Human telomerase RNA template sequence is a determinant of telomere repeat extension rate
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Running title: Telomerase RNA as a determinant of extension rate

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SUMMARY

Human telomerase is a specialized reverse transcriptase that utilizes an integral RNA subunit to template the synthesis of telomeres. In the present study, we demonstrate that the human telomerase template sequence not only determines the composition, but also the rate of synthesis, of telomere repeats. Mutagenesis of the template sequence identified variants that reconstitute enzymes with repeat extension rates that were either faster or slower than wild type template. Changes in extension rate could not be attributed solely to altered heteroduplex melting strongly suggesting that specific interactions between telomerase template, protein and products contribute significantly in determining repeat extension rate. Furthermore, some substitutions that had no effect on extension rate led to striking increases in repeat processivity, indicating that processivity and extension rates can be regulated independently of each other. Our results suggest that telomerase RNA template sequence is a key determinant of telomerase’s contribution to telomere length regulation.

INTRODUCTION

Protection of chromosome termini by telomere repeats is one of several strategies eukaryotic cells have developed to maintain the integrity of their genomes. The biosynthesis of telomeres, which are composed of short, repeated sequences, is accomplished by the action of telomerase, a specialized reverse transcriptase. Telomerase is a ribonucleoprotein complex, its active form minimally consisting of a catalytic protein subunit, the telomerase reverse transcriptase (TERT), and an integral RNA subunit, telomerase RNA (TR). The TERT protein contains the polymerase activity, which utilizes a short, well-defined region within the TR as the template for telomeric repeat synthesis. To facilitate synthesis of defined repeats of telomeric DNA, telomerase has evolved the ability to copy the template sequence in TR in a reiterative action. Consequently, telomerase can remain associated with the DNA strand being extended for many rounds of template copying, thus processively synthesizing many telomeric repeats. In vitro, repeat synthesis by human telomerase has been shown to be highly processive, with nearly all products of a given cycle of template copying being further extended rather than released by the enzyme at the end of each round (1)(Drosopoulos, unpublished data).

Mechanistic models of repeat synthesis by telomerase include successive cycles of enzymatic copying to the end of templating sequence followed by translocation and repositioning of the 3’ end of the nascent product with the 3’ end of the templating segment of TR (2,3). In addition to templating repeat synthesis, the TR assists in realignment of translocated product at the end of each round of template copying. Most TRs contain partial repeats of the templating sequence 3’ to the templating region, referred to as the alignment region. Through base-pairing interactions with alignment nucleotides, the translocated product can be properly positioned for a new round of template-directed polymerization. Sequences within the RNA also form secondary structures, which allow for template recognition (4) and
define template boundaries for terminating each cycle of telomere synthesis (5-7). Furthermore, regions within the TR have been shown to contribute to repeat processivity, presumably through base-pairing interactions between telomeric products and TR during translocation (8,9) or protein-TR-product interactions (10,11).

It has been shown that telomerasers can tolerate significant variation in the template sequences they copy. Template alterations including single point mutations, deletions, additions, as well as complete template substitutions, have been made in the TRs of several organisms, predominantly ciliates (12-19) and yeast (20-22) but also including human TR (hTR) (23-28) and these changes generally appear in the synthesized products. However, these modifications can result in significant changes in the in vitro and in vivo behavior of the enzyme. Thus, the sequence being copied and the resulting products contribute to enzyme activity, with the particular composition of the template sequence rendering specific properties required for efficient in vitro and in vivo function.

In the present study, we have examined the effects of modification of the templating and adjoining alignment sequences of human TR (hTR) on the biochemical behavior of human telomerase. hTR template mutants were made with substitutions in either the templating region alone, alignment region alone, or in both. Our results reveal that alterations in the template region can directly influence the overall repeat extension rate as well as rate of synthesis at individual template positions, probably by modulating interactions between telomerase protein, hTR and telomerase products. Our results further show that product/template composition can independently affect the rate of repeat translocation during template copying and repeat processivity.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides.** Oligonucleotide substrates were purified via denaturing polyacrylamide gel electrophoresis (PAGE) followed by gel extraction and ethanol precipitation.

**Cloning of hTERT.** Full-length hTERT cDNA was cloned from human testes cDNA library (Clontech, Palo Alto CA) via PCR. Two segments, a 5’ segment and a 3’ segment were independently amplified and ligated together by the unique MluI site in hTERT to form the complete full-length cDNA. The oligonucleotides used for the 5’ PCR were ACAAGATTCCGTCACCAGCGCCGCTCC CCGCTGCGGA (EcoHTRT4) and TGCCAGAGAGAGCGACGGCACCCTCCAAAGAG G (HTRT9), and for the 3’ PCR, GCAGGACGCTGAGCCGAGTGGA (Mlu h T E R T u p ) and AGTCAGATCTCTGACGAATTCTCCCA GGATGGTCTTGAAGTCTGA (3’hTERT Eco). The full-length hTERT cDNA was cloned into the in vitro expression plasmid pCITE4b (Novagen, Madison, WI) as follows. The pCITE4b vector was digested with MscI and BglII to remove the entire MCS and all epitope tags. The full-length hTERT cDNA fragment along with a linker containing a FLAG epitope sequence was inserted into the pCITE vector to yield the plasmid pNFLAGhTERT. The hTERT nucleotide sequence in pNFLAGhTERT was verified against the published sequence (GenBank accession # NM 003219) and was then used to express N-terminally tagged full-length hTERT protein in vitro in a coupled transcription-translation system.

