Regulation of Catalytic Activity and Processivity of Human Telomerase†

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ABSTRACT: The ends of eukaryotic chromosomes are specialized sequences, called telomeres comprising tandem repeats of simple DNA sequences. Those sequences are essential for preventing aberrant recombination and protecting genomic DNA against exonucleolytic DNA degradation. Telomeres are maintained at a stable length by telomerase, an RNA-dependent DNA polymerase. Recently, human telomerase has been recognized as a unique diagnostic marker for human tumors and is potentially a highly selective target for antitumor drugs. In this study, we have examined the major factors affecting the catalytic activity and processivity of human telomerase. Specifically, both the catalytic activity and processivity of human telomerase were modulated by temperature, substrate (dNTP and primer) concentration, and the concentration of K^+. The catalytic activity of telomerase increased as temperature (up to 37 °C), concentrations of dGTP, primer, and K^+ were increased. However, the processivity of human telomerase decreased as temperature, primer concentration, and K^+ were increased. Our results support the current model for human telomerase reaction and strengthen the hypothesis that a G-quadruplex structure of telomere DNA plays an important role in the regulation of the telomerase reaction.

The ends of eukaryotic chromosomes have a specialized sequence, termed telomeres, consisting of repeating guanine-rich DNA sequences (*1*, *2*). Telomeres are essential for preventing aberrant recombination and exonucleolytic DNA degradation (*1*, *3*, *4*). They also prevent the loss of terminal bases at the 5′-end of each strand following the completion of the linear chromosomal DNA replication (*5*). In various human somatic cells, telomeric DNA shortening during growth results in a limited replicative capacity, eventually leading to cellular senescence (*6*). The loss of telomeric repeats after each cell division may be a biological clock limiting the proliferative life span of somatic cells (*6*−*8*). In the presence of viral oncogenes or somatic mutations that block cellular senescence, cells continue to divide and telomere erosion proceeds until there are few telomeric repeats remaining. The activation of telomerase, however, allows a variety of tumor cells to develop an unlimited replicative capacity.

Telomerase, a ribonucleoprotein enzyme, was first discovered on the basis of distinctive telomere extension activity in the ciliate *Tetrahymena* and, subsequently, in the human HeLa cell line (*9*−*11*). This enzyme compensates the sequence loss that results from incomplete terminal replication by using a short RNA motif as a template for the synthesis of telomeric DNA. The RNA moiety of telomerase from various ciliates and two mammalian sources (human and mouse) has been cloned and sequenced (*12*, *13*). Both mammalian telomerase RNA are approximately 500 nucleotides in length. The protein components of telomerase have been identified first in *Euplotes* and budding yeast, in which the p123 and EST2 genes encode the catalytic subunit of the telomerases in *Euplotes* and budding yeast, respectively (*14*, *15*). Recently, an essential protein component of human telomerase has been identified independently by Cech's group and Weinberg's group (*16*, *17*). The genes are now called hTERT[1] (human telomerase reverse transcriptase), and the expression of the hTERT was shown to correlate with the telomerase activity in telomerase-negative and -positive cells. These recent studies clearly indicated that the telomerase catalytic component is encoded by the hTERT genes. Importantly, human telomerase has been proposed as a novel and potentially highly selective target for antitumor drug design, since telomerase is known to be expressed in human tumors and not in most somatic cells (*18*−*21*).

The current model for the mechanism of telomeric DNA synthesis by telomerase was first proposed by the Blackburn group (*1*, *2*, *22*). According to their model, shown in Figure 1, the template domain of telomerase RNA base pairs with the 3′-end of telomeric DNA (TTAGGG in human). This alignment allows primer extension using the RNA as a template and results in the addition of four telomeric nucleotides (TTAG). Next, the extended DNA terminus unpairs from its RNA template, and the newly extended 3′-end is repositioned on the template without dissociation of primer from enzyme, becoming available for another round of elongation by telomerase in which six nucleotides (GGTTAG) are added to the 3′-end of the telomeric DNA. One

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[1] Abbreviations: BME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; EDTA, ethylenediaminetetraacetic acid; hTERT, human telomerase reverse transcriptase; PMSF, phenylmethanesulfonyl fluoride.

