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TTAGG-repeat telomeres and characterization of telomerase in the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Abstract

Telomeres are maintained usually by telomerase, a specialized reverse transcriptase that adds this sequence to chromosome ends. In this study, telomerase activity was detected in the in different somatic tissues, such as midgut and fat bodies, by the telomeric repeat amplification protocol (TRAP) in *Spodoptera exigua*. The structure of the telomeres of *S. exigua* was evaluated by sequence analysis of the TRAP products, revealing that the telomerase synthesized a (TTAGG)n repeat. The presence of a telomerase reverse transcriptase (TERT) subunit coding gene has been cloned, sequenced and expressed in vitro successively. Notably, the *S. exigua* telomerase (SpexTERT) gene structure lacks the N-terminal GQ motif. Telomerase contains a large RNA subunit, TER, and a protein catalytic subunit, TERT. Here we report an in vitro system that was reconstructed by all components of the telomerase complex, a purified recombinant SpexTERT without a N-terminal GQ motif and a mutant human telomerase RNA (TER), showed telomerase activity. Together, these results suggest the GQ motif is not essential for telomerase catalysis.

Keywords: *Spodoptera exigua*, telomeres, telomerase, GQ motif.

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Introduction

Telomeres are repetitive DNA sequences at the end of linear chromosomes in eukaryotes (reviewed in Blackburn, 1991). Most insect species have adopted the pentanucleotide repeats of TTAGG as their telomeres (Sahara *et al.*, 1999; Sasaki & Fujiwara, 2000). Most somatic cells meet their "Hayflick limit" through the gradual shortening of telomeres caused by the absence of telomerase activity, after telomeres reach their critical length senescence onsets (Hayflick, 1965; reviewed in Aubert & Lansdorp, 2008). Therefore, telomeres work as a molecular clock for cell aging.

Telomerase, a ribonucleoprotein enzyme, is responsible for the complete replication of chromosomal termini in most eukaryotes (Greider & Blackburn, 1989). There is little or no telomerase activity in most normal somatic cells, whereas immortalized, cancer, stem and germ cells express telomerase and keep telomerase activity high, adding telomere tandem repeats to the ends of their chromosomal DNA (reviewed in Blasco, 2005, 2007).

Telomerase activity has been studied in a variety of organisms (Gomes *et al.*, 2011). Some arthropod species such as the lobster *Homarus americanus*, grow continuously throughout their lifespans; this growth is mainly attributed to lobsters maintaining high telomerase activity in all of their differentiated tissues throughout their lives (Klapper *et al.*, 1998). Similarly, in the cricket *Teleogryllus taiwanemma*, telomerase is expressed and

kept at high activity levels in most tissues (Sasaki & Fujiwara, 2000). The previous study proved TTAGG-telomerase activity in a wide range of insect orders, including Isoptera, Blattaria, Lepidoptera, Hymenoptera, Trichoptera, Coleoptera, and Sternorrhyncha. But telomerase activities were not detected in Orthoptera, Zygentoma, and Phasmida (Sasaki & Fujiwara, 2000; Korandova *et al.*, 2014). In addition, telomerase activity has been detected neither in the domestic silkworm (*Bombyx mori*) nor in the flour beetle (*Tribolium castaneum*) despite their telomerase reverse transcriptases (TERTs) being identified and characterized by genome sequencing and BLAST (Okazaki *et al.*, 1993; Osanai *et al.*, 2006). The crystal structure of the catalytic subunit of *T. castaneum* telomerase (TcTERT) has provided the evidence for the formation of an active telomerase elongation complex (Gillis *et al.*, 2008). The active *T. castaneum* telomerase ribonucleoprotein (RNP) complex was reconstituted in vitro by mixing the recombinant TcTERT and the total RNA of *T. castaneum* (Schuller *et al.*, 2011). However, there is still lack of direct evidence for the functionality of the telomerase and its activity in insects. Here we report a new coding sequence for *Spodoptera exigua* telomerase (SpexTERT) with active telomerase activity with a (TTAGG)n telomere pattern.

Telomerase complexes consist of two protein components, the TERT subunit, which performs DNA binding and catalysis, and the RNA component, which is the template for reverse transcription. A typical TERT usually consists of GQ, CP, QFP, T and RT motifs (Lee *et al.*, 2003; O'Connor *et al.*, 2005; Jacobs *et al.*, 2005). GQ motifs are responsible for the repeat processivity of yeast and human TERTs, and loss of the GQ motif is strongly believed to cause the low telomerase activity of the silkworm TERT (BmoTERT). Mutations in the conserved residues of the GQ motif have been confirmed to be lethal in yeast (Friedman *et al.*, 2003). All of the known functional TERTs have GQ motifs in their N-terminal regions and these residues are phylogenetically conserved. This is consistent with the discovery of a BmoTERT that is not functional (Osanai *et al.*, 2006). But, this is challenged by our finding of the SpexTERT. There is no GQ motif or TEN domain in SpexTERT's N-terminal yet it possesses a strong telomere repeat $(TTAGG)_n$ addition processivity as detected by a TRAP assay. We hypothesize that GQ motifs or TEN domains do not influence the catalytic activity of telomerase directly but possibly indirectly. The results are described here.

Material and Methods

Preparation of extracts from tissues and cell lines

Fat bodies and midguts were dissected from larvae of *S. exigua, S. litura* and *Helicoverpa armigera* raised in our lab. Approximately 1 ml of each tissue was washed in cold Ringer's solution and centrifuged at 2500 g for 5 min; the supernatant was removed, and this step was repeated for at least three times. The supernatant was removed again, and 1.5 ml of 1× CHAPS lysis buffer (10 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 0.5% Chaps, 10% glycerol and 1 mM DTT) with 0.52 μL β-Mercaptoethanol, 0.8 μL RNase inhibitor (50 U/μL) and 1.5 μL PMSF (10 mg/mL in ethanol) was added. The mixture was vibrated gently and placed on ice for 30 min. The mixture was vibrated several times during lysis. Afterwards the mixture was centrifuged at 12 000 g for 20 min at 4 °C, the supernatant was carefully collected and stored at -100 °C.

