Effect of dGTP Concentration on Human and CHO Telomerase†

Ira P. Maine,‡,§ Shih-Fong Chen,||,⊥ and Bradford Windle*,‡

Department of Cancer Biology and Department of Experimental Therapeutics, The Institute for Drug Development, The Cancer Therapy & Research Center, 8122 Datapoint Drive, #710, San Antonio, Texas 78229

Received July 9, 1999; Revised Manuscript Received September 22, 1999

ABSTRACT: Human telomerase produces a long ladder of six-base repeat additions to a primer, while CHO telomerase primarily adds only one or two repeat additions to a primer. Under the standard assay conditions, the concentration of dGTP is very low, so we investigated the effects of increasing dGTP concentration on human and CHO telomerase activities. Increasing dGTP concentration over a range of 1.5−50 μM caused the human telomerase to produce longer primer extension products until products were so large that no ladder pattern was apparent. Increasing dGTP concentration resulted in CHO telomerase producing one to eight repeat additions, though still not as many repeats as produced by human telomerase even under low dGTP conditions. CHO telomerase produced a six-base ladder pattern comparable to human telomerase only after raising the dGTP concentration to 500 μM under conditions in which the dATP concentration was low. Primer challenge experiments showed the human telomerase exhibited ∼100% processivity at both low and high concentrations of dGTP, and thus increasing dGTP concentration appeared to affect only the extension rate. In contrast, CHO telomerase exhibited low processivity under low concentrations of dGTP and increased processivity at higher dGTP concentrations. One explanation for the low processivity of CHO was found in CHO telomerase's inability to extend the GGTTAG permuted primer under nonprocessive conditions, while able to extend the other five permuted primers. Competition studies of different permuted primers indicated that the GGTTAG primer cannot interact with the nonprocessive CHO telomerase. A model is proposed for explaining the nonprocessive behavior of CHO telomerase.

The ribonucleoprotein complex telomerase is a telomere terminal transferase that has been detected in the majority of cancer cell types, as well as in germline cells (*1, 2*), yet is lacking from many normal somatic cells. Mammalian telomerase catalyzes the addition of TTAGGG repeats to telomeric sequences. This activity is involved in maintaining telomeres and chromosome stability (*3*). Telomerase expression has been shown to be responsible at least in part for the immortalization of mammalian cells (*4*−*8*).

Telomerase synthesizes the TTAGGG repeats using an RNA[1] template that contains a partially redundant sequence complementary to TTAGGG. Telomerase extends the primer until reaching the end of the template sequence. The primer is then unwound from the template and either dissociated from the nonprocessive telomerase or held by the processive telomerase for re-priming at the beginning of the template (*9*−*12*). Human telomerase extends a primer in a processive manner, producing the characteristic six-base ladder pattern when analyzed by polyacrylamide gel electrophoresis. Mouse telomerase produces one or two repeat additions under standard assay conditions, consistent with a mostly nonprocessive activity (*13, 14*).

There is currently a poor understanding of what determines whether telomerase is processive or nonprocessive. Nothing is known of the biological significance of a cell having processive versus nonprocessive telomerase. Our investigations focused on the nonprocessive hamster telomerase with comparison to the processive human telomerase. The studies presented in this paper show how the dGTP concentration can dramatically affect processivity in addition to the rate of primer extension. Our results of primer utilization provide an explanation for how telomerase can act nonprocessive.

EXPERIMENTAL PROCEDURES

Telomerase Preparation. Telomerase extracts were prepared from the CHO cell line UA21 and from 293S cells (a human immortal embryonal kidney cell line) by using a freeze−thaw procedure. UA21 cells were grown to 80% confluence in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), and 293S cells were grown in suspension to ∼5 × 10^5 cells/mL in MEM (Joklik modified; Sigma) with 10% FBS. Cells were harvested and washed in cold washing buffer [10 mM *N*-(2-hydroxyethyl)piperazine-

† This work was supported by Sanofi-Winthrop, NCI Grant 1U19CA67760, and NCI Grant P30CA54174.

* To whom correspondence should be addressed. Phone: (210) 616-5872. FAX: (210) 692-7502.

‡ Department of Cancer Biology.

§ Current address: University of Michigan, PATH 7520 MSRB1, 1150 W. Medical Center Rd., Ann Arbor, MI 48109.

|| Department of Experimental Therapeutics.

⊥ Current address: Piedmont Research Center, 860 Aviation Parkway, Suite 700, Morrisville, NC 27560.

[1] Abbreviations: TRAP, telomere repeat amplification protocol; MEM, minimal essential media; FBS, fetal bovine serum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; U, unit(s); DEAE, diethylaminoethyl; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dTTP, deoxythymidine triphosphate.

10.1021/bi991596+ CCC: $18.00
Published on Web 10/29/1999

N′-2-ethanesulfonic acid, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol] and centrifuged at 6000*g* for 2 min at 4 °C in an AM50.14 rotor (Jouan). The cells were then resuspended in cold lysis buffer [10 mM Tris-HCl, pH 7.2, 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM PMSF, 23 μM pepstatin (Sigma), and 1 μM leupeptin (Sigma)] at a cell density of 5×10^7 cells/mL and subjected to five freeze−thaw cycles in liquid nitrogen and 37 °C water. RNAGuard was then added to the mixture (40 U/mL), and the cell extracts were produced from the supernatant obtained by centrifuging the cell debris and cell nuclei at 15000*g* for 30 min at 4 °C in an AM2.21 rotor (Jouan).

Telomerase from hamster and human cells was partially purified by ion exchange chromatography on DE52 diethylaminoethyl cellulose (DEAE; Whatman) preequilibrated in elution buffer (10 mM Tris-HCl, 5 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM PMSF). Telomerase was eluted with a 0−1.0 M KCl gradient in elution buffer. Fractions with telomerase activity were pooled, dialyzed against elution buffer, frozen in 10% glycerol in liquid nitrogen, and stored at −70 °C.