**Cloning of human telomerase template RNA (hTR) cDNA.** The 451 nt full length hTR sequence was amplified from HeLa cell total RNA via RT-PCR using the primers CTGACTGCTCAGGGTTGGAGGGTGGGC C C T G ( H T R 1 ) a n d T A G C G A A T T C G C C G A T G T G T G A G C C G A GTCC (HTR). The amplified sequence was cloned into pBluescript II KS(+) (Stratagene, La Jolla, Ca) to generate the plasmid phTRF. The hTR sequence in phTRF was verified against the published sequence (GenBank accession # U86046) to ensure the integrity of the sequence.
Site directed mutagenesis of hTR. A cassette strategy (29) was used to generate mutants of hTR. An intermediate vector was generated from phTR by PCR in which the region of hTR encompassing nucleotides 36-64 was replaced with a pair of BsmBI endonuclease sites, resulting in a self excising sequence based on downstream cleavage by type IIIs restriction endonucleases. By digesting this plasmid, designated phTRFdel, with BsmBI, the self excising segment is released and a vector backbone with cohesive ends is generated, into which new sequences can readily be introduced. phTRFdel was digested with BsmBI, dephosphorylated with calf intestine alkaline phosphatase, and the 3.4 kb vector backbone agarose gel purified. Pairs of complementary oligonucleotides containing modified human (MH) hTR template sequences (for example, TGGCCATTTTTTGTCTTCCCCTTCCTGAG A A G G  ( M H 1  T o p )  a n d  A C G C C C C T T C C A G G A G G A G G A G A C A A A AAATG (MH1 Bottom)) were used to make the mutant MH1 (Figure 1)) were phosphorylated and annealed, and the annealed cassettes ligated into the phTRFdel backbone. The resultant constructs were transformed, isolated and sequenced to confirm the presence of the desired changes and the absence of unintended modifications.

Transcription and purification of human telomerase RNA. To prepare full length hTR RNA, phTR (or its mutant derivatives) was first digested with FspI, releasing a 615bp fragment containing the hTR sequence under T7 promoter control. The 615bp FspI fragment was then gel purified and used as a template for in vitro transcription of hTR RNA. Transcription reactions were performed with gel purified template using a MegaScript transcription kit (Ambion) according to manufacturer’s recommendations. Transcribed RNA was electrophoretically separated on 8M urea - 4% polyacrylamide gels and the RNA excised from gel. Gel slices were crushed and RNA eluted in 0.3M NaAcetate for 1 hr at 37°C. The eluted material was then phenol extracted and ethanol precipitated. The recovered, purified hTR RNA was dissolved in 10mM Tris-Cl pH 7 and stored at -80°C until needed.

In vitro reconstitution of human telomerase. Telomerase activity was reconstituted in vitro by synthesizing hTERT protein in the presence of purified hTR RNA using a TnT coupled transcription-translation kit. Reactions containing pNFLAGhTERT (0.77pmol (0.04µg)/µl) and purified hTR RNA (0.82pmol (0.12µg)/µl) were assembled as recommended by the manufacturer, with the exception that both minus methionine and minus cysteine amino acid mixes were used here. In vitro reconstitutions were carried out at 30°C for 3.5 hrs then flash frozen in liquid nitrogen and stored at -80°C until needed. Aliquots of the reconstitutions were also analyzed via SDS PAGE to quantitate the in vitro synthesized hTERT protein.

Telomerase activity assay. Telomerase activity was measured via a direct primer extension assay using radiolabeled primer and unlabeled deoxynucleotides. Typical primer extension reactions with radiolabeled primer contained 2µl in vitro reconstituted (IVR) telomerase, 1 pmole 5'-32P end-labeled substrate primer d(TTAGGG)3, 1mM dATP, 1mM dGTP and 1mM TTP in PE reaction buffer (50mM Tris-Cl pH 8.3, 50mM KCl, 2mM DTT, 3mM MgCl2 and 1mM Spermidine) in a total volume of 10µl. Reactions were incubated at 30ºC for 30 min then terminated by adding an equal volume of stop solution (RNase A (100µg/ml) in 10mM Tris-Cl pH 8.0, 20mM EDTA) and incubating at 37°C for 10 min. Reaction products were then isolated from terminated reactions by first treating with an equal volume of PK/SDS solution (proteinase K 0.2mg/ml 0.3%SDS in 10mM Tris-Cl pH 8.0) for 10 min at 37°C followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitation. The recovered reaction products were dissolved in Urea PAGE Sample buffer (95% formamide/20mM EDTA) and resolved on 8M urea - 10% polyacrylamide gels. The gels were then dried and reaction products detected and analyzed via phosphoimager (Molecular Dynamics) using ImageQuant software (Molecular Dynamics). Relative activities were determined by measuring the total amount of extended substrate primer for each reaction, correcting for
background and normalizing against unextended primer (which represented >95% of the recovered material) followed by expressing values thus obtained as a percent of wild type activity. Primer positioning assays were carried out as described above except deoxy- (dNTP) and/or dideoxynucleotides (ddNTP) were present at 0.5mM.

**Competitive primer challenge assay.** A competitive primer challenge (“bind and chase”) assay similar to that described by Bryan et al (30) was employed to assess processive DNA synthesis by IVR telomerase. IVR hTERT (8µl) was combined with 5-32P-labeled substrate primer d(TTAGGG)3 (4 pmole) in 1x PE reaction buffer (total volume, 24µl) and incubated for 5min at 30°C to allow TERT to bind the substrate primer. 16µl of 80 µM unlabeled (cold) competitor (chase) primer plus 2.5mM dATP, 2.5mM dGTP and 2.5mM TTP (in 1x PE salts) was then added to initiate a reaction time course of primer extension. The reaction mixture was incubated at 30°C and aliquots (10µl) were removed at 0 to 5 min and combined with an equal volume of stop solution to terminate the reaction. As a control, the effectiveness of the unlabeled competitor primer was tested by combining labeled primer (1 pmole), unlabeled primer (160 pmole) and dNTPs (1.25 mM) in 1.25x PE buffer first and then adding IVR hTERT (2µl). This mixture was incubated at 30°C generally for 5 min unless otherwise indicated, allowing for extension of labeled primer in the presence of competitor to be measured. The products of the primer extension reactions were then processed and analyzed via standard protocol described above for telomerase activity assay.

