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Enzymatic Activity of Endogenous Telomerase Associated with Intact Nuclei from Human Leukemia CEM Cells[1]

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Telomerase, a telomere-specific DNA polymerase and novel target for chemotherapeutic intervention, is found in many types of cancers. Telomerase activity is typically assayed using an exogenous primer and cellular extracts as the source of enzyme. Since the nuclear organization might affect telomerase function, we developed a system in which telomerase in intact nuclei catalyzes primer extension. Telomerase activity in isotonically isolated nuclei from human CEM cells shows low processivity (addition of up to four TTAGGG repeats). In contrast, telomerase activity which leaks into a 500 g postnuclear supernatant and the activity in a CHAPS extract are highly processive. The nucleotide inhibitor, 7-deaza-dGTP, seems to be more inhibitory against the nuclei-associated enzyme compared to telomerase from cytoplasmic extracts. However, 7-deaza-dATP and ddGTP are less inhibitory against nuclei-associated telomerase. The results suggest that the association of telomerase with the nuclear chromatin affects telomerase activity. Examination of telomerase activity in a more natural nuclear environment may shed new light on the telomerase function and provide a useful system for the evaluation of new telomerase inhibitors. © 1999 Academic Press

Telomeres are nucleoprotein structures thought to stabilize and protect the ends of eukaryotic chromosomes (1–5) Telomeric DNA comprises several thousand base pair long repetitive DNA sequences with $(TTAGGG)_n$ repeats as the prevailing motif in human cells (6–9)]. Due to the inability of the conventional replication process to synthesize DNA in 3′ to 5″ direction, a number of bases at the 3′ end of each strand are not replicated and each round of replication results in a shortening of the telomeric DNA (10–13). The ability to compensate for telomere shortening may dictate whether cells can continue to divide, senesce, or die.

Telomere length is usually maintained by telomerase, a telomere-specific ribonucleoprotein reverse transcriptase capable of adding telomeric sequence onto the 3′ end of the chromosome, although telomerase-independent telomere maintenance may exist (10, 14, 15). Telomerase activity has been associated with actively dividing cells such as germline and stem cells but is not present in normal somatic cells (16, 17). Since many types of cancer show telomerase activation, telomerase has attracted a considerable attention as a potential target for chemotherapeutic intervention (14, 16–20). For these reasons, an extensive research effort is being carried out to characterize telomerase in various cancers and to identify telomerase inhibitors, which may specifically target cancer cells.

The current knowledge of telomerase catalysis and its sensitivity to inhibitors is based entirely on examining this activity in cell extracts using artificial oligonucleotide template. It is possible, however, that solubilized telomerase in the extracts may not faithfully reflect the activity of an endogenous enzyme in cell nucleus interacting with telomere structures and other chromatin proteins. Telomeres, and possibly telomerase, interact with both the nuclear envelope and the nuclear matrix via telomere-associated proteins (21–24). The effects of such complex interactions on telomerase activity remain unknown.

In this study, we demonstrate that catalysis by the endogenous telomerase activity in nuclei isolated from human CEM cells can be detected using a non-amplification telomerase assay. The nuclei-associated telomerase activity differed from the activity of the

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; 7-deaza-dATP, 7-deaza-2′-deoxyadenosine; 7-deaza-dG, 7-deaza-2′-deoxyguanosine; dNTPs, deoxyribonucleotides; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; MEM, minimal essential medium; PMSF, phenylmethylsulfonyl fluoride; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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enzyme leaking from nuclei in both processivity and response to inhibitors.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were from Genosys (Woodlands, TX), dNTP's and RNA guard from Pharmacia-Upjohn (Kalamazoo, MI), [α-^{32}P]dGTP and [α-^{32}P]dATP from Dupont NEN (Boston, MA), and PMSF, pepstatin A, leupeptin, triton X-100, EGTA, RNase A, and other chemicals from Sigma-Aldrich (St. Louis, MO).

Isolation of intact nuclei. Nuclei isolation was carried out similar to previously published procedures (21–23, 25–27). Human CEM cells were grown to 1×10^6 cells/ml in Joklik's Minimal Essential Medium with 10% fetal bovine serum. Typically, 3×10^8 cells were harvested and washed with cold phosphate buffered saline (10 mM KH_2PO_4/K_2HPO_4, pH 7.5, 140 mM NaCl). All the subsequent steps were carried out at 0-4°C. The cell pellet was resuspended in 15 ml of Nuclei Isolation Buffer (2 mM KH_2 PO_4, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mM EGTA at pH 6.9) and centrifuged at ~200 g for 5 min. The pellet was resuspended in 300 μl Nuclei Isolation Buffer followed by addition of 2.7 ml of Nuclei Isolation Buffer with 0.4% Triton X-100 and 10 U/ml RNAGuard RNase inhibitor Pharmacia-Upjohn (Kalamazoo, MI). The cells were allowed to lyse on ice for 30 min. The lysed cells were gently resuspended and diluted with 15 ml Nuclei Isolation Buffer without Triton X-100. The mixture was centrifuged at $300 \times$ g for 13 min. The pellets containing intact nuclei were resuspended in 900 μl Nuclei Isolation Buffer without Triton X-100, typically resulting in $\sim 1.5 \times 10^8$ nuclei/ml, and used immediately in telomerase reaction.

