxy-(dG), 3′-deoxy, ribo-, or 2′,3′-dideoxyguanosine (ddG) were synthesized using standard solid-phase chemistry with the appropriate column, followed by purification through a 15% polyacrylamide gel containing 7 M urea. The primer was

identified by UV shadowing, excised, eluted into 0.5 M NH_4 acetate (pH 8.0) and 1 mM ethylenediaminetetraacetic acid, and purified through a C-18 Sep-Pak cartridge (Waters Corp). Primer−telomerase complex formation, labeling, and processing of reactions were identical as described above.

Primers ending in 3′-amino- or 3′-azido- were generated by incubating 25 pM telomerase and 100 nM primer 18AGG (Table 1), with an additional 250 μM of the guanosine triphosphate analogue, for 30 min at 22 °C. Challenging, labeling, and processing of reactions were identical as described above. To verify that the guanosine triphosphate analogues were incorporated, two additional reactions containing 25 pM telomerase were incubated with 20 μM of the 24GGG primer for 30 min at 22 °C. For each, an 18-μL aliquot was removed and labeled with a 2-μL nucleotide mix containing 2 mM dTTP and 10 μCi [α-^{32}P] dATP (3000 Ci/mmol; NEN) alone or with the additional 250 μM guanosine triphosphate analogue for 5 min. Quantification of the +3 and +4 products showed that greater than 98% of the labeled primer incorporated the guanosine triphosphate analogue.

Competition between Primer 18GGG and Thio-Phosphoramidate GRN163. Telomerase containing extract (G5000PW pool, 0.5 fmol/20 μL reaction) was incubated with 100 nM primer 18GGG in telomerase reaction buffer (see above) for 30 min at 22 °C. Complexes were then challenged by the addition of 0.5−50 nM thio-phosphoramidate GRN163 (Table 1). After an additional 30-min incubation, 18-μL aliquots were removed, labeled, and processed as described above. The products were separated on an 18% (1:19 bisacrylamide) polyacrylamide gel containing 7 M urea, 115 mM tris(hydroxymethyl) aminomethane, 15 mM boric acid, and 0.6 mM ethylenediaminetetraacetic acid. The gel (140 × 170 × 0.8 mm) was electorphoresed at 21 W until the bromophenol blue migrated off the gel. The gel was fixed in 5% trichloroacetic acid (w/v), rinsed in water, dried, and analyzed with a PhosphorImager (Molecular Dynamics).

RESULTS

We have purified human telomerase from 293 cells that overexpress the catalytic (hTERT) subunit of telomerase. These cells were found to possess about 5-fold more activity than the parental 293 cells (unpublished observation, R. Pruzan). Starting with whole cell extracts, we have purified telomerase using an affinity selection step (*22*, *23*) followed by size-exclusion chromatography. The resultant material was enriched approximately 10 000-fold for telomerase. The only enzymatic activity in the extracts capable of labeling an exogenous primer was deemed as telomerase by virtue of its RNA dependence, its sensitivity to a highly specific antitemplate telomerase inhibitor oligophosphoramidate GRN163 (17, Figure 5), as well as the correctly predicted products generated by incubating the purified extract along with primers having different 3′-ends with the appropriate [α-^{32}P] dNTP (data not shown and Figure 2).

The affinity of an enzyme for its substrate can be assessed from its dissociation constant (K_D). The K_D for a given substrate can be determined by varying its concentration and measuring the amount bound to enzyme as a function of its concentration. Alternatively, the dissociation rate (k_{off}) of enzyme bound substrates can be used to determine relative affinities of the substrates for the enzyme. Measuring the k_{off} is often a much more experimentally tractable approach and was chosen when determining primer affinities for telomerase. We developed a primer-binding assay that depended on telomerase activity, which provided us with a highly specific assay, since only primers bound to the active site of the enzyme were tallied. Initially, a saturating amount of the primer of interest was incubated with enzyme. After reaching equilibrium, the enzyme−primer complex was challenged with a large excess of a competitive primer having a different length than the original one. Aliquots were removed at various time intervals and pulse-labeled with dTTP and [α-^{32}P] dATP, which resulted in either the original primer or the challenge primer producing a discrete end-labeled product. These products were then resolved by denaturing polyacrylamide gel electrophoresis and analyzed by PhosphorImager scanning. The amount of labeled products were normalized to the amount of starting material at time zero and expressed graphically, which in turn was used to derive dissociation rates for the various primers.

When the association rate of the primer with enzyme (and the catalytic rate) is much greater than the dissociation rate, either the disappearance of the extended original primer with time, or conversely, the appearance of the extended challenge primer can serve to monitor the dissociation rate of the primer (Figure 1C). This property allowed us to examine the binding of primers that could not be extended, for example, those lacking a 3′-OH terminal group. Employing the same challenge primer allowed us to measure a variety of different primers using a consistent set of conditions. The technique is illustrated in Figure 1, where the intensity of the band representing the product of the initial enzyme−primer complex decreases as a function of time. A concomitant increase in the band representing the product of challenge primer is also seen (Figure 1A,B). The amount of product resulting from the initial enzyme−primer complex (21-mer in Figure 1A and 27-mer in Figure 1B) is assigned the value of 100% (time zero). The amount of complex remaining at subsequent times is expressed as a fraction of that initial amount. The 100% value for the challenge primers product is obtained by pulse-labeling a preequilibrated mixture of the initial primer and challenge primer (200-fold excess of the latter). When telomerase is prebound with the 18GGG primer (see Table 1 for sequence of primers) and challenged with the 24GGG primer, pulse-labeled products 3-nt longer than the original primers result from the addition of TTA. Thus, the 18GGG and 24GGG primers are converted into 21- and 27-nt products, respectively. Identical results were obtained when the band intensities of the 21- or the 27-nt products are quantitated individually and used to derive the dissociation rate of 18GGG (Figure 1C). This is noteworthy since some of the primers used in our study cannot be labeled using dTTP and [α-^{32}P] dATP, thus only the appearance of the challenge primer is visible. Furthermore, when the reciprocal experiment is done, first incubating telomerase with 24GGG followed by a challenge with an excess of 18GGG (Figure 1B,D), an equivalent rate of dissociation for 24GGG was observed, indicating that 18GGG is already longer than the minimal length requirement necessary to stabilize its interaction with the enzyme and that additional nucleotides at the 5′-end of the primer do not provide any added stability.

