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Reconstitution of telomerase activity utilizing human catalytic subunit expressed in insect cells☆

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Abstract

Telomerase is a specialized reverse transcriptase responsible for maintaining the termini of linear chromosomes. The human enzyme is a ribonucleoprotein complex minimally comprising a catalytic protein moiety (hTERT) and an RNA subunit (hTR) which acts as the template for the reverse transcriptase reaction. Here we report expression of recombinant hTERT protein in insect cells utilizing a baculovirus expression system. The recombinant hTERT protein reconstitutes telomerase activity in the presence of hTR, either when co-expressed in insect cells or when added in vitro. Reconstitution of telomerase activity using this system will facilitate further analysis of the biochemical and biophysical properties of this enzyme.

Keywords: Telomerase; Carcinogenesis; Cell aging; Anticancer drug

Telomerase is a ribonucleoprotein essential for replicating the termini of linear chromosomes in most eukaryotes [1,2]. The human enzyme adds $(TTAGGG)_n$ repeats at the telomere using its RNA component (hTR, for human telomerase RNA) as template [2,3]. Most normal somatic cells lack telomerase activity and their telomeres are observed to shorten gradually with each cellular division [4]. The hypothesis that this shortening was the mitotic clock which ultimately triggers senescence [5] was confirmed by the immortalization of a variety of human somatic cells by telomerase transduction [6,7]. Telomerase activity is observed and telomere length is maintained in the great majority of primary human tumor cells [8,9]. In addition, activation of telomerase is usually required for the immortal phenotype of tumor cells [5,10]. Consequently, telomerase modulation has become a major objective in research and development on degenerative diseases and cancer.

Following the cloning of the RNA component of human telomerase [11] reconstitution of telomerase activity in vitro was first accomplished using nuclease-treated 293 cell extracts and synthetically produced hTR [12]. With the cloning of the gene for the catalytic subunit of human telomerase (hTERT, for human telomerase reverse transcriptase) [13–16] an in vitro coupled transcription/translation reaction was used to show that expression of hTERT protein and hTR alone were necessary and sufficient for generation of telomerase activity [17,18]. However, generation of active enzyme in this system may have required additional factors present in the transcription/translation lysate [19]. The poor expression and reconstitution of telomerase in this in vitro system limited its utility [17,18]. Detailed biochemical studies of the enzyme require a more efficient

☆ *Abbreviations:* hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA; TRAP, telomeric repeat amplification protocol; bp, base pairs; kb, kilo bp; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenglycol bis(2-aminoethyl ether)-tetraacetic acid; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)propanesulfonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; m.o.i., multiplicity of infection.

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and easily scaled method for hTERT expression and telomerase reconstitution.

We now report expression of recombinant hTERT protein in insect cells utilizing a baculovirus-driven expression system. Telomerase activity could be reconstituted in vitro, by mixing the recombinant protein with hTR, or in vivo, by co-expressing the protein and RNA components in the insect cells. To our knowledge this is the first report of functional reconstitution of a RNP enzyme in a baculovirus expression system. The ability to reconstitute telomerase activity using this system facilitates further analysis of the biochemistry and biology of this enzyme.

Materials and methods

Cells. Spodoptera frugiperda (Sf21), insect cells were purchased from Invitrogen (San Diego, CA). They were maintained at 27 °C in TNH-FH medium (Pharmingen, San Diego, CA).

Plasmids. Plasmid pGRN164, which harbors the hTR gene under the control of the T7 RNA polymerase promoter, was used for in vitro transcription of hTR [17]. Plasmid pGRN165 which carries the native form of the hTERT gene at the *Eco*RI site of pBluescript II SK (+) was utilized for construction of baculoviral vectors.

