METHOD

Expression of hTERT and hTR in cis reconstitutes an active human telomerase ribonucleoprotein

FRANÇOIS BACHAND,1,2 GEORGE KUKOLJ,3,4 and CHANTAL AUTEXIER1,2
1Department of Anatomy and Cell Biology, McGill University, Montréal, Québec, H3A 2B2 Canada
2Bloomfield Centre for Research in Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis, Jewish General Hospital, Montréal, Québec, H3T 1E2 Canada
3Department of Microbiology and Immunology, McGill University, Montréal, Québec, H3A 2B4 Canada

ABSTRACT
Telomeres in eukaryotic cells are generally synthesized and maintained by the ribonucleoprotein (RNP) telomerase. This enzyme is composed of at least two subunits, the telomerase reverse transcriptase (TERT) and the telomerase RNA. Human telomerase activity can be reconstituted in vitro by the expression of the telomerase protein catalytic subunit (hTERT) in the absence of recombinant human telomerase RNA (hTR) or in a rabbit reticulocyte lysate (RRL) system. The hTERT and hTR subunits are independently expressed in vivo, and little is known about the mechanism of their assembly. To facilitate recombinant telomerase RNP formation and reconstitution, we engineered a construct, termed hTERT-hTR cis, in which the 3′ end of the hTERT coding sequence was extended by the addition of the sequence encoding hTR. Expression of the hTERT-hTR cis construct in vitro (in RRL) and in vivo (in the yeast Saccharomyces cerevisiae) produced hTERT-hTR transcripts of the predicted size. Active human telomerase was reconstituted by hTERT-hTR cis expression in both RRL and S. cerevisiae. Assembly of functional human telomerase by the bicistronic expression of the protein and RNA components may facilitate the overexpression and reconstitution of this enzyme in heterologous systems.

Keywords: cis expression; rabbit reticulocyte lysate; reverse transcriptase; RNP reconstitution; S. cerevisiae

INTRODUCTION
Telomerase is a ribonucleoprotein (RNP) that synthesizes DNA repeats at the physical ends of eukaryotic chromosomes. This enzyme is minimally composed of two subunits: the telomerase reverse transcriptase (TERT), which is the catalytic protein component, and the telomerase RNA, which provides the template for telomere synthesis (Nugent & Lundblad, 1998). Human telomerase is active in a number of transformed and tumor cell lines where it acts to maintain telomere length, yet is not active in most normal diploid human cells (Kim et al., 1994; Autexier & Greider, 1996; Shay & Bacchetti, 1997). Tumorigenic conversion of normal cells in vitro was recently accomplished by the ectopic expression of the oncogenic allele of H-ras, the SV40 large T antigen, and human TERT (hTERT) (Hahn et al., 1999a). The result of this and other studies suggests that telomerase and telomere maintenance may be critical for the formation of certain tumors in vivo (Bryan et al., 1997; Hahn et al., 1999a). The expression of dominant negative, catalytically inactive mutants of hTERT in certain cancer cell lines blocks the tumorigenic phenotype of these cell lines by activating the apoptosis cascade (Hahn et al., 1999b; Zhang et al., 1999). For these reasons, telomerase is an attractive target for anticancer therapy, and the development of telomerase inhibitors is an area of intensive research (Autexier, 1999).

Reconstitution of human telomerase activity has, thus far, been reported in four systems. First, addition of in vitro-synthesized human telomerase RNA (hTR) to micrococcal nuclease-treated, partially purified 293 cell extracts was used to study the regions of hTR that are essential for the reconstitution of an active telomerase enzyme (Autexier et al., 1996). Second, expression of hTERT in the presence of recombinantly transcribed hTR in rabbit reticulocyte lysates (RRL) reconstitutes a
Telomerase RNP reconstituted by hTERT-hTR in cis

RESULTS AND DISCUSSION

Expression of hTERT and hTR from the same RNA molecule (cis) may facilitate the production of active telomerase in different expression systems, as described for other reverse transcriptase/RNA template complexes (Lanford et al., 1995). To determine whether bicistronic expression of hTERT and hTR could reconstitute an active telomerase RNP, we designed a construct containing both hTERT (3.5 kb) and hTR (0.5 kb) sequences expressed as a single 4.0-kb transcript (hTERT-hTR cis construct; Fig. 1). This construct contains sequences beyond the 3’ end of the hTERT open reading frame that include the hTR sequence, known to be essential for human telomerase activity in vitro (Weinrich et al., 1997; Beattie et al., 1998).