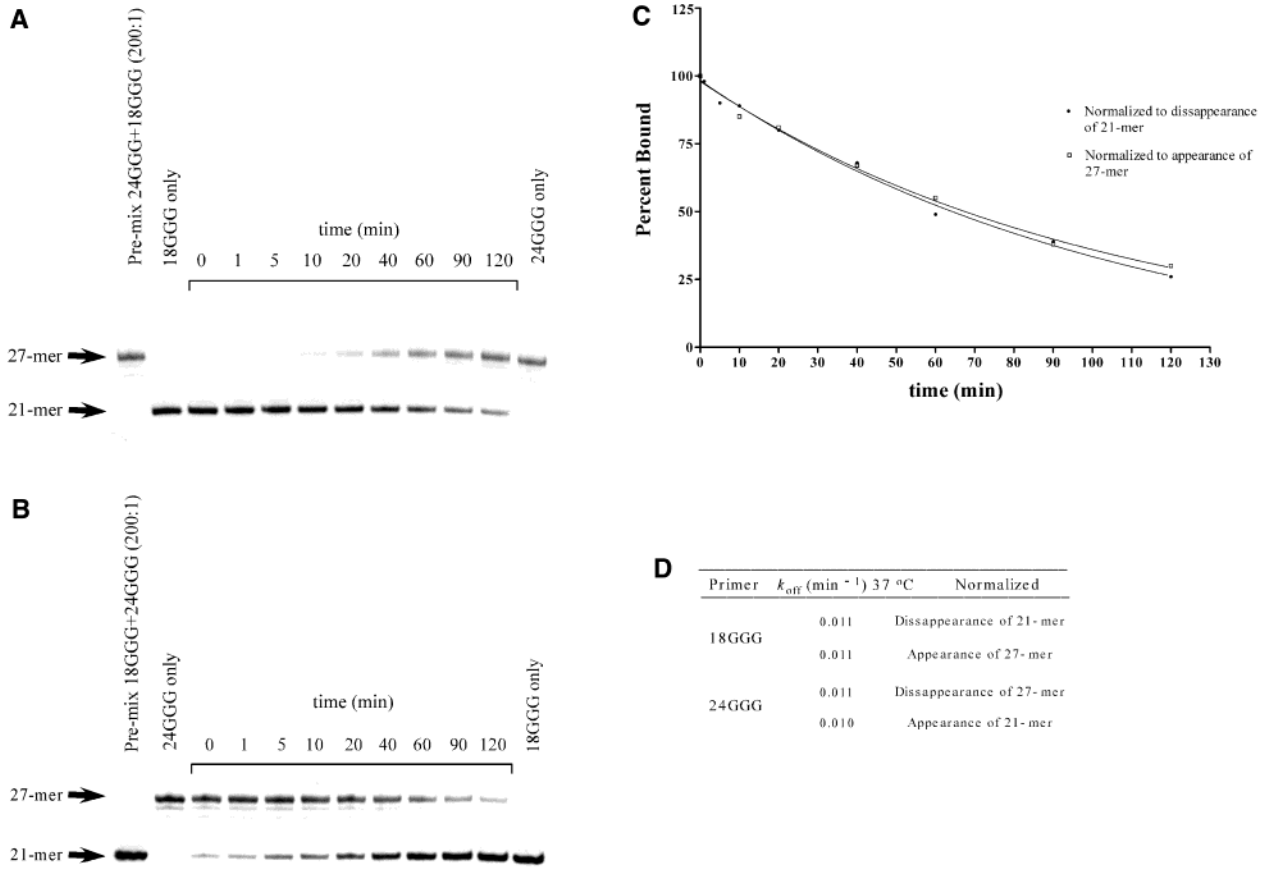
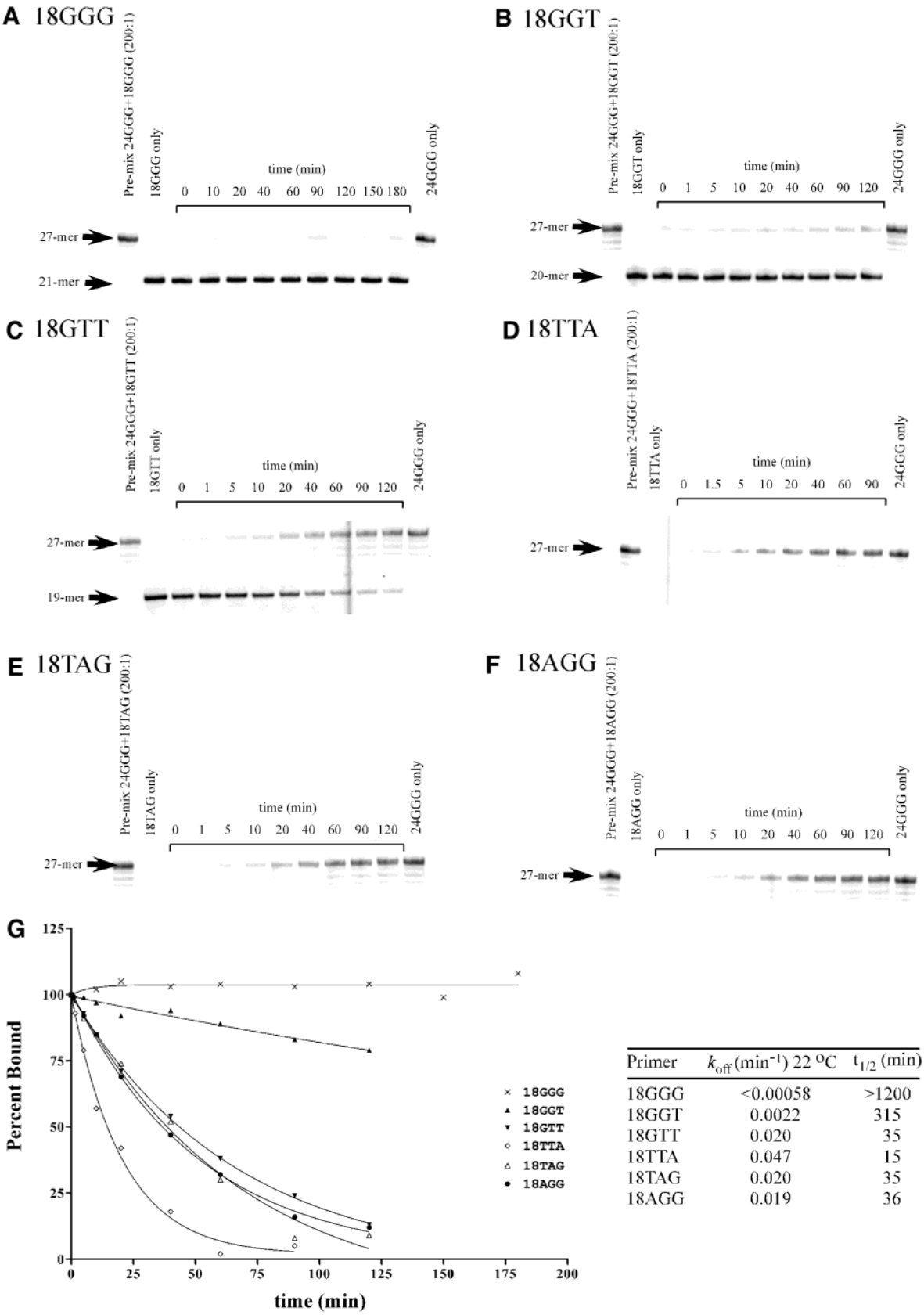