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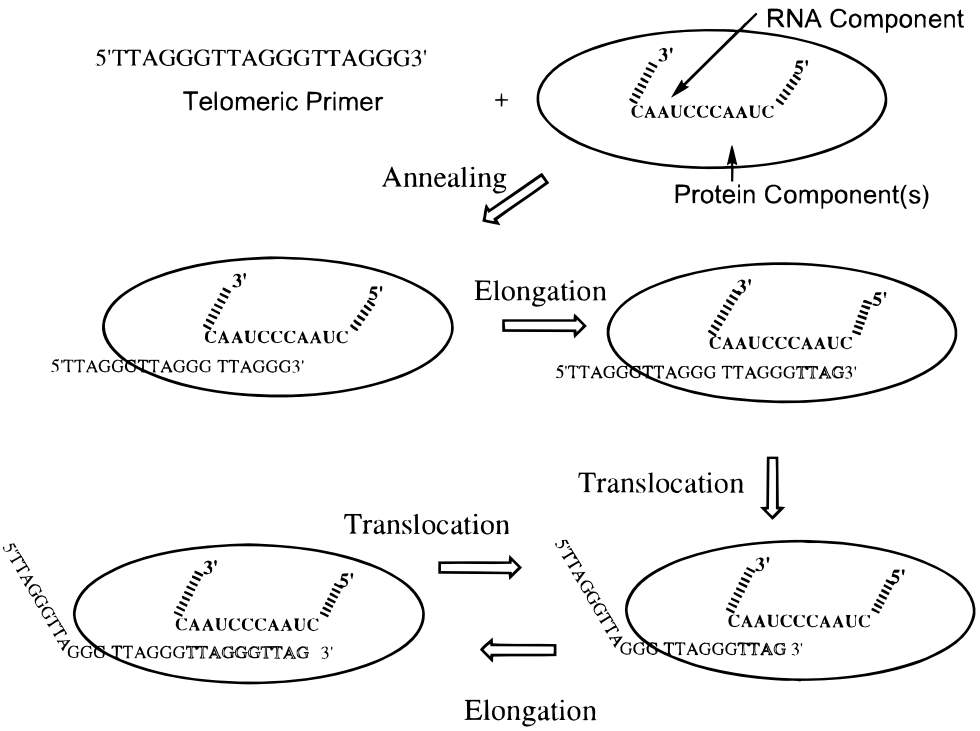

FIGURE 1: Model for processive extension of telomeric repeats by human telomerase.

of the most striking features of the telomerase reaction involves not only copying of an internal template but also an efficient translocation event that occurs after the last 5′ residue of the RNA template has been copied into DNA. The translocation step has been deduced from the processive nature of the telomerase reaction in a cell-free assay (*1*, *22*). Thus, telomerase initiates synthesis on a telomeric sequence DNA primer and continues to elongate the first primer up to hundreds of nucleotides before dissociation (*1*, *4*).

In this study, we set out to understand the biochemical aspects of the telomerase reaction by examining major factors affecting the catalytic activity and processivity of human telomerase, such as temperature, salts, and substrates. Our results indicate that both the catalytic activity and processivity of human telomerase can be modulated by temperature, substrate (dNTP and primer) concentration, and G-quadruplex interacting agents such as K^+. These results are anticipated to be useful in future studies, such as the characterization of telomerase activity in various tumor cells and discovery of potential telomerase inhibitors that interfere with the telomerase activity.

MATERIALS AND METHODS

Materials. Streptavidin-coated Dynabeads suspension (Dynabeads M-280 Streptavidin) was purchased from the Dynal Company and 5′-biotinylated $(TTAGGG)_3$ primer from Genosys. [α-^{32}P]dGTP was from NEN Dupont. The X-ray film, intensifying screens, and developing chemicals were from Kodak.

Preparation of Cell Lysate (S-100). Cell extracts were prepared from 1.2×10^9 cultured cells as previously described (*18*). Briefly, cultured HeLa cells (National Cell Culture Institute, MN) were washed once in phosphate-buffered saline (PBS) and resuspended in ice-cold washing buffer that contained 10 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM dithiothreitol. Cells were pelleted at 10000*g* for 1 min at 4 °C, resuspended in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol (BME), 1 mM dithiothreitol, and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) along with 10% glycerol (10^6 cells/20 μL of buffer), and incubated for 60 min on ice. Cell lysates were centrifuged for 1 h in an ultracentrifuge at 100000*g* at 4 °C. The resulting supernatant (S-100 extract) was then adjusted to 20% glycerol and stored at −80 °C.

Telomerase Assay. The assay was performed using 5′ biotinylated $(TTAGGG)_3$ as a telomere primer instead of $(TTAGGG)_3$, which is normally used. The reaction mixture (20 μL) contained 50 mM Tris-OAc (pH 8.5), 50 mM KCl, 1 mM $MgCl_2$, 5 mM BME, 1 mM spermidine, 1 μM telomere primer, 2.4 μM [α-^{32}P]dGTP (800 Ci/mmol), 1 mM dATP, and 1 mM dTTP with 4 μL of S-100 (*10*). The reaction mixtures were incubated at 37 °C for 1 h, unless otherwise specified. The reactions were terminated by adding 20 μL of Dynabeads suspension, and the reaction products were immobilized to Dynabeads following the procedure suggested by the manufacturer (*22*). The immobilized reaction products were washed at least five times with washing buffer [1 M NaCl and 10 mM Tris-HCl (pH 7.5)] to eliminate the [α^{32}P]dGTP background and nonspecific high molecular weight DNA. The reaction products were dissociated from the Dynabeads by incubating the bead-reaction product complex in 200 μL of guanidine-HCl solution (final 5.0 M) at 90 °C for 20 min. Following ethanol precipitation, telomerase reaction products were analyzed by 8% denaturing polyacrylamide gel electrophoresis. Gels were dried on filter paper and developed by autoradiography on a sensitive film (Kodak Biomax-MS). Telomerase activities