Telomerase Assay. The telomerase assay conditions were similar to those used in previously published procedures (*15*). A standard 40 μL reaction typically contained 20 μL of purified telomerase from the CHO or 293S cells, with a reaction buffer of 50 mM Tris−OAc, pH 8.5, 50 mM K−OAc, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 mM $MgCl_2$, 2 mM dATP, 2 mM dTTP, 1.56 μM [α-^{32}P]dGTP (800 Ci/mmol), and 1.5 μM 5′-biotin-labeled telomeric oligonucleotide primer. The reactions were incubated for 1 h at 30 °C, followed by the addition of 50 μL of stop solution (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 100 mg/mL RNase A). The biotinylated primers were isolated by binding to M280 streptavidin magnetic beads (Dynal) and magnetic separation. The biotin-primers were eluted from the beads using gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.02% xylene cyanol) and heated for 5 min at 70 °C. The free beads were removed by magnetic separation. The oligonucleotide products of the telomerase reaction were separated by electrophoresis on an 8% denaturing polyacrylamide sequencing gel. The incorporation of ^{32}P was detected by Phosphor-image analysis (Molecular Dynamics) and X-ray film (Kodak) autoradiography.

RESULTS

Telomerase Synthesizes Longer Products with Increasing dGTP Concentration. Human and CHO telomerases exhibit different patterns of primer extension under standard assay conditions. Figure 1 shows the long six-base ladder produced by the human telomerase (lane 1). CHO telomerase shows extensions primarily to the first repeat (lane 2), though many times there is activity in the second repeat, and occasionally a faint yet long six-base ladder. As for human telomerase activity, the CHO telomerase activity was sensitive to pretreatment with RNase as shown in lane 3, consistent with the RNA-dependent telomerase. The inability of the CHO telomerase to efficiently produce a long ladder is similar to mouse telomerase (*14*), which has been described as nonprocessive. These patterns of activity for human and CHO

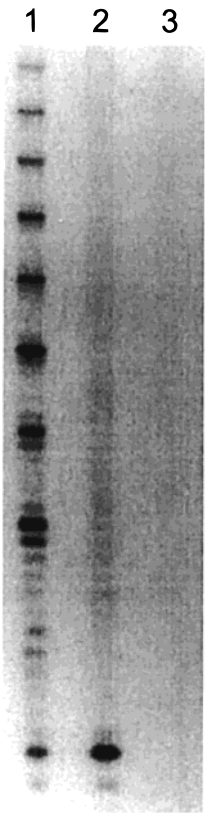

FIGURE 1: Comparison of human and CHO telomerases. Telomerase activity from HeLa cells and CHO cells was analyzed by incorporation of ^{32}P-labeled dGTP and separation by polyacrylamide gel electrophoresis. Lane 1 shows the telomerase activity from HeLa as multiple repeat extensions in a six-base ladder of bands, and lane 2 shows the telomerase activity from CHO cells as just one repeat extension in a single band. Lane 3 shows the absence of activity for the CHO telomerase preparation pretreated with RNase.

telomerases were observed in the standard telomerase assay reaction, which contains 1.56 μM dGTP. The low concentration is due to the dGTP being the ^{32}P-labeled nucleotide and there being a practical limit to how much can be added to a reaction. It is possible that the unusually low concentration of dGTP contributes to the appearance of the particular ladder sequences added by the telomerases. We investigated how increasing the dGTP concentration would affect human and CHO telomerase activity and their properties.

Human telomerase exhibited a dramatic size increase in the labeled products as dGTP concentration was increased from 1.56 to 50 μM (Figure 2A, lanes 1−5). Unlabeled dGTP was used to increase the dGTP concentration, and, therefore, the specific activity of the label was reduced. This accounts for the total ^{32}P signal incorporated going down as the dGTP concentration was increased. Under 50 μM dGTP conditions, the ladder was no longer visible, and only a smear of bands greater than 500 bp was visible. Lanes 6 and 7 show the same reactions as in lanes 1 and 5 but pretreated with RNase in which the activity is eliminated, consistent with telomerase. We confirmed that the longer labeled primers produced at 50 μM dGTP progressed from the six-base ladder by following a time course from 1 to 60 min (Figure 2B). The mean length of the extended primers was determined from plotting extension rates against dGTP concentrations in a double reciprocal plot to reveal a K_m of 4 μM (data not shown). The rate of polymerization at 50 μM dGTP was