The cell line IOZCAS-SpexII-A (SpexII-A) has been established and cloned from the fat body of *S. exigua* larvae (Zhang *et al.*, 2006; Zhang *et al.*, 2009). The cells were maintained in TNM-FH medium containing 10% inactivated fetal bovine serum in T25-cm^2 tissue culture flasks (Corning, NY, USA) at 27 °C. The osmolarity and pH of the medium were 368 mosM and 6.2, respectively. The cells from the stable cell line at passage 28 were harvested in log growth by centrifugation, and the cell pellets were washed three times in Ringer's solution. SpexII-A cell extracts were prepared identically using approximately 5×10^6 cells.

Human HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2% L-glutamine. Total RNA of the human telomerase (hTER-RNA) was isolated from approximately 5×10^6 cells of HeLa using TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

Insect telomeric repeat amplification protocol (TRAP) assay

Modified TRAP protocol of Takashi Sasaki and Haruhiko Fujiwara was used (Sasaki & Fujiwara, 2000) to detect insect telomeric repeats (TTAGG)n, as follows. Telomerase activity was determined by the polymerase chain reaction (PCR) procedure. TS primer was used as the forward primer and Bm-Cxa primer was used as the reverse primer (Table 1). The 10× TRAP buffer was composed of 200 mM Tris/HCl (pH 8.3), 15 mM $MgCl_2$, 630 mM KCl, 0.1% BSA, 0.5% Tween 20 and 10 mM EGTA. The 50 μL reverse transcription reaction mixture (RTRM) was composed of 2 μL TS primer (10 mM), 5 μL 10×TRAP buffer, 2 μL dNTPs (2.5 mM), 10 μL of cell or tissue extract (containing 10 μg of protein) and 31 μL H_2O. The RTRM was incubated at 30 °C for 60 min and then extracted with phenol-chloroform and precipitated with ethanol. The 50 μL PCR reaction mixture was composed of a 5 μL extraction from the RTRM, 2 μL dNTPs (2.5 mM), 2 μL TS primer (10 mM), 2 μL Bm-Cxa primer (10 mM), 0.5 μL EasyTaq DNA Polymerase (Transgen Biotec, 5 U/μL), 5 μL 10×TRAP buffer and 33.5 μL H_2O. We used a two-step PCR at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s and 55 °C for 30 s and then slowly reduced the reaction temperature to 30 °C. The PCR products were then resolved on a 15% nondenaturing polyacrylamide gel at 180 volts for 50 min (Falchetti *et al.*, 1999; Sasaki & Fujiwara, 2000; Rego & Marec, 2003). The Gel Logic 200 imaging system (Eastman Kodak Company) was used to visualize the banding patterns. Primers were synthesized from Sangon (Shanghai) Biotec (see Table 1).

Table 1. Primers used for sequencing *S. exigua* telomerase reverse transcriptase

No.	Primer name	Primer sequence (5′-3′)	Length (bp)	Usage
1	TS	AATCCGTCGAGCAGAGTT	18	TRAP*
2	Bm-Cxa	GTGTAACCTAACCTAACC	18	TRAP*
3	RVVDDYL	AGRGTTGTKGATGAYTAYTT	20	Degenerate PCR
4	Oligo dT	TTTTTTTTTTTTTTTTT	18	Degenerate PCR
5	SpexTERT_F	TAGAAAACATTCGAACATTTCTGCTTCT	28	SpexTert Clone
6	SpexTERT_R	TTACCTCAAAACATGTTTATTTTATTACAA	30	SpexTert Clone
7	Universal Primer A	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	45	5′- & 3′-RACE
8	Nested Universal Primer A	CTAATACGACTCACTATAGGGC	22	5′- & 3′-RACE
9	MHTERC_Res	GGAGCAAAAGCACGGCGCCTACGCCCTTCTCAGTTAGGTTAGACAAAAAATGGCCACCA	59	hTER-RNA Mutation
10	T7_HTERC_Up	CGGAATTCTTAATACGACTCACTATAGGGTTGCGGAGGGTGGGCCTGG	48	hTER-RNA Mutation
11	HTERC_Down	GCATGTGTGAGCCGAGTCCTGGGTGCACGTC	31	hTER-RNA Mutation

*Sasaki and Fujiwara, 2000

Cloning and sequencing of TRAP products

The TRAP products from SpexII-A cells were purified using the EasyPure PCR Purification Kit (Transgen Biotec.), cloned into the pEasy-T1 vector (Transgen Biotec.) and transformed into Trans109 Chemically Competent Cells (Transgen Biotec.). At least three positive colonies were selected and sent to BGI Co., Ltd. for sequencing.

cDNA synthesis and 5′- & 3′-RACE for SpexTERT clones

SpexII-A cell RNA was prepared using the Trizol RNA isolation protocol. First-Strand cDNA Synthesis was performed using the TransScript II Reverse Transcriptase (Transgen Biotec.) enzyme and protocol. 5′- & 3′-RACE was performed with the SMARTerTM RACE cDNA Amplification Kit (Clontech).

Primers were designed to clone genes of SpexTERT (Table 1). The degenerate PCR reaction was performed at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 30 °C-50 °C for 30 s, and 72 °C for 1-2 min followed by a step at 72 °C for 5 min. 50 μL of reaction buffer was composed of 5 μL 10× PCR buffer, 4 μL dNTPs (2.5 mM each), 2 μL each of forward (RVVDDYL) and reverse primers (Oligo d (T)) (10 mM), 0.5 μL EasyTaq DNA polymerase (Transgen Biotec.), 1 μL genomic DNA or a cDNA template and 35.5 μL H_2O. The PCR reaction for full length SpexTERT clone was performed at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min followed by a step at 72 °C for 5 min by primers of SpexTERT_F and SpexTERT_R.

Sequence analysis

The SpexTERT sequence was submitted to the NCBI GenBank (Accession Number KC347571). We used the Linux version of CLUSTALX 2.1 and DNAMAN 7.0 for sequence alignments (Tlarkin *et al*., 2007).

Expression of SpexTERT

SpexTERT was cloned into PET28b, and then transformed into BL21 (DE3) Rosetta cells (Transgen Biotec.). Rosetta cells were then incubated in LB kanamycin selective agar medium at 37 °C for 12 h. The monoclonal colonies were then picked and incubated in 5 ml LB kanamycin selective liquid medium at 37 °C for 5 h. The colonies were then sub-cultured in 500 ml LB kanamycin selective liquid medium and incubated at 37 °C until reaching a OD600 of 0.6. Liquid cultures were induced with 0.1 mM IPTG for 15 h. and then centrifuged at 4000 g for 10 min, to remove as much supernatant as possible. Cell lysis was conducted using the Bacterial Protein Extraction Reagent (Cwbiotech, Beijing.), and the SpexTERT protein was purified using a 6x His-Tagged Protein Purification Kit (Cwbiotech, Beijing.). The protein contents (Lowry *et al*., 1951) were determined following standard assays.

