Template definition by *Tetrahymena* telomerase reverse transcriptase

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The ribonucleoprotein enzyme telomerase extends chromosome ends by copying a specific template sequence within its integral RNA component. An active recombinant telomerase RNP is minimally composed of this RNA and the telomerase reverse transcriptase (TERT) protein, which contains sequence motifs conserved among viral reverse transcriptases (RTs), flanked by N- and C-terminal extensions specific to TERTs. We have used site-directed mutagenesis to explore the roles of *Tetrahymena* TERT in determining features of telomerase activity in general and in establishing the boundaries and use of an internal RNA template in specific. We identify a new ciliate-specific motif in the TERT N-terminus required for template definition. Moreover, several residues in reverse transcriptase motifs 1, 2, A and D are critical for specific aspects of internal template use. Our results indicate that the unique specificity of telomerase activity is conferred to a reverse transcriptase active site by TERT residues both within and beyond the RT motif region.

Keywords: reverse transcriptase/RNP/telomerase/TERT/*Tetrahymena*

Introduction

In most eukaryotic cells, telomere maintenance requires the enzyme telomerase. The telomerase ribonucleoprotein (RNP) recognizes chromosome 3' ends and elongates them by reverse transcription of a template sequence within the integral RNA component (reviewed in Greider, 1995). This *de novo* synthesis of one strand of telomeric repeats can balance the repeat loss inherent to replication of linear chromosomes by RNA primer-dependent DNA polymerases. Maintaining a relatively constant telomere length also requires telomere-bound proteins that regulate telomerase activity in a chromosome-specific fashion (reviewed in Shore, 1997; Collins, 2000).

Although most single-cell eukaryotes produce active telomerase and maintain telomeres constitutively, many cells in multicellular organisms have telomerase activity insufficient for telomere maintenance. For example, telomeric repeats in cultured primary human cells erode from each telomere at ~100 base pairs per cycle of cell

division, forcing the cells to enter a genome-protective proliferative senescence at a critical minimum telomeric repeat number (Harley, 1995; Bodnar *et al.*, 1998). In contrast, most human cancers are characterized by strong telomerase activity (Kim *et al.*, 1994), which is required for culture viability (Hahn *et al.*, 1999; Zhang *et al.*, 1999). In highly proliferative normal tissue as well, such as skin and blood, telomerase activation appears to be required for sufficient cellular renewal within a single life-span (Mitchell *et al.*, 1999). However, because primary cells divide less and generally have longer telomeres than cancer cells, inhibition of telomerase in an adult human may still reduce cancer cell viability in a relatively specific manner.

Only two telomerase components are known to be essential for properly templated telomerase activity assayed on oligonucleotide substrates: the telomerase RNA and the telomerase reverse transcriptase (TERT) protein. TERT was initially identified in *Saccharomyces cerevisiae*, encoded by one of several genes required for telomere length maintenance, and independently in *Euplotes aediculatus*, as a subunit of the purified telomerase RNP (Lingner *et al.*, 1997). Sequence alignments and mutagenesis studies indicate that TERT contains reverse transcriptase (RT) active site motifs (Lingner *et al.*, 1997). However, several features of the telomerase enzyme distinguish it from other RTs. Most significant is the stable association of an RNA molecule with the active enzyme; only a sequence within the telomerase RNA can be recognized and used as a template (Greider and Blackburn, 1989). Catalysis by the RNP observes strictly defined template boundaries, copying only a specific set of residues. The overall activity, fidelity and processivity of template copying are dependent on telomerase RNA sequences both within and outside of the template (reviewed in Collins, 1999).

Active recombinant telomerase can be reconstituted by production of recombinant TERT and telomerase RNA in rabbit reticulocyte lysate (Weinrich *et al.*, 1997). The recombinant RNP from the ciliate *Tetrahymena thermophila* displays activity that, while not identical to that assayed in extracts of *Tetrahymena*, recapitulates the hallmarks of telomerase reverse transcription (Collins and Gandhi, 1998). The reconstituted enzyme has endogenous-like nucleotide incorporation and nucleolytic cleavage activities, observes the proper template boundaries, and performs multiple rounds of repeat addition on a single primer, indicating that it has repeat addition processivity. The recombinant activity can be detected by direct incorporation of radiolabeled dNTPs, allowing comparison of both the amount and distribution of products. A previous investigation using this recombinant *Tetrahymena* telomerase expression system characterized the contribution of telomerase RNA residues to catalytic

activity (Licht and Collins, 1999). Substitutions in different regions of the 159 nucleotide *T.thermophila* telomerase RNA can alter specific features of activity such as nucleotide or repeat addition processivity. A small subset of RNA sequence changes, in residues near the template 5' end, inhibits the interaction of telomerase RNA with TERT (Licht and Collins, 1999; C.Lai and K.Collins, submitted).

The high sequence divergence between TERTs and RTs coupled with relatively low sequence similarity among TERT orthologs has left the functions of most of the residues within the large TERT protein unknown. Here we examine residues in TERT that are required for RT activity and for the unique properties of telomerase. Our results reveal single amino acid substitutions of TERT that can alter 5' and 3' template boundaries, nucleotide and repeat addition processivities, and nucleotide selectivity. We identify a previously unrecognized motif in the TERT N-terminal region that is critical for template definition and demonstrate that conserved residues of the RT active site motifs can have either telomerase-specific or general RT roles. Our results indicate that TERT contributes directly to RNA template definition and the characteristics of template use.