Relative repeat extension rates were calculated by comparing the modal size of the radiolabeled products associated with actively polymerizing complexes, at a given time point. Specifically, the population of products containing substrate primers that could be further elongated during the chase period (as observed at later time points) was selected for quantitation (for example, see wild type lanes in Figures 4A and 4B). This population consisted primarily of substrate primers associated with active complexes, along with a minor population of stalled/dissociated complexes. Individual band intensities of this group of bands were measured, the percentage of the total for each band determined and these values used to establish a modal product band in this population of bands. The relative extension rates were then calculated by dividing the number of repeats in the modal band by time. In these studies, relative repeat extension rates were calculated from the 3-minute time point of a given experiment, and were expressed relative to wild type. The choice of 3-minute time point was based on the reasoning that it allowed one to easily distinguish the products of actively polymerizing complexes (which corresponded to the most abundant products with more than five repeats added) from relatively abundant products resulting from low efficiency translocation (characteristically seen during the first few rounds of template copying), as well as for accurate size determination. Repeat processivity was calculated as a processivity index, i.e., the ratio of long products (>500 nucleotides) to total products (products <500 nucleotides + products >500 nucleotides). All values obtained were corrected for background and normalized against unextended primer. Relative processivity values are calculated as the processivity index of a given enzyme compared to that of wild type.

**RESULTS**

**Enzymatic activity of reconstituted human telomerase template mutants**

Telomeric repeat synthesis by human telomerase is characterized by reiterative cycles of template copying. In vitro products of such polymerization are observed as six nucleotide ladders of extension synthesis, each band of the ladder representing enzyme pausing, stalling, or dissociation at the end of a cycle of template copying. In general only minor product bands are seen between the major “rungs” of the ladder, implying that compared to reinitiating new rounds of template copying, copying across the template sequence is accomplished with relative ease. In addition, the abundance of the minor “intra-repeat” products is relatively uniform, indicating that polymerization/pausing
across every template position occurs with roughly equal efficiency. This suggests that human telomerase evolved its wild type templating/alignment sequence to be inherently optimal for extension synthesis. In order to examine this notion, hTR template mutants were created and examined (Figure 1). In order to maintain hybridization potential and to avoid mismatches during realignment, the identical changes were made in both the templating and alignment sequences in these mutants. To avoid disrupting the natural termini of the template and alignment sequences, the sites of modification were limited to template nucleotides 49AAU47 and alignment nucleotides 55AAU53 in hTR. In addition, leaving the terminal nucleotides 50C, 51C (template) and 52C (alignment) unaltered would allow the use of a common substrate primer d(TTAGGG)₃, based on the natural telomeric sequence to test the activity of the mutants.

Telomerase activity of the mutant enzymes was measured via a direct primer extension assay. As observed from the results in Figure 2, all of the mutant templates were active to some extent, although the level of activity varied from minimally active to wild-type-like. Specifically, the amount of d(TTAGGG)₃ primer elongation by the MH3 and MH4 mutants was, generally, very similar to wild-type. The next most active, with somewhat less than wild type activity, were the MH5, MH8 and MH9 enzymes. The mutants MH1, MH2, MH6 and MH7 exhibited relatively little activity, yet were nevertheless able to extend the substrate primer significantly. As these relative activities were determined with a single primer and, in the case of the mutants, some substrate/template mismatches were present for the first repeat of synthesis, it was possible that these mismatches could, at least partially, have resulted in the reduced overall activity observed with some of the mutants. Therefore, we tested the effect of using perfectly matched primers with mutants MH1 and MH2, two of the least active mutants. When assays were performed with the perfectly complementary primer to MH1 (d(GAAGGG)₃) or separately, with a primer to MH2 (d(AAAGGG)₃), we found no significant difference in relative overall activity of these mutants, compared to wild type (and the MH3, MH8 and MH9 mutants), from that determined with the standard wild type primer (data not shown). This indicated that the overall activities exhibited by the mutants with the standard d(TTAGGG)₃ primer seemed inherent to the enzymes and were not significantly influenced by altered DNA-RNA interactions in the template region resulting from primer mismatches during initial (first round) primer extension. Moreover, the ability of all of these mutants to perform telomeric elongation demonstrates that human telomerase, like the other, better studied telomerases of ciliates and yeasts, is quite tolerant of variation in the template sequence being copied.

More importantly, it was also evident from the primer extension reactions that the mutants could synthesize long products, an indication of processive polymerization by telomerase. Most of the mutants were capable of elongating the substrate primer by more than one hundred repeats over the course of the thirty minute reaction. The relatively active mutants MH3, MH4, MH8 and MH9 all appeared to produce a significant proportion of long products, observed as dark, slow migrating patches at the top of the lanes (Figure 2). Based on the proportion of long products (>500 nucleotides) to total products, an index of repeat processivity, it appears that these mutant enzymes were at least as processive as wild type. Interestingly, the MH1, MH2 and MH6 mutants, in spite of not being very active, were also able to produce a relatively high proportion of long products (Figure 2). These results indicate that repeat processivity is not necessarily related to overall activity, as once these mutant enzymes successfully add a few repeats, they become very efficient at additional extension. The only enzyme that was unable to synthesize appreciable amounts of long extension products was the MH7 mutant, indicating a possible defect in repeat processivity.