Reconstitution of active human telomerase by bicistronic expression of hTERT and hTR in rabbit reticulocyte lysates

To demonstrate that the hTR sequence expressed from the hTERT-hTR cis plasmid is an integral part of the 4.0-kb transcript, we expressed the constructs in RRL and harvested total RNA for Northern blot analysis (Fig. 2). Using an hTR-specific probe, no signal was detected in total RNA preparations from control RRL or RRL programmed with DNA encoding only hTERT (Fig. 2A, lanes 8 and 7, respectively). Addition of in vitro-synthesized hTR to an RRL reaction programmed with DNA-encoding hTERT (Fig. 2A, lane 6) revealed an RNA that comigrates with the hTR positive control (Fig. 2A, lane 3).Northern blot analysis of RRL reactions programmed with the hTERT-hTR cis constructs produced 4.0-kb signals with either an hTR- or hTERT-specific probe (Figs. 2A and 2B, respectively, lanes 4–5). Both hTERT and hTR are included in the 4.0-kb transcript when expressed in the context of the hTERT-hTR cis construct in RRL, consistent with combined hTERT and hTR sequences of 3.5 and 0.5 kb, respectively. Furthermore, synthesis of hTAR (0.5 kb) from this construct was undetectable by Northern blot analysis, suggesting no cryptic internal transcription initiation.

Extracts from differently programmed RRL reactions were assayed for telomerase activity to determine whether expression of hTR in cis of hTERT produces a functional human telomerase RNP. The N-terminus of hTERT is preceded by a small epitope (T7-tag) that is specifically recognized by a monoclonal antibody that can be used to immunoprecipitate hTERT from RRL extracts. Immunoprecipitates were prepared from RRLs programmed with different DNA constructs and analyzed for telomerase activity, hTERT protein levels, and hTR immunoprecipitation (Figs. 3A, 3B, and 3C, respectively). Immunoprecipitates from RRL reactions that expressed hTERT in the presence of gel-purified hTR reconstitute human telomerase activity (Fig. 3A, lane 3).
However, the expression of hTERT in the absence of hTR (Fig. 3A, lane 2), or the addition of hTR to RRL without the hTERT-expressing construct (Fig. 3A, lane 1) does not reconstitute activity. Reconstituted human telomerase activity was recovered from the immunoprecipitates of RRL expressing the wild-type (WT) hTERT with sense hTR in the bicistronic construct (Fig. 3A, lane 5). When a catalytically inactive mutant of hTERT (containing a point mutation at amino acid 868 that changes a conserved aspartic acid residue to an asparagine in motif C of hTERT) (Fig. 3A, lane 6), or hTR in the antisense orientation (Fig. 3A, lane 7) was expressed in the context of the cis construct, no telomerase activity was detected from the immunoprecipitates. These latter results indicate that this reconstitution system requires catalytically active hTERT with a functional hTR sequence. RNAse treatment of immunoprecipitate prepared from an RRL reaction programmed with the bicistronic construct eliminated the distinct hTR-containing RNAs (Fig. 3C, lane 8), and correlated with the loss of telomerase activity (Fig. 3A, lane 8). These results indicate that in vitro expression of hTERT and hTR from the same transcript reconstituted an active human telomerase RNP. The reconstitution of human telomerase by hTERT-hTR-cis expression also suggests that the human telomerase RNA assembles with hTERT to form an active RNP despite the 3,500 nt of hTERT-mRNA sequence at its 5' end.