Results

Selection of conserved residues for substitution

To identify residues that contribute to the overall level or the specific features of telomerase activity, we generated a set of *T.thermophila* TERTs carrying single amino acid substitutions. Substitutions were chosen using two major criteria. First, using sequence alignments of either all TERT orthologs or only ciliate TERTs, we selected amino acids with potential evolutionary conservation. Previous sequence alignments of TERTs and other RTs (Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Bryan *et al.*, 1998) identified TERT motifs that are shared with RTs (motifs 1, 2, A, B', C, D and E), a telomerase-specific motif found in all TERTs (motif T) and a motif shared by TERTs of ciliated protozoa including a cysteine shared by all TERTs (motif CP; Figure 1A). We identified two additional blocks of sequence conservation outside these motifs, conserved either in all TERTs (motif T2; Figure 1B) or only in ciliate TERTs (motif CP2; Figure 1C). Conserved residues from each of the motifs and from regions between motifs were selected for substitution. Secondly, we biased the selection for residues most likely to contact RNA directly. Based on the literature describing protein–RNA interactions, we chose amino acid side chains frequently involved in either hydrophobic base stacking or electrostatic interactions. Almost all selected amino acids were originally replaced with alanine. For some positions, multiple sequence substitutions were examined to determine the significant features of the side chain.

TERT proteins were expressed in rabbit reticulocyte lysate, assembled with purified recombinant telomerase RNA, then assayed for telomerase activity in the presence of all four dNTPs and either (TG)₈T₂G or (TG)₈T₂G₄ (see Materials and methods). The overall activity of telomerase RNPs with 54 site-specific TERT amino acid substitutions is summarized in Table I for both primers and is shown in Figure 2 for the primer (TG)₈T₂G₄. With this primer, two

dTTPs and one dGTP are added as synthesis proceeds to the template 5' end (to a product length of primer +3; Figure 1D). If repeat addition is processive, some percentage of primer +3 product will be extended by addition of a second, 6 nucleotide repeat (total product length of primer +9). An equivalent level of protein synthesis of all TERT variants was confirmed for the experiments included in Table I and Figure 2 by comparing [³⁵S]methionine incorporation, using SDS–PAGE and phosphorimager analysis (data not shown). Activity assays with both primers were also performed with recombinant telomerase RNPs assembled by co-expression of telomerase RNA with each TERT variant, rather than by addition of RNA after TERT expression. The relative activities of the various TERTs and all qualitative changes in activity induced by TERT amino acid substitutions were similar when examined in telomerase RNPs assembled by either reconstitution protocol (data not shown).

Quantitative changes in telomerase activity

A surprising number of amino acids in RT motif regions B' and E that are conserved among TERTs and RTs could be altered to alanine without destroying or qualitatively altering the activity of the recombinant catalytic core (Table I; Figure 2). In contrast, substitutions in motifs 1, 2, A and C had strong quantitative and qualitative effects. Varying results were also obtained from substitution analysis of conserved residues in the N-terminal TERT-specific extension (there is very weak sequence conservation among TERT C-terminal extensions). Substitution in the T and CP motifs revealed few side chains critical for activity, as described here and in a previous study of the human TERT motif T (Weinrich *et al.*, 1997). In contrast, substitutions in the T2 and CP2 motifs had strong quantitative and qualitative effects. A detailed description of quantitative changes produced by substitutions in *Tetrahymena* TERT (including comparison with complementary changes in human TERT and other RTs) is available in the Supplementary material, available in *The EMBO Journal* Online.

Single amino acid TERT substitutions can qualitatively alter RNP activity

Direct visualization of the products of the telomerase reverse transcription reaction allowed us to detect alterations in specific aspects of enzymatic activity, with or without an overall effect on the level of product synthesis. We categorized the qualitative activity changes observed among the 54 individual TERT amino acid substitutions to facilitate additional investigation (Table I). First we describe the classifications, and then report on the more detailed characterization of each class. A single Class 1 enzyme gave predominant products that were one nucleotide longer than the wild-type products of first repeat synthesis in reactions with either primer (Figure 3). This TERT substitution is in the newly identified ciliate-specific N-terminal motif CP2 (R237A). Three Class 2 enzymes demonstrated differential primer use, showing much less activity in reactions with the primer (TG)₈T₂G, which aligns near the template 3' end, than in reactions with the primer (TG)₈T₂G₄, which aligns near the template 5' end (Figure 4). These Class 2 enzymes include TERTs with a substitution in motif CP2 (Y231A) or in motif 1