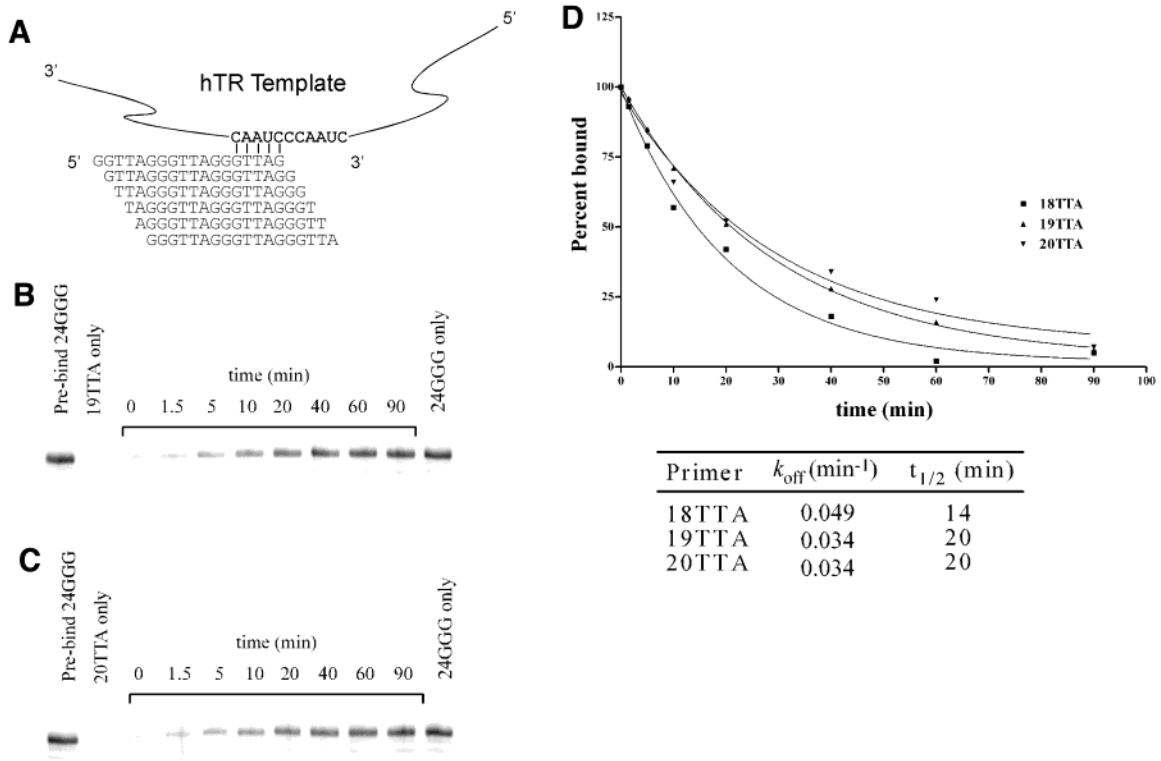
FIGURE 1: Assay for measuring dissociation rate of primers from telomerase. Telomerase containing extract (20 μL) was incubated with primer (100 nM) for 30 min at 22 °C in a total reaction volume of 200 μL as described in Materials and Methods. The mix was equilibrated at 37 °C after which the challenge primer (20 μM) was added. Aliquots (18 μL) were removed at times indicated and added to a nucleotide mix (2 μL) containing 10 μCi [α-^{32}P] dATP and 2 mM dTTP and pulse-labeled for 5 min. The samples were extracted and analyzed on a 20% denaturing polyacrylamide gel as described in Materials and Methods. (A) Enzyme incubated with 18GGG and challenged with 24GGG. (B) Enzyme incubated with 24GGG and challenged with 18GGG. (C) Bands depicted in panel A were quantitated by PhosphorImager analysis and expressed as a percentage of complex bound based on the sample in which the challenge primer was premixed with the initial primer. (D) Data from experiments depicted in panels A and B were analyzed as in panel C, and dissociation rates were determined from the graphs using the Prism GraphPad software.

Dissociation Rates of Permutated Telomeric Primers. Human telomerase is able to extend a variety of primers with different efficiencies in cell free assays (*21*), although the elements involved in their recognition have not been well-defined. We have begun to investigate the determinants of primer recognition and specificity using the two-primer method described above. Using 24GGG as the competitor, we measured the k_{off} of six different 18-nt telomeric primers, representing the six possible permutations of a repetitive TTAGGG sequence. The sequences of these primers are listed in Table 1, and the dissociation rates for these primers from telomerase can be seen in Figure 2. These data are presented graphically along with a summary of the derived k_{off} values for these primers (Figure 2G). When telomerase is incubated with 18GGG, 18GGT, and 18GTT, in the presence of dTTP and [α-^{32}P] dATP, the enzyme will generate products of 21-, 20-, and 19-nt, respectively (Figure 2A−C). Primers 18TTA, 18TAG, and 18AGG cannot be labeled with dTTP and [α-^{32}P] dATP (Figure 2D−F). Therefore, the appearance of the 27-nt band originating from the labeling of the competitor primer 24GGG was used for determining the k_{off} values for this group of primers. The comparison between the six permutated 18-mers was carried out at room temperature (~22 °C) to capture those primers that had rapid dissociation rates. The k_{off} values ranged from 0.047 min^{-1} for the least stable 18TTA to <0.00058 min^{-1} for the most stable 18GGG (Figure 2A−G). The dissociation rate of the latter primer was not measurable at room temperature. It was followed for 20 h, after which a half-life for this primer−telomerase complex was still not reached (data not shown); hence, we ascribe a rate of <0.00058 min^{-1}, which represents a $t_{1/2}$ of >20 h. Three of the primers (18GTT, 18AGG, and 18TAG) had similar k_{off} values of about 0.02 min^{-1} (Figure 2, panels C, E−G), and 18GGT had an intermediate k_{off} (0.002 min^{-1}) that was about 10-fold slower than the previous three (Figure 2C−G). Thus, the exact 3′-register of the TTAGGG hexanucleotide repetitive sequence is extremely important for its recognition by the enzyme. Telomeric primers can theoretically form between five and 11 base-pairs with the RNA template of telomerase (Figure 3A). A primer terminating in TAG can be depicted as fully aligned with the template in a pretranslocation register or partially aligned, poised to be extended (ref *18*, Figure 3A). Dissociation rates therefore do not correlate with the number of potential base-pairs that a primer can form with the template. It is noteworthy that 18TTA, which can potentially form the most complete duplex with the RNA template, has the most rapid k_{off} ($t_{1/2}$ ~15 min,