Mutant hTER-RNA and In vitro reconstitution of SpexTERT

hTER-RNA was isolated from the cultured cells of HeLa. Using primers MHTERC_Res, T7_HTERC_Up and HTERC_Down, hTER-RNA, (TTAGGG)n six nucleotide repeat template was mutated into the (TTAGG)n five nucleotide repeat similar to that of *S. exigua* (Feng *et al*., 1995). A linear T7 DNA template was acquired by amplifying the mutant hTER-RNA gene by PCR using primers containing the T7 promoter, accordingly. First step, T7_HTERC_Up and MHTERC_Res were used as primers and HeLa cell cDNA as template to form a 113bp PCR product in which the (TTAGGG)n six nucleotide repeat template was mutated into the (TTAGG)n five nucleotide repeat. Second, a 476bp PCR product was generated when cDNA from the HeLa cells was as template and the former PCR product of 113bp as the upstream primer and the HTERC_Down as the downstream primer. The PCR reaction protocols are the same with that in section 2.4. The PCR product was then cloned, sequenced and blasted. In vitro transcription of the mutant hTER-RNA was performed using the T7 RNP protocol (Thermo Fisher Scientific Inc.). After reconstitution of the SpexTERT protein and RNA component, traditional TRAP assays for telomerase activity were conducted (Beattie *et al*., 1998; Sasaki & Fujiwara, 2000). In vitro reconstitution complex was performed by mixing recombinant His-tagged SpexTERT protein (0.5 μg/μL) with four concentration of mutant hTER-RNA in RNase-free tubes with RNase inhibitor added in, and then incubated at 37 °C for 60 min in the presence of RNasin to prevent RNA degradation. Four concentration of mutant hTER-RNA in the reconstitution complex was 5.8 ng/μL, 0.58 ng/μL, 0.29 ng/μL and 0 ng/μL, respectively.

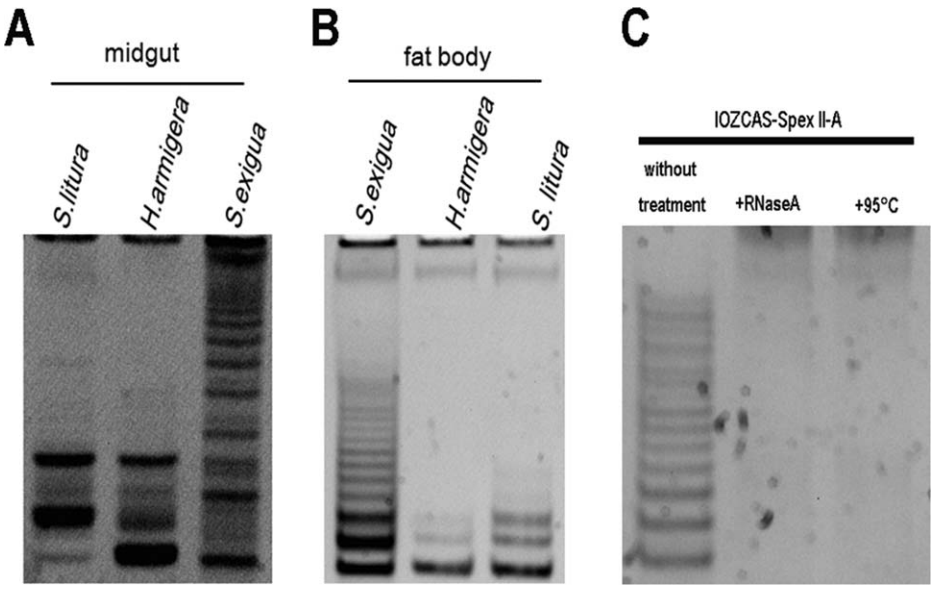

Figure 1. Telomerase activity detection in midgut (A) and fat body (B) of *S. litura*, *H. armigera* and *S. exigua*. A regular TRAP pattern was observed using *S. exigua* protein extracts in contrary to *S. litura* and *H. armigera*. (C) Telomerase activity was abolished in extracts of IOZCAS-SpexII-A cells using different treatments - RNase A, heat inactivation (95 °C for 20 min).

Results

Detection of telomerase activity in SpexII-A cells and all three tissues

Telomerase activities were detected ex vivo. TRAP pattern was observed in SpexII-A cells and the midgut and fat body tissues of *S. exigua*, and an non-TRAP pattern was observed from the tissue extracts of *S. litura, and H. armigera* (Fig. 1A and 1B). The sequences of TRAP products show that sequences added by telomerase are (TTAGG)n repeats (Fig. 2A). In addition, two experiments were conducted that included heating at 94 °C for 20 min and an RNase A digestion (Fermentas) at 37 °C for 50 min. TRAP pattern was abolished after both treatments, indicating the telomerase activity was heat and RNase sensitive (Fig. 1C).

Cloning of the SpexTERT gene

SpexTERT was cloned, which has no GQ motif in its gene structure, using degenerate PCR and RACE. Several degenerate PCR experiments were conducted using degenerate primers and cDNA or genomic DNA as a template. PCR products produced using RVVDDYL and oligo (dT) primer pairs display a band at approximately 900bp, a length corresponding to the *B. mori* telomerase reverse transcriptase BmoTERT target band (Fig. 3A). The 900bp product was purified and cloned into the pEasy-T1 vector and then being sequenced. Results show the sequence shares approximately 40% nucleotide similarity to corresponding parts of the BmoTERT gene sequence. Thus, we are sure this 900bp band is part of the TERT gene of *S. exigua*. Then, 5′- & 3′-RACE were conducted using the SMARTerTM RACE cDNA Amplification Kit (Clontech) and several sequencing primers was designed for whole mRNA sequencing. The RACE experiments revealed a 2278-bp mRNA sequence containing only one potential open reading frame (ORF) of 2127-bp (Fig. 3B and Fig. 4). The newly found gene was named SpexTERT after data processing and was submitted to GenBank (GenBank RefSeq KC347571).