Fig. 1. TERTs contain multiple conserved motifs in the N-terminal extension and the RT domain. Multiple sequence alignments (MSAs) of TERTs from a variety of organisms indicate that TERTs contain the RT motifs 1, 2 and A–E (Lingner *et al.*, 1997; Nakamura *et al.*, 1997), previously identified telomerase motifs T and CP (Nakamura *et al.*, 1997; Bryan *et al.*, 1998), and two new motifs T2 and CP2 shown here. (A) Location of conserved motifs in *T. thermophila* TERT. Regions with a high proportion of conserved residues are indicated by boxes. Scale bar, 100 amino acids. Dots indicate the 40 unique sites substituted in this study. Functions are assigned to motifs as described in the Results. (B and C) MSAs of newly identified motifs T2 and CP2. Motif T2 is found in all TERTs and motif CP2 is found only in ciliate TERTs. Dots indicate substituted residues. (D) Primer extension along the telomerase RNA template. Template and primer residues are in capital letters whereas telomerase-added nucleotides are in lower case. The *T. thermophila* template (RNA Template) and primers used in the initial screen (DNA Primers) are shown. The primer (TG)₈T₂G₄ is shown after extension to the end of the template (First repeat addition product). A product of first repeat addition can realign at the template 3' end and be extended by a second repeat (Repeat addition processivity product).

(K532A, K532R). Two Class 3 enzymes generated only very short products in reactions with either primer, including some radiolabeled products of primer size in reactions with the primer (TG)₈T₂G₄ (Figures 2 and 5). These Class 3 enzymes arise from TERT substitutions in motif 1 (K538A) or motif 2 (R543A). Three Class 4 enzymes also produced products shorter than those of the wild-type enzyme but differed from Class 3 enzymes in the prominent accumulation of product corresponding to

dissociation or pausing two nucleotides short of the template 5' end (Figure 5). These enzymes include TERTs with substitutions in several RT motifs: motif 2 (R543K), motif A (D624A) and motif D (K849A). A single Class 5 enzyme generated predominant products that had slightly slower mobility than wild-type first repeat synthesis products (Figure 2), particularly in reactions with the primer (TG)₈T₂G (data not shown). Unlike Class 1 enzyme products, however, Class 5 enzyme products were

Table I. Summary of substitution data

Motif ^a	Seq ^b	Sub ^c	Activity ^d	Class	Motif ^a	Seq ^b	Sub ^c	Activity ^d	Class	Motif ^a	Seq ^b	Sub ^c	Activity ^d	Class
T2	a	F158A	–		T	A	T479A	++		B'	a	Q767N	++	
T2	A	Q168A	+		T	A	E480D	+++		B'	A	Q773A	++	
T2	A	Q168E	+++		T	A	E480Q	++		B'	A	S778A	++	
T2	A	Q168N	+		(T)	A	R492A	++		C	a	R812A	–	
CP2	C	R226A	+		(T)	a	K493A	+++		D	A	K849A	++	4
CP2	C	F230A	++		1	C	K532A	+ / +++	2	D	A	K849R	+++	
CP2	C	Y231A	+ / ++	2	1	C	K532R	+ / +++	2	D	a	F854A	++	
CP2	C	C232A	+++		1	A	R534A	–		E	a	W876A	–	
CP2	C	H234A	+++		1	A	R534K	++		E	a	W876F	+	
CP2	C	R237A	+	1	1	A	K538A	+ / +++	3	E	a	G878A	++	
CP	A	C331A	+++		1		K539A	+++		E	C	S880A	+++	
	A	F379A	++		2	C	F542A	–		E	C	D882A	++	
		R381A	+++		2	A	R543A	+ / ++	3	E	C	D882N	+++	
	a	P389A	++		2	A	R543K	+++	4	E		N884A	+++	
	a	W433A	++		A	a	Y623A	+	5	E		N884D	–	
	a	F462A	+++		A	A	D624A	++	4	E	a	T885A	++	
(T)	C	R473A	++		B'	a	Q767A	++				K910A	+++	
T	A	Y477F	+++		B'	a	Q767E	+				K1077A	+++	

^aPreviously and newly identified motifs are indicated. (T) indicates that the residue falls within an extended motif T but was not included as part of this motif when originally identified (Nakamura *et al.*, 1997).

^bSeq indicates the extent of sequence conservation among TERTs for the substituted amino acid as follows: A, absolutely conserved; a, almost absolutely conserved; C, conserved among ciliates. *Tetrahymena thermophila* F462 is W in all other organisms, but because the amino acid at this position is almost absolutely conserved, the 'a' designation is still used. See Figure 1B for species list.

^cSub indicates the *T. thermophila* TERT amino acid substitution. Substitutions to alanine are in bold.

^dActivity relative to wild-type enzyme was quantitated by phosphorimager and assigned as follows: +++, one-third or more of wild type; ++, one-tenth to one-third of wild type; +, less than one-tenth of wild type but reproducibly detectable; –, very low or undetectable. Activity was quantitated for both (TG)₈T₂G and (TG)₈T₂G₄ primers and was usually similar (indicated by one score only) but differed in a few cases [indicated as score for (TG)₈T₂G/score for (TG)₈T₂G₄].

irregular in size and fuzzy in appearance. The TERT substitution inducing Class 5 activity is in motif A (Y623A).