Construction of baculoviral vectors. For hTERT, a 3.5 kb *Eco*RI fragment from pGRN165 was inserted into the *Eco*RI site of pVL1392 (Pharmingen), giving rise to pGRN471. For hTR expression from the polyhedrin promoter, a 687 bp *Xho*I/*Sph*I fragment from pGRN293 was cloned into the transfer vector pBAC1 (Novagen) to create pGRN524. To allow for expression initiating at the +1 position of hTR, and to remove unnecessary leader sequences, a fragment of hTR was generated by PCR that contained a *Bam*HI site just 5′ of the +1 position of hTR. This fragment was digested with *Bam*HI and *Xba*I and cloned into pGRN524 to give pGRN527. For expression of hTR under control of the U1 promoter, a 1358 bp *Bgl*II/*Sph*I fragment containing full-length hTR behind the U1 promoter [20] (a generous gift of John Rossi, Beckman Research Center of the City of Hope) was cloned into pBAC1 to create pGRN525.

For addition of the rabbit β-globin [21] and hammerhead terminators [22] to hTR, PCR fragments were generated that contained full-length hTR bounded at the 5′ end by a *Bam*HI site, and at the 3′ end by the specific terminator sequence followed by a *Eco*RI site. These PCR fragments were cloned into pVL1393 (Pharmingen) to give pGRN534 (rabbit β-globin) and pGRN533 (hammerhead). For the U1 terminated hTR, the *Bam*HI/*Bbs*I fragment of pGRN527 was shuttled into pVL1393. The *Xba*I fragment of this construct was then replaced by the *Xba*I fragment of pGRN293 which contained the 3′ end of hTR followed by the U1 termination sequence [20] to give pGRN532.

Expression of hTERT protein and hTR. Viruses were generated from the transfer vectors described above by established protocols [23]. These viruses were used to infect exponentially growing Sf21 cells at a m.o.i. of 0.1 and 1. Cells were harvested at 24, 48, 72, and 96 h post-infection by centrifugation and washed once in PBS and then centrifugation pellets were frozen or lysed directly for analysis.

Western blotting. SDS/PAGE was performed in 4–20% (w/v) gradient gels according to the method of Laemmli [24,25]. After electrophoresis, proteins were transferred to nitrocellulose membranes and treated with the anti-hTERT mouse monoclonal antibody, 1A4, which was raised from a bacterially expressed C-terminal fragment of hTERT (unpublished). For the secondary antibody, we used peroxidase-conjugated goat anti-Mouse IgG, Fc specific (Sigma). Generation and detection of the chemiluminescent signals were performed using the SuperSignal West Dura system (Pierce).

Extraction of hTERT proteins from Sf21 cells. All the steps after harvesting of hTERT-expressing Sf21 cells were carried out at 4 °C and supplemented with, Complete (Roche Molecular Biochemicals), a cocktail of protease inhibitors. The cytoplasmic fraction from 5×10^6 cells was extracted by incubating in 1 ml of CHAPS buffer (10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.5% (w/v) CHAPS, and 10% (v/v) glycerol) on ice for 30 min. Cells were centrifuged at 12,000 rpm for 30 min and the supernatant was removed. The nuclear pellet was suspended in 1 ml KCl lysis buffer (50 mM Tris–HCl (pH 7.5), 420 mM KCl, 5 mM $MgCl_2$, 6 mM dithiothreitol, 0.1 mM EDTA, 10% (w/v) sucrose, and 20% (v/v) glycerol) and sonicated until the visible aggregates were cleared. After a brief centrifugation, the nuclear extracts were used for complex formation with hTR.

RNA preparation and analysis. RNA was prepared from 1×10^6 Sf21 cell equivalents by standard methods [25] using Proteinase K treatment followed by DNase I digestion of cytoplasmic extracts. The hTR standard RNA was generated by in vitro transcription and quantitated by measuring trace levels of incorporated [^{35}S]UTP [5]. RNA samples from 10^5 cell equivalents were analyzed for hTR on Northern blots using the Northern-Gly Max protocol (Ambion). Hybridization was carried out at 55 °C with 100–200 fMol of a full-length hTR antisense riboprobe generated by in vitro transcription and labeled with ^{32}P-GTP to a specific activity of 100,000 cpm/fmol. Washed blots were analyzed with a phosphorimager.

Reconstitution of telomerase complexes. The telomerase RNA component hTR was synthesized in vitro using pGRN164 linearized with *Fsp*I in the Ribomax RNA transcription system (Promega). After incubation at 37 °C for 2 h, synthesized hTR was minimally purified using a Quick Spin G-50 Sephadex Column (Roche Molecular Biochemicals). Nuclear extracts of hTERT from Sf21 cells were mixed with hTR and incubated at 30 °C for 90 min to allow for complex formation. These telomerase complexes were then directly used for detection of telomerase activity as described below.