Mutations in template residues can affect copying at unmodified template positions

In addition to differences in catalytic activity, the observed banding patterns of the elongation
products synthesized by most of the mutants varied significantly from that of wild type hTR (see Figure 2 – panels A and B and their enlarged sections). In order to establish exact templating positions corresponding to particular product bands, primer extension reactions were performed where dGTP was replaced with ddGTP to determine initial primer positioning. When assayed under these conditions, elongation of the d(TTAGGG)₃ primer by wild type enzyme results in termination after addition of four nucleotides, including the terminal ddGMP (Figure 3, lane WTdd), indicating primer 3’ terminal G binding to C₅₀ of the template. This is the primary predicted alignment based on fewest primer/template mismatches. Similarly, ddGTP termination of primer extension by the mutant enzymes indicated that the predominant binding preference (with the exception of MH₆ and MH₇) was alignment of the substrate primer terminus to C₅₀. The MH₆ mutant primarily aligned primer terminal G with C₅₁ while the MH₇ mutant displayed a fairly equal usage of two alignments, one with primer terminus binding to C₅₁ and one with terminal binding to C₅₀.

Based on the d(TTAGGG)₃ substrate primer positioning, the specific sites at which product bands were accumulating during template copying were identified. The mutants seemed to be divided into two classes defined by their propensity to generate distinct product patterns. The MH₃, MH₄, MH₅ and MH₇ mutants were similar to wild type in that their major products could be observed in clear patterns repeated in regular six nucleotide intervals, however (with the exception of MH₇), there were significant differences in the patterns themselves (Figure 2). Specifically, repeat synthesis by the MH₃ (A₄₈U) mutant was characterized by many abundant products early (within the first 4 to 5 repeats) which, similar to wild type, settled into a pattern with a single major product at the end of the repeat. However, the relative difference between the major product and minor product bands was much less than observed with wild type. Even greater changes from the wild type pattern were seen with the MH₄ and MH₅ mutants, which produced ladders of a doublet or a triplet, respectively. In addition to the major end of repeat product observed with wild type hTR, a two nucleotide longer major product is seen for both MH₄ and MH₅, corresponding to template copying at C₅₀ (Figure 2A, c.f. lanes WT, MH₄ and MH₅). A third major product, corresponding to template copying at C₅₁, is also observed for MH₅ (Figure 2A, lane MH₅). It is striking that the sites where template copying is being affected are not the sites of template mutation (U₄₇ and A₄₈ for MH₄ and U₄₇ for MH₅). In fact, no apparent change is detected when copying at the altered positions in these mutants. Although a similar observation had previously been observed in Tetrahymena telomerase (16), this is the first demonstration via direct primer extension of such an effect for human telomerase.

The other group of mutants, MH₁, MH₂, MH₆, MH₈ and MH₉ enzymes, did not produce regularly repeated 6-base ladders but uniform accumulation at every position. These enzymes initially synthesized products in a weak to modest pattern, which subsequently became much less distinct after a few rounds of template copying (Figure 2, lanes MH₁, MH₂, MH₆, MH₈ and MH₉). This behavior was the most pronounced in the MH₂ mutant, where any discernible pattern quickly disappeared after the first two rounds of template copying. The MH₉ mutant exhibited a slight variation in behavior relative to the other mutants of this group. The major synthesis products of template copying by the MH₉ mutant almost appear to be shifting from round to round and finally to no readily perceptible pattern. The ability of mutations in hTR template nucleotides to influence the copying at sites other than at the mutated positions suggests that interactions with templating nucleotides are occurring outside of the polymerization site which can affect copying.

**Alterations in the template region can directly affect repeat extension rates**

The accumulation of products of a given size can be indicative of either pausing during template copying or enzyme dissociation from the growing product. Because repeat synthesis
by human telomerase is highly processive, it is possible to distinguish transiently paused products from those either associated with non-productively stalled enzyme or completely dissociated from the enzyme via competitor challenge (“bind and chase”) assays. Since pausing events are transient, accumulated products at positions where pausing has occurred will, in time, continue to be extended further when “chased” by competitor.

To further characterize the modified template copying behavior observed in the standard telomerase assays, primer extension reactions were performed with hTR mutants under bind and chase conditions (Figure 4). In general, patterns observed in the bind and chase assay reflected patterns seen in the standard assay (compare Figures 2 & 4) – thus, pause sites were generally sites of stalls/dissociations. The main exceptions were observed with the MH4, MH7 and MH9 mutants. In addition to the product bands seen in the standard assays, an extra pause at C51 can be observed in the product band patterns of the MH4 and MH7 mutants (Figure 4). (In the case of MH7, it is likely that the extra band is actually end of repeat product from primers initially aligned with C51 (see above)). The MH9 mutant, which displayed no real distinguishable pattern in the standard assay, exhibited a distinct strong pause at C51 and a weaker but noticeable end of repeat pause as well (Figure 4).

The competitive bind and chase assays provided valuable measurements of telomeric repeat extension rate. Relative repeat extension rates, determined from an average of the rates at the three minute time point of the bind and chase assays (Figure 4) are shown in Table 1. A comparison of these rates reveals significant differences between the mutant and wild type enzymes, with the mutant extension rates ranging from less than half (e.g., MH7) as fast to over twice (e.g., MH4) as fast as wild type. Specifically, the extension rates for the MH1, MH2, MH8, and MH9 mutants were close to wild type. Since the MH9 mutant was relatively active compared to wild type (Figure 2), an extension rate that was fairly similar to wild type seems appropriate. In contrast, there was no correlation of overall activity to extension rate observed with the MH1 and MH2 variants, which displayed only a fraction of the enzymatic activity of wild type, yet were able to synthesize repeats near or at wild type rate. This was particularly unexpected as the extension rates determined for the two other low activity mutants, MH6 and MH7 were also considerably less than wild type (68% and 47% of wild type, respectively). The lack of a specific correlation between overall enzymatic activity and extension rate was also evident with mutants exhibiting increased extension rates. The MH3 and MH4 mutants demonstrated wild type levels of activity, while MH5 was somewhat less active, in the standard primer extension assay (Figure 2). However each of the mutants performed repeat synthesis at a rate roughly twice as fast as wild type. Perhaps even more surprising is that the mutants MH4 and MH5 have double the extension rate of wild type in spite of a higher frequency of pausing while copying across the template. It would have been expected that the additional slowdowns in polymerization reflected in the extra product bands should have reduced extension rate.