Primer	k_{off} (min^{-1}) 22 °C	$t_{1/2}$ (min)
18GGG	<0.00058	>1200
18GGT	0.0022	315
18GTT	0.020	35
18TTA	0.047	15
18TAG	0.020	35
18AGG	0.019	36

FIGURE 2: Dissociation rates of the six permutated sequences of primer $(TTAGGG)_3$. Telomerase containing extract (20 μL) was incubated with primer (100 nM) for 30 min at 22 °C in a total reaction volume of 200 μL as described in Materials and Methods. Following the addition of the 24GGG challenge primer (20 μM), aliquots (18 μL) were removed and added to a nucleotide mix (2 μL) containing 10 μCi [α-^{32}P] dATP and 2 mM dTTP and pulse-labeled for 5 min. The samples were extracted and analyzed on a 20% denaturing polyacrylamide gel as described in Materials and Methods. The following primers were initially incubated with telomerase in panels A−F, 18GGG, 18GGT, 18GTT, 18TTA, 18TAG, and 18AGG. (G) Bands depicted in panels A−F were quantitated by PhosphorImager analysis and expressed as a percentage of complex bound based on the sample in which 24GGG was added together with 18-nt primer. k_{off} values for primers are depicted in panel G as determined from the graphs using the Prism GraphPad software.



Primer	k_{off} (min^{-1})	$t_{1/2}$ (min)
18TTA	0.049	14
19TTA	0.034	20
20TTA	0.034	20

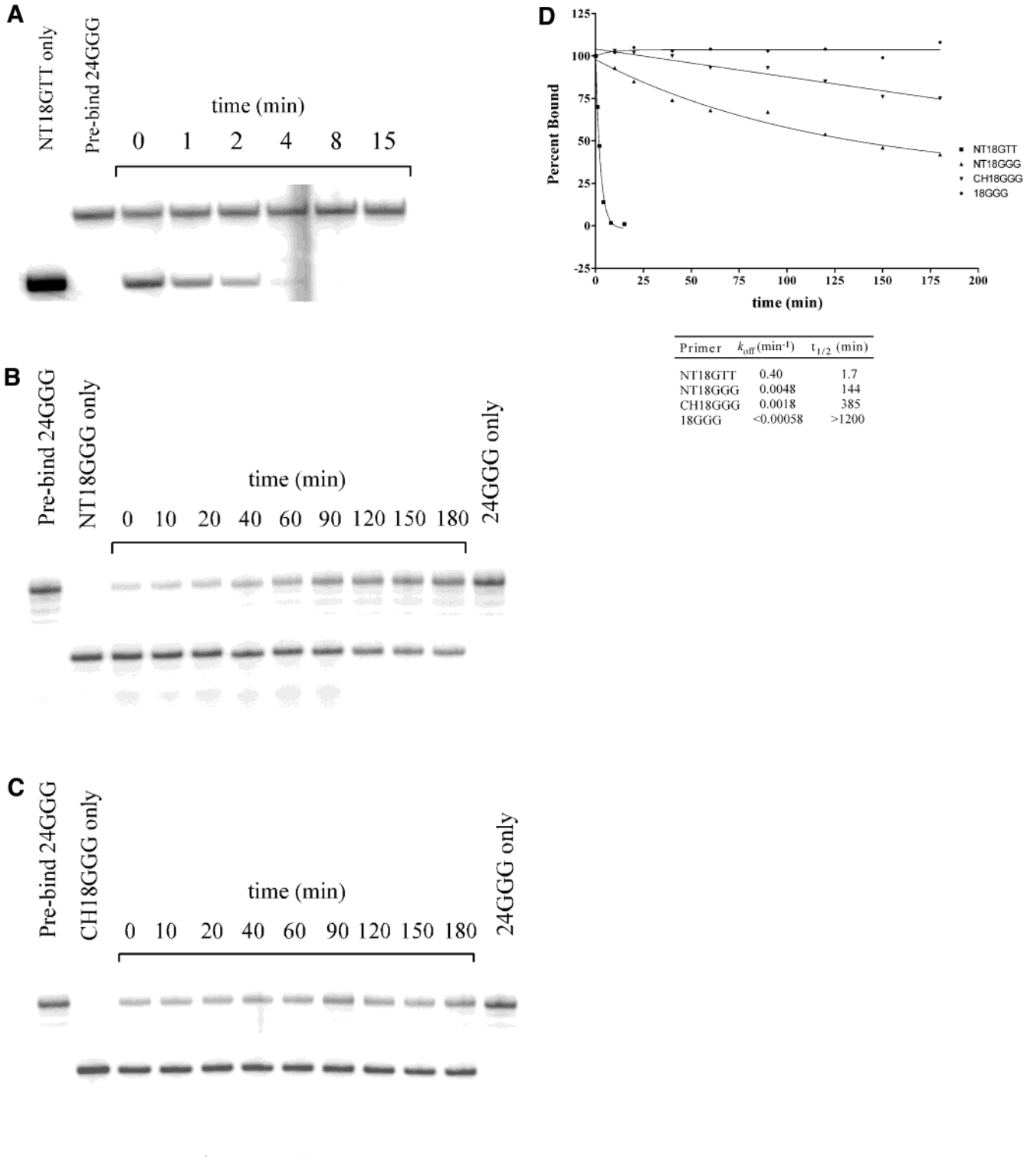
FIGURE 3: Primer's affinity for telomerase depends on its register with respect to the template. Dissociation rates for primers were determined as described in Materials and Methods and in legend to Figure 2. (A) Schematic of the template region of hTR is depicted together with primers having the six possible sequence permutations of $(TTAGGG)_3$ and their possible alignment with the template. (B) Dissociation of 19TTA. (C) Dissociation of 20TTA. (D) Bands depicted in panels B and C were quantitated by PhosphorImager analysis and expressed as a percentage of complex bound based on the sample in which 24GGG was added together with 19- or 20-nt primer. Also shown is the dissociation rate of 18TTA taken from Figure 2G. k_{off} values for primers depicted in panel D are determined from the graphs using the Prism GraphPad software.