Measurement of telomerase activity. Telomeric repeat amplification protocol (TRAP) was performed using the TRAPEZE Telomerase Detection Kit (Intergen). A sample of each extract was boiled for 3 min as a control for PCR artifacts. Every assay included a telomerase-positive control extract (293 cells). All telomerase reactions were done in a total volume of 50 μl; 15 μl of this was loaded on a 12.5% non-denaturing polyacrylamide gel and electrophoresed for 45 min at 250 V. Gels were stained for 30 min in SYBRGreen I (Molecular Probes) and analyzed with a FluorImager SI (Amersham Pharmacia Biotech). Alternately, TRAP was performed according to standard protocols [26] using a radiolabeled TS primer; detection and analysis were performed on a phosphorimager.

Results

Expression and solubilization of hTERT protein in insect cells

Expression of hTERT in a number of heterologous systems has been reported [17,27,28]. Limited amounts of protein have been obtained and levels of reconstituted enzyme have been low. We chose to express the protein and RNA subunits of telomerase in a baculovirus-driven insect cell expression system. For hTERT expression, a number of promoters and transfer vectors were tested (not shown), but the best results were obtained in Sf21 cells with the polyhedrin promoter of the pVL1392 vector. Two to three rounds of viral amplification were required to observe significant expression of the protein,

which could be detected by immunoblot analysis using a mouse monoclonal antibody directed against a fragment of hTERT (Fig. 1A).

A number of methods were tested for solubilization of the protein. Simple lysis of infected cells in CHAPS buffer released only a small portion of the expressed hTERT (Fig. 1A). Best results were obtained by lysis in CHAPS buffer and removal of cytoplasmic fractions by centrifugation. The nuclear pellet was then suspended in KCl lysis buffer (Materials and methods), sonicated until the visible aggregates cleared, and briefly centrifuged. The resulting extract contained about 20-fold more hTERT than found in the initial CHAPS lysate (Fig. 1A). Significant quantities of the hTERT expressed in the insect cells, up to approximately 20 mg/L, remained insoluble after this extraction; this fraction of hTERT could only be solubilized in the presence of strong denaturing agents (not shown).

Maximal hTERT expression was obtained when cells were infected at low multiplicities of infection and harvested 72 h after infection (Fig. 1A). Higher m.o.i. result in an earlier peak of protein expression, but the overall hTERT levels were lower. This probably indicated continued growth of the insect cell culture during the early stages of the infection at lower m.o.i., resulting in a larger total pool of infected cells at 72 h post-infection. Harvest at later time points did not significantly increase overall hTERT expression, though somewhat more of the protein appeared to shift into the insoluble pool.

Reconstitution of telomerase activity in vitro

Although hTERT protein was solubilized at only low levels by CHAPS extraction, this fraction showed telomerase activity when incubated in the presence of in vitro transcribed hTR (Fig. 1B). On the other hand, greater telomerase activity was observed in the presence of hTR after solubilization of hTERT protein by KCl lysis buffer followed by sonication (Fig. 1B, lane 4). One cell equivalent was sufficient to detect telomerase activity (Fig. 2).

The amount of activity derived from the recombinant protein per infected cell compared very favorably with native sources of the protein. Detection of telomerase activity in telomerase-positive tumor cells such as 293 cells generally requires 10–100 cell equivalents in a TRAP assay [26]. Nevertheless, the specific activity of hTERT expressed in the insect cells is very poor. Quantitative immunoblotting (not shown) indicated that over 5×10^6 copies per insect cell of hTERT were released by KCl lysis. This represents at least a 10^3–10^4-fold increase over estimated levels of hTERT in tumor cells (Pruzan and Trager, unpublished results). Clearly much of this protein is not forming active enzyme when reconstituted in vitro. To make more effective use of the

