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One-step affinity purification protocol for human telomerase

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ABSTRACT

Human telomerase is a ribonucleoprotein (RNP) enzyme, comprising protein components and an RNA template that catalyses telomere elongation through the addition of TTAGGG repeats. Telomerase function has been implicated in aging and cancer cell immortalization. We report a rapid and efficient one-step purification protocol to obtain highly active telomerase from human cells. The purification is based on affinity chromatography of nuclear extracts with antisense oligonucleotides complementary to the template region of the human telomerase RNA component. Bound telomerase is eluted with a displacement oligonucleotide under mild conditions. The resulting affinity-purified telomerase is active in PCR-amplified telomerase assays. The purified telomerase complex has a molecular mass of ~550 kDa compared to the ~1000 kDa determined for the telomerase RNP in unfractionated nuclear extracts. The purification protocol provides a rapid and efficient tool for functional and structural studies of human telomerase.

Human telomerase, a specialized reverse transcriptase, synthesizes telomeric DNA and contributes to the maintenance of functional telomeres in immortal or highly proliferative cells such as germline cells, hematopoietic precursors and tumor cells (1,2). The template for telomeric repeat synthesis is provided by the RNA subunit. Telomerase RNAs from ciliates, yeast and mammalian cells, including the RNA component of human telomerase (hTR), have been cloned and sequenced (3–5). In addition, several protein components associated with telomerase activity have been identified. These include p80 and p95 from *Tetrahymena* (6), p43 from *Euplotes* (7) and TP1/TLP1 from mammalian cells (8,9). Most recently, the cDNAs encoding the catalytic subunit of telomerase were cloned and the deduced amino acid sequences of yeast (10), *Euplotes* (11) and human telomerase (12–14) contain several sequence motifs that are hallmarks of the catalytic region of reverse transcriptases.

Despite these recent successes, the composition of the functional telomerase ribonucleoprotein (RNP) within human tumor cells has not been resolved so far. The unique composition of telomerase as an RNP enabled us to develop a rapid affinity purification protocol that allows an efficient one-step purification of human telomerase.

Affinity purification of telomerase. For analytical purification, 2 ml of nuclear extract (corresponding to 5×10^8 cells) in buffer A (20 mM HEPES pH 7.9, 1 mM EDTA, 300 mM KCl, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF) + 0.5% Triton X-100 was used. Yeast tRNA (50 µg/ml) and 1 nmol affinity oligonucleotide [5′-biotin-CTAGACCTGTCATCA-rmeG-$(rmeU)_2$-rmeA-$(rmeG)_3$-$(rmeU)_2$-rmeA-rmeG-3′ (rme = 2′*O*-methyl ribonucleotides)] were added per 1 ml of extract and incubated for 5 min at 30°C. This oligonucleotide/extract mixture was added to 250 µl of Ultralink-immobilized Neutravidin Plus beads (Pierce), preblocked twice with buffer A containing 0.5 mg/ml BSA, washed once with buffer A and incubated for 10 min at 30°C. After 2 h incubation at 4°C, the column material was collected by centrifugation for 5 min at 1000 r.p.m. at 4°C and the supernatant was removed. After three washes with 4 ml of buffer A + 0.5% Triton X-100 (washes one to three), the beads were rinsed with buffer A containing 600 mM KCl (wash four), followed by two washes with buffer A at 25°C for 5 min (washes five and six). Telomerase was eluted by addition of a 2.5-fold excess of displacement oligonucleotide (5′-CTAACCCTAACTGATGACAGGTCTAG-3′) in buffer A containing 0.15% Triton X-100. Following a 30 min incubation at room temperature, the eluate was removed from the beads by centrifugation for 5 min at 1000 r.p.m. The elution of bound telomerase was repeated twice more under the same conditions, while in the last elution step 0.5% Triton X-100 in buffer A was used.

Telomerase assay. Telomerase activity was determined using a PCR-based assay (1). The reaction products were extracted with phenol/$CHCl_3$, precipitated with EtOH and resolved on 6% acrylamide/7 M urea sequencing gels. For quantitative analysis, the reaction products were supplemented with 200 µg/ml calf-thymus DNA and precipitated with 5% TCA. The precipitated nucleic acids were collected on glass fibre filters (Millipore) and the total amount of incorporated ^{33}P-dCMP was measured by liquid scintillation counting. To obtain a linear correlation of

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activity and protein concentration, nuclear extract and affinity eluates were diluted 400- and 200-fold, respectively.

Detection of hTR. RNA was extracted from protein fractions by phenol/chloroform and precipitated with 2.5 vol of EtOH, 0.1 vol of 7.5 M NH_4AC and 3 µg of yeast tRNA. The precipitate was washed with 70% EtOH, dissolved in water and electrophoresed on a formaldehyde/1.2% agarose gel in MOPS buffer. The RNA was transferred to Nytran 12 N membrane (Schleicher & Schuell) and hybridized with an hTR probe as described (5). Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics). *In vitro* transcribed hTR loaded on the same gel was used as a standard.