Figure 2G). Thus, significant interactions apart from base-pairing with the template must be involved in stabilizing the interaction between primer and enzyme. Given the identical length of all six primers, one trivial explanation for the rapid dissociation rate of 18TTA was the loss of a potential stabilizing contact between the 5′-end of the primer and hTERT. As depicted in Figure 3A, 18TTA would align with the 5′-end of the template region, which could potentially distance its 5′-end from a putative binding site on hTERT. To rule out this possibility, two additional primers, 19TTA and 20TTA (Table 1), were synthesized. These primers should maintain their 3′-nucleotide alignment with the template, while having the identical 5′-end as 18GTT and 18GGT, which have respective 2- and 20-fold lower dissociation rates as compared to 18TTA (Figure 2G). Only a minor decrease in the k_{off} was observed (less than 2-fold) when the 5′-end of 18TTA was extended (Figure 3B−D). Taken together, these data indicate that a telomerase−primer complex may be stabilized by an intricate set of interactions; some that may reside at or near the 3′-end of the primer. The extremely stable complex observed with 18GGG and the rapid change in stability, with respect to a single nucleotide change of the register of the primer, would be compatible with the 3′-terminus having a different topology in relation to the hTERT protein domain.

Sites of Telomerase−Primer Interactions Are Separable. Two sites of primer interaction with telomerase have been previously proposed: an anchor site interacting with the 5′-end of the primer and a template site interacting with the 3′-end of the primer (*21*, *24*). Data supporting this model was obtained by experiments photocross-linking a primer with the *Euplotes* enzyme (*25*).

To discern the different contributions to the primer complex stability in the human enzyme, we compared the k_{off} values of four primers, 18GGG, CH18GGG, NT18GGG, and NT18GTT (Table 1). As mentioned above, 18GGG forms an extremely stable complex with telomerase at room temperature. NT18GTT is an 18-nt nontelomeric primer ending with GTT-3′ (Table 1) that can be extended by telomerase. This primer is used in the common TRAP assay (*26*). It binds only weakly to the enzyme, having a k_{off} of about 0.4 min^{-1} (Figure 4A). In fact, we found NT18GTT to bind telomerase with an affinity similar to a random oligomer, when monitoring the end-labeled primer's ability to bind the enzyme using a nitrocellulose filter-binding assay (data not shown). Primer 18GTT (Table 1), by contrast, has a k_{off} of about 0.02 min^{-1} (Figure 2G). This affinity difference may be attributed to its ability to form additional base-pairs with the template region or to a stronger interaction with the anchor site of hTERT.

To separate the stability contributions of the primer's register from those of either the template or the anchor site interaction, we compared the affinity of three primers ending in the GGG-3′ register. We designed a primer (NT18GGG, Table 1) identical to NT18GTT that ended with TGGG-3′ instead of AGTT-3′. This primer is not complementary to the template region with the exception of the last three G's. The A was converted to T to eliminate one extra base-pair. NT18GGG was found to dissociate from telomerase with a k_{off} of 0.0048 min^{-1} (Figure 4B,D). Thus, similar to primers



Primer	k_{off} (min^{-1})	$t_{1/2}$ (min)
NT18GTT	0.40	1.7
NT18GGG	0.0048	144
CH18GGG	0.0018	385
18GGG	<0.00058	>1200

FIGURE 4: Nontelomeric primer ending in GGG has a high affinity for telomerase. Dissociation rates for primers were determined as described in Materials and Methods and in the legend to Figure 2. Comparison of dissociation rates of NT18GTT (A), NT18GGG (B), or CH18GGG (C) (Table 1). (D) Graphical representation of dissociation rates from panels A−C together with that of 18GGG from Figure 2G. k_{off} values for primers depicted in panel D as determined from the graphs using the Prism GraphPad software.

with a telomeric sequence, the register ending with GGG-3′ confers a dramatic increase to the enzyme−primer stability. CH18GGG was designed as a chimeric primer, 11 5′-nucleotides are isosequential to NT18GTT, and seven 3′-nucleotides matched those of 18GGG. It is fully complementary to the seven 3′-nucleotides of the hTR template region. Replacing four nucleotides of NT18GGG with ones complementary to the template reduces the k_{off} of the primer between 2−3-fold (Figure 4B−D), which indicates that most of the potential additional base-pairs are likely not formed. Comparing the k_{off} values for 18GGG and CH18GGG reveals the former is at least 3-fold more stable than the latter