Substitutions in two motifs affect synthesis specifically at the template 5' or 3' end

To investigate the enzymatic basis for the qualitative changes in activity described above, we varied the activity assay reaction conditions. The Class 1 enzyme was examined under conditions designed to test whether the aberrantly long products of first repeat synthesis result from copying beyond the normal template 5' boundary, through the adjacent position U42. Wild-type and Class 1 enzymes were assayed with the primer (TG)₈T₂G or (TG)₈T₂G₄, [α -³²P]dGTP, and dTTP in the presence or absence of dATP (Figure 3). Wild-type telomerase product profiles were identical in reactions with or without dATP, because dATP is not incorporated in copying the standard template (Figure 3, lanes 1, 2, 5 or 6). The most prominent product with the primer (TG)₈T₂G had a length of primer +6, while the most prominent product with the primer (TG)₈T₂G₄ had a length of primer +3. The Class 1 enzyme also produced products with appropriate lengths of (TG)₈T₂G +6 and (TG)₈T₂G₄ +3 in the absence of dATP (Figure 3, lanes 4 and 8). In the presence of dATP, however, products with lengths of (TG)₈T₂G +7 and (TG)₈T₂G₄ +4 were synthesized (Figure 3, lanes 3 and 7). These results suggest that the motif CP2 substitution R237A allows use of an extra telomerase RNA position, U42, as template. This position is not used by wild-type telomerase as template but can be used if additional nucleotides are inserted between the normal template 5' end at position C43 and the TERT binding motif 5' of

the template (see below). Little if any repeat addition processivity is catalyzed by the telomerase RNP containing R237A TERT when assayed in the presence of dATP (Figure 3, lanes 3 and 7). Since repeat addition processivity depends upon the release of product DNA from the template 3' end and repositioning at the 5' end, incorporation of residues that interfere with alignment at the 5' end reduces processivity. Incorporation of dATP by TERT R237A thus reduces repeat addition processivity because the repositioned product cannot be elongated.

Class 2 enzymes (Y231A, K532A, K532R) were tested under several conditions designed to resolve why these enzymes are less active in synthesis directed by the template 3' end than the template 5' end [(TG)₈T₂G₄ product > (TG)₈T₂G product]. Wild-type and Class 2 enzymes were assayed using a set of primers with varying 3' lengths of telomeric sequence but with a constant 5' end (Figure 4A). The varying 3' lengths also changed the telomeric repeat permutation at the primer 3' end and thus altered primer binding in two ways: the template position for first dNTP addition moved 5' on the template and the length of the primer–template hybrid increased. With these primers, the gel mobility of the first radiolabeled product increased with primer length, but the gel mobility of the product of complete synthesis of the first repeat was the same. With wild-type enzyme, all four primers (TG)₈T₂G, (TG)₈T₂G₂, (TG)₈T₂G₃ and (TG)₈T₂G₄ stimulated comparable levels of activity (Figure 4A, WT lanes). A similar affinity for different 3' end permutations has been shown previously for the endogenous enzyme and derives from the fact that an increasing length of hybrid is required to move increasingly 5' template positions into the active site (Wang *et al.*, 1998). With each of the Class 2 enzymes, all

Fig. 2. Primer extension activity from substituted TERTs reconstituted with telomerase RNA *in vitro*. Each of the 54 TERT substitutions was expressed in rabbit reticulocyte lysate, reconstituted with recombinant telomerase RNA and assayed for extension of (TG)₈T₂G₄ in the presence of all four dNTPs. Wild-type (WT) and substituted TERTs are indicated, along with motif location of substitutions where applicable. Products marked +3 and +9 are extended by three and nine nucleotides; these positions correspond to extension to the end of the template during first and second repeat addition, respectively. See Table I for quantitation of activity on (TG)₈T₂G₄ (shown here) and (TG)₈T₂G (not shown).

three of the primers (TG)₈T₂G₂, (TG)₈T₂G₃ and (TG)₈T₂G₄ stimulated more activity than the shortest primer (TG)₈T₂G (Figure 4A, Y231A, K532A and K532R lanes). These results suggest that Class 2 enzymes, with TERTs substituted in motifs CP2 and 1, either poorly use template position C48 or poorly form or use a three base-pair primer-template hybrid. The very weak repeat addition processivity for Class 2 enzymes is consistent with an inability to use the 3' end of the template following product repositioning in addition to during first repeat synthesis.

As one test to resolve the possibilities above, we examined whether increasing the concentration of the shortest primer (TG)₈T₂G would rescue its activity deficiency. The standard 1 μM primer concentration used in the assays previously shown is in excess of that required for maximal activity with the wild-type enzyme. Therefore, with the wild-type enzyme, a 10-fold increase in concentration of the primer (TG)₈T₂G or (TG)₈T₂G₄ stimulated no additional product synthesis (Figure 4B, lanes 1, 2, 6 and 7). Similarly, the activity of Class 2 enzymes was not enhanced in assays with higher concen-

Fig. 3. Class 1 TERT R237A copies past the 5' template boundary. Telomerase RNPs assembled from WT or R237A TERTs were assayed in the presence or absence of dATP. Assays with (TG)₈T₂G (lanes 1–4) and (TG)₈T₂G₄ (lanes 5–8) are shown. Predominant products are identified by the number of nucleotides added. The schematic shows telomerase RNA sequence (wild-type template underlined), primers used (capital letters) and nucleotides added (lower case letters) in reactions with R237A TERT in lanes 3 and 4, or 7 and 8.