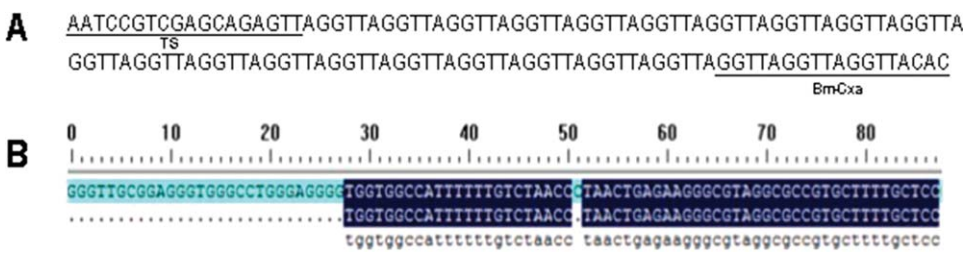

Figure 2. (A) The sequences of TRAP products show the sequences added by telomerase of IOZCAS-SpexII-A cells are (TTAGG)n repeats by traditional TRAP assays with TS as the forward primer and Bm-Cxa as the reverse primer. (B) A 113bp PCR product was generated in which the (TTAGGG)n six nucleotide repeat template was mutated into the (TTAGG)n five nucleotide repeat by using T7_HTERC_Up and MHTERC_Res as primers and HeLa cell cDNA as template.

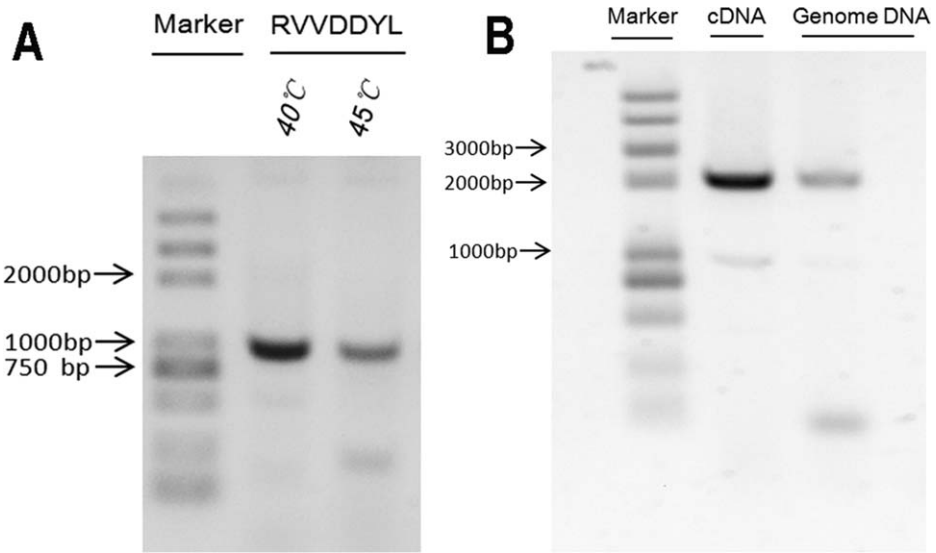

Figure 3. (A) Partial SpexTERT gene cloned by degenerate PCR using RVVDDYL (See Table1) as forward primer and oligo dT as reverse. Lane1, DNA marker. Lane 2-3, bands of degenerate PCR product with annealing temperature of 40 °C and 45 °C, respectively. (B) PCR product of SpexTERT gene complete sequences at 55 °C using cDNA (Lane 2) and genome DNA (Lane 3) as template respectively (Numbers of Lanes are counted from left to right, respectively).

Characterization of SpexTERT

The resultant full-length protein of *S. exigua* telomerase (SpexTERT, GenBank RefSeq AGH33838.1) is 708 amino acids and has 23% identity and 35% similarity to human *Homo sapiens* telomerase (HsTERT, GenBank RefSeq EAX08171.1). The armyworm's telomerase has 54% identity and 73% similarity to BmoTERT. The armyworm's telomerase has 25% identity and 40% similarity to the flour beetle's *T. castaneum* telomerase (TcTERT, GenBank RefSeq NP_001035796.1). Gel imaging and sequencing results show the SpexTERT gene has no introns like BmoTERT and many other TERT genes (Fig. 3B). It has one ATG codon in the 5'UTR, but it is not suitable for ribosome binding or for translation start. Therefore, this gene should have a unique translation start site and a unique gene product as well.

Multiple amino acid sequence alignments of the N-terminal, the central and C-terminal regions of TERT proteins show that the SpexTERT gene has the CP, QFP, T, 1, 2, A, B', C, D and E motifs but lacks the GQ motif according to the histogram (Fig. 4).

Expression, reconstitution and detection activity of the SpexTERT protein in vitro

TERT is the known coding sequence for the catalytic subunit of telomerase, which has telomerase activity. SpexTERT was a new report of an (TTAGG)n telomere pattern in insect species. SpexTERT was cloned into PET28b, expressed in BL21 (DE3) Rosetta cells and purified with a His-tag column (see Fig. 5A). The (TTAGGG)n six nucleotide repeat sequence was mutated, from human telomerase RNA, to a (TTAGG)n five nucleotide repeat sequence similar to the telomere repeats of *S. exigua* (Feng *et al.*, 1995). According to the designed primers containing the T7 promoter (Tabel 1), we amplified the mutant hTER-RNA gene by PCR, and acquired a linear T7 DNA template for transcription in vitro (Fig. 5B).

The reconstitution of complex was performed. A 113bp PCR product was generated in which the (TTAGGG)n six nucleotide repeat template was mutated into the (TTAGG)n five nucleotide repeat by using T7_HTERC_Up and MHTERC_Res as primers and HeLa cell cDNA as template (Fig. 2B). After reconstitution of the SpexTERT protein and the RNA component, traditional TRAP assays for telomerase activity were conducted (Beattie *et al.*, 1998; Mitchell *et al.*, 2010). The step-wise ladder of telomerase products can be seen in lane 1, but still, there are some bands shown in lane 2 and lane 3 respectively. No bands shown in Lane 4 which sample is the negative control without mutant hTER-RNA in the reconstitution complex (Fig. 5C). The results showed that we successfully expressed and purified the SpexTERT protein and the mutant hTER-RNA. Moreover, TRAP experiments showed the recombinant SpexTERT protein and mutant hTER-RNA complex had telomere segment lengthening activity and that SpexTERT had reverse transcriptase activity in vitro. These results further suggest that telomerase activity was detected in the fat body tissues and cells of *S. exigua*.