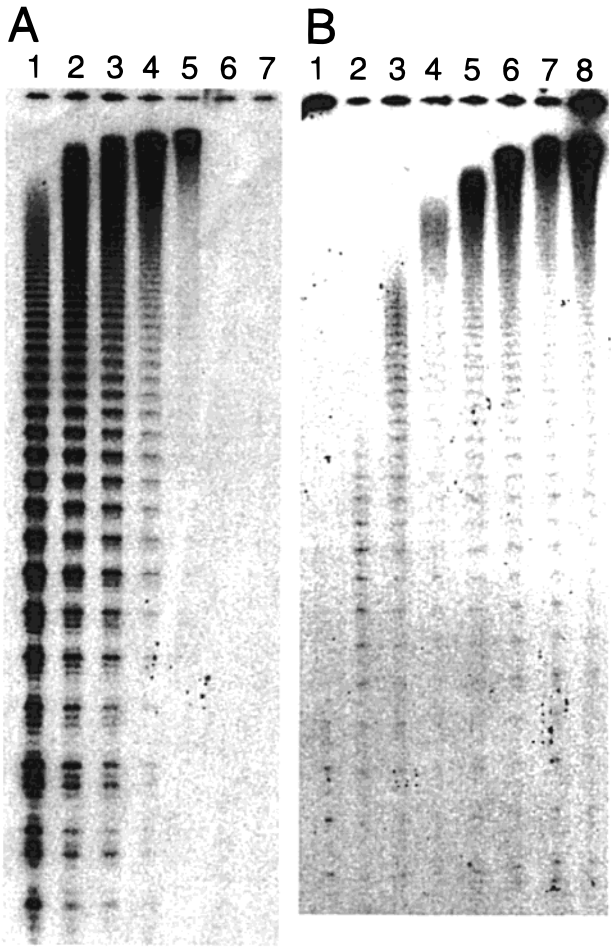

FIGURE 2: Effect of dGTP concentration on human telomerase activity. (A) Human telomerase was incubated with $(TTAGGG)_3$ primer at constant dATP and dTTP concentrations, and increasing dGTP concentrations. Telomerase activity was analyzed by incorporation of ^{32}P-labeled dGTP and separation by polyacrylamide gel electrophoresis. The final concentrations of dGTP in lanes 1−5 were 1.56, 4, 8, 15, and 50 μM, respectively. Lanes 6 and 7 had 1.56 and 50 μM dGTP plus pretreatment with RNase. (B) Time course for telomerase activity with the $(TTAGGG)_3$ primer at 50 μM dGTP. Incubation times were 1, 3, 6, 10, 15, 30, and 60 min.

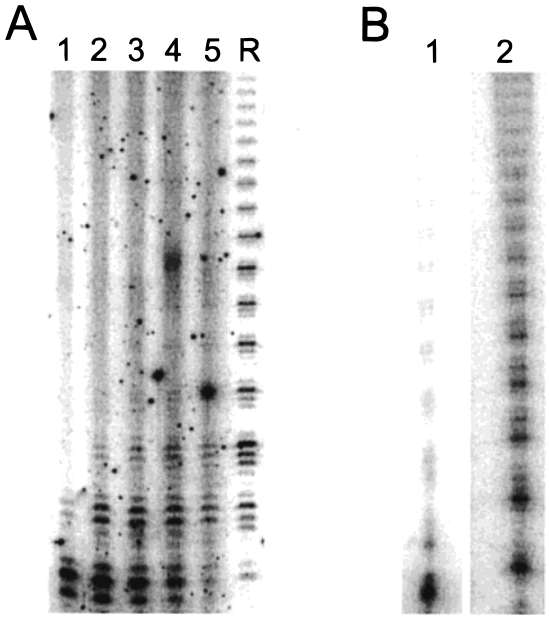

FIGURE 3: Effect of increasing dGTP concentration on CHO telomerase activity. (A) CHO telomerase was incubated with $(TTAGGG)_3$ primer at constant dATP and dTTP concentrations, and increasing dGTP concentrations. Telomerase activity was analyzed by incorporation of ^{32}P-labeled dGTP and separation by polyacrylamide gel electrophoresis. The final concentrations of dGTP in lanes 1−5 were 1.56, 4, 8, 15, and 50 μM, respectively. The right lane (R) shows human telomerase activity for reference, under 1.56 μM dGTP conditions, the same conditions as in lane 1. (B) CHO telomerase was incubated with $(TTAGGG)_3$ primer at 1.56 μM dGTP, using dGTP as the ^{32}P-label, shown in lane 1. Lane 2 shows a long ladder of repeat additions from CHO telomerase activity at 500 μM dGTP and 1.56 μM dATP, using dATP as the ^{32}P-label.

calculated to be an average of ~50 bases per minute, which is significantly faster than the rate of 1−2 bases per minute at 1.56 μM dGTP but is still relatively slow.

The analysis of CHO telomerase with increased dGTP concentrations also demonstrated an increase in product size. Figure 3A shows that the increased dGTP concentration results in CHO telomerase no longer stopping at one or two repeat additions (lane 1) but adding as many as four to eight repeat additions (lanes 4 and 5). However, even at 50 μM dGTP, CHO telomerase did not produce the long six-base ladder observed for human telomerase. The dGTP concentration could not be increased beyond 50 μM without reducing the specific activity of the label to the point where signal could not be detected. We therefore used a different strategy for increasing the dGTP concentration. We switched the ^{32}P-labeled nucleotide from dGTP to dATP. This allowed us to increase the dGTP concentration to 500 μM; however, now the dATP concentration is at 1.56 μM. Figure 3B shows CHO telomerase activity under the two labeling conditions (low [dGTP] and high [dGTP] but low [dATP]). Lane 1 shows CHO telomerase activity under 1.56 μM dGTP conditions compared to the long ladder of bands from CHO telomerase activity at 500 μM dGTP using the $(TTAGGG)_3$ primer in lane 2. This result indicates that CHO telomerase is capable of producing a long ladder similar to human telomerase. Merely the choice of labeled nucleotides made the difference in categorizing the type of telomerase activity. Human telomerase, in contrast, did not show a dramatic change in the ladder length when high [dGTP] and low [dATP] were used. One similarity between CHO telomerase and human telomerase was the interesting two-base shift in the pausing site at high dGTP and low dATP conditions (compare the positions of the starting bands in lanes 1 and 2), as previously observed for human telomerase (*16*).

High dGTP Concentration Increases Telomerase Extension Rate and Processivity. Two explanations could account for the increase in the product lengths accompanying the high dGTP reaction conditions. The longer extension products could merely result from an increase in the extension rate of telomerase as would be expected when a rate-limiting substrate's concentration is increased. However, the longer products could, in addition, result from an increase in the processivity of telomerase. We investigated the effects of dGTP concentration on processivity by performing a competitive primer challenge experiment. This experiment involved using a biotinylated $(TTAGGG)_3$ telomerase substrate primer that could be isolated from an oligonucleotide competitor for analysis of activity. The approach of using a ^{32}P-end-labeled substrate primer was not successful apparently due to insufficient telomerase activity (data not shown).