These results provide the first demonstration that template sequences directly influence the rate of telomeric repeat extension synthesis. Furthermore, the greatest effects on repeat synthesis rate were felt when modifications were closer to the ends of the alignment and templating regions, i.e., nucleotides C52 and C46.

Both templating and alignment regions contribute to repeat extension rate

In order to determine whether increases in extension rate and changes in product banding patterns were mainly resulting from properties of the modified nascent products, the modified alignment regions, or both, variants of the double mutants were created where only templating nucleotides or alignment nucleotides were modified (Figure 1). This set was limited to mutants that had displayed wild type or near wild type enzymatic activity (MH3, MH4, MH5, MH8 and MH9).
When tested in the standard primer extension assay, both template (t) series and alignment (a) series mutants exhibited significant differences when compared to their double mutant counterparts. These included changes in enzymatic activity, product patterns and ability to synthesize long products (Figure 5). While some mutants (i.e. MH3a, MH9a, MH3t and MH9t) displayed near parental (double mutant) activity levels when compared to wild type, the relative activity of others was substantially reduced (compare Figures 2 and 5). The MH3a, MH4a, MH5a, MH4t, MH5t and MH8t mutants all showed considerably less enzymatic activity relative to wild type than did their parental counterparts (compare Figures 2 and 5), particularly the MH4a, MH4t and MH8t mutants. In no instances did any a- or t-series variant appear more active than its tandemly mutated parent.

The product band patterns generated by the singly modified alignment mutants differed considerably from those of the double mutants. All of the a-series mutants exhibited a typical wild type pattern (Figure 5), likely reflecting that they all were copying the same wild type template sequence. However, there was some slight variation in pattern among the mutants, as small to moderate amounts of product accumulation one nucleotide before and/or after the major end of repeat product was also seen. All of the t-series mutants, in contrast, displayed distinctly different patterns, indicative of different template sequences being copied. Product patterns of the MH3t and MH5t mutants generally resembled those of the parental MH3 and MH5 mutants, respectively (compare Figures 2 and 5). Similarly, the lack of a distinct pattern observed for MH8t mutant mirrored the behavior observed with the MH8 mutant (Figure 2 lane MH8 vs. Figure 5 lane MH8t). The MH9t product pattern, however, seemed to be somewhat more well-defined than that of the MH9 parent, with end of repeat product accumulation and a pronounced lack of product at template position A48. A comparison of the pattern of the limited repeats the MH4t mutant was able to synthesize (Figure 5, lane MH4t) to that of MH4 mutant (Figure 2, lane MH4) shows these mutants differed as well. Thus, these results suggest that the sequence modifications in the templating region make a more significant contribution to the product patterns than modifications in the alignment region.

The biggest apparent difference between the single and double mutants was in their ability to synthesize long products. The MH3a, MH4a, MH5a, MH4t and MH5t mutants, unlike their double mutants counterparts, were unable to synthesize long (>100 repeat) products. In fact, these mutants appeared only able to elongate the substrate primer by five to fifteen repeats (Figure 5), indicating severe processivity defects. The MH8a, MH3t and MH8t mutants were able to synthesize some long products (Figure 5), although to a substantially lesser extent than their doubly mutated parents (Figure 2). Conversely, MH9a and MH9t (Figure 5, lanes MH9a and MH9t, respectively) were the only variants capable of long product synthesis at near parental levels (Figure 2, lane MH9), particularly the MH9t mutant.

Competitive bind and chase assays were also performed with the single mutants in an attempt to measure relative extension rates (data not shown). It was possible to determine relative extension rates for only one of the mutants (MH9a) from the bind and chase assays due to the severe processivity defects previously observed (see above). The extension rate of the MH9a mutant was approximately 60% of wild type, slightly lower than the relative rate observed for the MH9 parent. Although reduced processivity prevented extension rates for most mutants to be measured at after three minutes, it was possible to observe active extension at one minute for two mutants, MH3a and MH5a. At this time point, it appeared that the MH3a and MH5a mutants extend at a rate considerably less than that of wild type (data not shown), in contrast, to the parental MH3 and MH5 mutants, which elongated primer at approximately twice the rate of wild type enzyme (Table 1).

These results demonstrate that both alignment and templating elements contribute to extension rate and processivity, as evidenced by the clear need for both template and alignment sequences to be identical for the observed increased
extension rates as well as increases in processivity to be observed. However, based on these experiments, it was difficult to assess the exact contribution of template versus alignment to extension rate because of decreases in processivity, particularly in the variants of the increased extension rate mutants MH4 and MH5. As with the tandem mutants, the most profound effects on extension rate and processivity were observed when sequence changes were closer to the ends of the alignment or templating regions.

**Substitutions at template positions can markedly influence repeat processivity without affecting extension rate**

The results depicted in Figure 4 revealed that the degree to which pauses led to stalls/dissociations, indicative of processivity, varied among the enzymes. Therefore, we chose to examine processive synthesis at longer time points in competitive bind and chase assays, which allowed for processivity differences between enzymes to be more clearly discerned. The results of these assays revealed considerable differences between the wild type and mutant enzymes (Figure 6). Several mutants displayed significant increases in relative processivity (measured as processivity indexes (see Experimental Procedures)) when compared to the wild type enzyme (Figure 6). Specifically, the relative processivities of the MH1, MH6, MH8 and MH9 mutants were 2- to 4-fold higher than that of wild type, which was clearly reflected in the substantial accumulation of longer products (>500 nucleotides) and relative absence of shorter repeats seen with these mutant enzymes, particularly with the MH1 and MH8 mutants (Figure 6). This effect was not mediated by extension rate as the MH1, MH8 and MH9 mutants all displayed extension rates that were similar to wild type (Table 1). In fact, there seemed to be no correlation of processivity to extension rate. The MH3, MH4, and MH5 mutants had all displayed extension rates that were approximately twice that of wild type, yet two of the three (MH3 and MH4) were more processive than wild type while the other (MH5) was less processive. The two slowest enzymes, MH6 and MH7, also differed significantly in their relative processivities, as the MH6 mutant displayed more than a 2-fold increase over wild type while the MH7 enzyme was approximately 4-fold less processive than wild type (Figure 6). These results demonstrate that template substitutions can markedly influence processivity, even when the identical changes are simultaneously made in both copied and alignment nucleotides. Furthermore, these results show that changes in processivity and extension rate can occur independently of each other.