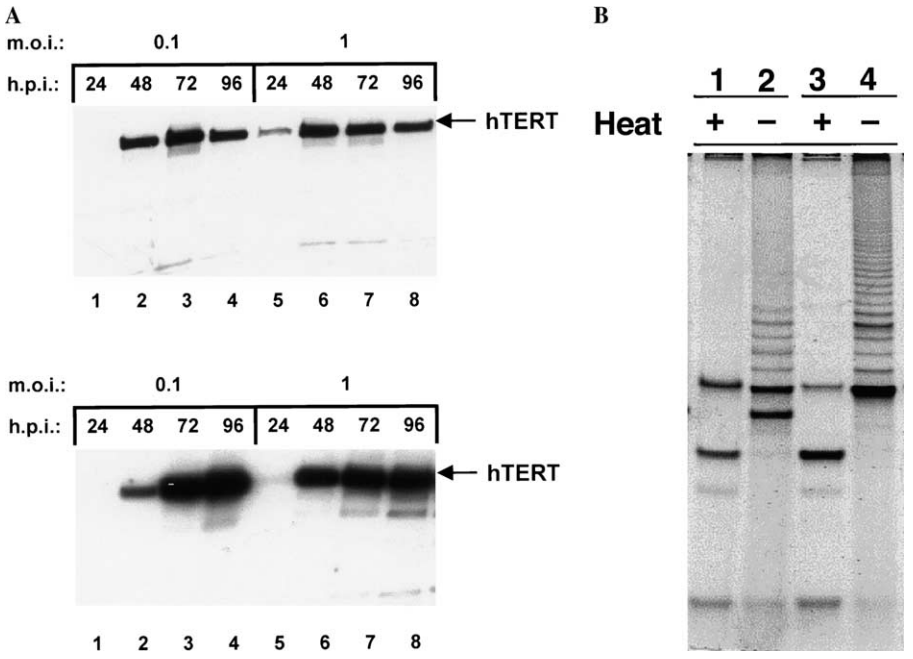

Fig. 1. hTERT expression and telomerase reconstitution. (A) Western blot analysis using monoclonal anti-hTERT 1A4. Top panel: CHAPS extracts from 1.5×10^4 virally infected Sf21 cells. Bottom panel: KCl extracts from 5×10^3 infected cells. Lanes 1–4 show extracts made from cells infected at an m.o.i. of 0.1 and harvested at 24, 48, 72, and 96 h after infection (h.p.i.). Lanes 5–8 show extracts made from cells infected at a m.o.i. of 1 and harvested at the same time points. (B) TRAP activity after telomerase reconstitution. hTERT present in CHAPS (lanes 1 and 2) or KCl (lanes 3 and 4) extracts from 100 infected Sf21 cells was reconstituted with 1 ng (6.7 fMol) of in vitro transcribed hTR. The reconstituted enzyme was assayed by TRAP. Lanes 1 and 3 are negative controls; extracts heat-denatured prior to TRAP. Data shown are representative of multiple experiments.

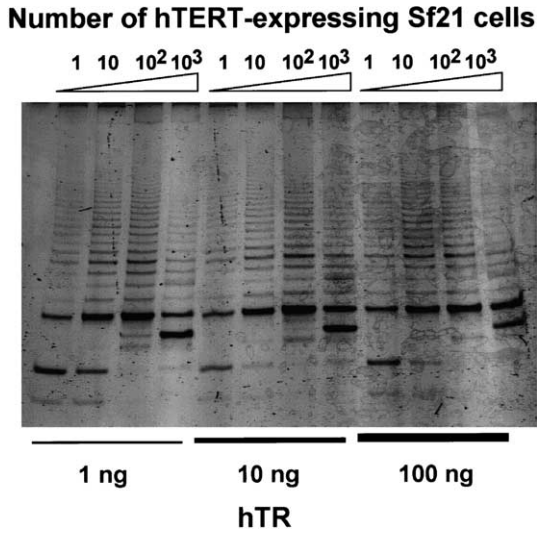

Fig. 2. Reconstitution of telomerase activity from the hTERT protein expressed in one Sf21 cell. hTERT protein was extracted from Sf21 cells using KCl lysis buffer followed by sonication. The extract derived from the number of Sf21 cells indicated above each lane was mixed with the amount of hTR indicated below for complex formation and assayed by TRAP. Data shown are representative of multiple experiments.

