 Nucleic Acids Research, 1999, Vol. 27, No. 21 **4269–4275**

Identification of an essential proximal sequence element in the promoter of the telomerase RNA gene of *Tetrahymena thermophila*

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Received June 7, 1999; Revised and Accepted September 17, 1999

ABSTRACT

Telomerase is a ribonucleoprotein reverse transcriptase that synthesizes and maintains telomeric DNA. Studies of telomeres and telomerase are facilitated by the large number of linear DNA molecules found in ciliated protozoa, such as *Tetrahymena thermophila*. To examine the expression of telomerase, we investigated the transcription of the RNA polymerase III-directed gene encoding the RNA subunit (TER1) of this enzyme. A chimeric gene containing the *Glaucoma chattoni TER1* transcribed region flanked by 5′ and 3′ *Tetrahymena* regions was used to identify promoter elements following transformation of *Tetrahymena* cells. Disruption of a conserved proximal sequence element (PSE) located at –55 in the *Tetrahymena TER1* 5′ flanking region eliminated expression of the chimeric gene. In addition, mutation of an A/T-rich element at –25 decreased expression markedly. A gel mobility shift assay and protein–DNA cross-linking identified a PSE-binding polypeptide of 50–60 kDa in *Tetrahymena* extracts. Gel filtration analysis revealed a native molecular mass of ~160 kDa for this binding activity. Our results point to a similar architecture between ciliate telomerase RNA and metazoan U6 small nuclear RNA promoters.

INTRODUCTION

The DNA sequence of telomeres, the nucleoprotein structures that cap eukaryotic chromosomes, is synthesized in part by the enzyme telomerase. In the absence of telomerase activity, telomeres shorten in successive generations, resulting in chromosome fusions, cell cycle arrest, senescence and, ultimately, cell death (1–3). Telomerase is active and telomeric DNA is maintained in cells with unlimited proliferative capacity (unicellular eukaryotes, germline cells, and the majority of cancer cells; reviewed in 4). Consequently, understanding how telomerase activity is regulated is a topic of intense investigation, as it is likely to have implications for human aging and cancer.

Ciliated protozoa are ideal organisms for investigating both the structure and regulation of telomerase, because they contain a large number of chromosome ends that are synthesized *de novo* during a specific stage in the ciliate life cycle. Upon conjugation of starved ciliates, a new macronucleus develops from a copy of the germline micronucleus, in a process that requires extensive chromosome fragmentation and telomere addition (5). Telomerase is a ribonucleoprotein complex, containing a reverse transcriptase subunit (TERT) and an RNA subunit that provides the template for telomeric DNA synthesis. An increase in telomerase activity, such as observed during ciliate conjugation, requires the concerted expression of the RNA and TERT genes. During mating of *Tetrahymena thermophila*, telomerase RNA levels increase ~3-fold and induction of TERT is substantially greater (6,7). Recent studies demonstrate a role for coordinated synthesis of TERT and telomerase RNA in mammalian cells (8).

Genes encoding the telomerase RNA subunit have been cloned from a variety of organisms, including many ciliated protozoa. While human and mouse telomerase RNA subunit genes are likely to be transcribed by RNA polymerase II (9,10), the *T.thermophila TER1* gene is transcribed by RNA polymerase III (pol III), based on the presence of an oligo(U) stretch at the 3′-end of the short RNA and α-amanitin sensitivity in run-on transcription using isolated nuclei (11). Sequence-comparison of a number of *TER1* 5′-flanking regions from ciliated protozoa suggests that these genes may contain upstream elements reminiscent of the vertebrate U6/7SK class of pol III-transcribed loci (12,13). Unlike genes encoding tRNAs and 5S ribosomal RNA, that include essential intragenic control regions, the vertebrate U6/7SK-type pol III promoters contain upstream proximal sequence elements (PSEs) and TATA boxes, and lack intragenic elements (14).

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In this study, we investigated the role of upstream sequences in expression of the *TER1* gene in *Tetrahymena*. Our results demonstrate that the *T.thermophila TER1* promoter contains an essential PSE centered at –55 and an important A/T-rich element at –25. Furthermore, we characterized a multi subunit protein that binds to the PSE.

