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Telomerase repeat amplification protocol (TRAP) activity upon recombinant expression and purification of human telomerase in a bacterial system

Debra T. Hansen [a, 1], Thirumagal Thiyagarajan [a, 2], Amy C. Larson [a], Jeffrey L. Hansen [a, b, c, *]

[a] Medical University of South Carolina, Department of Biochemistry and Molecular Biology, 173 Ashley Avenue, Charleston, SC, 29425, USA
[b] A.T. Still University, School of Osteopathic Medicine in Arizona, 5850 E. Still Circle, Mesa, AZ, 85206, USA
[c] Barrow Neurological Institute, Phoenix, AZ, 85013, USA

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ABSTRACT

Telomerase biogenesis is a highly regulated process that solves the DNA end-replication problem. Recombinant expression has so far been accomplished only within a eukaryotic background. Towards structural and functional analyses, we developed bacterial expression of human telomerase. Positive activity by the telomerase repeat amplification protocol (TRAP) was identified in cell extracts of *Escherichia coli* expressing a sequence-optimized hTERT gene, the full-length hTR RNA with a self-splicing hepatitis delta virus ribozyme, and the human heat shock complex of Hsp90, Hsp70, p60/Hop, Hsp40, and p23. The Hsp90 inhibitor geldanamycin did not affect post-assembly TRAP activity. By various purification methods, TRAP activity was also obtained upon expression of only hTERT and hTR. hTERT was confirmed by tandem mass spectrometry in a ~120 kDa SDS-PAGE fragment from a TRAP-positive purification fraction. TRAP activity was also supported by hTR constructs lacking the box H/ACA small nucleolar RNA domain. End-point TRAP indicated expression levels within 3-fold of that from HeLa carcinoma cells, which is several orders of magnitude below detection by the direct assay. These results represent the first report of TRAP activity from a bacterium and provide a facile system for the investigation of assembly factors and anti-cancer therapeutics independently of a eukaryotic setting.

1. Introduction

Telomerase extends the 3′ ends of chromosomes by the addition of a telomere repeat sequence [1]. In the absence of telomerase, telomeres become incrementally shorter with each round of replication, leading to chromosome instability, cell senescence and death [2]. Accordingly, most cancer cells require the abnormal production of telomerase to become immortalized [3,4]. Experimental anti-cancer therapeutics inhibit telomerase by direct binding or by interfering with assembly or regulation [5]. Therefore, fundamental insights into the assembly and regulation of human telomerase may facilitate the development of anti-cancer therapies.

Dozens of proteins have been implicated in the assembly, activity, localization and regulation of human telomerase (reviewed in Refs. [6,7]). The core telomerase enzyme consists of the catalytic protein subunit (hTERT) [8,9], the telomeric-repeat-encoding template RNA (hTR) [10], and the accessory proteins dyskerin, NHP2, NOP10 and GAR1 [11,12]. Mass spectrometry analyses of purified telomerase [13,14] have also identified telomerase co-purification with the dyskerin/NHP2/NOP10 complex, the heterogeneous nuclear ribonucleoprotein subunits hnRNP U and hnRNP C, Sm proteins, and the four NTPases NAT10 (*N*-acetyltransferase 10), GNL3L, RuvBL1/pontin and RuvBL2/reptin. Several studies also implicate the Hsp90 chaperone complex in both the assembly and activity of telomerase, as Hsp90 and p23 interact with hTERT and remain bound to active holoenzyme [15,16]. Telomerase also undergoes complex sub-organellar localization [17] and is further regulated at the telomere by the shelterin complex of proteins [18,19].

* Corresponding author. A.T. Still University, School of Osteopathic Medicine in Arizona, 5850 E. Still Circle, Mesa, AZ, 85206, USA.
E-mail address: JHansen@atsu.edu (J.L. Hansen).
[1] Present address: Center for Applied Structural Discovery, The Biodesign Institute, Arizona State University, Tempe, AZ, 85287-5901, USA.
[2] Present address: Division of Hematology & Oncology, Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, 38103-3403, USA.