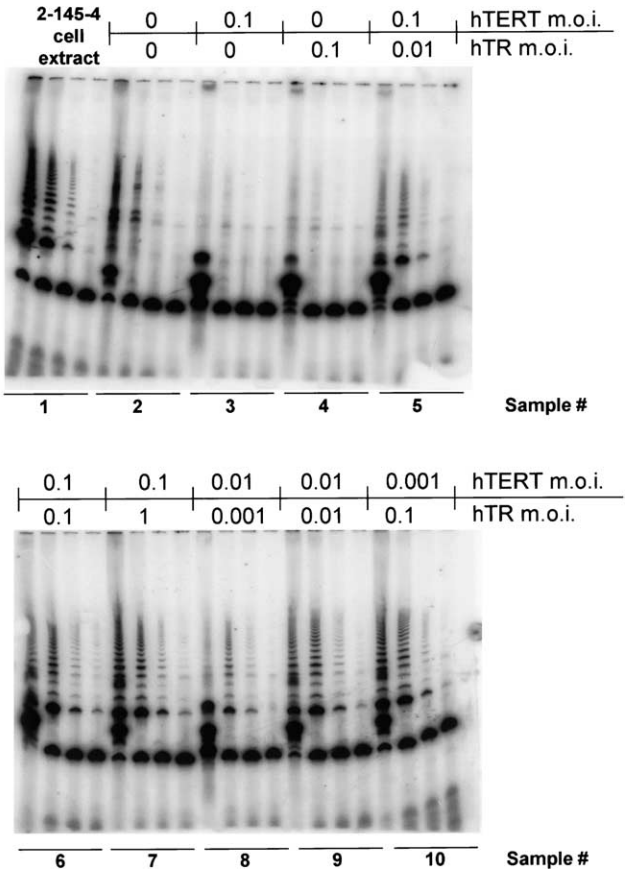

Fig. 3. Generation of recombinant telomerase by co-expression of hTR and hTERT in Sf21 cells. Sf21 cells were infected with pGRN471 (hTERT) and pGRN527 (hTR) viruses at various m.o.i. (samples 2–10). Cells were lysed in CHAPS buffer and telomerase activity of 2000, 200, 20, and 2 cell equivalents was assayed by TRAP (four lanes per sample, respectively). As a positive control, the same cell equivalents of a CHAPS extract prepared from 2-145-4 cells (293 cells overexpressing hTERT) were also subjected to TRAP analysis (sample 1). Data shown are representative of multiple experiments.

hTERT expression levels observed in the insect cells, we attempted to co-express hTR in Sf21 cells. We hoped to take advantage of one attractive feature of baculovirus-driven expression: the ability to super-infect insect cells with two or more viruses in order to achieve functional reconstitution of a multi-subunit enzyme [23].

hTR expression and telomerase reconstitution in vivo

A number of hTR expression constructs were prepared, utilizing a variety of viral or cellular promoters, full-length or truncated hTR, and transcription termination signals. When insect cells were co-infected with viruses coding for expression of hTR and hTERT, telomerase activity could be observed in CHAPS extracts equivalent to as few as two cells (Fig. 3). Cells infected with hTR or hTERT viruses alone had no detectable telomerase activity, confirming that both components are required for reconstitution of telomerase activity. As observed for hTERT, levels of enzyme production were maximal when viral m.o.i. were low. To our knowledge this is the first report of functional reconstitution of telomerase or any RNP enzyme in a baculovirus expression system.

The recombinant telomerase activity observed in insect cells co-expressing hTERT and hTR was comparable to that observed in human 293 cells which have been engineered to overexpress hTERT (Fig. 3). Immunoblot analysis demonstrated that hTERT expression levels were not significantly affected by co-expression of hTR (not shown). To determine whether hTR expression was limiting the generation of active telomerase, hTR expression in the insect cells was assessed by Northern blotting (Fig. 4). Expression levels using either the mammalian U1 or the baculoviral polyhedrin promoter were similar (Fig. 4A) and generated heterogenously sized hTR molecules. These constructs, which contain minimal native sequence 3′ of the mature hTR coding, may terminate poorly using the viral transcriptional termination signals that follow the polyhedrin promoter. Previous studies have indicated that baculoviral transcriptional termination sequences function weakly [21]. We therefore created a series of constructs bounded at their 3′ end by transcriptional termination sequences derived from the rabbit β-globin or the human U1 gene, or bounded by a hammerhead ribozyme encoding sequence designed to cleave at its own 5′-end. All of these constructs were placed under the polyhedrin promoter. Co-infection of each of these constructs with an hTERT expression virus generated