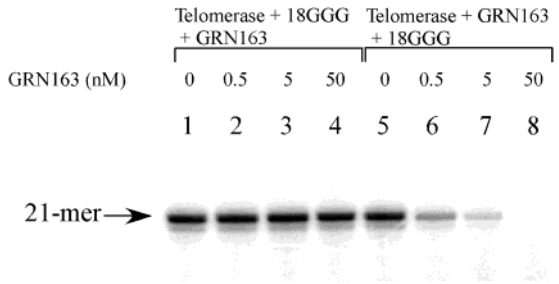
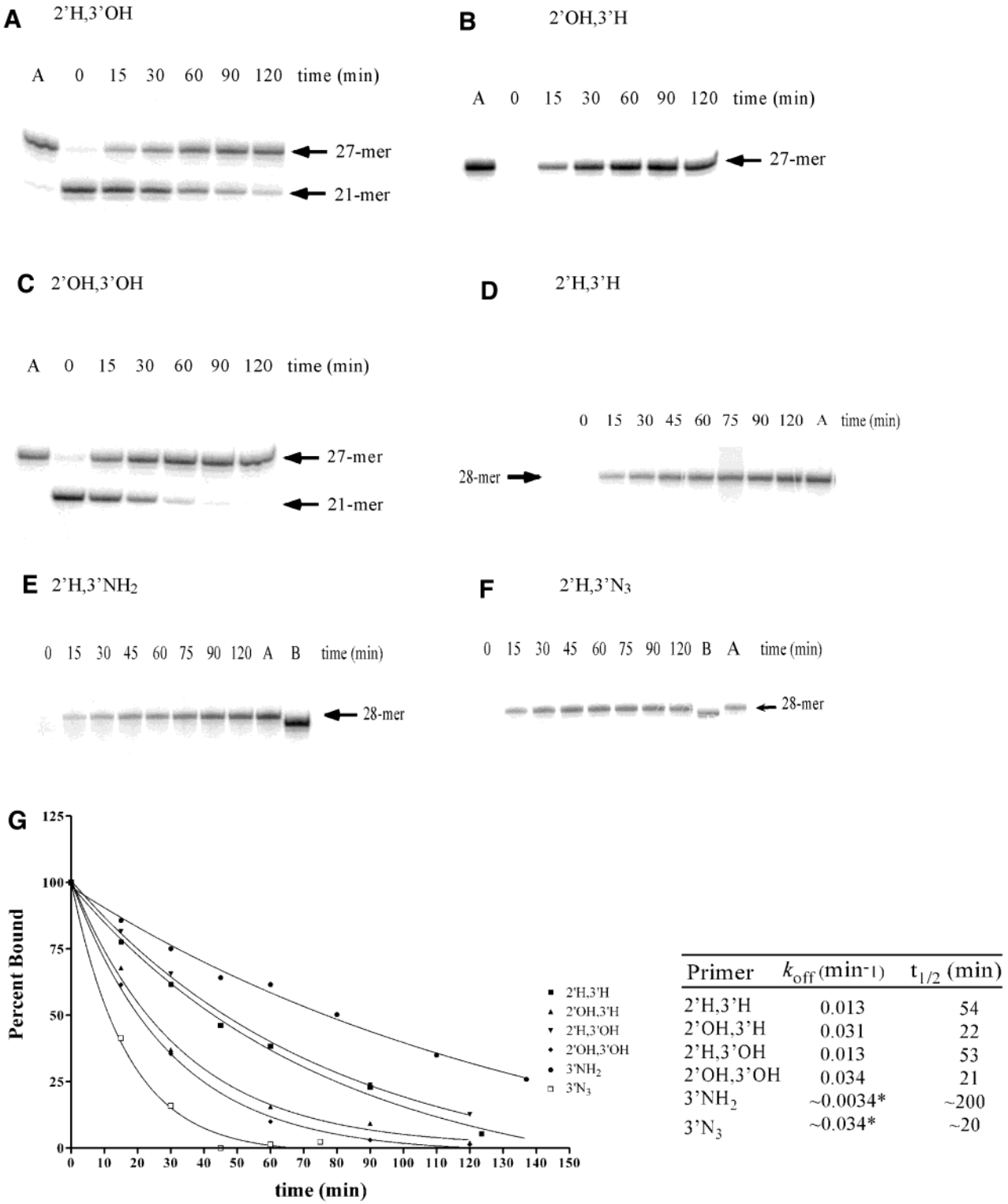


FIGURE 5: 18GGG primer prevents binding of template antagonist GRN163. Purified telomerase (0.5 fmol) was preincubated with the telomeric primer 18GGG (100 nM) for 30 min at room temperature and then challenged with GRN163 at concentrations that varied from 0.5 to 50 nM (lanes 1−4). In lanes 5−8, the enzyme was first incubated with GRN163 for 10 min, after which primer 18GGG was added. The enzyme complex was pulse-labeled for 5 min with dTTP (200 μM) and [α-^{32}P] dATP (10 μCi, 3000 Ci/mmol).

(<0.00058 and 0.0018 min^{-1}, respectively; Figure 4D), indicating a preference for telomeric sequences at the 5′-end of the primer. While TTAGGG-based telomeric sequences contribute to the stability of the telomerase−primer complex (10-fold or greater reduction in k_{off}), adding stability through contacts at both the 5′-end and through an interaction with the template, the alignment of the 3′-end of the primer in the active site represents a previously unidentified and potentially more significant interaction in providing stability to the complex. Substituting acyclovir or 3′-azido-dG for the 3′-ultimate nucleotide in 18GGG destabilizes the complex that the primer forms with the enzyme at room temperature, such that k_{off} values 0.0068 and 0.0022 min^{-1} are observed respectively (data not shown). While the former modification affects both base-pairing and stacking interactions, the reduced stability of the latter substitution suggests a disruption of an interaction in the sugar domain of the nucleoside since 3′-azido-dG can base-pair similar to a 3′-OH-dG.

18GGG Competes with hTR Template Antagonist GRN163. The stability of a primer−telomerase complex depends on the aggregate of elaborate interactions between the enzyme and the primer. We have compared the binding of 18GGG, whose 3′-terminal nucleotide register was found to have the most stable interaction with telomerase, to that of an extremely potent and specific telomerase template antagonist, GRN163. The latter is a 13-nt thio-phosphoramidate oligonucleotide complementary to nucleotides 42−54 of hTR that is a competitive inhibitor of telomerase, having an IC_{50} value of 0.4 nM (*17*). When GRN163 was prebound to purified telomerase, it prevented binding and labeling of 18GGG in a dose-dependent manner (Figure 5, lanes 5−8). If however, the order of addition was reversed, 18GGG was prebound to the enzyme, and GRN163 was added, then the complex was refractory to binding and inhibition by GRN163 (Figure 5, lanes 1−4). This demonstrates the very stable nature of the 18GGG primer complex and supports our finding that these two oligonucleotides compete at least partially for the same binding site (data not shown). GRN163 is a hTR template antagonist, and it interacts primarily with hTR, whereas 18GGG is stabilized significantly by interactions with the protein component of telomerase. This was demonstrated by binding either ^{32}P-labeled 18GGG or ^{32}P-labeled GRN163 to telomerase and comparing the migration of the resultant complexes formed on a native gel (unpublished observation). While both the primer and the template antagonist labeled a complex that migrated similar to an endogenously labeled telomerase complex, treatment of the GRN163-labeled complex with proteinase K resulted in a shift of that complex to a position on the gel similar to that of labeled hTR, whereas the same treatment of an 18GGG-labeled complex resulted in the disappearance of any primer complex (unpublished observation).