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To support structural and functional studies, we pursued a recombinant expression system with a high capacity for manipulation and overexpression. Active human telomerase has so far been achieved only using eukaryotic systems, mainly in human cells or extracts [20,21] and in rabbit reticulocyte lysates [22,23], and also in yeast [24,25]. Here we demonstrate for the first time [25,26] telomerase activity in a bacterium, as detected by the telomerase repeat amplification protocol (TRAP[3]). *Escherichia coli* naturally lacks telomeres due to the circular chromosome. Since this recombinant system lacks endogenous telomerase assembly components, it provides unique possibilities for studying the biogenesis, enzymology and structure of human telomerase.

2. Materials and methods

2.1. Plasmid construction

Plasmids used in this study are listed in Supplementary Table 1. DNA and sequences for plasmids associated with TRAP activity have been deposited with the DNASU Plasmid Repository at Arizona State University [27]. Clones were generated by ligation of PCR products into one of four vector types based on antibiotic resistance and origin of replication (Supplementary Table 1). The novel empty vectors pET-BAD and pRSF-BAD allow expression from the arabinose-inducible promoter and *Escherichia coli rrnB* transcription terminator from pBAD18 [28]. Sequences were verified using the Nucleic Acid Analysis Facility at the Medical University of South Carolina.

The hTERT cDNA [24] (encoding GenBank accession no. AF015950), from Chantal Autexier (McGill University, Québec, Canada), was cloned in tagged and untagged forms and with various promoters, ribosome binding sites, and termination sites. Three synthetic hTERT genes removed secondary structure and rare *E. coli* codons: hTERTr5 (Celtek Bioscience; GenBank accession no. FJ600487; Supplementary Fig. 1) altered the 5-prime 2250 base pairs; hTERTr5TAA (Supplementary Fig. 2) additionally included mutations in the last 70 base pairs plus the TGA stop codon was changed to TAATAA; and hTERTr6 (GeneArt; Supplementary Fig. 3) was optimized for the entire gene. Cloning of hTERTr5 into pGEX-6P-3 (GE Healthcare) yielded pGEX-6P-3-GST-hTERTr5TAA, which effectively deleted the first, third and fourth residues of hTERT. Removal of one of two T7 promoters and its associated multiple cloning site (MCS) from pETDuet-1 (Novagen) yielded the "noMCS2" plasmids. The hTR DNA [10] was obtained from Isabel Kurth and Joachim Lingner (Swiss Institute for Experimental Cancer Research, Switzerland). All hTR constructs contained a 3-prime, 85-base pair hepatitis delta virus ribozyme [29]. The DNA sequence corresponding to the hTR-ribozyme transcript is detailed in Supplementary Fig. 4. The pCDF-90-23-70-60-40-Trx plasmid encoded human Hsp90 [30,31] (from Sophie Jackson, University of Cambridge, UK), p23 [32] (from Bill Sullivan and David O. Toft, Mayo Clinic College of Medicine, USA), Hsp70 [33] (from Sue Fox and Richard I. Morimoto, Northwestern University, USA), p60/Hop [34] (from David F. Smith, Mayo Clinic Scottsdale, USA), Hsp40 [35] (from Taeho Ahn, Chonnam National University, Korea) and *E. coli* thioredoxin (TRX, from pT-Trx [36], from Shunsuke Ishii, RIKEN Tsukuba Life Science Center, Japan). The cDNA encoding the putative telomerase-inhibitory protein LPTS-L (liver-related putative tumor suppressor) [37] was from Mujun Zhao (Chinese Academy of Sciences, China). The RIG plasmid, from Wim Hol (University of Washington, USA) expresses rare *E. coli* tRNAs for Arg, Ile and Gly [38]. Into RIG was also cloned the four human box H/ACA small nucleolar ribonucleoproteins (snoRNPs): dyskerin [39] (from Inge Krebs and Annemarie Poustka, German Cancer Research Center, Germany), GAR1 [40], NHP2 [41] and NOP10 [41] (from Vanda Pogačić and Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Switzerland).