Fig. 4. Class 2 TERTs have a defect in primer affinity at the 3' end of the template. (A) Class 2 TERTs elongate the primer (TG)₈T₂G inefficiently. RNPs assembled from WT, Y231A, K532A and K532R TERTs were assayed for extension of the primers (TG)₈T₂G, (TG)₈T₂G₂, (TG)₈T₂G₃ and (TG)₈T₂G₄, indicated by their variable 3' ends. Nucleotides added to primer (TG)₈T₂G are indicated on the left, whereas nucleotides added to primer (TG)₈T₂G₄ are indicated on the right. (B) The Class 2 defect in the use of (TG)₈T₂G is due to decreased primer affinity. RNPs assembled from WT and Y231A TERTs were assayed for extension of primers (TG)₈T₂G (lanes 1–5) or (TG)₈T₂G₄ (lanes 6–10) at primer concentrations from 1 to 100 μM. Products extended to the end of the template are indicated. The amount of product from complete first repeat synthesis is indicated for each primer and enzyme, normalized to activity at the highest primer concentration.

trations of the primer (TG)₈T₂G₄ (Figure 4B, lanes 8–10; other Class 2 enzymes, data not shown). In contrast, the activity of Class 2 enzymes was dramatically enhanced

(>20-fold) in assays with higher concentrations of the primer (TG)₈T₂G (Figure 4B, lanes 3–5; other Class 2 enzymes, data not shown). These results and others not

Fig. 5. Class 3 and Class 4 TERTs copy an incomplete template. (A) Class 3 TERTs have reduced nucleotide addition processivity. WT, Class 3 (K538A, R543A) and Class 4 (R543K, D624A, K849A) TERTs were assembled with telomerase RNA and assayed for extension of (TG)₈T₂G in reactions with nucleotide concentrations indicated. Products elongated to the end of the template have a length of primer +6. The specific activity of the radiolabeled dGTP was decreased when total dGTP concentration was raised to 25 μM due to addition of unlabeled dGTP. (B) Class 4 TERTs copy the 5' end of the template inefficiently. WT and R543K TERTs were co-expressed with wild-type or 5'+2U telomerase RNA and then assayed for extension of (TG)₈T₂G in reactions with 3.3 μM dGTP and 200 μM dTTP with or without 200 μM dATP. The schematics illustrate predominant products with the wild-type template (lanes 3 and 4) or with the 5'+2U template (lanes 7 and 8) in the presence of dATP.

shown favor the possibility that a less stable three base-pair primer–template hybrid is formed at the template 3' end. However, we cannot exclude the possibility that template mispositioning contributes at least partially to this phenotype.

Substitutions in RT motifs induce specific template use defects

The predominant first repeat addition product of (TG)₈T₂G with wild-type enzyme is primer +6 (Figure 5A, lane 1), reflecting synthesis to the 5' end of the template. Atypically short product synthesis was induced in Class 3 and 4 enzymes by substitutions in several RT motifs: motif 1 (K538A, Class 3), motif 2 (R543A, Class 3; R543K, Class 4), motif A (D624A, Class 4) and motif D (K849A, Class 4). The accumulation of first repeat products shorter than primer +6 could occur for a number of reasons, including a defect in nucleotide addition processivity. In this case, product dissociates from the

enzyme before all six first repeat nucleotides are added. To investigate the enzymatic defect in greater detail, we first assayed activity in reactions with the primer (TG)₈T₂G and different concentrations of dGTP or dTTP (Figure 5A). If nucleotide addition processivity is reduced because of a decreased nucleotide binding affinity, assays with an elevated nucleotide concentration should yield increased product lengths. This was observed for the Class 3 enzyme with motif 1 substitution K538A, for which increasing dGTP concentration from 5.0 to 25 μM did indeed substantially change the product profile from lengths of mostly primer +1 to lengths of primer +1 to +4 (Figure 5A, compare lanes 2 and 8). For other enzymes, including wild type, this change in dGTP concentration increased repeat addition processivity but did not otherwise substantially alter the product profile (Figure 5A, compare lanes 1, 3–6 with 7, 9–12). The increased repeat addition processivity was expected based on previous studies (Collins and Gandhi, 1998), because the K_m for dGTP stimulation of

repeat addition processivity ($\sim 5 \mu\text{M}$) is higher than the K_m for dGTP incorporation ($<1 \mu\text{M}$) for wild-type recombinant enzyme. Although the nucleotide addition processivity of the Class 3 enzyme with K538A substitution was improved by the increase in assay dGTP concentration, this change did not restore the product profile to that produced by wild-type enzyme. Instead, this enzyme at high dGTP concentration (Figure 5A, lane 8) adopted the product profile of a Class 4 enzyme (lanes 5 and 6), with accumulation of products two nucleotides shorter than that expected. This would correspond to a halt in template copying two nucleotides short of the template 5' end, across from template position A44. This Class 4 product profile did not become like wild type if dTTP concentration was increased above the standard $200 \mu\text{M}$ in the activity assay (data not shown). These results suggest that the TERT substitution K538A reduces nucleotide binding affinity, but that this substitution as well as those of the Class 4 enzymes also introduces a template copying problem not derived from altered nucleotide binding affinity.