Telomerase assay using intact nuclei. The telomerase assay was a modification of the standard non-amplification protocol (28–30). The assay mixture (total of 60 μl) was composed of 50 mM Tris-acetate pH 8.5, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 mM $MgCl_2$, 1 mM dATP, 1 mM dTTP, 5 μl [α-^{32}P]dGTP (800 Ci/mmol), 1 μM 5′-biotinylated oligonucleotide primer, $(TTAGGG)_3$, and 10-60 μl of freshly isolated nuclei suspension. To analyze the telomerase activity released into the supernatant, the appropriate amounts of nuclei preparations were centrifuged at $500 \times$ g and the post-nuclear supernatant was gently removed and used in the telomerase reaction instead of nuclei suspension. The reaction mixtures were incubated for 1 h at 30°C or time indicated in time course reactions and stopped by incubation for 5 min in 125 mg/ml RNase A, 10 mM Tris-EDTA, pH 8. The mixtures were centrifuged at 1000*g* for 15 min at 4°C to release primer extension products into the supernatant and remove the nuclei which could interfere in the subsequent steps. The supernatants were incubated for 30 min with 20-30 μl of streptavidin coated beads (Dynal Inc., Lake Success, NY) that were washed with buffer containing 1 M KCl, 40 mM Tris-HCl, 1 mM Na_2EDTA with dGTP. The beads were then washed twice with 150 μl binding and washing buffer followed by 3-5 washes with 150 μl of $2\times$ SSC + 0.1% SDS. Telomerase products were extracted from the beads by incubation at 90°C with 5.7 M guanidinium hydrochloride and ethanol precipitated. The [α-^{32}P]dGTP labeled telomerase products were separated by electrophoresis on an 8% polyacrylamide sequencing gel and detected by X-ray film (Biomax MS, Kodak, Rochester, NY) autoradiography. Telomerase isolation and assay from CHAPS cytoplasmic extracts were performed as outlined previously (28, 29). Telomerase products on X-ray films were quantitated using a laser densitometer and ImageQuant software (Molecular Dynamics San Jose, CA).

RESULTS

Telomerase activity in isotonically isolated nuclei. To examine the catalytic activity of endogenous nuclear telomerase, we employed nuclei isolated under isotonic conditions. Isotonic conditions have been shown to preserve the highly condensed chromatin structures (31–33) and the activity of other nuclear chromatin associated enzymes such as topoisomerases (25–27) and DNA polymerase α (34). We analyzed telomerase activity in isotonic nuclei isolated from human CEM leukemic cells using a direct telomerase assay. This direct assay allows one to assess telomerase processivity, i.e., the actual number of telomeric repeats added to the primer (telomerase "ladder") (28–30). Whereas the commonly used "TRAP" assay based on amplification of the telomerase products also generates a ladder, the size distribution of these amplified products is not related to the original distribution of telomerase additions (35). Telomerase activity was detected in intact nuclei as judged by a ladder of radiolabeled products pertaining to the extension of the 5′-biotinylated $(TTAGGG)_3$ input primer (Fig. 1A). The sites of enzyme pausing, reflected by individual bands in the ladder, corresponded to the additions of 6-base repeat units. Interestingly, the majority of signal observed in intact nuclei corresponded to telomerase products up to 46 bases or $(TTAGGG)_7$ttag (Fig. 1A). Thus, the enzyme associated with nuclei exhibited a relatively low processivity, pausing the primer extension after the addition of up to 4 full telomerase repeats. As expected for telomerase being a ribonucleoprotein, primer extension was completely abolished by RNase A.