Discussion

Detection of telomerase activity in SpexII-A cells and all three tissues

Establishment of insect cell lines for this study has been a difficult task. A notable phenomenon was found in establishing insect lines in our lab. The larval fat body

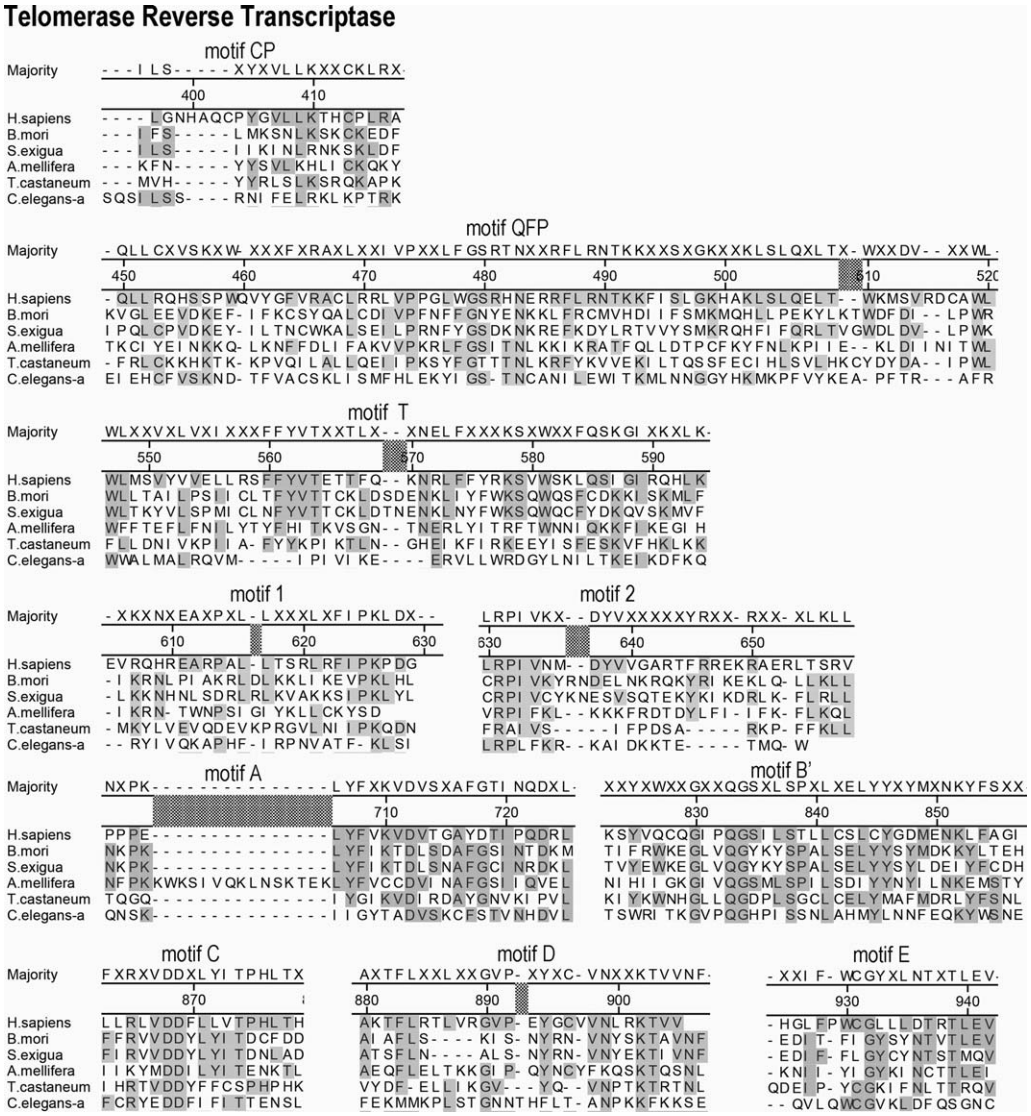

Figure 4. Multiple amino acid sequence alignment of the N-terminal, the central and C-terminal regions of TERT proteins. The alignment includes TERT sequences from *H. sapiens* (*Homo sapiens* ABB72674.1), *B. mori* (*Bombyx mori* NP_001037666.1), *S. exigua* (*Spodoptera exigua* AGH33838.1), *T.castaneum* (*Tribolium castaneum* NP_001035796.1), *A. mellifera* (*Apis mellifera* NP_001035771.1) and *C. elegans* (*Caenorhabditis elegans* CAB09413.1). Shaded residues represent similar amino acids.

tissues of three noctuids (*S. exigua, S. litura* and *H. armigera*) were used and primary cells were separated from each tissue and cultured them without any treatment. After several generation passages, only cells from *S. exigua* survived and had the potential to be a continuous cell line, primary cells from *S. litura* and *H. armigera* usually died. The experiment was repeated several times with replicable consequences.

Overall, robust telomerase expression is present in insects not only in somatic tissues but also in germ and pluripotent stem cells as observed in human tissues (Krupp *et al*., 2000; Lang *et al.,* 2004; Donate and Blasco, 2011). The TRAP pattern of the two *S. exigua* tissues indicated the telomerase in these tissues possesses repeat addition processivity, whereas the non-TRAP pattern of *S. litura* and *H. armigera* tissues indicated they do not, and that of the three species studied, telomerase activity is the highest in *S. exigua*. Primary cells derived from the larval fat body of *S. exigua* are much easier to immortalize than primary cells derived from *S. litura* and *H. armigera* in our lab maybe it is because in *S. exigua* high telomerase activity overcomes the restrictions of the “Hayflick limit” (Hayflick & Moorhead, 2008). Results also suggest that SpexII-A cells may use a telomerase to solve their telomere shortening problem (Fig. 1C). The non-TRAP pattern in *S. litura* and *H. armigera* indicated their telomerase activity has been partially lost (Fig.