**DISCUSSION**

The ability of telomerase to produce telomeric repeats derives from its ability to reiteratively copy a defined templating region in its intrinsic RNA subunit. In the case of human telomerase, this is an efficient process, based on the highly processive nature of repeat synthesis displayed by the enzyme *in vitro*. We generated hTR template mutants with sequences designed to allow processive repeat synthesis, i.e., with identical changes in both templating and alignment sequences. Each mutant was designed to synthesize repeats in some variation of the sequence GGNNNG (Figure 1). All of these mutants were found to be enzymatically active to some extent, confirming the flexibility of human telomerase in template copying previously reported by others (23-28). More unexpected, however, was the effect on template copying at unmodified positions observed in some of these mutants, previously observed only in Tetrahymena (16). In some instances, i.e. with MH4, MH5 and MH9, pauses and stalls/dissociations at unaltered sites were as strong as or stronger than those at the end of each round of template copying (Figures 2 and 4). In other cases, i.e., with MH1, MH2, MH6 and MH8, pausing/stalling/dissociation became relatively evenly distributed with no particularly dominant site (Figures 2 and 4). Product pattern changes indicate shifts in the sites and events that constitute the slow steps of repeat synthesis. Increases in nucleotide insertion efficiency at a mutated site and/or decreases in insertion...
efficiency at an unmodified site could account for such shifts. The relatively high nucleotide concentrations used in our assays rule out decreased insertion efficiency as a basis for these shifts. Furthermore, it would be expected that increases in insertion efficiency at the altered sites would increase the accumulation of the major, end of template product over any minor products, which was not observed.

An alternative possibility would be that movement of the templating nucleotide through the catalytic active site and/or repeat product translocation has somehow been affected. Many of the pattern changes involve positions that are 3’ of the mutated template position. It is possible that upstream interactions between the TERT protein and the single stranded template region are influenced in a sequence-dependent manner. This could lead to sequence-specific differences in the rate of movement of the single stranded region through the active site or changes in template positioning. Differences in the nascent product/template heteroduplex may also account for product accumulation at unmutated positions, although this would require the protein to be able to sense sequence composition in the duplex. If this were the case, product accumulation at unaltered sites could represent sequence-specific restriction to duplex movement or duplex-directed changes in template positioning. While we favor the idea of protein-template (with or without product) interactions being mechanistically responsible for the template directed effects on template copying, alternate explanations are possible. Perhaps the changes in the composition of the product may influence its ability to form secondary structures (e.g., G-G hairpins) proposed to promote repeat translocation (31,32), which in turn may influence extension/translocation rate. Alternatively, product composition may affect its ability to enter/interact with anchor sites or lagging product sites thought to exist in telomerases. Altered interactions with these putative sites were proposed to explain similar unexpected template copying effects observed in Tetrahymena (15,16). Moreover, it is possible that some of the mutant patterns result from the combined influence of more than one of the above proposed mechanisms. Ultimately, the definitive explanation for these effects on template copying await further studies, including more in depth structural information about telomerase.

The rate of extension by human telomerase is determined by the combined effects of template copying, translocation and realignment, factors directly influenced by both copied and uncopied sequences in the template region. The characteristic accumulation of synthesis products at six nucleotide intervals indicates that nascent repeat translocation and realignment is the slowest step of repeat synthesis, implying that repeat synthesis rate is mainly determined by product translocation/realignment rate. We find that template sequence composition can also have a significant influence in determining the rate of repeat synthesis. We have identified template sequences that reconstitute enzymes with extension rates either faster than or slower than wild type telomerase. Of the mutants studied, those whose sequences were modified at positions closer to the 5’ end of the alignment and templating regions (i.e., nucleotides C52 and C46) displayed the greatest changes in extension rate. While there were no readily apparent, specific sequence elements associated with extension rate, it seemed that substituting C residues, especially close to nucleotides C46/C52, resulted in reduced extension rate. The two slowest mutants, MH6 and MH7, both share the U47C/U53C substitutions. The MH1 and MH8 mutants, exhibiting the next slowest extension rates after MH6 and MH7, share the A49C/A55C mutations. It is tempting to speculate that increased C content results in more GC basepairs in the nascent product/template duplex, which would require greater energy to melt, and this, in turn, manifests as reduced extension rate. Calculation of free energies for heteroduplex formation based on nearest neighbors (33) does reveal a significant increase in duplex strength (at 37°C) for MH6 (-1.1 kcal mol⁻¹), MH7 (-1.5 kcal mol⁻¹) and MH8 (-1.1 kcal mol⁻¹) compared to the wild type sequence. However, there is only a negligible difference in duplex free energy (-0.1 kcal mol⁻¹) between MH1 and wild type. Moreover, simple thermodynamic
considerations do not explain the significant increases in extension rate observed with the MH4 and MH5, which had virtually no change (+0.1 kcal mol\(^{-1}\) for MH4) or a slight gain (-0.4 kcal mol\(^{-1}\)) in duplex stability relative to wild type. Similarly, the significant free energy change calculated for MH2 (+1.8 kcal mol\(^{-1}\)) would not predict that the extension rate of this mutant would be the same as wild type as was observed.