The Class 4 enzyme pattern of product accumulation has some resemblance to that of a previously characterized dGTP misincorporation reaction in which RNA position C46 is copied multiple times in a template slippage-mediated reaction (reviewed in Collins, 1999). A single round of template slippage generates a T_2G_5 product 3' end, which is more likely to dissociate, resulting in a strong band at +4 when a primer ending in TTG is used. To test whether template slippage was enhanced in the Class 4 enzymes relative to wild type, elongation of the primer $(\text{TG})_8\text{T}_2\text{G}$ was assayed in the presence of dGTP only (Figure 5A, lanes 13–18). With only dGTP present, the wild-type TERT extends the primer to +3. If Class 4 TERTs had enhanced dGTP misincorporation, elongation with dGTP only would result in products longer than primer +3. However, incorporation beyond primer +3 was inefficient, indicating that the Class 4 defect is not due to template slippage. When assayed with the primer $(\text{TG})_8\text{T}_2\text{G}_4$ in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$ and unlabeled dGTP, all Class 3 and 4 enzymes extended the primer predominantly by addition of a single dTTP, while wild-type enzyme incorporated two dTTPs and one dGTP to reach the template end (data not shown). Together, these results suggest that the Class 4 enzymes experience an inhibition of template copying upon reaching template position A44 and frequently dissociate without copying position A44 or C43.

We sought to determine whether the inefficient copying of A44 by Class 4 enzymes was due to its sequence or its position within the template. To discriminate between these possibilities, we reconstituted the R543K TERT (the Class 4 TERT with the highest specific activity) with a telomerase RNA containing a template lengthened at its 5' end. The telomerase RNA 5'+2U has two U residues inserted between the template and the TERT binding site 5' of the template (Figure 5B, bottom). Wild-type TERT assembled with the 5'+2U RNA copied to the normal 5' template boundary in assays without dATP (Figure 5B, compare lanes 1 and 5) but copied both of the two inserted U residues in assays with dATP (compare lanes 3 and 7). With the 5'+2U RNA in the absence of dATP, R543K TERT efficiently copied the template to the wild-type

template 5' end (Figure 5B, lane 6). This was a dramatic rescue of the failure to copy past RNA position A44, which normally results in substantial accumulation of shorter products in the presence or absence of dATP (Figure 5B, lanes 2 and 4). With the 5'+2U RNA in the presence of dATP, R543K TERT remained unable to copy as much of the expanded template as wild-type TERT, stopping one nucleotide short of the new 5' template end (Figure 5B, lane 8). These results indicate that R543K TERT copies the 5' end of the template poorly, independent of the particular template sequence. We conclude that the Class 4 defect is due to inefficient use of the template residues adjacent to the 5' template boundary.

Finally, we investigated the enzymatic defect in the Class 5 enzyme with the motif A substitution Y623A. The analogous substitution in human TERT, Y717A, generates a recombinant enzyme that was judged inactive by TRAP assay (Weinrich *et al.*, 1997). In the direct primer extension assays of our initial screen, the Y623A substitution induced the synthesis of first repeat addition products that appeared longer than wild-type products in reactions with both primers $(\text{TG})_8\text{T}_2\text{G}$ and $(\text{TG})_8\text{T}_2\text{G}_4$. However, this product profile was not dependent on the presence of dATP (data not shown). Thus, the Y623A enzyme is unlikely to use an extended template. A second possibility for the slower mobility of Class 5 enzyme products was that ribonucleotides rather than deoxyribonucleotides were incorporated into product DNA. Although ribonucleotides were not added to the recombinant telomerase activity assays, they were present as part of the diluted reticulocyte lysate used for protein synthesis. Also, TERT Y623 (motif A) corresponds to an RT residue previously implicated in deoxynucleotide selectivity. In the crystal structures of a fragment of Moloney murine leukemia virus (MMLV) RT (Georgiadis *et al.*, 1995) and human immunodeficiency virus (HIV-1) RT covalently trapped with primer–template and dNTP (Huang *et al.*, 1998), the side chain of the phenylalanine or tyrosine residue at this location is positioned such that it would sterically select against a ribose 2' hydroxyl group. Indeed, substitution of this amino acid (F155V) in MMLV RT decreases discrimination against rNTP binding by 100-fold, roughly equalizing the K_m for dNTPs and rNTPs (Gao *et al.*, 1997).

To investigate whether the Y623A TERT substitution decreases the dNTP selectivity of the recombinant enzyme, we first immunopurified the RNP from reticulocyte lysate. We then assayed activity with the primer $(\text{TG})_8\text{T}_2\text{G}_4\text{T}$ and $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$. Wild-type recombinant enzyme, like endogenous *Tetrahymena* telomerase (Collins and Greider, 1995), demonstrated a substantial preference for dGTP over rGTP (Figure 6, WT TERT lanes 1–9). With rGTP (lanes 6–9), as with $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$ alone (lane 1), primer was elongated by only one nucleotide (primer +1). In the presence of dGTP (Figure 6, lanes 2–5), a complete first repeat and additional repeats were synthesized (primer +2, +8). In contrast, recombinant telomerase with Y623A TERT incorporated either dGTP or rGTP with a similar K_m (Figure 6, Y623A TERT lanes 1–9). In addition, the catalytic activity of Y623A TERT was similar with rGTP and dGTP. This lack of nucleotide discrimination is unparalleled even by F155V MMLV RT, which retains a catalytic bias against

Fig. 6. Class 5 TERT Y623A does not discriminate rGTP from dGTP. Immunopurified RNPs assembled from WT and Y623A TERTs were assayed for extension of $(\text{TG})_8\text{T}_2\text{G}_4\text{T}$ with $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$ and unlabeled dGTP or rGTP. Products resulting from incorporation of a single dTTP (+1) or from copying to the end of the template (+2 and +8) are indicated.

rNTPs despite a similar K_m for rNTPs and dNTPs (Gao *et al.*, 1997). The Y623A TERT substitution did increase the K_m for dGTP incorporation slightly (~5-fold) and also reduced overall activity slightly (also ~5-fold). We conclude that the major qualitative change in activity of the Class 5 enzyme derives from a loss of dNTP/rNTP selectivity.