2.2. Transformation and growth of E. coli

Expression was in the RNase E deficient (*rne131*) *E. coli* strain BL21Star(DE3) (Invitrogen). Plasmids were co-transformed by electroporation (BioRad Gene Pulser Xcell) with selection on LB Lennox medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) containing 1.5% agar plus antibiotics as needed (50 μg/mL ampicillin, 34 μg/mL chloramphenicol, 50 μg/mL spectinomycin, 25 μg/mL kanamycin). With four plasmids, half-strength antibiotics were used in liquid medium. Overnight cultures (5 mL or 100 mL) were grown at 37 °C with shaking at 225 rpm. For expression of human telomerase, 2.5 mL or 20 mL of the overnight culture was inoculated into 100 mL or 750 mL medium, respectively, in baffled flasks (250 mL or 2 L). At an absorbance (600 nm) of 0.3, growth temperature was changed to 30 °C, and after 30 min the cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Anatrace) and/or 0.2% arabinose (Alfa Aesar). Two hours after induction, cells were pelleted at 4000 × *g*, washed twice in 1X PBS (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3), flash-frozen in liquid nitrogen, and stored at −80 °C.

2.3. Preparation of E. coli cell extracts

Milli-Q Synthesis-purified water (18 MΩ) and 4 °C were used for purifications and assays unless noted. Pelleted frozen cells from 2 mL of culture were suspended by vortex in 0.2 mL CHAPS Lysis Buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol [BME], 0.5% CHAPS, 10% glycerol) from the TRAPeze system (Chemicon) plus 0.1 units/μL RNasin Ribonuclease Inhibitor (Promega), passed 10 times through a 28 gauge needle (BD #309309), and incubated on ice 30 min. Centrifugation at 12,000× *g* for 20 min yielded 0.16 mL of extract supernatant. Protein concentration was determined by Bradford. For positive controls, A549 cells (provided by Besim Ogretmen, Medical University of South Carolina, USA) and HeLa cells were lysed and stored in CHAPS Lysis Buffer at −80 °C as 25 ng protein per μL and 5000 cells/μL, respectively.

2.4. Purifications using glutathione S-transferase (GST) tag

Frozen *E. coli* cell pellets from 750 mL of culture were lysed by sonication (1 min cycle of 2 s on, 4 s off, at 30% amplitude; Fisher Model 500 sonicator) on ice in, per g of cells, 10 mL of Buffer A (1X PBS, pH 7.3, 2 mM dithiothreitol, 0.02% NaN_3) plus 1 mM benzamidine-HCl, 0.5% CHAPS (Anatrace), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 20,000× *g* for 30 min, the supernatant was filtered (0.45 μm PVDF) and loaded onto a 1 mL GSTrap FF column (Glutathione Sepharose Fast Flow, GE Healthcare) that had been washed with 6 M guanidine-HCl and equilibrated in Buffer A. Elution was with 2 mL of 50 mM Tris-HCl, pH 8.5, 140 mM NaCl, 0.02% NaN_3 and 40 mM reduced glutathione. Where indicated, enzyme was concentrated on an Amicon Ultra 4 (10,000 MWCO), exchanged 10-fold into Buffer B (25 mM Tris-HCl, pH 7.5, 140 mM KCl, 1 mM $MgCl_2$, 0.05 mM Na_2EDTA, and 5 mM BME), and exchanged 10-fold into the same buffer plus 30% glycerol.

[3] Abbreviations used: hTERT, human telomerase protein subunit; hTR, human telomerase RNA component; snoRNP, small nucleolar ribonucleoprotein; TRAP, telomerase repeat amplification protocol.