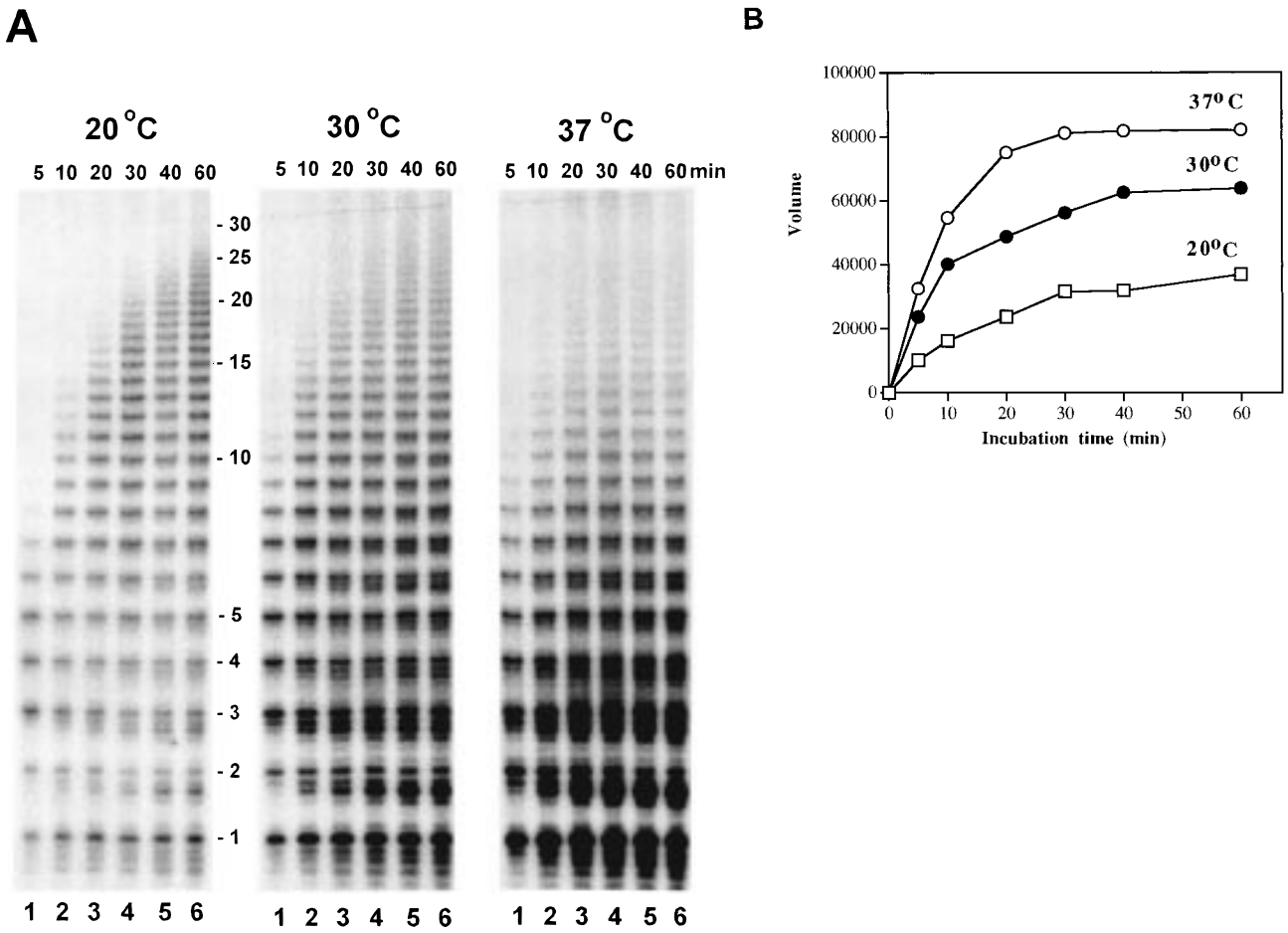

FIGURE 2: Effect of temperature on the catalytic activity and processivity of human telomerase. (A) Time course of telomerase reactions at three different temperatures. Telomerase reactions were carried out at 20, 30, and 37 °C for a given period of time under the standard reaction conditions. Numbers refer to the number of extensions of the telomeric repeat. In the first extension, the major band corresponds to the 22 bp species produced by the addition of four telomeric nucleotides (TTAG) to 18 bp primer, $(TTAGGG)_3$. In the following extensions, six telomeric nucleotides (5′-GGTTAG) were added to telomeric primers, resulting in the generation of 6 bp ladders (28, 34, and 40 bp, etc.). (B) Graphical representation of data from A. Total amount of [α^{32}P]dGTP incorporated into extension products of primer was quantified using Image Quant and plotted against incubation times.

were quantified by densitometric analyses (ImageQuant, Molecular Dynamics) of the autoradiographs.

RESULTS

In this study, we used a modified conventional assay method to measure telomerase activity, as described in Materials and Methods. In a typical telomerase assay, a characteristic pattern of product bands is produced corresponding to additions of telomeric repeats to a substrate primer. This modified method allows us to measure telomerase activity in a quantitative manner and to distinguish the processive activity from the nonprocessive activity of telomerase. Therefore, this assay has been used throughout this study to examine major factors affecting the catalytic activity and processivity of human telomerase.