Discussion

Telomerase as a unique RT

At the outset of this study, we had hypothesized that the complex interplay of protein and RNA function in the telomerase RNP reflected a structural adaptation of the reverse transcriptase active site. The TERT active site, after all, must use an internal template and an atypical substrate. Results from both this study and mapping of the RNA-binding domain of TERT (C.Lai and K.Collins, submitted) do not support this hypothesis. High affinity RNA binding and 5' template definition both require amino acid sequences in the TERT N-terminal extension. These results suggest that the specialization of telomerase for use of an internal template was accomplished in evolution by the combination of a polymerase module (motifs 1, 2, A–E) and a novel domain with appropriate RNA binding specificity (the N-terminus) that could place the template in the polymerase active site.

We show that in the TERT subunit, 5' template definition requires the side chain of an arginine in the N-terminal motif CP2. TERT R237A substitution induces a bypass of the normal template 5' end (Class 1). This activity change suggests that R237 helps to establish the correct spacing between the TERT binding motif 5' of the template and the intended template 5' end. The involvement of a ciliate-specific TERT motif in template definition is consistent with the ciliate-specific conservation of template-adjacent telomerase RNA structure and suggests that non-ciliate telomerases define their 5' template boundary differently. Establishment of the *Khuyveromyces lactis* 5' template boundary requires a telomerase RNA base pairing interaction (Tzfati *et al.*,

2000). It remains to be seen whether the *K.lactis* TERT actively recognizes this structure to mark a template 5' end or if duplex formation alone acts as a simple steric block.

Efficient use of the wild-type 5' end of the template, compromised in the Class 4 enzymes, requires residues in motifs 1, 2, A and D. To copy the template 5' positions, the template sequence must be moved through the active site. This displacement may induce some destabilization of the product–template hybrid, ultimately favoring product release from the template. Thus, the selective inhibition of product synthesis at the template 5' end observed with Class 4 enzymes could derive from a deficiency in the movement of the template through the active site, an overly destabilized hybrid, or constraints on the as yet uncopied template.

A template use deficiency specific to the template 3' end was created by substitutions in either the N-terminal motif CP2 or the RT region motif 1 (Class 2). Because this failure to use the template 3' end efficiently was rescued by elevated primer concentration (Figure 4) but not by altering template length or positioning (data not shown), this activity defect appears to result from a reduced stability of a short primer–template hybrid rather than from template mispositioning. This reduced stability could reflect loss of an interaction between TERT and the hybrid, or between TERT and DNA alone.

Telomerase has some features of a typical RT

This work demonstrates that the TERT N-terminus is important for telomerase-specific features of RT activity such as use of an integral template. But how different is the function of the central region of telomerase from that of any other RT? Recombinant TERT is unable to use an annealed primer–template substrate (Collins and Gandhi, 1998). One interpretation of this result was that specialization of the telomerase active site altered features standard among other RTs. The minimal extent of sequence conservation between TERTs and other RTs supported this hypothesis. However, an opposite conclusion is implied in the results presented here. Because substitutions of conserved amino acids in TERT motifs 1, 2 and A induce activity changes that can be explained by the roles of the cognate viral RT amino acids in the RT active site, the telomerase active site is likely to have substantial architectural similarity with that of other RTs. This may be particularly true for the nucleotide binding pocket.

One prominent feature of the RT nucleotide binding pocket is the presence of a tyrosine or phenylalanine five residues after the conserved aspartic acid of motif A. In this position, a large amino acid side chain discriminates rNTPs from dNTPs. This function has been previously demonstrated for MMLV RT F155 (Gao *et al.*, 1997) and predicted structurally for HIV-1 RT Y115 (Huang *et al.*, 1998). We show here that Y623 performs the same function for *T.thermophila* TERT. The Y623A TERT substitution reduces discrimination against rGTP, allowing highly efficient rGTP incorporation. Thus, motif A in TERT and other RTs is likely to be positioned in a similar manner relative to the sugar of the incoming nucleoside triphosphate. Considering also the aspartic acid residues in motifs A and C that are responsible for metal ion coordination, key residues of the entire palm domain appear similarly positioned in TERT and RTs.