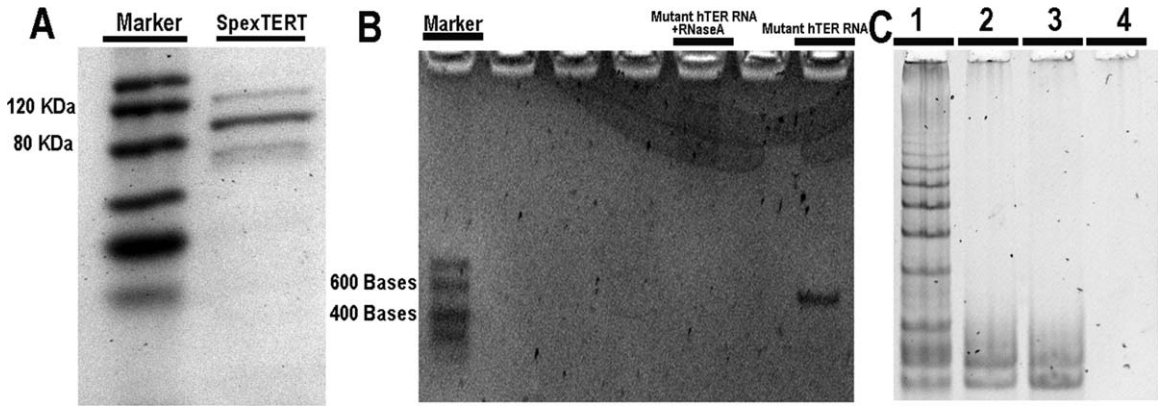

Figure 5. (A) SpexTERT purification after expression by SDS-PAGE. Lane 1: Protein marker. Lane 2: SpexTERT protein expressed by bacterial system and purified by 6x His-Tag. (B) Mutant hTER-RNA. Lane 1: Marker. Lane 2 to lane 4 and lane 6, none. Lane 5, mutant hTER-RNA with RNase A treatment. Lane 7, mutant hTER-RNA. (C) TRAP image of reconstitution complex of SpexTERT protein and mutant hTER-RNA. Concentration of SpexTERT protein was 0.5 μg/μL from Lane 1 to Lane 4. Concentration of mutant hTER-RNA in the reconstitution complex was 5.8 ng/μL. 0.58 ng/μL, 0.29 ng/μL and 0 ng/μL, respectively.

1B). Above all, *S. exigua* expresses telomerase and keeps a high telomerase activity and therefore its cells are relatively easy to establish as cell lines while that of *S. litura* and *H. armiger* do not. This may be explained by the pro-source relationship of these species because telomerase activity in *B. mori* cannot be detected by TRAP assays either (Osanai *et al.*, 2006).

Characteristics of SpexTERT

Five telomerases have been identified to date in insects (*A. mellifera*, *A.pisum*, *B. mori*, *B. mandarina*, *T. castaneum*) (Honeybee Genome Sequencing Consortium, 2006; Osanai *et al.*, 2006; Robertson and Gordon, 2006; Monti *et al.*, 2011). The presence of a telomerase coding gene of *S. exigua* has been identified successively, which synthesizes (TTAGG)n patterned telomeric tandem repeat. The telomerase activity has not been detected in all investigated tissues of the domestic silkworm *B. mori*. While we have confirmed SpexTERT to be active ex vivo by TRAP assay. Gene structure analysis shows there is no GQ motif at the N-terminus of SpexTERT which is similar to the TERT's of *B. mori* and *T. castaneum*. Despite lacking the GQ motif, SpexTERT is still active ex vivo, and its gene lacks introns similarly to BmoTERT and the *T. castaneum* TERT gene (Osanai *et al.*, 2006). This indicates the GQ motif is not essential for telomerase catalysis and that BmoTERT's inactivity is not due to the loss of the GQ motif. The GQ motif has been reported to have interactions with telomeric DNA and telomerase RNA in some species. However, this is challenged by our discovery of SpexTERT. There is no GQ motif or TEN domain in the N-terminal of SpexTERT, but a TRAP assay with SpexTERT detects the addition of TTAGG repeats to the telomere, indicating SpexTERT has strong telomerase activity.

The GQ motif is in the conserved N-terminus region of TERT and is responsible for recruitment of Est3p, a telomerase regulatory protein and nonspecific nucleic acid binding in budding yeast (Xia *et al.*, 2000; Friedman *et al.*, 2003), interactions with proteins that interacts with telomeric DNA and telomerase RNA in humans (Lee *et al.*, 2003; Moriarty *et al.*, 2004), interactions with telomerase RNA in *T. thermophila* (O'Connor *et al.*, 2005), and repeat processivity in yeast and human TERTs (Moriarty *et al.*, 2004). The GQ motif has been found in the N-terminal structure of TERTs of many species (Xia *et al.*, 2000) and is reported to have an important function in maintaining telomeres through telomerase elongation (Blaine *et al.*, 2001). As far as we know, all functional TERTs have GQ motifs or N-terminal domain of TERT (TEN domains) in their N-terminal regions and these residues are phylogenetically conserved. This is consistent with the discovery that there are no GQ motifs or TEN domains in BmoTERT, which is not functional (Osanai *et al.*, 2006). The discoveries in this study may provide new explanations about the functions of GQ motifs (Osanai *et al.*, 2006). The GQ motif, in addition to participating in the catalysis function of the telomerase complex, may function as an internal receptor or recruiter on the complex in some nucleus pathway. The GQ motif may also be involved in the formation of the correct structure of the telomerase complex. The loss of telomerase activity and repeat processivity might be caused by a change in the complex structure rather than the loss of the GQ motif. The absence of high-resolution data for the full-length catalyst unit of the enzyme, a complete structure of the telomerase complex and its additional components is a major limiting factor (Petrova

Table 2. G-quadruplexs and GQ motifs in TERTs of 13 species

Species name	Telomere sequences	G-quadruplex Formation	Existence of conserved GQ motif in TERT
B. mori	TTAGG	-	-
A. mellifera	TTAGG	-	-
S. exigua	TTAGG	-	-
T. castaneum	TCAGG	-	-
C. elegans	TTAGGC	-	-
C. albicans	ACGGATGTCTAACTTCTTGGTGT	-	+
H. sapiens	TTAGGG	+	+
M. musculus	TTAGGG	+	+
G. gallus	TTAGGG	+	+
X. laevis	TTAGGG	+	+
D. rerio	TTAGGG	+	+
T. thermophila	TTGGGG	+	+
S. pombe	G_{2-8}TTAC(A)	+	+

(-) means no G-quadruplex formation or no existence of GQ motif in TERT respectively while (+) means the contrary.

et al., 2014). Investigations of the BmoTERT telomerase RNA component should be conducted to determine the cause of its low activity (Greider & Blackburn, 1989; Osanai *et al.*, 2006). We must identify the RNA component of the *S. exigua* telomerase complex and will recombine the catalysis unit and RNA component in vitro to test its activity (Beattie *et al.*, 1998). However, we have not yet attained the RNA sequence. It is gratifying that there were reports about successfully reconstruction of telomerase activity using TERT and RNA component from different species (Laetitia *et al.*, 2012). We therefore mutated the reverse transcript template of hTER which is (TTAGGG)n six nucleotide repeat sequence to a (TTAGG)n five nucleotide repeat sequence, acquired this mutated hTER DNA template. Then the DNA template was transcripted into mutated hTER RNA using T7 RNA transcriptase and expressed the SpexTERT protein in vitro and recombined the two components. TRAP bands show the complex is active. These results support the hypothesis we mentioned above.