Calculation of cellular hTR content. 5×10^6 cells were lysed with 1 ml of TRIzol™ (Gibco-BRL) and total cellular RNA was isolated as described by the manufacturer. Aliquots of the isolated RNA (corresponding to different cell numbers) were loaded on a 1.2% agarose gel and hTR was detected and quantified as above. *In vitro* transcribed hTR loaded on the same gel was used as a standard. The observed signals were proportional to the amount of hTR loaded.

Glycerol gradients. An aliquot of 200 µl of nuclear extract or affinity purified telomerase fraction was fractionated on 10–40% glycerol gradients containing 20 mM Tris–HCl, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 3 mM $MgCl_2$, 250 mM KCl, 3% sucrose and 0.5 mM DTT. Glycerol gradients were poured in tubes (13 × 51 mm, 5 ml) and centrifugation was performed in a SW 50.1 Ti rotor (Beckman) at 37 000 r.p.m. for 15 h at 4°C. Marker proteins (thyroglobulin, 669 kDa; β-Amylase, 200 kDa; and BSA, 66 kDa) were run in parallel. Fifteen fractions (330 µl each) were collected from the bottom of the tubes.

Affinity purification of hTR. To obtain a highly purified preparation of human telomerase, we adopted a method developed for the purification of snRNPs (15). Antisense inhibition experiments revealed a 14 nt domain of hTR, including the entire template region and adjacent 3′ nucleotides, to be accessible to inhibitory hybridization within the RNP complex (data not shown). Based on these results, an affinity bait for human telomerase was designed. This affinity oligonucleotide was complementary to nucleotides 46–59 of hTR and included 12 random nucleotides 5′ to the hTR-specific sequence. The presence of a biotin residue at the 5′ end allowed immobilization to an avidin column. Nuclear extracts from HeLa cells were incubated with the affinity oligonucleotide as described earlier. Following binding and extensive washing steps, active telomerase was eluted from the affinity matrix by addition of a displacement oligonucleotide complementary to the affinity oligonucleotide over its entire length. Since the displacement oligonucleotide can form a thermodynamically more stable duplex to the affinity oligonucleotide than does hTR, it triggers the release of the telomerase RNP from the affinity matrix. Efficient eluation

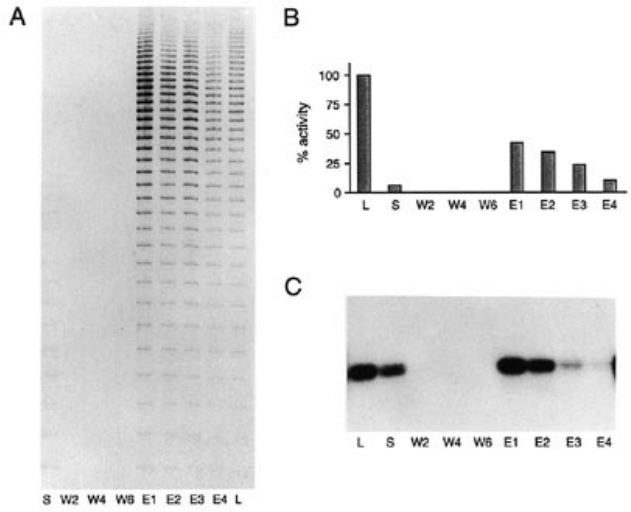

Figure 1. Affinity purification of hTR. Nuclear extract (L) from HeLa cells was incubated with an affinity oligonucleotide complementary to the template region of hTR. After binding to Neutravidin beads and removal of the supernatant (S), the resin was washed extensively, resulting in fractions W1–W6. Telomerase was eluted by addition of a displacement oligonucleotide in the presence of 0.15% Triton X-100 (E1–E3) or 0.5% Triton X-100 (E4). (**A**) Telomerase activity assay. Aliquots of the indicated fractions (2 µl each, diluted 1:200) were tested for telomerase activity by the PCR-based telomerase assay. Reaction products were resolved on a 6% sequencing gel. (**B**) Quantitation by TCA precipitation. Reaction products obtained by the PCR-based telomerase assay were precipitated with TCA, and the amount of incorporated ^{33}P-dGMP was measured by scintillation counting. Telomerase activity in individual fractions is indicated as % activity compared to the load (100%). (**C**) Northern blot analysis of hTR. RNA was extracted from 30 µl of the load and 50 µl each of supernatant, wash and eluate fractions, separated by denaturing gel electrophoresis and the presence of hTR was determined by northern blot hybridization.

of telomerase required the addition of a 2.5-fold molar excess of the displacement oligonucleotide and the presence of 0.15–0.5% Triton X-100. All four eluate fractions contain significant telomerase activity (Fig. 1, lanes E1–E4). For a quantitative analysis of telomerase activity, ^{33}P-labelled reaction products of the telomerase assay were precipitated with TCA and quantified by liquid scintillation counting. As shown in Figure 1B and in Table 1A, 96% of the activity was present in the eluate fractions, and only minor activity remained in the supernatant. The distribution of hTR during affinity purification was determined by northern blot analysis and allowed a second way to track the telomerase RNP. Quantitation of the hTR signal showed that ~65% of the RNA was present in the eluate fractions E1–E4, whereas 33% remained in the supernatant (Fig. 1C and Table 1B). A calculation of the amount of hTR present in RNA preparations isolated from HeLa cells suggests that a single cell contains ~3000–5000 molecules of hTR.