Results from motif 1 and 2 substitutions suggest that the incoming dNTP triphosphate group is also bound in a similar manner in TERT and RTs, in this case relative to the surface of the dNTP binding pocket formed by the fingers domain. Structural studies of HIV-1 RT suggest that the fingers domain closes onto a nucleotide bound in the correct configuration before its addition to a primer-template duplex (Huang *et al.*, 1998). This movement brings the conserved lysine of motif 1 (HIV-1 RT K65, *T.thermophila* TERT K538) and the conserved arginine of motif 2 (HIV-1 RT R72, *T.thermophila* TERT R543) into contact with the γ and α phosphate groups, respectively. HIV-1 RT motif 2 R72 also interacts with the incoming base. In this context, it is particularly interesting that the two TERT substitutions with the most severe impact on nucleotide addition processivity are K538A and R543A (Class 3). In HIV-1 RT, K65A substitution moderately inhibits activity (Harris *et al.*, 1998). The HIV-1 R72A substitution dramatically reduces nucleotide addition processivity (Sarafianos *et al.*, 1995) and second dNTP addition occurs with a much slower rate than first dNTP addition. With TERT R543A, most product accumulates from a single dNTP addition as well: (TG)₈T₂G elongation by one [α -³²P]dGTP (Figure 5), (TG)₈T₂G₄ elongation by one [α -³²P]dTTP (data not shown), or (TG)₈T₂G₄ elongation by one [α -³²P]dGTP after telomerase-mediated nucleolytic removal of the 3' primer residue (Figure 2). In summary, our results suggest that despite the lack of flanking sequence homology, key residues in motifs 1, 2 and A play strikingly similar roles in TERT and other RTs.

Materials and methods

Recombinant telomerase production

Expression plasmids were derived from pT7159 (Autexier and Greider, 1994) for telomerase RNA or p133CITE (Collins and Gandhi, 1998) for TERT. Site-specific mutagenesis was performed by limited linear amplification of plasmid DNA with *Pfu* polymerase using a complementary oligonucleotide pair including the intended sequence change. Telomerase RNA coding regions were re-sequenced completely. TERT coding regions were partially or completely re-sequenced and analyzed as multiple independent clones to ensure the specificity of the phenotype for each amino acid substitution. Some TERT coding regions were also subcloned into HisHAp133CITE for immunopurification (Licht and Collins, 1999).

Telomerase was produced by co-expression of telomerase RNA and TERT in rabbit reticulocyte lysate (Promega TNT) or by expression of TERT alone followed by assembly with purified telomerase RNA (Collins and Gandhi, 1998). Co-expression reactions contained equal masses of telomerase RNA and TERT expression plasmids, which yielded approximately equimolar amounts of both components. For reconstitution by addition of purified telomerase RNA, the lysate expression reaction containing recombinant TERT (2–3 μ l) was supplemented with an excess of telomerase RNA (35–100 ng) and incubated for 10–15 min (Licht and Collins, 1999). For immunopurification from lysate, N-terminally HA-epitope tagged TERT was purified as described (Licht and Collins, 1999).

Telomerase activity assays

We chose initial assay conditions that facilitated the identification of changes in a wide variety of aspects of telomerase activity. For each substituted TERT, two separate assays were conducted using oligonucleotide primers directed to anneal either towards the template 3' end [(TG)₈T₂G] or towards the template 5' end [(TG)₈T₂G₄]. Thus, the relative use of two different template regions was compared. In the initial screen, activity was assayed in the presence of [α -³²P]dGTP and dTTP, dATP and dCTP. Wild-type telomerase incorporates only dGTP and dTTP, but the presence of dATP and dCTP permitted the detection of extension beyond the normal 5' template boundary. Finally, the dGTP

concentration in the initial activity assay was adjusted to stimulate an intermediate extent of repeat addition processivity.

Samples for activity assays were brought to 10 μ l with T2MG (20 mM Tris-HCl pH 8, 1 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol). Other reaction components were added to obtain a 20 μ l final assay volume in 1 $\times$ telomerase assay buffer (50 mM Tris-acetate pH 8.0, 2 mM MgCl₂, 10 mM spermidine, 5 mM β -mercaptoethanol). Standard assays contained 1 μ M [α -³²P]dGTP (NEN, 800 Ci/mmol), 2 μ M unlabeled dGTP, 200 μ M dTTP and if indicated 200 μ M dATP and/or dCTP. In assays using radiolabeled dTTP, reactions contained 2–3 μ M [α -³²P]dTTP (NEN, 800 Ci/mmol) and 10 μ M unlabeled dTTP. DNA oligonucleotide primers were used at 1 μ M final concentration unless otherwise indicated. Reactions were incubated at 30°C for 1 h. Product DNA was extracted, precipitated and analyzed by electrophoresis on 9 or 12% (19:1) acrylamide, 7 M urea, 0.6 $\times$ TBE gels. Quantitation was performed by phosphorimager analysis using software from the manufacturer (Fuji).

Supplementary material

Supplementary material to this paper is available in *The EMBO Journal* Online.

Acknowledgements

We thank Jill Licht, Carla Schultz and Doreen Cunningham for reagents, Dorothy Shippen for unpublished data, and Donald Rio, James Berger and members of the Collins laboratory for comments on the manuscript. This work was supported by a National Science Foundation Graduate Fellowship (M.C.M.), the Biology Fellows Program at University of California, Berkeley (J.K.L.) and National Institutes of Health grant GM54198 (K.C.).

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*Received April 17, 2000; revised June 26, 2000;
accepted June 30, 2000*