**In vivo expression of the hTERT-hTR cis construct reconstitutes a functional human telomerase in S. cerevisiae**

Human telomerase activity can be reconstituted by co-expressing a GST-hTERT fusion and hTR in the yeast S. cerevisiae (Bachand & Autexier, 1999). We cloned the hTERT-hTR cis construct under the control of GAL1-responsive elements to determine the ability of the bicistronic expression of hTERT and hTR to reconstitute active human telomerase in vivo in yeast. Total RNA was extracted from yeast grown in the presence of galactose (induced) or glucose (repressed). RNA preparations harvested from yeast that contained the hTR-expression vectors were analyzed by Northern blot using an hTR-specific probe (Fig. 4A). Total RNA from galactose-cultured yeast containing separate hTERT
and hTR expression vectors harbors the expected 0.5-kb hTR transcript (Fig. 4A, lanes 3–4). Galactose-induced yeast containing the bicistronic construct (either hTERT WT or DN) express the predicted GST-hTERT-hTR cis 4.5-kb transcript (Fig. 4A, lanes 1–2). hTR-specific RNAs were not detected in total RNA preparations from uninduced yeast grown in glucose (Fig. 4A, lanes 5–8), demonstrating the specificity of the expression system. In addition, sequences corresponding to hTR were not detected in RNA preparations from galactose-cultured yeast that lacked the hTR-expression vector (data not shown).

Protein extracts from galactose-cultured yeast transformed with different DNA constructs were subjected to immunoprecipitation with an anti-GST serum to determine whether the bicistronic expression of hTERT and hTR in S. cerevisiae reconstitutes a functional human telomerase RNP. Immunoprecipitates from extracts that were prepared from yeast transformed with the GST-hTERT-hTR cis construct contained telomerase activity at levels comparable to (or greater than) immunoprecipitates from extracts of yeast expressing the

![Figure 2](image-url) **FIGURE 2.** Bicistronic expression of hTERT and hTR in rabbit reticulocyte lysates. Northern blots of total RNA harvested from equivalent volumes of RRL reactions (lanes 4–8) and probed for hTR- (A) or hTERT-specific (B) sequence. In vitro (IVT)-transcribed RNAs (lanes 1–3) were included as positive controls. RNA markers (in kilobase pairs) are indicated on the left.

![Figure 3](image-url) **FIGURE 3.** Reconstitution of functional human telomerase by the bicistronic expression of hTERT and hTR in RRL. Equal volumes of RRL reactions programmed with different DNA constructs [labeled above A] were subjected to immunoprecipitation (IP) with a monoclonal anti-T7 antibody. The washed beads were analyzed for telomerase activity (A), hTERT protein levels (B), and hTR coimmunoprecipitation (C). A: Telomerase activity was analyzed by the TRAP assay, and 100 ng of partially purified 293 cell extracts were used as a positive control (lane 9). IC: internal PCR control; WT: wild-type hTERT; DN: D868N hTERT mutant. B: Protein levels were analyzed by SDS-PAGE and autoradiography of [S^{35}]-methionine-labeled hTERT. C: hTR coimmunoprecipitation was analyzed by Northern blotting. In vitro-synthesized hTR (lane 10) and hTERT-hTR cis (lane 11) RNAs were used as positive controls. RNA markers are indicated on the right (in kilobase pairs).
two components from different plasmids (Fig. 4B, lanes 4 and 2, respectively). Both the GST-hTERT and the GST-hTERT-hTR cis constructs are expressed from a high copy 2-μm plasmid, whereas hTR is expressed from a centromeric plasmid. The higher expression of hTR in the cis system may be responsible for the efficient reconstitution observed with the bicistronic expression of hTERT and hTR in S. cerevisiae. Alternatively, the expression of hTERT and hTR in cis in S. cerevisiae may promote a better assembly of the human telomerase RNP than the expression of GST-hTERT and hTR from two distinct plasmids.