In parallel to samples with intact nuclei, telomerase reactions were carried out with supernatant obtained by a gentle centrifugation of nuclear suspensions. A profound telomerase activity was found in this post-nuclear supernatant (Fig. 1A, lane 3). Surprisingly, in contrast to nuclei-associated activity, telomerase in post-nuclear supernatants was highly processive, adding often more than 10-20 telomere repeat units, reminiscent of telomerase from cytoplasmic extracts (Fig. 1B). Most of the leakage of highly processive solubilized enzyme probably occurred during the centrifugation step, given the marginal amount of high molecular weight extension products in reactions with intact nuclei. However, some nuclei preparations showed small levels of higher molecular weight products probably due to the presence of soluble telomerase before centrifugation. It needs to be underscored that the post-nuclear supernatants were always obtained from the same batch of nuclei suspension which was used directly in the extension rections. Thus, enzyme release to supernatants during centrifugation makes it possible to use reactions with post-nuclear supernatants as quasi-internal controls emphasizing differential behaviour of the nuclei-associated and solubilized enzyme.

The reaction time course for the nuclear activity differed slightly from the activity released into post-nuclear supernatant (Fig. 2) which was similar to the

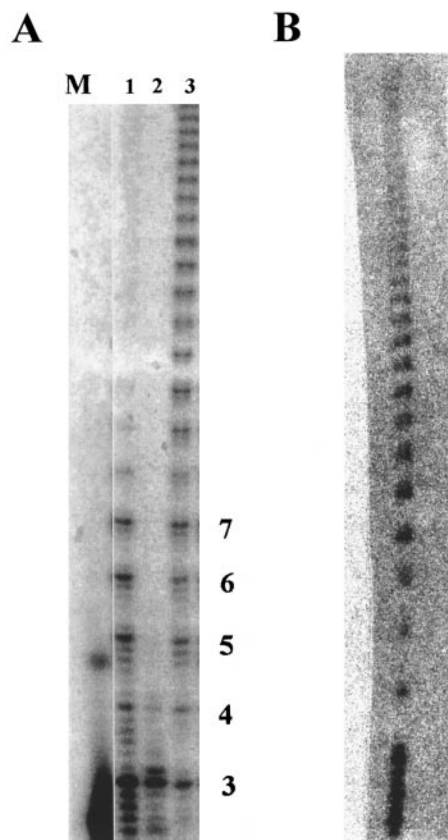

FIG. 1. Telomerase activity in intact nuclei and post-nuclear supernatants. Telomerase extension reactions used the 5′ biotinylated $(TTAGGG)_3$ primer. (A) Reactions with intact nuclei (lanes 1, 2) and post-nuclear extract (lanes 3). Reactions with intact nuclei in the presence (lane 2) of 0.125 μg/μl RNase A. The reactions contained 40 μl of nuclei suspension or post-nuclear supernatant obtained as described under Materials and Methods. Lane marked "M" (19 base marker) shows the position of the 5′ biotinylated $(TTAGGG)_3$ primer used in the reactions that was 3′ labeled with α-^{32}P cordycepin and terminal transferase. The n values beside the gel refer to the number of full repeats in the extension products $(TTAGGG)_n$ttag. (B) Telomerase reaction using CHAPS cytoplasmic extract obtained from CEM cells as previously described (28, 29). The data shown are representative for several independent experiments.

time course of telomerase in cellular extracts ((28–30) and data not shown). The differences in processivity of telomerase associated with intact nuclei were not markedly affected by varying reaction times. Also, increasing the amount of nuclei in the reaction did not increase the processivity of the nuclei-associated enzyme (data not shown). In the case of the post-nuclear supernatant, increasing time resulted in the synthesis of an increasing amount of products with >10 TTAGGG repeats. Moreover, increasing amounts of post-nuclear supernatant produced larger molecular weight products (data not shown).

Telomerase in cytoplasmic extracts tends to pause mainly at the first G of the TTAGGG repeat (29). The comparison of the extension ladder obtained with $(TTAGGG)_3$ input primer and an alternative primer,

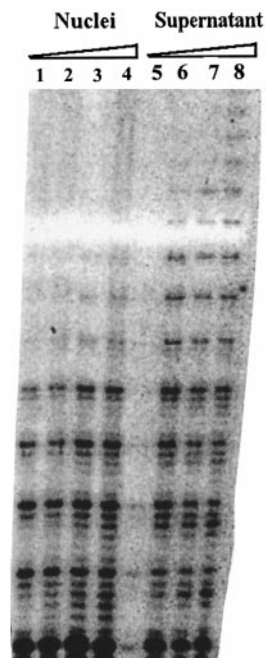

FIG. 2. The time course of telomerase reactions catalyzed by either intact nuclei or postnuclear supernatant. The reactions contained 40 μl of nuclei suspension (lanes 1–4) or 40 μl postnuclear supernatant (lanes 5–8) obtained as described under Materials and Methods. For other details see legend to Fig. 1. Reaction times for were 10 min (lanes 1, 5); 20 min (lanes 2, 6), 30 min (lanes 3, 7), and 60 min (lanes 4, 8).