Effect of Temperature on Telomerase Activity. To examine the effect of temperature on the catalytic activity and processivity of human telomerase, the reaction mixtures were incubated at three different temperatures (20, 30, and 37 °C) during the indicated periods of time, as described in Materials and Methods. Reaction products were then analyzed by denaturing polyacrylamide gel electrophoresis (Figure 2A). The amount of radioactivity incorporated into primers was quantified from Figure 2A and plotted in Figure 2B. This time-course experiment at different temperatures has revealed that the catalytic activity (represented as total incorporation of [α^{32}P]dGTP into primers) of human telomerase increased significantly as the reaction temperature increased. The catalytic activity reached a maximum at 37 °C and then gradually decreased as the temperature was increased further (data not shown). In contrast, the processivity of the telomerase reaction significantly decreased as the temperature increased. The average length of extension products is much shorter at the highest temperature (37 °C) than at lower temperatures. More than 30 repeats of the TTAGGG sequence were observed after the 1 h incubation at 20 °C, indicating that telomerase might possess a high degree of processivity in vivo as long as the telomerase enzyme maintains its complex with reaction products.

Effect of Primer Concentration on Telomerase Activity. We next determined if primer concentrations could affect the catalytic activity and processivity of human telomerase. As illustrated in Figure 3, the catalytic activity of telomerase increased gradually with increasing concentrations of primer and reached a maximum at ~1 μM, possibly due to the increase in the available primer concentration (see Figure 3, panels A and B). The intensity of major bands at each extension was measured and then plotted versus numbers of

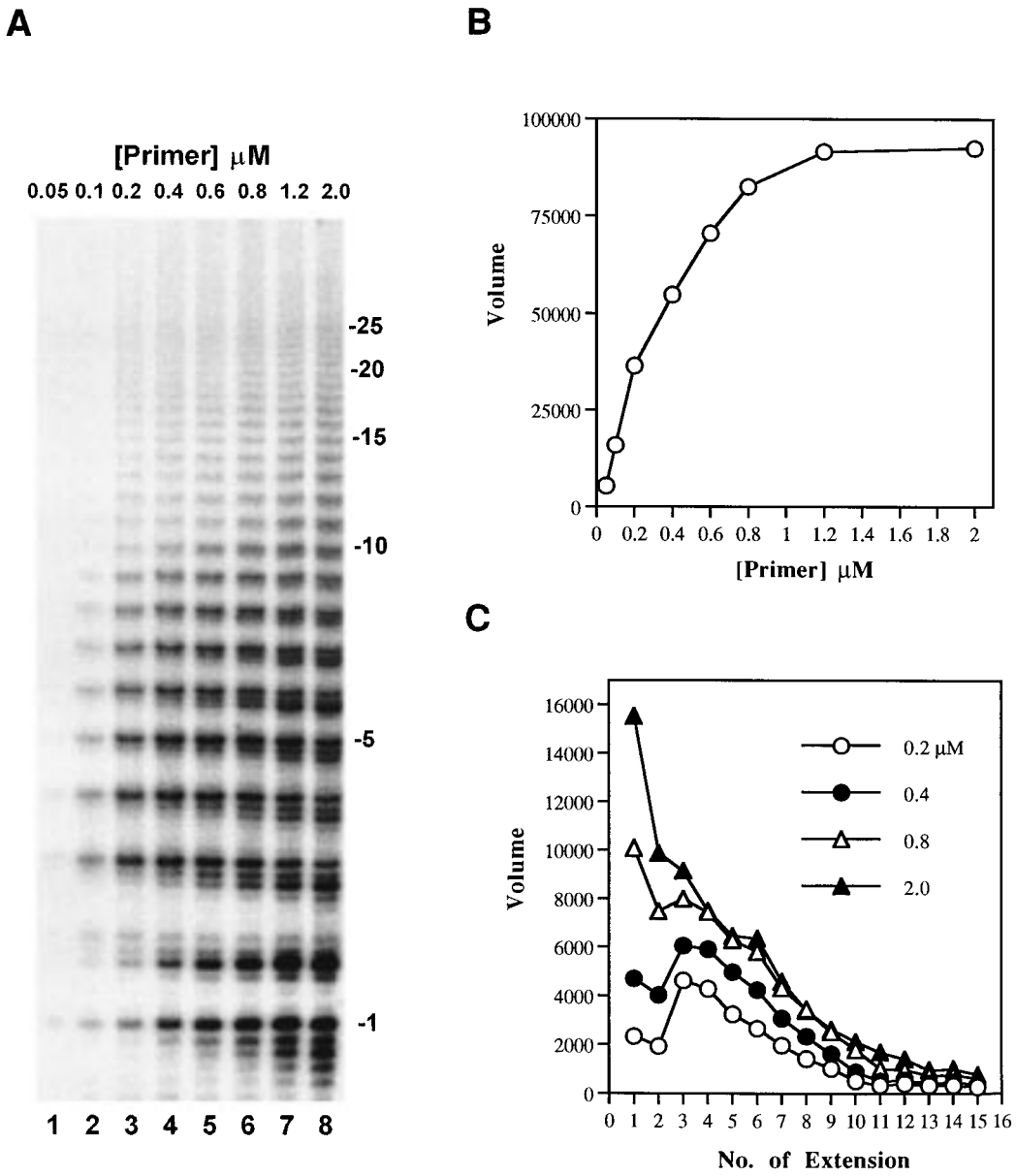

FIGURE 3: Effect of primer concentration on the catalytic activity and processivity of human telomerase. (A) Telomerase reactions were carried out under standard reaction conditions with primer concentrations of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, and 2.0 μM. (B) Effect of primer concentration on the catalytic activity (total volume) of telomerase. (C) Telomerase processivity at various primer concentration (μM). Volume represents the volume of each extension.