Effect of 3′-Terminus Modifications on Primer Binding. To explore potential interactions between the 3′-end of the primers and telomerase, we have examined the k_{off} values of several primers ending in GGG-3′ in which the sugar residue of the terminal nucleoside was modified. Because of the high stability of the complex formed between telomerase and 18GGG, we were unable to define dissociation rates at room temperature and have therefore conducted the experiments comparing the primers with modified sugars at 37 °C. At that temperature, 18GGG has a k_{off} of 0.013 min^{-1} (Figure 6A,G). We tested the effect of having either an H or an OH in the 2′- or 3′-positions. A primer with a 2′-OH and a 3′-H was found to have a modest increase in its k_{off} (0.031 min^{-1}; Figure 6B,G). This difference could either indicate a stabilizing influence of a 3′-OH group or a destabilizing one of a 2′-OH group. We then tested the binding of a primer having hydroxyl groups at both the 2′ and 3′-positions and found it to have a nearly identical k_{off} as the previous primer, 0.034 min^{-1}, which indicated the 2′-OH has a modest destabilizing effect on the binding of the primer (Figure 6C,G). This conclusion was confirmed by determining the k_{off} of a terminal dideoxy primer, which had stability similar to 18GGG (0.013 min^{-1}; Figure 6D,G). We also surveyed two additional 3′-modifications: a 3′-azido and a 3′-amino. Both of these terminal modifications were created by incubating telomerase with 18AGG followed by the addition of either 3′-NH_2-dGTP or 3′-N_3-dGTP (250 μM). While both of these nucleotides are chain terminators and thus do not allow labeling of the original primer, we can infer the analogues were readily incorporated at the end of the original primer as the challenge primer (24GGG) was extended to a 28-nt pulse-labeled product as compared to a 27-mer that was seen when the nucleotide triphosphate was absent (Figure 6E,F). At the same time we observed, however, that the presence of a dGTP analogue in the reaction mixture reduced the affinity of 18GGG by approximately 2-fold (data not shown) and have adjusted the k_{off} values for these two primers to reflect this fact (Figure 6G, bottom panel). At 37 °C the destabilizing effect of the azido substitution is similar to the 2′-OH substitution, 2−3-fold (Figure 6G, bottom panel). In contrast, the 3′-amino modified primer was moderately stabilized (approximately 4-fold) relative to its OH counterpart (Figure 6G, bottom panel). The more basic nitrogen atom in the 3′-position appears able to form an additional interaction with the protein subunit. We surmised that the additional stability of the 3′-amino primer was a consequence of the basicity of nitrogen. We tested this supposition by measuring the effect that acetylating the 3′-amino group had on the primer's affinity. Acetylation increased the k_{off} to about 0.014 min^{-1}, which was similar to that of 18GGG (data not shown). This supports the notion that the nucleophilic electron pair of the nitrogen is responsible for the more stable interaction of the 3′-amino primer. Thus, while the exact interaction between the 3′-end of 18GGG and the enzyme is still undefined, it is clear



Primer	k_{off} (min^{-1})	$t_{1/2}$ (min)
2'H,3'H	0.013	54
2'OH,3'H	0.031	22
2'H,3'OH	0.013	53
2'OH,3'OH	0.034	21
3'NH_2	~0.0034*	~200
3'N_3	~0.034*	~20

FIGURE 6: Dissociation rates of primers terminating in GGG register having modifications in their terminal sugar moiety. Dissociation rates for the indicated primers were determined as described in Materials and Methods and in the legend to Figure 2 except that experiments were conducted at 37 °C. The 3′-NH_2 and 3′-N_3 primers were generated by incubating telomerase and 18AGG (100 nM) with the respective nucleotide triphosphates (250 mM) for 30 min at room temperature. Lanes A and B represent reactions where 24GGG (20 μM) was added along with the 18AGG primer without or with the addition of nucleotide triphosphate, respectively. The k_{off} of 18GGG was increased approximately 2-fold in the presence of guanosine nucleoside triphosphates, and hence the k_{off} reported for the 3′-NH_2 and 3′-N_3 primers was adjusted in the table of panel G to reflect this fact and is indicated in the bottom panel of panel G (*).

that 2′ or 3′ groups that affect charge, nucleophilicity, and steric requirements can all influence the primer's affinity, and thus, the stability of a primer in this register results from an aggregate of interactions.

DISCUSSION

Telomerase is a ribonucleoprotein that catalyzes the synthesis of TTAGGG repeats onto the ends of chromo-

somes. The template for these repeats is an intrinsic part of the RNA subunit of the enzyme. In vitro, the enzyme can use short single-stranded DNAs as the substrate for the repeated nucleotide addition. We have determined the K_m for the 18-nt primer, $(TTAGGG)_3$ (at 37 °C), to be about 2 nM, which indicates a highly specific interaction with this substrate (unpublished data, R. Pruzan). We were interested in examining the interactions between telomerase and the substrate primer that contribute to this specificity. A binding assay was established that depended on pulse-labeling the primers, which had the advantage of recording only primers bound to the enzyme's active site. We measured the dissociation rates of 18-nt primers that had the six permutated sequences of TTAGGG. The most interesting finding was the nearly 100-fold range in k_{off} values that were observed between the different primer registers of TTAGGG repeats. A primer ending in GGG-3′ formed a complex with telomerase that was stable at room temperature ($t_{1/2}$ greater than 20 h) as compared to one ending in TTA-3′ that dissociated from the enzyme with a $t_{1/2}$ of about 15 min. The differences in affinity could neither be attributed to differences at the 5′-end of the primer nor solely to changes in base-pairing with the template. While the added potential GC base-pair for 18GGG versus 18AGG could potentially explain the added stability (−2.1 kcal mol^{-1}; ref *28*) for the former primer, that trend is not followed when subsequent nucleotides are added at the 3′-end of the primer (Figure 2G).

Primers 18GGT and 20TTA have identical 5′-ends, and 20TTA has the potential to form two additional base-pairs, yet 18GGT has a 15-fold higher affinity for the enzyme. Thus, it appears that the entire template is not involved with base-pairing to the primer and that interactions between the primer and the protein subunit hTERT are likely the key determining factors involved in stabilizing the enzyme−primer complex. A further indication that there is limited base-pairing between the template of hTR and a primer substrate came from data comparing the primers NT18GGG with CH18GGG. While the latter primer has the potential to form four additional base-pairs with the template of hTR, only a modest decrease in the k_{off} (2−3-fold) was observed for CH18GGG, suggesting that base-pairing has only a minor role in primer stabilization. Energetically, these four additional base-pairs would be expected to result in greater than a 2000-fold increase in stability (∼-4.6 kcal mol^{-1}; ref *28*); thus, they are most likely not occurring. Previous studies with *Euplotes* telomerase have indicated that the entire template is not used for base-pairing with the primer, and it was suggested that the amount of duplex formed between the primer and the template may be constant, similar to a transcription complex (*27*). Such a model where minimal base-pairing occurs between the 3′-end of the primer and the template can more easily explain the modest observed difference in affinity between 18TAG and 18TTA (∼3-fold) as compared with the enormous difference expected (∼7.9 kcal mol^{-1}) if the primer could form a complete 11-base-pair duplex with the template. On the basis of our results, we favor a model that involves minimal base-pairing between primer and template, and one where most of the energy of stabilization comes from primer−protein contacts. At room temperature, the 18-nt telomeric primers had k_{off} values ranging from 0.047 to <0.00058 min^{-1}, which translate into ΔG values ranging from −12.4 to −15.0 kcal mol^{-1} (*28*, *29*). These values clearly dwarf the energy contributions resulting from base-pairing. This was further illustrated when the nontelomeric primers were examined. The NT18GGG and NT18GTT primers had respective k_{off} values of 0.0048 and 0.40 min^{-1}, which represent ΔG values of −13.7 and −11.1 kcal mol^{-1}. While the three 3′-nucleotides may be important for alignment of the primer with the template, the contribution from these base-pairs toward stabilization of the primer would be insignificant. Furthermore, we observed that the telomerase−primer complex becomes more labile as the ionic strength of the buffer increases, which again indicates that protein−nucleic acid interactions are the predominant force stabilizing the complex (G. Wallweber, unpublished data); a more stable complex would be expected if the majority of the stabilizing energy were the result of duplex formation.