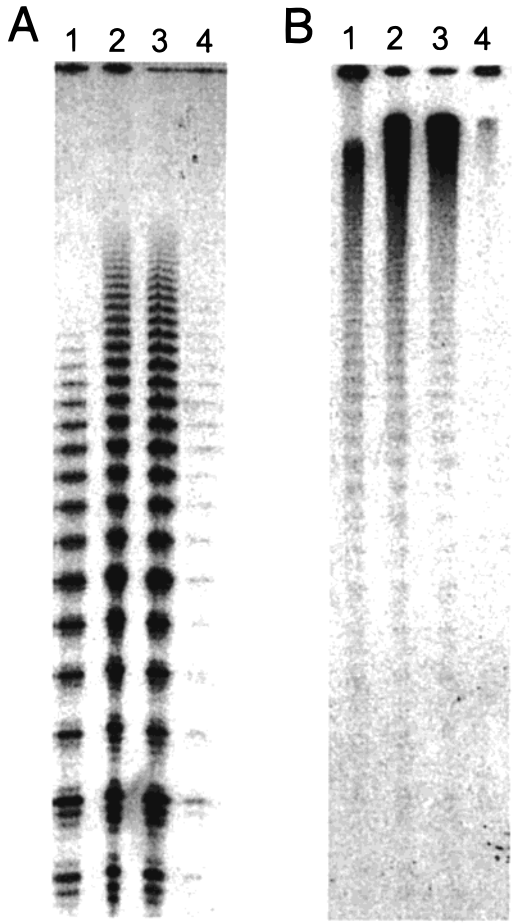

FIGURE 4: Primer competition study of human telomerase. (A) Human telomerase activity was analyzed using a biotinylated $(TTAGGG)_3$ primer at 1.56 μM dGTP after a 15 min incubation, as shown in lane 1. The activity shown in lanes 1−4 is only from the biotinylated primer at the saturating concentration of 1.5 μM. Lane 2 shows longer products and more signal after a 30 min incubation under the same conditions. Lane 3 shows no effect of adding a nonbiotinylated competitive primer, $(TTAGGG)_3$, at 20 μM, 15 min into a 30 min reaction. Lane 4 shows the small amount of telomerase activity visible after 30 min when 20 μM competitor primer is present at the beginning of the incubation, and demonstrates the competitive ability of the competitor primer. (B) Human telomerase activity was analyzed using a biotinylated $(TTAGGG)_3$ primer at 50 μM dGTP after a 15 min incubation, as shown in lane 1. Lane 2 shows longer products and more signal after a 30 min incubation under the same conditions. Lane 3 shows no effect of adding a nonbiotinylated competitive primer, $(TTAGGG)_3$, at 20 μM, 15 min into a 30 min reaction. Lane 4 shows the small amount of telomerase activity visible after 30 min when 20 μM competitor primer is present at the beginning of the incubation.

The rationale for this experiment is that if telomerase acts processively, then once bound to the original biotinylated primer it would continue to extend that primer even when excess primer competitor with no biotin is subsequently added. If telomerase acts nonprocessively, then the primer competitor would reduce or eliminate further incorporation of label and extension to the original biotinylated primer by competing for the free telomerase that turns over.

Figure 4A shows this experiment conducted with human telomerase under 1.56 μM dGTP reaction conditions. Lane 1 shows the amount of signal and lengths of products after 15 min. This incubation allows telomerase to attach to the primer and begin extending. An additional 15 min incubation resulted in more signal and longer products (lane 2). The additional incorporation of label and the further extension of the primer represent telomerase activity from both nonprocessive and processive activities. Adding a 13-fold excess of $(TTAGGG)_3$ competitor DNA 15 min into the reaction, followed by another 15 min incubation, still resulted in the increased signal and longer products specific for the biotinylated primer (lane 3). This additional activity and extension of the primer represents essentially only the processive telomerase activity. If telomerase was synthesizing DNA nonprocessively with a distributive mode of action, then the excess competitor primer would have interfered with the continued extension of the biotinylated primer. The ratio (or percentage) of the processive activity to the total processive plus nonprocessive activity is a rough measure of the processivity. This analysis of human telomerase at low dGTP concentrations indicated that approximately 100% of the telomerase activity is processive. A reaction in which the excess competitor primer is added simultaneously with the biotinylated primer demonstrates that the competitor primer effectively competes with the biotinylated primer, reducing most activity (lane 4). Use of two nontelomeric sequence primers, 5′TGACACTAGTCTTGTACA and 5′TGTAGCCAACGCCATGCC, showed very little or no telomerase activity, respectively, and they were incapable of competing for CHO telomerase or human telomerase (data not shown). However, not all primers lacking TTAGGG repeats are inactive with telomerase. The primer used in the telomere repeat amplification protocol (TRAP) assay, 5′AATCCGTCGAGCAGAGTT, is an excellent substrate and competitor for telomerase (*1*).

Similar results were observed when the competition experiment was conducted at 50 μM dGTP (Figure 4B). Signal within a smear was evident after the 15 min incubation (lane 1). An additional 15 min resulted in more signal in longer products (lane 2), and this was not blocked by the excess competitor (lane 3). The results of these experiments indicate that human telomerase is ~100% processive at both low and high dGTP concentrations. Therefore, the longer primer extensions under increased dGTP concentrations were due only to an increase in extension rate by human telomerase.

The fact that 100% of the telomerase enzyme molecules remain bound to the original primer during the reaction time indicates that there is one active enzyme per extended primer. We can take advantage of this observation to estimate the number of active telomerase molecules present. Thus, if we calculate the number of extended primers, we can then calculate how many active molecules of telomerase are present in an extract of 10^6 cells. The number of extended primer molecules was calculated from the average length of the extended primers and the amount of dGTP incorporated. Using these calculations, we estimate that there are approximately 10 active telomerase molecules per cell.