extension at each primer concentration (Figure 3C). Interestingly, the proportion of shorter products increased as the primer concentration increased, reflecting that the processivity of telomerase enzyme can be decreased by an increase of primer concentration in reactions. In addition, the presence of excess primer resulted in a change in the pattern of bands produced at each extension.

Effect of dGTP Concentration. The effect of nucleotides on the catalytic activity and processivity of human telomerase was also examined in this study. As a representative nucleotide, dGTP was used in the experiment described in Figure 4. In this experiment, the concentration of dGTP was varied from 0.75 to 18 μM with excess dATP and dTTP (1 mM each). As shown in Figure 4A, both the catalytic activity and processivity of telomerase increased as the concentration of dGTP was increased. This must be due to subsaturating dGTP concentrations used in these experiments. The amount of telomerase reaction products was linearly dependent on the dGTP concentration until it reached a maximum at ~15 μM dGTP (see Figure 4B). However, the pattern of bands produced at each extension did not change as the dGTP concentration was varied. The titration of dGTP over 20 μM did not affect the amount of telomerase reaction products formed during the reaction, and other nucleotides, dATP and dTTP, also increased the processivity and catalytic activity of the telomerase enzyme as the concentration of each nucleotide was increased (data not shown).

Effect of K^+ Concentration on Telomerase Reaction. The inhibition of telomerase by G-quartet DNA structures has been noted previously (*24*). In this study, the extent of telomere elongation by human telomerase was examined in the presence of K^+. As illustrated in Figure 5A, the catalytic activity of telomerase gradually increased as the KCl concentration increased up to 100 mM. However, the overall length of telomerase products was much shorter in the presence of high K^+ than low K^+ concentration, indicating