Telomerase activity can be reconstituted in vitro by the addition of the RNA component after the translation of the catalytic subunit is complete (Weinrich et al., 1997; Bachand & Autexier, 1999; Holt et al., 1999; Licht & Collins, 1999). However, the proximity of hTR to hTERT, provided in the bicistronic construct, may promote efficient RNP folding or assembly during translation of hTERT from the hTERT-hTR cis transcript, as previously described for the HBV reverse transcriptase (Lanford et al., 1995). DNA replication of HBV is initiated by the recognition of the pregenomic RNA by the viral-encoded RT (Wang & Seeger, 1992; Wang et al., 1994). Evidence indicates that this recognition occurs best in cis, whereby the reverse transcriptase binds to its own mRNA, including the epsilon stem-loop structure (Wang et al., 1994). Our hTERT-hTR bicistronic system mimics the HBV RT in that the interaction of an RNA element with the RT is essential for RNP formation and for the expression of the reverse transcriptase in an enzymatically active form in heterologous systems (Wang & Seeger, 1992; Lanford et al., 1995). HBV reverse transcriptase RNP formation is dependent on two molecular chaperones, Hsp90 and p23, both in vitro and in vivo (Wang et al., 1994; Hu & Seeger, 1996; Hu et al., 1997). Recent results have suggested that Hsp90 and p23 may be implicated in the assembly of the human telomerase RNP; however, there is no evidence that these molecular chaperones are required for the reconstitution of human telomerase activity in S. cerevisiae.
for the physical interaction of hTERT with hTR (Holt et al., 1999).

In this study, we demonstrated that a functional human telomerase RNP can be reconstituted by expressing both hTERT and hTR from a single transcript in vitro in RRL, and in vivo in the yeast S. cerevisiae. The potential of this reconstitution system for future studies on telomerase is diverse. Bicistronic expression of hTERT and hTR simplifies the previous RRL reconstitution system in that the production of recombinant hTR is not required. Consequently, large-scale screening of telomerase inhibitors using the RRL expression system can be accelerated by the use of the bicistronic hTERT-hTR construct. Such a construct may also facilitate the establishment of active recombinant telomerase in transgenic organisms and simplify the generation of telomerase activity from a variety of sources. The ability to reconstitute an active human telomerase RNP through bicistronic expression of the protein and RNA subunits will be useful for future genetic and biochemical studies of this enzyme.

MATERIALS AND METHODS

Construction of plasmids

The construction of the WT and the DN hTERT expression plasmids was previously described (Bachand & Autexier, 1999). To generate the hTERT-hTR cis construct, the hTR coding sequence was amplified by PCR from pGRN33 (Feng et al., 1995) using the 5’ primer 5’-CGCGGATCCGGGACGGCCAGCGACCAGGCTGTCGG3’ and the 3’ primer 5’-CGCGGATCCGGCAGCTAGTGTGAGCCGAGTCCTGGGT-3’, both containing a BamHI site. The BamHI-digested hTR-PCR product was cloned into the BamHI site of the pBluescript II SK (Stratagene) in both sense and antisense orientation resulting in the BSSK/hTR and BSSK/hTR vectors, respectively. The WT hTERT cDNA was PCR amplified from the pGRN121 vector using the 5’ primer 5’-CCGGAATTCTATCGGGCAGGCTGCCGCTTCCGTTGG-3’ and the 3’ primer 5’-CCGGAATTCTCACTCGCAGAGTGTCTTG-3’, both containing EcoRI sites. The EcoRI-digested hTERT-PCR product was cloned into the EcoRI site of both the BSSK/hTR and the BSSK/hTR vectors, respectively. Cloning of the WT hTERT DN mutant was performed by the same procedure but using the pET28b/ hTERT(DN) construct as a template for PCR amplification (Bachand & Autexier, 1999).

To construct the pET28c/hTERT(WT)-hTR, pET28c/ hTERT(DN)-hTR, and the pET28c/hTERT(WT)-hTR plasmids, the respective 4.0-kb pair hTERT-hTR DNA fragments were excised from BSSK/hTERT(WT)-hTR, BSSK/ hTERT(DN)-hTR, and BSSK/hTERT(WT)-hTR by HindIII-NotI digestions. Each of the HindIII-NotI hTERT-hTR fragments were cloned into a pET28c vector (Novagen) that was previously digested with HindIII and NotI.

To generate the yeast expression plasmids pEGK/ hTERT(WT)-hTR and pEGK/hTERT(DN)-hTR, a 2.5-kb *MluI*-HindIII fragment from pEGK/hTERT (Bachand & Autexier, 1999) was replaced with 3.1-kb *MluI*-HindIII fragments from the pFhBb/hTERT(WT)-hTR and pFhBb/hTERT(DN)- hTR constructs (a detailed description of these plasmids will be given elsewhere). The construction of the yeast hTERT expression plasmids was previously described (Bachand & Autexier, 1999).