CHO telomerase activity was also analyzed under low and high dGTP concentrations. Figure 5A shows that after 15 min, the expected products were observed under 1.56 μM dGTP conditions (lane 1), which increased in signal intensity and increased in length to some extent after an additional 15 min (lane 2). The excess competitor primer eliminated most of the increase in signal during the additional 15 min (lane 3). Therefore, CHO telomerase does not display significantly high processivity under 1.56 μM dGTP condi-

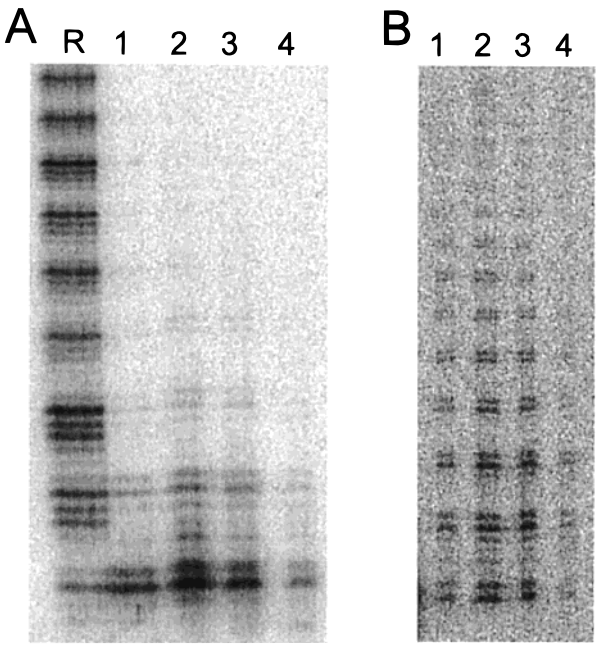

FIGURE 5: Primer competition study of CHO telomerase. (A) CHO telomerase activity was analyzed using a biotinylated $(TTAGGG)_3$ primer at 1.56 μM dGTP after a 15 min incubation, as shown in lane 1. Human telomerase activity is shown in the left lane (R) for reference. The activity shown in lanes 1−4 is only from the biotinylated primer at the saturating concentration of 1.5 μM. Lane 2 shows slightly longer products and more signal after a 30 min incubation. Lane 3 shows the effect of adding a nonbiotinylated competitive primer, $(TTAGGG)_3$, at 20 μM, 15 min into a 30 min reaction, in which a reduction in activity is observed compared to lane 2. Lane 4 shows the small amount of telomerase activity visible after 30 min when 20 μM competitor primer is present at the beginning of the incubation. (B) CHO telomerase activity was analyzed using a biotinylated $(TTAGGG)_3$ primer at 50 μM dGTP after a 15 min incubation, as shown in lane 1. Lane 2 shows longer products and more signal after a 30 min incubation. Lane 3 shows the small effect of adding a nonbiotinylated competitive primer, $(TTAGGG)_3$, at 20 μM, 15 min into a 30 min reaction. Lane 4 shows the small amount of telomerase activity visible after 30 min when 20 μM competitor primer is present at the beginning of the incubation.

tions, in contrast to human telomerase. The measure of processivity was determined to be ~8% for CHO telomerase under low dGTP concentrations. This amount of activity is approximately the expected amount of activity not competed by a 13-fold excess of competitor primer. Figure 5B shows that at 50 μM dGTP, extension products of multiple repeat additions were produced after 15 min (lane 1). An additional 15 min resulted in more signal and longer products (lane 2), which could not be completely blocked by the competitor primer (lane 3). Analysis of processivity indicates that the CHO telomerase is ~50% processive under high dGTP concentrations. Therefore, increasing dGTP concentrations increase processivity of CHO telomerase.

CHO Telomerase Fails To Extend a DNA Primer That Ends with the Sequence 5′-(GGTTAG)-3′ under Nonprocessive Conditions. Telomerase has the novel feature that the number of bases added within the first unit of extension to a telomeric primer and its extended ladder is determined by the sequence at the 3′ end of the primer (*17*). Therefore, telomeric primers of the same size but with circularly permuted repeats are extended to different lengths by telomerase. The CHO telomerase was analyzed for this telomerase-specific property. A set of six biotinylated circularly permuted primers based on the telomeric six-base repeat was used in the CHO telomerase reaction, $(TTAGGG)_3$, $(TAGGGT)_3$, $(AGGGTT)_3$, $(GGGTTA)_3$, $(GGTTAG)_3$, and $(GTTAGG)_3$. Because each of the primers is the same length, termination of DNA elongation at the same nucleotide position relative to the internal telomerase RNA template will result in products whose sizes vary by one base as presented in Figure 6A. Therefore, all extensions should end with the sequence 5′GGTTAG3′ stopping at the first G residue of the repeat, the stopping or pausing site for human and mouse telomerase (*14*, *15*). The expected "step ladder" pattern was observed with CHO telomerase under 1.56 μM dGTP (lanes 1−6), but with one exception. The $(GGTTAG)_3$ primer did not appear to be extended as seen by the lack of signal in lane 5. The expected band for this extended primer is six bases longer than the primer and would be the longest of the extensions. The lack of signal for the $(GGTTAG)_3$ primer is even more significant when one considers that the six-base extension should have incorporated 3 times as many radiolabeled G residues as the first four primers (lanes 1−4). The $(GTTAGG)_3$ primer signal intensity (lane 6) was also consistently less than that of the first four primers. The reduced signal for the $(GTTAGG)_3$ primer was also significant because its extension should have incorporated twice as many radiolabeled G residues as each of the first four primers. Quantitation of the signal of the expected bands from CHO telomerase-mediated extension of the permuted primers under nonprocessive conditions is presented in Figure 6B. These data show that the primer that ends 3′ with GGTTAG is consistently extended with poor efficiency, averaging only ~4% of the activity of the TTAGGG primer under nonprocessive conditions.

The same analysis with the circularly permuted primers was conducted under the conditions of 500 μM dGTP and 1.56 μM dATP in which CHO telomerase is more processive. We found significantly different results compared to nonprocessive conditions. The long six-base ladder was observed, as expected under the high dGTP concentration, and each permuted primer was extended equally (Figure 6C, lanes 1−6). Therefore, the $(GGTTAG)_3$ primer was capable of being extended by CHO telomerase, indicating that the nonprocessive CHO telomerase selectively extends the other primers over the $(GGTTAG)_3$ primer.