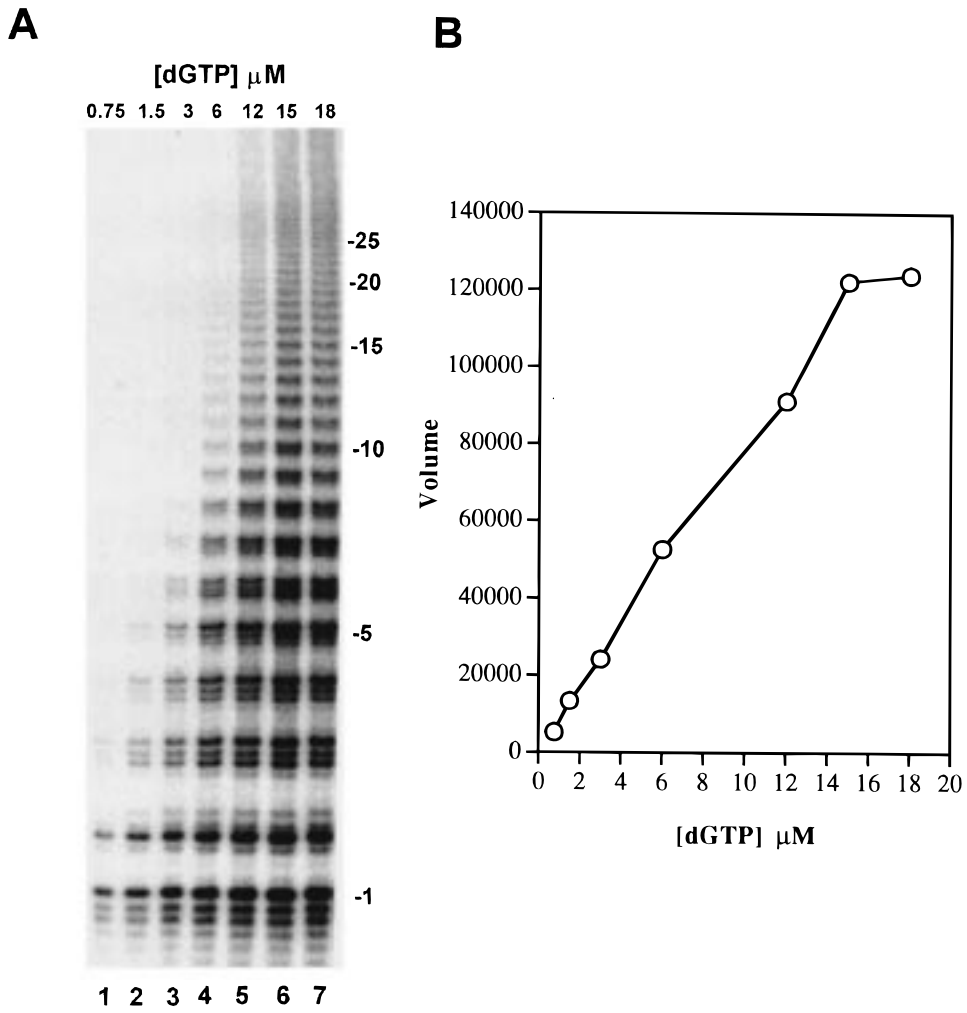

FIGURE 4: Effect of dGTP concentration on the catalytic activity and processivity of human telomerase. (A) Telomerase reactions were carried out with dGTP concentrations of 0.75, 1.5, 3.0, 6.0, 12, 15, and 18 μM under standard reaction condition containing 1 mM dATP and 1 mM dTTP. [α^{32}P]dGTP was diluted 3-fold with cold dGTP and was used for the reactions. (B) Graphical representation of data obtained in panel A.

that the telomerase reaction became nonprocessive with increasing concentration of K^+. At each K^+ concentration, the intensity of major bands of each extension was measured and then plotted versus the K^+ concentration (Figure 5C). In the presence of a high concentration of K^+, the first, second, and third extension products accumulate, but at more than four extension products there is a significant decrease. This result indicates that the inhibition of telomerase by K^+ occurred only after primers are extended to a total of six repeats of TTAGGG. When telomerase reactions were performed at 30 °C, the similar pattern of inhibition was observed, indicating that the effect of K^+ on telomerase reaction does not depend on the temperature of the telomerase reaction (data not shown).

To confirm that K^+ modulates telomerase activity in two ways, i.e., increasing the catalytic rate of the polymerization reaction but decreasing its processivity, telomerase reactions were performed in the presence of dATP (in a form of [α^{32}P]-dATP) and dTTP without dGTP as nucleotide substrates. In this case, the telomerase reaction was prematurely terminated due to the absence of dGTP before proceeding to the translocation step after incorporation of 5′-TTA-3′ into the 3′-end of primer (see Figure 6A). This premature termination allowed us to monitor the effect of K^+ on the first round of telomeric repeat synthesis (the initiation step). The result of the experiment shown in Figure 6 supports the idea that K^+ increases the turnover rate of the telomerase enzyme during the reaction, since the catalytic activity in terms of dAMP incorporation by telomerase into the primer was significantly stimulated by K^+ in a concentration-dependent manner.

DISCUSSION

Human telomerase, together with *Tetrahymena* enzymes, is processive in in vitro cell-free assays, although the mouse enzyme reportedly acts distributively (*9*, *10*, *25*). The processive nature of the telomerase reaction suggested that telomerase can continue the addition of a repeat to the newly extended 3′-end without dissociation of the primer from the enzymes (see Figure 1). The catalytic rate of the telomerase polymerization reaction has been studied by varying the 5′-end sequence of the primer to understand the sequence-specific interaction between the primer and telomerase, using *Tetrahymena* enzymes (*26*). However, important questions from previous studies regarding the modulation of processivity and catalytic activity of human telomerase have yet to be addressed.

In this study, we used a modified conventional assay to measure telomerase activity, in which 5′-biotinylated