A TRAP assay of SpexTERT confirms it is active in vitro, which challenges former reports that the low telomerase activity of BmoTERT is caused by the loss of the GQ motif and that the GQ motif is necessary for transcriptase activity. We propose a hypothesis where neither GQ motifs nor TEN domains influence the catalytic activity of telomerase directly but rather do so indirectly in two possible ways. First, the GQ motif or TEN domain interacts with proteins needed for the assembly of the telomerase holoenzyme thus forming the correct protein formation and stabilizing the RNA-DNA-protein complex. Second, the GQ motif or TEN domain interacts with proteins that can recruit other proteins to promote the formation of a higher DNA structure such as a G-quadruplex, which stabilizes telomere DNA, or unwinds G-quadruplexes to allow telomerase to bind to telomeres (Paeschke *et al.*, 2008; Zhang *et al.*, 2010; Zhong *et al.*, 2012; Eckert & Collins, 2012). Current data on the structure of telomerase proteins is scarce. Out of thirteen species, eight have GQ motifs in their TERTs including *H. sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, *T. thermophila, C. albicans* and *Schizosaccharomyces pombe* and their telomere sequences are able to form G-quadruplexes (Table 2). Meanwhile, *B. mori, T. castaneum, S. exigua, Apis mellifera* and *Caenorhabditis elegans* do not have GQ motifs in their TERTs and their telomere sequences are unable to form G-quadruplexes (Table 2). According to the coadaptation of structure and function we believe that this hypothesized mechanism might be formed through biological evolution or change. Species (human, mouse, yeast, etc.) that have GQ motifs need the promotion of the formation or unwinding of G-quadruplexes because they have $N_{(1\text{-}7)}G_{(3+)}$ patterned telomere sequences in which a G-quadruplex can form an intramolecular formation (Huppert & Balasubramanian, 2005). Species such as insects and *C. elegans* those lack GQ motifs do not need the promotion of the formation or unwinding G-quadruplexes. That is because there telemere pattern is always like $[TT/CAGG]_n$ in which an intramolecular G-quadruplex cannot be formed. This pattern is found in most insects and $[TTAGGC]_n$ in *C. elegans* (Coleman *et al.*, 1993; Okazaki *et al.*, 1993; Zhang *et al.*, 1994; Sahara *et al.*, 1999; Rego & Marec, 2003; Traut *et al.*, 2007; Gomes *et al.*, 2011) Mainly, we have acquired new insights on GQ motifs and TEN domains, which may function as internal receptors or recruiters on the telomerase complex in a nucleus pathway. Additionally, we have found a new and possibly important function of the N-terminal of TERT that correlates with the regulation of G-quadruplexes on 3' overhangs of telomere DNA.

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References

Aubert, G. and Lansdorp, P.M. (2008) Telomeres and aging. *Physiol Rev* **88** (2): 557–579.

Beattie, T.L., Zhou, W., Robinson, M.O. and Harrington, L. (1998) Reconstitution of human telomerase activity in vitro. *Curr Biol* **8**: 177–180.

Blackburn, E.H. (1991) Structure and function of telomeres. *Nature* **350**: 569–573.

Blaine, N.A., Soma, S.R.B., Guo, C.H., Allyson, C.S. and Christopher, M.C. (2001) N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. *Mol Cell Biol* **21**: 7775–7786.

Blasco, M.A. (2005) Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* **6** (8): 611–622.

Blasco, M.A. (2007) Telomere length, stem cells and aging. *Nat Chem Biol* **3** (10): 640–649.

Coleman, M.J., McHale, M.T., Arnau, J., Watson, A. and Oliver, R.P. (1993) Cloning and characterisation of telomeric DNA from *Cladosporium fulvum*. *Gene* **132**, 67–73.

Donate, L.E. and Blasco, M.A. (2011) Telomeres in cancer and ageing. Philos Trans R Soc London. *Ser B Biol Sci* **366**(1561): 76–84.

Eckert, B. and Collins, K. (2012) Roles of telomerase reverse transcriptase n-terminal domain in assembly and activity of tetrahymena telomerase holoenzyme. *J Biol Chem* **287**: 12805–12814.

Falchetti, A., Becherini, L., Martineti, V., Morelli, A., Benvenuti, S., Picariello, L. *et al*. (1999) Telomerase Repeat Amplification Protocol TRAP. A new molecular marker for parathyroid carcinoma. *Biochem Bioph Res Co* **265**: 252–255.

Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiu, C.P. *et al*. (1995) The RNA component of human telomerase. *Science* **269** (5228): 1236–1241.

Friedman, K.L., Heit, J.J., Long, D.M. and Cech, T.R. (2003) N-terminal domain of yeast telomerase reverse transcriptase: Recruitment of Est3p to the telomerase complex. *Mol Biol Cell* **14**: 1–13.

Gillis, A.J., Schuller, A.P. and Skordalakes, E. (2008) Structure of the *Tribolium castaneum* telomerase catalytic subunit TERT. *Nature* **455** (7213):633–637.

Gomes, N.M.V., Ryder, O.A., Houck, M.L., Charter, S.J., Walker, W., Forsyth, N.R. *et al*. (2011) Comparative biology of mammalian telomeres: Hypotheses on ancestral states and the roles of telomeres in longevity determination. *Aging Cell* **10**: 761–768.

Greider, C.W. and Blackburn, E.H. (1989) A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* **337**: 331–337.

Hayflick, L. (1965) The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* **37**(3): 614–636.

Honeybee Genome Sequencing Consortium. (2006) Insights into social insects from the genome of the honeybee Apis mellifera. *Nature* **443**(7114): 931–949.

Huppert, J.L. and Balasubramanian, S. (2005) Prevalence of quadruplexes in the human genome. *Nucleic Acids Res* **33**: 2908–2916.