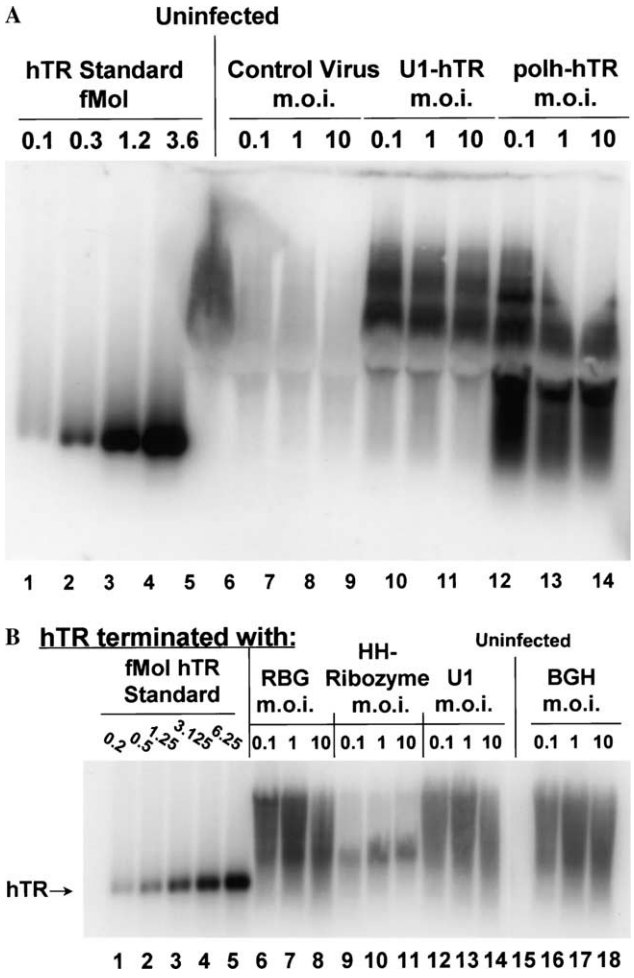

Fig. 4. Northern blot analysis of hTR expressed in Sf21 cells. RNA was prepared from 4×10^5 infected Sf21 cells and analyzed by Northern blot hybridization using a ^{32}P-labeled antisense probe to hTR. As a control for size and expression levels, known quantities of in vitro transcribed hTR were run alongside the experimental lanes. (A) RNA prepared from uninfected Sf21 cells (lane 5), control virus infected (lanes 6–8), or pBac-1 driving hTR expression under either the U1 (lanes 9–11), or polyhedrin (lanes 12–14) promoters was compared to in vitro transcribed hTR. Cells were infected at a m.o.i. of 0.1 (lanes 6, 9, and 12), 1 (lanes 7, 10, and 13), and 10 (lanes 8, 11, and 14). (B) Comparison of hTR termination sequences. RNA was prepared from Sf21 cells expressing hTR driven by the polyhedrin promoter and terminated by the rabbit β-globin terminator (RBG, lanes 6–8), a hammerhead ribozyme sequence (HH, lanes 9–11), the U1 terminator (lane 12-14), or the native terminator (BGH, lanes 16–18). As a negative control, RNA was prepared from uninfected Sf21 cells (lane 15).

active telomerase, at levels similar to those shown in Fig. 3 (not shown). Analysis of hTR expression driven by these viruses showed that only termination by a hammerhead ribozyme sequence designed to cleave at its own 5′ end resulted in the production of a discrete RNA (Fig. 4B). Expression of this form of hTR was weak; quantitative analysis showed that fewer than 5000 copies of the hTR/hammerhead transcript were produced per cell, compared with over 10^{10} copies of hTERT protein. Other constructs were expressed at somewhat higher levels, but the expressed RNA was mostly present in heterogeneous forms of unknown functionality (Fig. 4B). The low expression levels of hTR observed might be linked with the inefficient termination of the transcript; although significant hTR breakdown products were not observed, the long transcripts produced may be targets for rapid degradation by cellular or viral nucleases.