Since the rate of repeat synthesis in the wild type enzyme appears primarily determined by the repeat translocation rate (see above), perhaps the product composition is affecting protein-template-product interactions thought to occur during repeat translocation. The patterns of repeat synthesis by the actively polymerizing enzymes observed in the competitor challenge assays seemed to support this idea. Specifically, the change from the wild type product pattern seen in most of the mutants, where the end of repeat product is no longer the sole, dominant product, indicates sequence-induced changes in the status of repeat translocation as the main bottleneck to extension synthesis. If the rate of repeat translocation were unchanged in the mutants then one would expect the extension rate to be reduced by the additional slowdowns reflected in these pattern shifts. In fact, only two out of the nine mutants displayed significant decreases in extension rate. In the case of MH4 and MH5 in particular, a reduction in the impediment of repeat translocation seems to have been necessary as additional new, yet more rapidly passed barriers to product elongation (Figure 4) appear to have been generated these increased extension rate mutants. Therefore, our results strongly suggest that, in addition to heteroduplex melting properties, there are specific interactions between the telomerase protein, hTR template and telomerase products that are contributing significantly in determining repeat extension rate, possibly influencing repeat translocation.

The requirement for complementarity between telomeric repeat product and the template alignment region for optimal enzymatic activity and repeat processivity has been well established (8,9). Our results demonstrate that for maximal rate of repeat synthesis, proper basepairing between product and alignment residues is required, especially that involving nucleotide positions 53 and 54. In comparing the extension rates of tandemly substituted mutants to the ‘alignment only’ or ‘templating only’ variants, we found (to the limited extent measurable (see above)) a general reduction in the rate of the variant compared to that of the parent. This was particularly evident in the MH3 and MH5 series of mutants. Presumably, the mismatches in the product/alignment duplex of the single mutants reduced the potential extension rate demonstrated by the double mutant parents.

Another important finding revealed by our studies is that changes in the efficiency of repeat translocation, the main force that governs telomerase processivity (9), do not necessarily result in changes in extension rate or vice versa. The mutants MH1, MH8 and MH9 all have nearly wild type extension rate but are more processive than wild type enzyme (Figures 4 and 6). The MH5 mutant has an increased extension rate relative to wild type hTR, yet is less processive (Figures 4 and 6). These observations demonstrate two significant points. They underscore the importance of nucleotide sequence as a determinant of repeat translocation efficiency. They also indicate that other factors in addition to repeat translocation efficiency, such as repeat translocation rate (see above), have considerable influence in determining telomerase extension rate. Our findings likely reflect the idea that the efficiency of repeat translocation is related to the ability of the product to stay associated with the enzyme, while the rate of extension synthesis seems to be most closely linked to how quickly the template can reposition itself relative to the enzyme active site. Furthermore, the separable effects on extension rate and processivity suggest that discrete TR or protein elements are involved in product binding versus template repositioning during repeat translocation.

We have shown that given appropriate template sequence, human telomerase can perform processive extension synthesis at rates ranging from half as fast to twice as fast as with its native sequence. This would suggest that repeat
extension rate may have been evolutionarily optimized to add telomeric repeats at a rate that allows for proper biological function. The primary role of telomerase is to protect chromosomes by synthesizing the DNA framework onto which chromosome-capping telomere binding proteins such as TRF1 and TRF2 can assemble. Therefore, perhaps the amount of processive repeat synthesis achieved in a single encounter by telomerase bearing the wild type hTR sequence results in the optimal overhang size for proper looped structure formation and maintenance at the telomere terminus.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Charles Query and Michael Brenowitz for their helpful comments during the preparation of this manuscript. We also wish to thank Ms. Nitya Viswanathan for technical assistance and the AECOM DNA Sequencing Facility. This work was supported by Public Health Service grants K01 CA87542 (Howard Temin Award) to W. C. D. and R01 AI30861 to V. R. P.

REFERENCES

FIGURE LEGENDS

**Figure 1** A) Schematic representation of a template region of hTR. B) Template sequences of modified human (MH) hTR mutants. Changes from wild type sequence are indicated in bold and underlined. Suffixes “a” and “t” are used to designate singly mutated alignment region (nucleotides 52-56) or templating region (nucleotides 46-51) only variants of doubly modified mutants (MH1-MH9). Predicted repeat product is also shown.

**Figure 2** Primer extension by human telomerase template mutants. In vitro reconstituted telomerase template mutants MH1 to MH9 were assayed for telomerase activity via direct primer extension as described in Experimental Procedures. Phosphoimages of the PAGE-resolved reaction products are shown. Unextended substrate primer (P) within each reaction served as loading control (as it represented >95% of the recovered material). Quantitation of shorter phosphoimager exposures (not shown) confirmed equivalent sample loads. Lysate lane: extension reaction minus hTERT cDNA and hTR (i.e., IVR contained rabbit reticulocyte lysate only). Primer lane: d(TTAGGG)₃ substrate primer only. Marker sizes (in nucleotides) are indicated. hTR nucleotide positions are indicated over wild type sequence. Sequence changes from the wild type are underlined in each mutant. Positions of major accumulated products are indicated, in bolded type, under enlarged segments. The sequence of wild type product is also shown. A) Primer extension by mutants MH1 – MH5. B) Primer extension by mutants MH6 – MH9. Relative activities were determined as described in Experimental Procedures and are reported as percent wild type.

**Figure 3** Primer positioning by human telomerase template mutants. Primer extension reactions were carried out with IVR telomerase under standard conditions in the presence of 0.5 mM dATP, dGTP and dTTP (lanes marked “d”) or 0.5 mM dATP, ddGTP and dTTP (lanes marked “dd”). Unextended d(TTAGGG)₃ substrate primer was loaded in lane P. Potential substrate primer alignments with template RNA are shown as well as predicted elongation product (in lower case) terminated with ddG. Mispaired primer nucleotides are underlined.