The GGTTAG Primer Does Not Interact with CHO Telomerase under Nonprocessive Conditions. The GGTTAG primer may not get extended by the CHO telomerase because (1) the primer anneals with the template but polymerization does not occur, or (2) the primer does not anneal with the template or bind to telomerase to allow polymerization. We investigated these two possibilities by devising an experiment using the competition of two circularly permuted primers. We can monitor telomerase activity on each permuted primer simultaneously because the product size for each primer is different. The binding and extension of one permuted primer by telomerase would be expected to compete for the binding and extension of another permuted primer. We confirmed this by monitoring telomerase activity using the $(TTAGGG)_3$ primer at a saturating concentration of 1.5 μM as we increased the competing permuted $(GGGTTA)_3$ primer concentration from 0.15 μM up to 20 μM (Figure 7A). The $(TTAGGG)_3$ primer is extended to a 22-base product ending

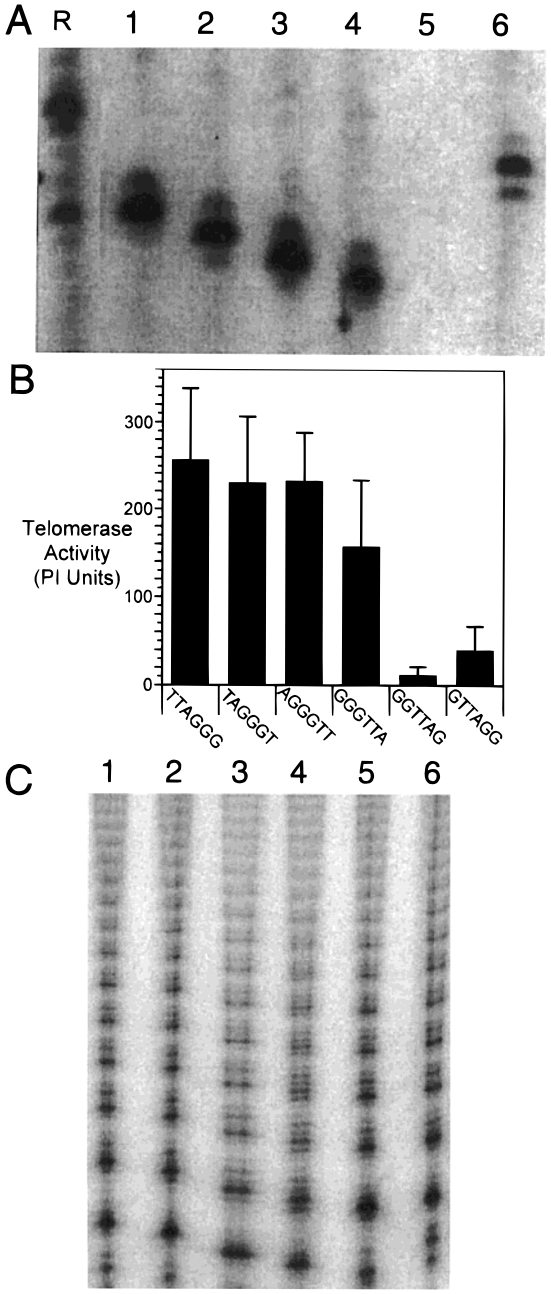

FIGURE 6: CHO telomerase activity using permuted primer. (A) CHO telomerase activity was analyzed under 1.56 μM dGTP conditions using the 6 circularly permuted telomeric primers, each 18 bases in size. The left lane (R) shows human telomerase activity with the $(TTAGGG)_3$ primer for reference. Lane 1 contains CHO telomerase activity using the $(TTAGGG)_3$ primer. Lane 2 contains CHO telomerase activity using the $(TAGGGT)_3$ primer. Lane 3 contains CHO telomerase activity using the $(AGGGTT)_3$ primer. Lane 4 contains CHO telomerase activity using the $(GGGTTA)_3$ primer. Lane 5 contains CHO telomerase activity using the $(GGTTAG)_3$ primer. Lane 6 contains CHO telomerase activity using the $(GTTAGG)_3$ primer. (B) The amount of labeled dGTP incorporated into the products shown in panel A was quantitated. A bar graph is shown in which the average signal from six separate analyses was determined and plotted. The repeat sequence is shown below each bar. The amount of activity was normalized for the number of labeled G residues incorporated. The error bars represent the standard deviation for each of the six values. PI Units are units from the phosphor-imager analysis. (C) CHO telomerase activity was analyzed using the six circularly permuted telomeric primers under 500 μM dGTP and 1.56 μM dATP conditions in which CHO telomerase is more processive (lanes 1−6). Activity for all primers including the $(GGTTAG)_3$ primer was evident.