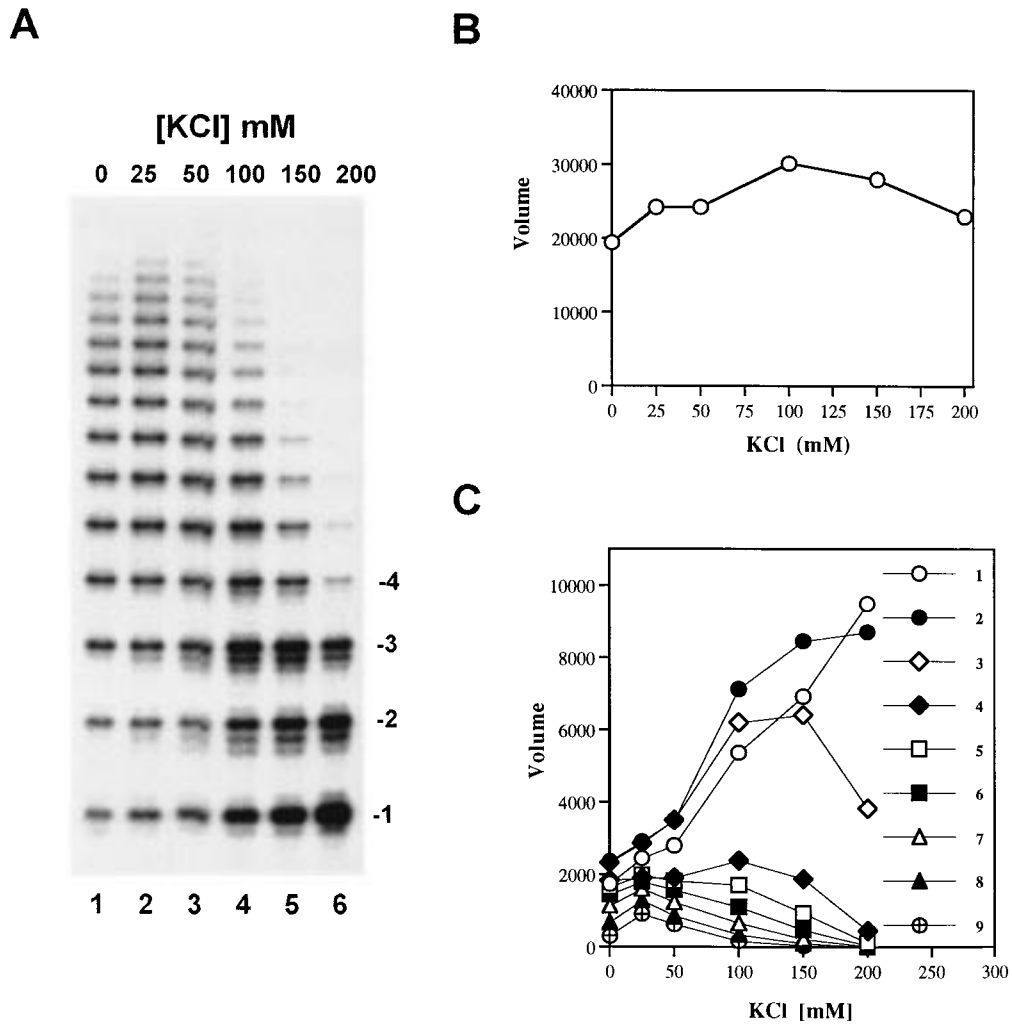

FIGURE 5: Effect of K^+ on the catalytic activity and processivity of human telomerase. (A) The standard telomerase reactions were carried out for 10 min at 37 °C with dGTP (^{32}P), dATP, and dTTP in the presence of 0, 25, 50, 100, 150, and 200 mM KCl, respectively (lanes 1−6). (B) Graphical representation about the effect of K^+ on the incorporation of ^{32}P into primers (as the catalytic activity). (C) Effect of K^+ on the amount of major bands of each extension.

$(TTAGGG)_3$ was used as a substrate primer in telomerase reactions. This method efficiently eliminates excess [α^{32}P]-dGTP and labeled high molecular weight DNA by selectively immobilizing telomerase reaction products including unreacted primers to streptavidin-coated Dynabeads (Dynal), as described in the Materials and Methods. Using this assay method, we have found from this study that the catalytic activity and processivity of human telomerase can be modulated by varying reaction conditions such as temperature, nucleotide and primer concentrations, and K^+ concentration.

The time-course experiment at different temperatures has revealed that the catalytic activity of human telomerase increased significantly as the reaction temperature increased until it reached 37 °C, while the processivity of the telomerase reaction significantly decreased. The greater accumulation of the short products at the elevated reaction temperature results from an increased rate of enzyme turnover during the reaction. This reflects a high probability of product dissociation from telomerase rather than proceeding on to the next round of polymerization events without dissociating from telomerase. In subsequent rounds, the telomerase enzyme initiates the binding process with new primers in order to proceed to the next polymerization. This result provides strong evidence that telomerase might possess a high degree of processivity in vivo as long as the telomerase enzyme forms a stable complex with the reaction products.

From this study, we also demonstrated that the concentration of primer affects the catalytic activity and processivity of human telomerase. The catalytic activity of human telomerase increased as the concentration of primer was increased. This is most likely due to the subsaturating concentrations of primer used in these experiments. However, the size of products became smaller as the primer concentration increased, reflecting a decrease in the processivity of telomerase. This observation could originate from competition for a common or adjacent binding site between telomerase reaction products and excess primers during the translocation, resulting in dissociation of the premature reaction products from telomerase.

We also determined if the concentrations of nucleotides could affect the catalytic activity and processivity of human telomerase. As a representative nucleotide, dGTP was used in this study. It is known that the processivity of *Euplotes aediculatus* telomerase decreases as the concentration of dGTP is increased (*27*). Also the same authors observed that the characteristic banding pattern generated by telomerase shifts in response to changes in dGTP concentration.