$(GGGTTA)_3$, confirms that the nuclei-associated activity shares this property of solubilized telomerase (Fig. 3). The products resulting from the use of $(GGGTTA)_3$ as the input primer were three bases shorter than those obtained with the $(TTAGGG)_3$ primer. This pattern corresponds to telomerase pausing after synthesizing $(GGGTTA)_n$g and $(TTAGGG)_n$ttag from $(GGGTTA)_3$ and $(TTAGGG)_3$ respectively (28–30).

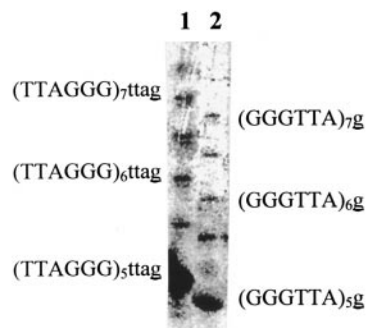

FIG. 3. Telomerase activity from intact nuclei extends both $(TTAGGG)_3$ and $(GGGTTA)_3$. Telomerase reactions contained 40 μl of intact nuclei suspension with either 5′-biotin-$(TTAGGG)_3$ (lane 1) or 5′-biotin-$(GGGTTA)_3$ (lane 2). Sequences of individual extension products consistent with band pattern are indicated in the figure with the terminal G underlined. For other details see legend to Fig. 1.

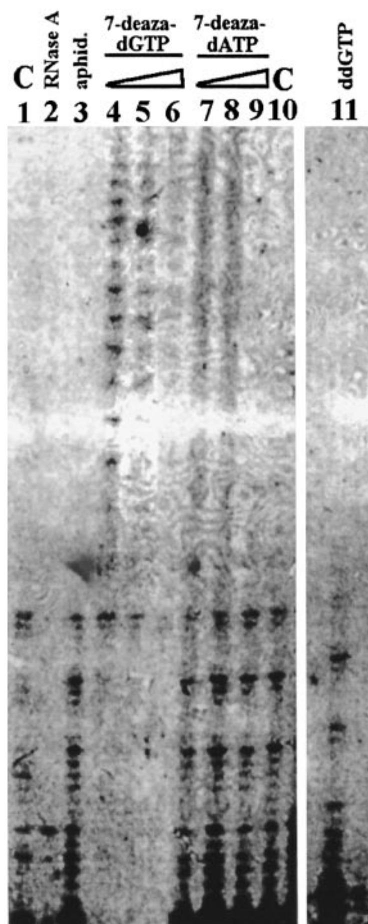

FIG. 4. Effect of the nucleotide analogs, 7-deaza-dGTP, 7-deaza-dATP, ddGTP, and a polymerase α inhibitor, aphidocolin on telomerase activity from intact nuclei. The following additions/inhibitors were used. Lanes 1, 2: control reactions without any inhibitors and with 0.125 μg/μl RNase A, respectively. Lane 3: aphidicolin at 100 μM. Lanes 4–6: 7-deaza-dGTP at 1, 5, and 10 μM, respectively. Lanes 7–9; 7-deaza-dATP at 25, 50, and 100 μM; respectively; and lane 11: ddGTP at 20 μM. For other details see legend to Fig. 1.

Effects of inhibitors on nuclear telomerase. To further characterize the nuclei-associated telomerase, we examined its susceptibility to the nucleotide analogs, 7-deaza-2′-deoxyguanosine-5′-triphosphate (7-deaza-dGTP) and 7-deaza-2′-deoxyadenosine-5′-triphosphate (7-deaza-dATP). As determined previously, 7-deaza-dGTP and 7-deaza-dATP were potent telomerase inhibitors (28).

Telomerase in intact nuclei was also affected by these nucleotide analogs but to a different extent than the solubilized enzyme. For example, 7-deaza-dGTP, which previously showed IC_{50} of 10 μM against solubilized enzyme (28), seemed to completely inhibit nuclear telomerase at concentrations as low as 1 μM (Fig. 4, lanes 4–6). Careful inspection of the respective lanes revealed the presence of a telomerase ladder with larger molecular weight oligonucleotides. Although the low signal/noise ratio in these experiments prevented clear-cut conclusions, it appears that the addition of 7-deaza-dGTP accentuated the products that were most likely a result of telomerase that leaked into the supernatant. These products were inhibited with an approximate IC_{50} value of 10 μM, i.e., similar to that observed with solubilized enzyme.