Jacobs, S.A., Podell, E.R., Wuttke, D.S. and Cech, T.R. (2005) Soluble domains of telomerase reverse transcriptase identified by high-throughput screening. *Protein Sci* **14**: 2051–2058.

Klapper, W., Heidorn, K., Kuhne, K., Parwaresch, R. and Krupp, G. (1998) Telomerase activity in 'immortal' fish. *Febs Lett* **434**: 409–412.

Korandová, M., Krůček, T., Vrbová, K., and Frydrychová, R.C. (2014) Distribution of TTAGG-specific telomerase activity in insects. *Chromosome Res* **22**(4): 495–503.

Krupp, G., Bonatz, G., and Parwaresch, R. (2000) Telomerase, immortality and cancer. *Biotechnol Ann Rev* **6**: 103–140.

Laetitia, T.F., Delphine, T.M.E., Johans, F., Denis, R., and Chantal, A. (2012) The human telomerase catalytic subunit and viral telomerase RNA reconstitute a functional telomerase complex in a cell-free system, but not in human cells. *Cell Mol Biol Lett* **17**(4): 598–615.

Lang, G.H., Wang, Y., Nomura, N. and Matsumura, M. (2004) Detection of telomerase activity in tissues and primary cultured lymphoid cells of *Penaeus japonicas*. *Mar Biotechnol* **6**: 347–354.

Lee, S.R., Wong, J.M. and Collins, K. (2003) Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. *J Biol Chem* **278**: 52531–52536.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., (1951) Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Mitchell, M., Gillis, A., Futahashi, M., Fujiwara, H. and Skordalakes, E. (2010) Structural basis for telomerase catalytic subunit TERT binding to RNA template and telomeric DNA. *Nat Struct Mol Biol* **17**: 513–518.

Monti, V., Giusti, M., Bizzaro, D., Manicardi, G.C. and Mandrioli, M. (2011) Presence of a functional (TTAGG)(n) telomere-telomerase system in aphids. *Chromosome Res* **19**(5): 625–633.

Moriarty, T.J., Marie-Egyptienne, D.T. and Autexier, C. (2004) Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer. *Mol Cell Biol* **24**: 3720–3733.

O'Connor, C.M., Lai, C.K. and Collins, K. (2005) Two purified domains of telomerase reverse transcriptase reconstitute sequence-specific interactions with RNA. *J Biol Chem* **17**: 17533–17539.

Okazaki, S., Tsuchida, K., Maekawa, H., Ishikawa, H. and Fujiwara, H. (1993) Identification of a pentanucleotide telomeric sequence, (TTAGG)n, in the silkworm Bombyx mori and in other insects. *Mol Cell Biol* **13**: 1424–1432.

Osanai, M., Kojima, K.K., Futahashi, R., Yaguchi, S. and Fujiwara, H. (2006) Identification and characterization of the telomerase reverse transcriptase of Bombyx mori (silkworm) and Tribolium castaneum (flour beetle). *Gene* **376**: 281–289.

Paeschke, K., Juranek, S., Simonsson, T., Hempel, A., Rhodes, D. and Lipps, H.J. (2008) Telomerase recruitment by the telomere end binding protein-b facilitates G-quadruplex DNA unfolding in ciliates. *Nat Struct Mol Biol* **15**: 598–604.

Petrova, O.A., Smekalova, E.M., Zvereva, M.E., Lamzin, V. and Dontsova, O.A. (2014) Identification of additional telomerase component of the yeast H. polymorpha is a step towards understanding the complex at the atomic level. *Dokl Biochem Biophys* **455**(1): 59–64.

Rego, A. and Marec, F. (2003) Telomeric and interstitial telomeric sequences in holokinetic chromosomes of Lepidoptera: Telomeric DNA mediates association between postpachytene bivalents in achiasmatic meiosis of females. *Chromosome Res* **11**: 681–694.

Robertson, H.M. and Gordon, K.H.J. (2006) Canonical TTAGG-repeat telomeres and telomerase in the honey bee, Apis mellifera. *Genome Res* **16**(11): 1345–1351.

Sahara, K., Marec, F. and Traut, W. (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosome Res* **7**: 449–460.

Sasaki, T. and Fujiwara, H. (2000) Detection and distribution patterns of telomerase activity in insects. *Eur J Biochem* **267**: 3025–3031.

Schuller, A.P., Harkisheimer, M.J., and Skordalakes, E. (2011) In vitro Reconstitution of the active *T. castaneum* telomerase. *J Vis Exp* **14** (53): e2799.

Tlarkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H. *et al*. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

Traut, W., Szczepanowski, M., Vitkova, M., Opitz, C., Marec, F. and Zrzavy, J. (2007) The telomere repeat motif of basal Metazoa. *Chromosome Res* **15**: 371–382.

Xia, J.Q., Peng, Y., Mian, S. and Lue, N.F. (2000) Identification of functionally important domains in the N-terminal region of telomerase reverse transcriptase. *Mol Cell Biol* **20**: 5196–5207.

Zhang, H., Zhang, Y., Qin, Q., Li, X., Miao, L., Wang, Y. *et al*. (2006) New cell lines from larval fat bodies of *Spodoptera exigua*: Characterization and susceptibility to baculoviruses (Lepidoptera: Noctuidae). *J Invert Path* **91**(1): 9–12.

Zhang, H., Zhang, Y., Qin, Q., Li, X., Miao, L., Wang, Y. *et al*. (2009) A cell strain cloned from *Spodoptera exigua* cell line (IOZCAS-Spex-II) highly susceptible to *S. exigua* nucleopolyhedrovirus infection. *In Vitro Cell Dev Biol-Animal* **45**: 201–204.

Zhang, M.L., Tong, X.J., Fu, X.H., Zhou, B.O., Wang, J., Liao, X.H. *et al*. (2010) Yeast telomerase subunit Est1p has guanine quadruplex promoting activity that is required for telomere elongation. *Nat Struct Mol Biol* **17**: 202–210.

Zhang, Y.J., Kamnert, I., Lopez, C.C., Cohn, M. and Edstrom, J.E. (1994) A family of complex tandem DNA repeats in the telomeres of *Chironomus pallidivittatus*. *Mol Cell Biol* **14**: 8028–8036.

Zhong, F.L., Batista, L.F.Z., Freund, A., Pech, M.F., Venteicher, A.S. and Artandi, S.E. (2012) Tpp1 OB-fold domain controls telomere maintenance by recruiting telomerase to chromosome ends. *Cell* **150**: 481–494.