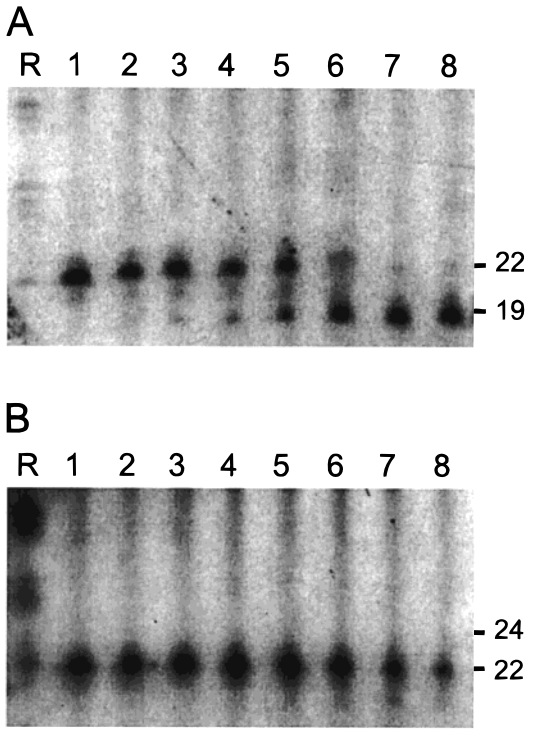

FIGURE 7: Permuted primer competition study with CHO telomerase. (A) CHO telomerase activity under 1.56 μM dGTP conditions is shown using the $(TTAGGG)_3$ primer with the $(GGGTTA)_3$ primer in competition. All lanes contain the $(TTAGGG)_3$ primer at 1.5 μM. The left lane (R) shows human telomerase activity for reference. Lane 1 has only the $(TTAGGG)_3$ primer, which results in a 22-base product. Lanes 2−8 show the effect of increasing amounts of the $(GGGTTA)_3$ primer, which results in a 19-base product. Lanes 1−8 contain the $(GGGTTA)_3$ primer at the following respective concentrations: 0, 0.15, 0.3, 0.75, 1.5, 5.0, 15.0, and 20.0 μM. The positions for the 19- and 22-base products are shown on the right. (B) CHO telomerase activity under 1.56 μM dGTP conditions is shown using the $(TTAGGG)_3$ primer with the $(GGTTAG)_3$ primer in competition. All lanes contain the $(TTAGGG)_3$ primer at 1.5 μM. The left lane (R) shows human telomerase activity for reference. Lane 1 has only the $(TTAGGG)_3$ primer which produces the 22-base product. Lanes 2−8 show the effect of increasing amounts of the $(GGTTAG)_3$ primer which should result in a 24-base product. Lanes 1−8 contain the $(GGTTAG)_3$ primer at the following respective concentrations: 0, 0.15, 0.3, 0.75, 1.5, 5.0, 15.0, and 20.0 μM. No 24-base product is visible in lanes 1−8. The positions for the 22- and 24-base products are shown on the right.

with TTAG (lane 1). Extension of the $(GGGTTA)_3$ primer yields a 19-base product clearly distinguishable from the 22-base product. As the $(GGGTTA)_3$ primer concentration was increased (lanes 2−8), the signal from the 22-base band specific for the $(TTAGGG)_3$ primer proportionally decreased as the signal for the 19-base band specific for the $(GGGTTA)_3$ primer proportionally increased. The telomerase activity on both permuted primers could be easily be detected when the primer concentrations were equal in concentration. At 20 μM, the $(GGGTTA)_3$ primer produced the maximum signal while virtually no signal can be seen for the $(TTAGGG)_3$ primer (lane 8). These results demonstrate that the two permuted primers compete for an initial binding site or active site of telomerase.

The same experiment was performed with the $(TTAGGG)_3$ primer and the $(GGTTAG)_3$ primer as competitor. The telomerase band specific for the $(GGTTAG)_3$ primer is expected to be 24 bases long (a 6-base addition). Figure 7B

shows the telomerase activity for the $(TTAGGG)_3$ primer as the $(GGTTAG)_3$ primer concentration was increased to 20 μM. The results show that there was very little effect of adding the $(GGTTAG)_3$ primer on the activity specific for the $(TTAGGG)_3$ primer, even when the $(GGTTAG)_3$ primer was at 13-fold excess at 20 μM (lane 8). There was also no detectable telomerase activity specific for the $(GGTTAG)_3$ primer even at 20 μM, consistent with data from Figure 6A.

We addressed the possibility that the $(GGTTAG)_3$ primer was slow to form a complex with telomerase by pre-reacting the primer under 1.56 μM dGTP assay conditions for 1 h with the CHO telomerase before assaying activity with the $(TTAGGG)_3$ primer. However, even under these conditions, the $(TTAGGG)_3$ primer was extended to its 22-base product with the same activity as for the mock pre-reaction (data not shown). Therefore, these experiments demonstrate that the nonprocessive CHO telomerase does not recognize the $(GGTTAG)_3$ primer as it does the other primers.

DISCUSSION

This report shows that the DNA extension rate for human telomerase increases with increasing dGTP concentration. However, even at relatively high concentrations of dGTP, telomerase is still a slow polymerase. The critical and necessary process of unwinding the primer from the template and repositioning of the primer for re-priming is likely to be the rate-limiting step that accounts for the slow rate. Considering all the steps involved in DNA synthesis, the increase in [dGTP] may affect not only the rate of incorporation of the nucleotide, but also the primer translocation process. This possible effect is supported by a report by Hammond and Cech (*18*) that showed increasing dGTP concentrations facilitated dissociation of dideoxy-blocked DNA primers from *Euplotes* telomerase template. The authors postulated that dGTP may stimulate the translocation step by increasing the unwinding rate of extended primers from the template. This effect of dGTP may also apply to human and CHO telomerases to account for the increase in extension rate and processivity we observed.

Human telomerase was found to be virtually 100% processive at low dGTP concentrations, while the CHO telomerase was mostly nonprocessive. The increase in dGTP concentration resulted in CHO telomerase becoming more processive as human telomerase remained processive. Our results for CHO telomerase agree with another study using *Euplotes* telomerase that showed increasing dGTP concentration caused an increase in processivity at concentrations up to 100 μM (*19*). A similar stimulatory effect of dGTP on processivity was also reported for reconstituted *Tetrahymena* telomerase. It was shown that a reconstituted telomerase activity producing one extension repeat at low dGTP concentration was capable of extending the primer up to five or more repeats under higher dGTP concentrations (*20*). Our analysis of low dATP concentrations showed that, in contrast to low dGTP concentrations, both human and CHO telomerases were processive.

Our studies of the extension of circularly permuted primers by CHO telomerase elucidated one possible explanation for the nonprocessive state of CHO telomerase. We found a striking and consistent inability of nonprocessive CHO telomerase to efficiently extend the primer ending with the first G residue in the TTAGGG sequence (i.e., GGTTAG) when compared to primers ending with other permutations, such as TTAGGG. CHO telomerase terminates the repeat synthesis of the active primers at the first G residue in the TTAGGG repeat, as does telomerase from human and mouse cells. Therefore, when telomerase unwinds the extended primer and attempts to re-prime for the start of processive synthesis, the same inability to extend a primer ending with GGTTAG should be encountered. The primers would be extended to end with GGTTAG and then become unreactive. This explanation, however, does not exclude the possibility that a slow extension rate could also play a role in limiting the number of repeat extensions by nonprocessive CHO telomerase.