In contrast to more potent effects of 7-deaza-dGTP, 7-deaza-dATP was considerably less potent against nuclei-associated telomerase. While 7-deaza-dATP inhibited solubilized telomerase with IC_{50} value of 60 μM (28), the analog had no effect up to 100 μM on the nuclei-associated enzyme (Fig. 4, lanes 7–10). Similarly, ddGTP at 20 μM had only a slight effect on nuclei-associated telomerase (Fig. 4, lane 11), while completely abolishing telomerase activity from cytoplasmic extracts (Fletcher, T., unpublished results).

In addition to telomerase inhibitors, the effect of aphidocolin, a DNA polymerase α and δ inhibitor, was examined. Whereas it was possible that nuclear DNA polymerases might compete with telomerase for nucleotide substrates, addition of aphidocolin had no effect on the telomerase ladder obtained with intact nuclei (Fig. 4, lane 3).

DISCUSSION

Thus far, telomerase activity has been investigated using cellular or nuclear extracts as the source of enzyme. In this study, we demonstrate for the first time that catalysis by the endogenous telomerase activity in intact nuclei from human tumor cells can also be detected. Nuclei-associated telomerase generates a range of extension products and, like its solubilized counterpart, tends to pause at the first G in the TTAGGG repeat. Intriguingly, the telomerase product distribution indicates that nuclei-associated enzyme is less processive than telomerase activity which leaks into the post-nuclear supernatant or the activity in cell extracts. The lack of high molecular weight products indicates also that the leakage of telomerase from isotonic nuclei prior to their centrifugation is minimal.

The low-processivity of intact nuclei-bound telomerase found in this study (addition of mostly up to four TTAGGG repeats) differs from the usual high processivity of the enzyme in cytoplasmic extracts (28–30, 36). The processivity of telomerase in the extracts is affected by various factors, such as monovalent cations, concentrations of dNTPs, nucleotide analogs, and G-quadruplex stabilizing compounds (28–30, 37–39). Since nuclei preparations are likely to contain some dNTPs, a trivial explanation for low processivity with nuclei might reflect altered final concentrations of unlabeled dNTPs. This explanation is unlikely, however, since essentially the same amounts of carried over dNTPs should be present in the post-nuclear supernatants. Some rodent cells contain an inherently non-processive telomerase which mostly adds only one

TTAGGG repeat (39). However, addition of only four TTAGGG repeats onto the $(TTAGGG)_3$ input oligonucleotide by human telomerase has not previously been observed.

The observed reduced processivity may be due to the association of the endogenous enzyme with nuclei since the enzyme is highly processive in post-nuclear supernatants. However, different forms of telomerase with varying degrees of processivity were fractionated from cells obtained from patients with AML (40). Thus, the high processivity of telomerase in the commonly used cytoplasmic extracts (28–30, 36) may reflect a property of the system or the presence of only the more highly processive enzyme rather than what actually exists in the nucleus. "Physiological" processivity of telomerase remains unknown. The caveat here is that the low processivity of nuclei-associated telomerase with an artificial substrate does not need to extend to the action on natural substrate-telomeres. Still, an analogous precedence for differential catalysis by nucleoprotein-associated vs. solubilized enzyme exists. Jackson and Cook (34) reported that DNA polymerase α which was tightly associated with nuclei under isotonic conditions differed from the dissociated soluble activity in DNA substrate requirements.

Nuclei-associated and solubilized forms of telomerase also vary in their response to the known telomerase inhibitors. Our results suggest that some inhibitors, such as 7-deaza-dGTP, may be more potent and some, such as 7-deaza-dATP, less potent against nuclei-associated enzyme. Although these results are preliminary, they alert of potentially profound differences in telomerase responses to telomerase inhibitors in various systems. Comparative kinetic studies with nuclei-associated and solubilized (both post-nuclear supernatant and cytoplasmic) forms of telomerase are required to ascertain the reason for the differences observed in this study.

The notion that telomerase association with the nucleus can be significant for enzyme properties is reinforced by other studies. A defined spatial pattern was observed for the binding of antibodies against the catalytic protein subunit of human and mouse telomerase (hTERT and mTERT) in the nuclei of HeLa cells (41, 42) suggesting a specific arrangement of nuclei-bound telomerase. Telomerase-associated proteins have been identified in addition to the catalytic subunits (41, 43–45). Also, a variety of telomere binding proteins, known to affect telomere length [for recent reviews see (46–49)], may work in concert to regulate telomerase function. Therefore, caution is needed in the interpretation of findings based solely on the released telomerase activity. On the other hand, exploration of the endogenous, nuclei-associated telomerase can provide further insight into telomerase biochemistry and, perhaps, a more realistic system for the evaluation of potential telomerase inhibitors.

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