In vitro transcription and translation

Plasmids under the control of the T7 promoter were included in coupled transcription/translation (Promega) reactions (10– 25 μL) at a final concentration of 25 ng/μL, with or without 10–20 ng/μL of gel-purified hTR.

RNA preparation and Northern blot analyses

The yeast strain YPH499 (Sikorski & Hieter, 1989) was transformed with the different expression plasmids using the lithium acetate method (Ausubel et al., 1998). Induction and repression of expression from GAL1-responsive elements using galactose and glucose, respectively, was described previously (Bachand & Autexier, 1999). Extraction of total RNA from yeast was performed using a method previously described (Schmitt et al., 1990). Briefly, transcription was induced or repressed using galactose or glucose, respectively, from 10-mL yeast cultures. The cells were harvested by centrifugation and resuspended in AE buffer (50 mM Na acetate, pH 5.3; 10 mM EDTA). After addition of 10% SDS and fresh phenol, mixtures were incubated at 65°C for 4 min and then rapidly chilled in a dry ice/ethanol bath. Following centrifugation, the aqueous phase was phenol/chloroform treated and the RNA precipitated. To extract RNA from RRL, 10 μL of the reaction were mixed with 40 μL of H2O, and RNA was extracted using 950 μL of TRIZOL reagent (GIBCO BRL) as described by the manufacturer. RNAs were separated by electrophoresis on 1% agarose/0.66 M formaldehyde/1× MOPS gel in 1× MOPS buffer (20 mM 3-[(N-morpholino)-propane sulfonic acid; 50 mM sodium acetate; 1 mM EDTA) and subsequently transferred to nylon membranes (Hybond; Amersham Pharmacia Biotech). Nylon membranes were incubated in Church buffer (1% BSA; 1 mM EDTA; 0.5 M NaHPO4, pH 7.2; 7% SDS) with hTR- or hTERT-specific probes. The hTR probe was generated by random hexamer radiolabeling of a BamHI restriction fragment (from BSSK/hTR) corresponding to full-length hTR DNA. The hTERT probe was generated with the same method using a 1,181-bp *MluI*-Xhol fragment from the BSSK/hTERT construct. Hybridizations were performed at 42–45°C.

Protein extraction and immunoprecipitations

Protein extraction from yeast cells was performed by glass bead lysis as previously described (Bachand & Autexier, 1999), with the exception that yeast cells were lysed in 10mM Tris- HCl (pH 7.5); 2.0 mM MgCl2; 1.0 mM EDTA; 5.0 mM β-mercaptoethanol; 150 mM NaCl; 1% NP-40; and 0.25 mM sodium deoxycholate. Immunoprecipitation experiments were performed as previously described using anti-T7 and anti-GST antibodies purchased from Novagen and Amersham Pharmacia Biotech, respectively (Bachand & Autexier, 1999). Antibody–protein complexes were precipitated using protein-
A-sepharose (Amersham Pharmacia Biotech) and washed extensively in lysis buffer. Sepharose beads were then resuspended in 100 µl of buffer A (Autexier et al., 1996), and telomerase activity, protein, and RNA analyzed by TRAP, SDS-PAGE/autoradiography, and Northern blot, respectively. In Northern blot analysis of coimmunoprecipitated RNA, 85% of the immunoprecipitated beads were resuspended in 200 µl of a solution containing 0.4 mg/ml proteinase K; 150 mM NaCl; 10 mM Tris-HCl (pH 7.5); 12.5 mM EDTA; and 1% SDS; and incubated at 37°C for 15–20 min. RNA was then extracted with phenol/chloroform and ethanol precipitated using 20 µg of glycogen carrier (GIBCO BRL).

Telomerase activity assays
Telomerase activity was assayed by a two-tube modified TRAP as described previously (Bachand & Autexier, 1999). The positive control used in TRAP assays consisted of partially purified 293 cell extracts prepared as previously described (Autexier et al., 1996).

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F Bachand, G Kukolj and C Autexier

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