Competition analysis of the GGTTAG primer with the TTAGGG primer showed that the GGTTAG primer does not interact with the CHO telomerase under nonprocessive conditions. Telomerase is speculated to have two primer-interactive sites: a binding site that holds the primer while being translocated, and the site of annealing to the RNA (*21*, *22*). It is possible that the binding site could also utilize a copy of the RNA for facilitating binding, since more than one RNA has been found associated with each telomerase complex in yeast (*23*). Our results suggest that the GGTTAG primer may not interact with the initial binding site, and we propose a lack of annealing with the RNA template sequence as one explanation.

The RNA sequence of the hamster telomerase template region is 5′-UCUAACCCUGAA-3′ and contains only eight contiguous bases capable of base-pairing (underlined) with a TTAGGG repeat primer (*24*). The mouse telomerase also acts nonprocessively and has only eight bases of template sequence (*24*). Interaction of the GGTAAG primer with the 3′ end of the template would potentially allow only 2 base pairs to form for priming. This is in contrast to 3 base pairs for the 9-base template used by the *Tetrahymena* telomerase (*10*) or 5 base pairs for the 11-base template potentially used by the human telomerase (*11*). These 2 base pairs are sufficient for priming since at high dGTP concentrations, the GGTTAG primer does anneal and is extended by CHO telomerase. One possible explanation for a lack of annealing at low dGTP concentrations is that the 3′ end of the nonprocessive telomerase template sequence may not be sufficiently accessible for GGTTAG primer base-pairing. The GTTAGG primer would have 3 base pairs when annealed, and this apparently is accessible and sufficient for priming, though the activity with this primer was relatively low. The other permuted primers would form 4−7 base pairs and appeared to have equal accessibility and activity with the CHO telomerase. Concentrations of dATP would appear not to affect this change in accessibility since even at low dATP concentrations, CHO telomerase appeared very processive. The effect of dGTP is not likely to be related to dGTP binding at the polymerase active site because this step of the primer extension process is before polymerization. We speculate that dGTP has an allosteric effect on the CHO telomerase that changes the accessibility of the template to the GGTTAG and GTTAGG primers. Hammond and Cech (*19*) have also suggested that dGTP may have an allosteric effect of *Euplotes* telomerase, affecting its processivity.

The importance of the dGTP-dependent shift in processivity of the CHO telomerase in the cell is not clear. We

note that normal dNTP concentrations in CHO nuclei range from 1 to 15 μM, with the highest level coinciding with S phase (*25*). It is possible that CHO cells increase the processivity of telomerase during S phase when telomere maintenance is required. Under conditions in which telomerase is nonprocessive, it appears that telomerase might perform a different function by ensuring all telomeric 3′ end sequences terminate with GGTTAG. It may be important for cells to have telomeres that always end with an exact sequence permutation for binding of proteins and telomere functionality.

REFERENCES

1. Kim, N., Piatyszek, M., Prowse, K., Harley, C., West, M., Ho, P., Coviello, G., Wright, W., Weinrich, S., and Shay, J. (1994) *Science 266*, 2011−2015.
2. Shay, J. W., and Bacchetti, S. (1997) *Eur. J. Cancer 33*, 787−791.
3. Blackburn, E. (1992) *Annu. Rev. Biochem. 61*, 113−129.
4. Zhu, J., Wang, H., Bishop, J. M., and Blackburn, E. H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3723−3728.
5. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhutz, A. J. (1998) *Nature 396*, 84−88.
6. Jiang, X. R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D., and Chiu, C. P. (1999) *Nat. Genet. 21*, 111−114.
7. Morales, C. P., Holt, S. E., Ouellette, M., Kaur, K. J., Yan, Y., Wilson, K. S., White, M. A., Wright, W. E., and Shay, J. W. (1999) *Nat. Genet. 21*, 115−118.
8. Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998) *Science 279*, 349−352.
9. Shippen-Lentz, D., and Blackburn, E. H. (1990) *Science 247*, 546−552.
10. Greider, C. W., and Blackburn, E. H. (1989) *Nature 337*, 331−337.
11. Feng, J., Funk, W., Wang, S., Weinrich, S., Avilion, A., Chiu, C., Adams, R., Chang, E., Allsopp, R., Yu, J., Le, S., West, M., Harley, C., Andrews, W., Greider, C., and Villeponteau, B. (1995) *Science 269*, 1236−1241.
12. Singer, M., and Gottschling, D. (1994) *Science 266*, 404−409.
13. Prowse, K., and Greider, C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4818−4822.
14. Prowse, K., Avilion, A., and Greider, C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1493−1497.
15. Morin, G. (1989) *Cell 59*, 521−529.
16. Fletcher, T. M., Salazar, M., and Chen, S. F. (1996) *Biochemistry 35*, 15611−15617.
17. Greider, C. W., and Blackburn, E. H. (1987) *Cell 51*, 887−898.
18. Hammond, P. W., and Cech, T. R. (1998) *Biochemistry 37*, 5162−5172.
19. Hammond, P. W., and Cech, T. R. (1997) *Nucleic Acids Res. 25*, 3698−3704.
20. Collins, K., and Gandhi, L. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8485−8490.
21. Melek, M., Davis, B. T., and Shippen, D. E. (1994) *Mol. Cell. Biol. 14*, 7827−7838.
22. Hammond, P. W., Lively, T. N., and Cech, T. R. (1997) *Mol. Cell. Biol. 17*, 296−308.
23. Prescott, J., and Blackburn, E. H. (1997) *Genes Dev. 11*, 2790−2800.
24. Blasco, M. A., Funk, W., Villeponteau, B., and Greider, C. W. (1995) *Science 269*, 1267−1270.
25. Skoog, L., and Bjursell, G. (1974) *J. Biol. Chem. 249*, 6434−6438.

BI991596+