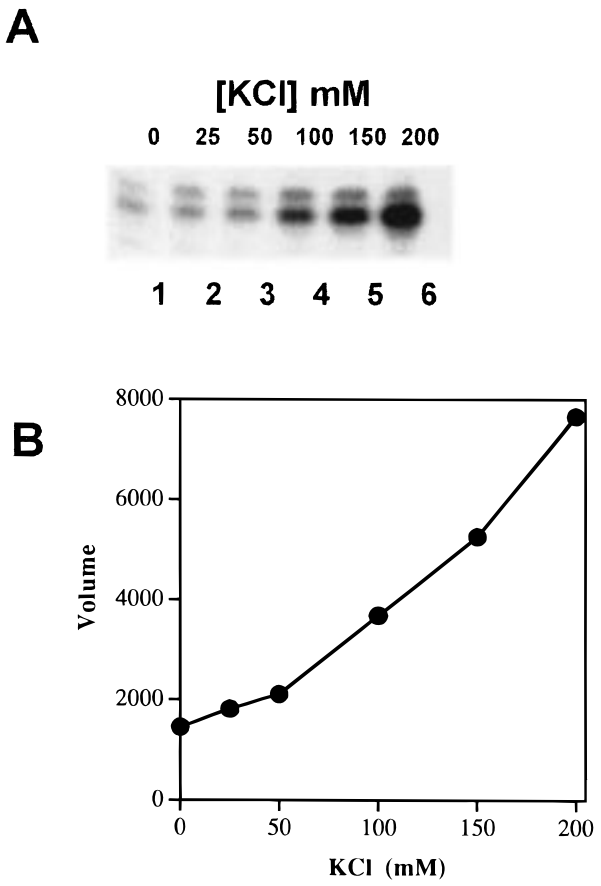

FIGURE 6: Effect of K^+ on the initiation reaction of human telomerase. (A) The telomerase reactions were carried out for 10 min at 37 °C with only with dATP (^{32}P) and dTTP as nucleotide substrates in the presence of 0, 25, 50, 100, 150, and 200 mM KCl, respectively (lanes 1−6). (B) Graphical representation of data obtained in panel A.

However, in the experiments described here (Figure 4), both the catalytic activity and processivity of telomerase increased as the concentration of dGTP was increased. This must be due to the subsaturating dGTP concentrations used in our experiments. The amount of telomerase reaction products was linearly dependent on the dGTP concentration until it reached a maximum at ~15 μM dGTP. In addition, the pattern of bands produced at each extension did not change as the dGTP concentration was varied. Other nucleotides, dATP and dTTP, showed the same effects on the processivity and catalytic activity of telomerase (data not shown).

One unexpected conclusion from our experiments is the role of K^+ in the modulation of human telomerase activity. The importance of a folded structure such as a G-quadruplex and its stabilization by K^+ in the regulation of telomerase activity has been previously noted (*24*). The same authors proposed that the formation of a G-quadruplex structure in a primer by K^+ inhibits its use as a substrate by telomerase. Furthermore, the octanucleotide T4G4, which does not fold, is a better primer than (T4G4)2, which can form a foldback structure. They also proposed that the folding of telomeric DNA into G-quadruplex structures seems to influence the extent of telomere elongation in vitro and might therefore act as a negative regulator of elongation in vivo. In our study, we used a primer oligonucleotide consisting of three telomeric repeats, which cannot form an intramolecular G-quadruplex structure in the telomerase reactions. Our results demonstrate that the catalytic activity of telomerase gradually increased with K^+ concentration (up to about 100 mM), whereas the telomerase reaction became nonprocessive with increasing concentrations of K^+. When the intensity of the major bands of each extension was measured at each K^+ concentration, and plotted versus K^+ concentration, there was a pronounced accumulation of the first, second, and third extension products followed by a significant reduction of further extension products in the presence of high concentrations of K^+. This observation indicates that the significant inhibition of telomerase by K^+ occurs only after primers are extended to six repeats of TTAGGG. Since the formation of a G-quadruplex structure requires at least four repeats of TTAGGG in the portion of the primer that is not base-paired to the RNA template, this result supports the conclusion that K^+ interacts mainly with quadruplex structures formed during the telomerase reaction. It is well established that G-rich sequences of telomere DNA can adapt unusual DNA secondary structures (*1*, *24*). While most of the telomeric repeats are contained within the duplex region, the extreme ends consist of a single stranded overhang averaging 200 nucleotides in length (*28*). Eventually, the formation of a G-quadruplex structure in the portion of single-stranded overhang of telomeric repeats could interfere with in vivo addition of telomeric repeats to a chromosome end by facilitating the dissociation of products from telomerase.

In addition to the effect of K^+ on the processivity, to confirm the effect of K^+ on the catalytic rate of polymerization reaction, telomerase reactions were performed in the presence of dATP and dTTP without dGTP as nucleotide substrates. This experiment confirmed the idea that K^+ increases the turnover rate of the telomerase enzyme during the reaction, since the catalytic activity in terms of dAMP incorporation by telomerase into the primer was significantly stimulated by K^+ in a concentration-dependent manner. The K^+ effect can also be produced by Na^+, but not by other cations (data not shown), in accord with a previous observation that Na^+ can also induce intramolecular folding of single-stranded telomeric DNA containing more than four repeats (*24*). In addition, the banding patterns of telomerase reaction products in the presence of high K^+ concentrations are very similar to those generated in the reaction with BSU-1051 and TMPyP4, known G-quadruplex interactive agents (*29*, *30*). A major difference observed was that there is no significant effect of BSU-1051 or TMPyP4 on the first and second telomerase-catalyzed extension of the 18-mer primer, while K^+ significantly stimulates the first and second telomerase-catalyzed extension. In sharp contrast, third and higher telomerase-catalyzed extensions are significantly inhibited (>50%) in the presence of 10 μM of BSU-1051 or TMPyP4. Thus, BSU-1051 and TMPyP4 appear to function as low molecular weight mimics of K^+ in stabilizing the G-quadruplex folding structure and, consequently, inhibiting telomerase activity (*29*, *30*).

Taken together, these data suggest that both the catalytic activity and processivity of human telomerase are modulated through unique mechanisms, which function in different ways. These results may be useful in future studies about the biochemical aspect of the telomerase reaction and the molecular mechanism of potential telomerase inhibitors.

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