Discussion

Weinrich et al. [17] successfully performed in vitro reconstitution of telomerase activity using hTR and hTERT that were synthesized in a in vitro transcription/translation coupled system consisting of T7 RNA polymerase and rabbit reticulocyte lysate. Recently, Bachand and Autexier [27] reported expression of hTERT as a fusion with glutathione *S*-transferase (GST-hTERT) in *Saccharomyces cerevisiae*. This appears to be the first report that described the heterologous expression of a catalytically active recombinant hTERT in a system other than rabbit reticulocyte lysate. More recently, Masutomi et al. and Wenz et al. [28,29] also reported hTERT expression in insect cells.

Our study is in broad agreement with the previous reports of baculoviral expression of hTERT, showing that the majority of the hTERT expressed in the insect cells is in an insoluble form. While some of this protein can be solubilized by sonication in the presence of high salt or detergent, the majority remains in a refractory form. We have found hTERT expression levels in Sf21 cells to be over 20 mg/L culture. This is good expression for an exogenous protein. However, over 95% of the expressed protein is only soluble under highly denaturing conditions; most of this protein is tightly associated with nucleic acids (not shown). For this reason, we attempted to render a greater proportion of the expressed hTERT both soluble and active by co-expression of the telomerase RNA component, hTR.

It has recently been shown that hTR is a member of the family of box H/ACA RNAs; the majority of these are snoRNAs which play a role in ribosome biogenensis [30]. The contribution made by this 3′ structural element to telomerase biogenesis is not well understood, though Mitchell et al. [30] indicate that this region is necessary in part for hTR stability. Processing mechanisms of the 3′ end of hTR in vivo are also unknown. Although hTR has been successfully expressed in mammalian cells, expression in heterologous systems has not yet been characterized. Nor is there great precedent for heterologous expression of structural RNAs, many of which are normally expressed at high levels. In the present study we failed to achieve high level expression of hTR, though the sequence was placed under the control of extremely powerful viral promoters. A major problem

was the lack of efficient termination of the hTR transcript. Termination of baculoviral transcripts has been reported to be inefficient [21] and none of the termination sequences used in this study was able to confer both efficient termination and high level expression to the hTR transcript. The low expression observed may have been a function of the degradation of the long transcripts, presumably unprotected by the normal translation machinery or by the relevant RNA binding proteins in the insect cells. It is possible that the inclusion of further 3′ distal sequences may contribute to the processing of box H/ACA RNAs. It is also possible that the normal processing of these RNAs is abrogated in virally infected insect cells, or that human H/ACA motifs are not efficiently recognized in insect cells.

We succeeded in reconstituting telomerase activity in vivo by co-expression of the protein and RNA components of the enzyme. Unfortunately, the solubility of hTERT in lysates was not significantly affected by co-expression of hTR. The activity generated was roughly equivalent to that observed in human 293 cells. Reconstituting the enzyme in vitro generated slightly greater activity. In this study, we showed that only one hTERT-expressing Sf21 cell is enough to confer telomerase activity after complex formation with hTR, suggesting that at least 10–50 times the amount of catalytically active hTERT exists in these Sf21 cells compared to telomerase-positive tumor cells, such as 293 cells. In contrast with published reports for reconstitution of the telomerase enzyme of *T. thermophilia* [31,32], reconstitution efficiency of the human enzyme using recombinant components in vitro is low. This may reflect the fact that the structure of the protein expressed in heterologous systems is only partially native. Alternatively, it may be that multiple contacts must be formed between the protein and RNA components of the enzyme, and some of these contacts may require the intervention of specialized folding machinery found in more native systems. Expression of hTERT in insect cells should provide a good source for providing adequate quantities of recombinant hTERT and telomerase for further biochemical studies.

Acknowledgments

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