**Figure 4** Competitive primer challenge assay with hTR mutants. Primer extension reactions were carried out under competitor challenge (“bind and chase”) conditions as detailed in Experimental Procedures. Following 5 min substrate primer binding, extension reactions were initiated and chased with excess (160 fold) cold competitor primer. Post-chase aliquots were taken at 0.5, 1, 3 and 5 min and analyzed via PAGE. Prechased lane: 5 min extension reaction where excess competitor primer (160 pmole) was added before IVR hTERT (WT hTR). Lysate lane: extension reaction with IVR minus hTERT cDNA and hTR. A) Competitive primer challenge with mutants MH1 – MH5. B) Competitive primer challenge with mutants MH6 – MH9. (Note: the band seen at approximately 48 nucleotides is nonspecific as it present in all samples including primer only) C) and D) Enlarged view of the area between 50 and 75 nucleotides of MH1 – MH5 and MH6 – MH9, respectively. The region of active polymerization for wild type is indicated by a vertical line to the left of the lane corresponding to the 3 min time point. Sequence of hTR templating region (nucleotides 46-51) is provided for reference. The sequence of the wild type product is also shown.

**Figure 5** Primer extension by human telomerase template variants containing only alignment or templating nucleotide mutations. IVR telomerase template mutants were assayed for telomerase
activity via direct primer extension as described in Experimental Procedures. Phosphoimages of
the PAGE-resolved reaction products are shown. Lysate lane: extension reaction minus hTERT
cDNA and hTR (i.e., IVR contained rabbit reticulocyte lysate only). Sequence changes from the
wild type are underlined in each mutant. The sequence of wild type product is also shown.
Positions of major accumulated products are indicated, in bolded type, under enlarged segments.
A) Primer extension by alignment region mutants.  B) Primer extension by templating region
mutants.

Figure 6 Competitive primer challenge assay with hTR mutants under extended elongation
conditions. Primer extension reactions were carried out under competitor challenge conditions as
detailed in Experimental Procedures. Post-chase aliquots were taken at 3 and 30 minutes and
analyzed via PAGE. Pre-chased lane: 30 min extension reaction where excess competitor primer
was added before adding the in vitro transcribed wild type hTERT enzyme. Relative processivity
values are calculated as the processivity index (determined as detailed in Experimental
Procedures) of a given enzyme compared to that of wild type. The processivity index of wild type
(shown in parentheses below the wild type lanes) is provided for reference.
Table 1. Relative extension rates of hTR mutants.

<table>
<thead>
<tr>
<th>HTR (3’- 5’ sequence)</th>
<th>Relative extension rate a (% WT±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT         CAAUCCCAAUC</td>
<td>1.00</td>
</tr>
<tr>
<td>MH1        C\textbf{CU}UC\textbf{CCU}UC</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td>MH2        \textbf{CU}U\textbf{CU}C\textbf{CU}UC</td>
<td>1.17±0.16</td>
</tr>
<tr>
<td>MH3        CA\textbf{UU}CC\textbf{UA}UC</td>
<td>1.97±0.24</td>
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<tr>
<td>MH4        CA\textbf{UA}CC\textbf{CA}UC</td>
<td>2.05±0.26</td>
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<tr>
<td>MH5        CAA\textbf{AC}CC\textbf{AA}AC</td>
<td>1.76±0.11</td>
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<tr>
<td>MH6        CA\textbf{UC}C\textbf{CA}U\textbf{UC}C</td>
<td>0.68±0.02</td>
</tr>
<tr>
<td>MH7        CAA\textbf{AC}CC\textbf{AA}CC</td>
<td>0.47±0.05</td>
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<tr>
<td>MH8        C\textbf{CA}U\textbf{U}CC\textbf{CA}UC</td>
<td>0.91±0.12</td>
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<tr>
<td>MH9        C\textbf{UA}U\textbf{U}CC\textbf{UA}UC</td>
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a Comparative repeat extension rates, expressed percent wild type, were determined as described in Experimental Procedures. Rates represent averages of two (MH6–MH9) or three (MH1–MH5) independent experiments.
### A

**Alignment**

```
P-5'-TTGGGTTAG
hTR ————3'-CAAUCCCAAUC-5'———
```

**Template**

```
Alignment
Templating
```

### B

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<tr>
<th>hTR</th>
<th>Sequence (3'-5')</th>
<th>Predicted repeat</th>
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<td>WT</td>
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</tr>
<tr>
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<td>GGGAAG</td>
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<td>Relative Activity</td>
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<td>18.4</td>
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**A**

- Marker Lysate MH3a WT
- MH4a MH5a MH8a MH9a
- WT: 3’ CAAUC 5’
- MH3a: CAUUC
- MH4a: CAUAC
- MH5a: CAAAC
- MH8a: CCAUC
- MH9a: CUAUC

**B**

- Marker Lysate MH3t WT
- MH4t MH5t MH8t MH9t
- WT: 3’ CCAAUC 5’
- MH3t: CCAUUC
- MH4t: CCAUAC
- MH5t: CCAAAC
- MH8t: CCCAUC
- MH9t: CCUAUC

---

**A**

- Marker Lysate MH3a WT
- MH4a MH5a MH8a MH9a
- WT: 3’ CCCAAUC 5’
- MH3a: CCAUUC
- MH4a: CCAUAC
- MH5a: CCAAAC
- MH8a: CCCAUC
- MH9a: CCUAUC

**B**

- Marker Lysate MH3t WT
- MH4t MH5t MH8t MH9t
- WT: 3’ CCCAAUC 5’
- MH3t: CCAUUC
- MH4t: CCAUAC
- MH5t: CCAAAC
- MH8t: CCCAUC
- MH9t: CCUAUC
Human telomerase RNA template sequence is a determinant of telomere repeat extension rate
William C. Drosopoulos, Roberto DiRenzo and Vinayaka R. Prasad

*J. Biol. Chem.* published online August 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M506319200

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