Table 1A. Determination of telomerase yield based on enzyme activity

Fraction	Protein (mg)	Activity (c.p.m. $\times 10^9$)	Specific activity (c.p.m. $\times 10^9$/mg protein)	Yield (%)	Purification (fold)
Nuclear extract	30	3.0	0.100	100	
Affinity supernatant	27.5	0.17	0.006	6	
Affinity eluate 1–3	0.022	2.9	132	96	1320

Table 1B. Determination of telomerase yield based on hTR measurement

Fraction	Protein (mg)	hTR (ng)	hTR (ng/mg protein)	Yield (%)	Purification (fold)
Nuclear extract	30	11.5	0.383	100	
Affinity supernatant	27.5	3.8	0.138	33	
Affinity eluate 1–3	0.022	7.5	341	65	890

Nuclear extract was prepared from 5×10^8 HeLa cells. The protein concentration was determined according to Bradford using BSA as standard. Telomerase activity and hTR concentrations were determined as described in the text.

Sedimentation properties of affinity-purified telomerase. Affinity-purified telomerase and nuclear extract were run in parallel on identical glycerol gradients. Fractions collected from the bottom of the gradients were assayed for the distribution of hTR (Fig. 2A and B) and telomerase activity (Fig. 2C and D). A co-migration of hTR with telomerase activity was found in both gradients (compare Fig. 2A with C and B with D, respectively). As shown in Figure 2D, affinity-purified telomerase sedimented with a native molecular weight of ~550 kDa, while telomerase present in nuclear extracts sedimented with a peak at ~1000 kDa (Fig. 2C). Conceivably, affinity purification results in the dissociation of loosely associated component(s) not required for the catalytic activity of telomerase. A 1000 kDa complex has been observed before in the characterization of the rat telomerase RNP (9). However, a recent reconstitution experiment with the cloned human telomerase components, hTR and hTERT, provides direct evidence that these two components comprise the minimal telomerase RNP (16). The calculated molecular weight of the monomeric complex is 280 kDa. Our results suggest that the telomerase RNP present in human tumor cells may constitute a large multisubunit 'holoenzyme' complex, only parts of which have been identified so far. Previous purification studies in ciliates, which contain ~100-fold more telomerase RNPs per cell, led to the identification of additional candidate telomerase subunits (6,7). Human homologs of these proteins as well as the telomerase associated protein TP1/TLP1 (8,9) may be part of the hTR RNP. The affinity chromatography protocol described here, which resulted in a one-step 890-fold purification of hTR and a 1300-fold enrichment of telomerase activity, in combination with conventional chromatography methods, may help in a determination of the subunit composition and the stoichiometry of human telomerase.

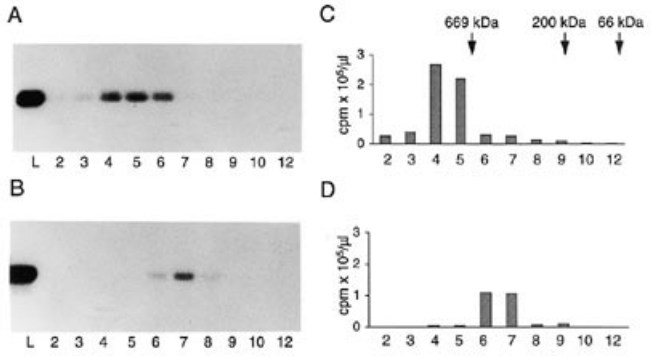

Figure 2. Glycerol gradient analysis of telomerase. Nuclear extract (**A** and **C**), affinity-purified telomerase (**B** and **D**) and marker proteins (Thyroglobulin, 669 kDa; β-Amylase, 200 kDa; and BSA, 66 kDa) were sedimented on 10–40% glycerol gradients run in parallel. (A and B) Northern blot analysis of hTR. RNA present in the load (L) and in individual fractions (2–10,12) was isolated, separated by denaturing gel electrophoresis and the presence of hTR was determined by northern blot hybridization. (C and D) Telomerase activity. Aliquots of the indicated fractions were tested for telomerase activity using the TCA precipitation protocol. Results are shown as specific activity of ^{33}P-labelled telomerase reaction products. In addition, the elution profile of marker proteins is indicated. The number of the individual fractions is indicated.

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