Observations from a different experimental approach also resulted in the similar conclusion: that the high affinity between telomerase and 18GGG was primarily the result of protein contacts. We compared the binding of this primer to telomerase with that of the potent hTR template antagonist, GRN163. Following binding to telomerase and subsequent treatment with proteinase K, we found that GRN163 remained associated with hTR, while 18GGG binding was lost (data not shown). While GRN163 formed a stable interaction with hTR and 18GGG presumably with hTERT, interestingly, their binding to telomerase was competitive with each other, indicating an overlap of the binding sites.

The variance in affinity with respect to the primer register was observed with the nontelomeric primers as well as with the telomeric series, which supported the idea of a significant interaction between the enzyme and the 3′-terminus of the primer. There was at least an 80-fold difference between the nontelomeric primer ending in GTT-3′ (NT18GTT) versus the one ending in GGG-3′ (NT18GGG). The difference in base-pairing (+1.1 kcal mol^{-1} for a GTT DNA−RNA duplex as compared with a GGG DNA−RNA duplex; ref *28*) once again cannot explain the difference in stability that was observed. The register ending in GGG-3′ may be optimally positioned in relation to the protein subunit to form a stabilizing interaction. In this simplistic model, primers ending in GGT-3′, GTT-3′, and TTA-3′ would be respectively more distant and increasingly rotated away from a given protein site and thus less able to form an interaction there. The TTA-3′ register is 3-nt removed from the putative site of interaction and has the weakest affinity. The next register, TAG-3′, can potentially bind in two different positions, the fully extended pretranslocation position or the post-translocation position. Primer 18TAG had a monophasic decay profile for its k_{off}, which we interpret as binding primarily in a single position (Figure 2G). Furthermore, the k_{off} of 18TAG determined using a competitive blocked primer and pulse-labeling with [α-^{32}P] dGTP were similar to the k_{off} determined in Figure 2 (data not shown); thus, we believe that we have measured the affinity of 18TAG in the post-translocation position. On the basis of our model, we would predict a primer bound in the pretranslocation position to be the same or weaker than the primer ending in TTA-3′. From a teleological point of view, a weak interaction in this register would be favorable to an incipient translocation following the addition of the first G nucleotide. The post-

translocation register is 2-nt distance from the optimal register (rotated in the opposite direction as compared to GTT-3′) and has a similar affinity to the primer ending in GTT-3′.

While it is difficult to directly compare the dissociation rates between the human enzyme with that of the *Euplotes* enzyme, as there were minor differences in the conditions used, it appears that primers for the human enzyme (in certain registers) are able to bind their cognate enzyme more tightly (*27*). This distinction might be expected given the apparent differences in processivity between the two enzymes. Interestingly, in *Euplotes* the k_{off} values for the different primers seem to be invariant with respect to the primer's register (*27*).

We conducted all of our binding assays under identical conditions with the exception of those that involved the primers having a terminal 3′-amino-G or 3′-azido-G. For these two primers, the 3′-terminal nucleotide was added by telomerase using 18AGG as the initial substrate together with either 3′-amino-dGTP or 3′-azido-dGTP. Since the presence of dGTP analogues was a modification in our binding conditions, we examined their effect on the k_{off} of the 18GGG primer. We observed an approximately 2-fold increase in the k_{off} for that primer and subsequently found that ddGTP as well as rGTP had a similar effect on 18GGG (unpublished observation, R. Pruzan). These observations resulted in our imputing a 2-fold increase in the k_{off} values for the primers with 3′-amino-dG and 3′-azido-dG. We did not explore our observations regarding the presence of nucleotides and their affect on primer affinity further. While the ddGTP is a readily accepted substrate that can be added to a primer, rGTP is not (unpublished observation, R. Pruzan) and raises the interesting question as to the possibility of a second nucleotide-binding site and whether nucleotide hydolysis may be involved in this phenomenon. Interestingly, dGTP was previously shown to affect the binding of certain primers to the *Euplotes* enzyme, and it was postulated that dGTP may provide a source of energy for translocation (*27*). Answering these interesting questions and others such as the effect of different nucleotides upon primers having different registers, however, will require a separate study and is beyond the scope of this work.

In a separate but related observation, we found that the k_{off} values for primers terminating with GGG-3′, GTT-3′, and TTA-3′ appeared to be the same whether their 3′-ends were generated by telomerase or were presented to the enzyme as exogenously synthesized primers. For example, starting with a primer ending with AGG-3′ and allowing the enzyme to extend this primer by a single nucleotide, using [α-^{32}P]-dGTP, resulted in a stable enzyme−primer interaction, similar to one ending in GGG-3′ (unpublished observations, G. Wallweber). Conversely, 18GGG, which forms a very stable complex with the enzyme, could be extended with dTTP and [α-^{32}P] dATP, and that extended primer ending in 3′-dA was observed to have a similar k_{off} as that of a primer ending in TTA-3′ (unpublished observations, G. Wallweber). This suggests there is no difference in primer recognition by an enzyme in an elongation mode as compared to an enzyme in a static mode. These experiments, however, were carried out at nucleotide concentrations well below the K_m of nucleotides and therefore are different than the previously mentioned experiments with dGTP analogues, which were conducted at concentrations of nucleotide well above its K_m.

While the 3′-end of the primer was important, we also observed a contribution to the enzyme−primer complex stability from telomeric sequences at the 5′-end of the primer, as well as a limited contribution from base-pairing, and thus are able to discern three separable regions of the primer that can interact with telomerase. In addition to the previously proposed template and anchor sites, we have identified an interaction at the 3′-end of the primer that can add 1−2 orders of magnitude to its affinity depending upon its 3′-register with respect to hTR. We have further defined the nature of this 3′-interaction by appending the 2′ and 3′ positions of the 3′-ultimate sugar residue. No single modification affected the affinity more than about 3−4-fold. A more systematic study involving various 3′-terminal nucleotides and the enzyme may be required.

Also, further studies including structural ones will be required to map and understand all the interactions involved between telomerase and its primer substrate. These, however, must await the production of large amounts of active enzyme.

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