MATERIALS AND METHODS

Plasmid constructions

The parent plasmid for these studies contains a chimeric gene with the *T.thermophila TER1* 5′ and 3′ flanking regions surrounding the *Glaucoma chattoni TER1* transcribed sequence (*Gc.TER1* in ref. 15) inserted into pCR2.1 (Invitrogen). Truncations in the 5′ flanking region were made at the following restriction sites: *Dra*I (*Gc.ter1*-Δ*135*), *Mbo*II (*Gc.ter1*-Δ*94*) and *Ssp*I (*Gc.ter1*-Δ*37*). Clustered mutations were generated by PCR with the parent plasmid using oligonucleotides that contained *Sal*I restriction sites to substitute for specified nucleotides within the PSE and potential TATA elements. These nucleotide changes are (mutations indicated in lower-case): *pse*: (–64)CA-GAgtCgacAAAA, *tata1*: (–43)CCAAgTcgacTCCA, *tata2*: (–32)CCACgTcgAcTAGA, *tata3*: (–20)GAAAgTcgacTAAG. Furthermore, an exact truncation of the 3′ flanking region (*Gc.ter1*-Δ*3*′) was constructed by PCR using the oligonucleotide 5′-ATACAAATCGAAATAGggtaccAAAAC-TTGGCATTC-3′ to create a *Kpn*I restriction site (lower-case letters), followed by ligation at the *Kpn*I site of the pCR2.1 plasmid. Similarly, a plasmid lacking the 3′-flanking region beyond position +222 (*Gc.ter1*-Δ*+222*) was constructed by PCR using the oligonucleotide 5′-TGA-TCCTATTAAAAT-ACGTCggtaccTAA-‘‘‘CTTGTTAAAACA-AATCC-3′ to create a *Kpn*I site at this location. The sequences of all plasmid inserts were verified using the dideoxy method.

In order to introduce the chimeric genes into *Tetrahymena* cells, the inserts from pCR2.1 were excised and subcloned into the polylinker of the high copy number, episomal plasmid prD4-1 (16). Purified plasmid DNAs were prepared by alkaline lysis of bacterial cells, CsCl gradient centrifugation and chromatography on Bio-Gel A5m resin (BioRad). DNA concentrations were determined spectrophotometrically using absorbance at 260 nm and verified by visual examination of ethidium bromide-stained agarose gels.

Transformation of *Tetrahymena* cells and RNA analysis

Transformation of wild-type *T.thermophila* cells (strains CU427, B2086, CU428) was performed by electroporation (17). After dilution to obtain potential single transformant lines, drug selection was applied by the addition of paromomycin to 100–120 μg/ml 12–15 h after electroporation. Clones of transformants were harvested 3–4 days after drug addition. Pooled transformants (12–15 single lines) were grown in liquid culture in 2% PPYS (2% proteose peptone, 0.2% yeast extract, 0.03% sequestrine) at 30°C with shaking overnight, and then in 50 ml cultures for ~15 h. Total RNA was prepared using TRI REAGENT (Molecular Research Center, Inc.). Fifteen micrograms of RNA were electrophoresed on 6% polyacrylamide/7 M urea/TBE denaturing gels, and electrotransferred to Qiabrane Nylon Plus membrane (Qiagen). Filters were probed with random-primed radiolabeled PCR fragment for *GcTER1* (15), stripped and reprobed with a kinased oligonucleotide complementary to *T.thermophila* 5S RNA (5′-TTCGAATGGGATCCGGT-GTTTTCACCTTAG-3′). The *GcTER1* probe was hybridized at 65°C in Rapid-hyb buffer (Amersham) for 1.5 h, and washed sequentially with 2× SSC, 0.1% SDS for 20 min at room temperature, followed twice with 1× SSC, 0.1% SDS for 15 min at 65°C. The 5S oligonucleotide probe was hybridized at 42°C in Rapid-hyb buffer for 1 h, and washed in 5× SSC, 0.1% SDS for 20 min at room temperature, followed with 1× SSC, 0.1% SDS at 42°C. Relative radioactivity in specific bands on the blots was quantitated using a Fujix BAS2000 PhosphorImager (Fuji).

Preparation of extracts from *Tetrahymena* cells

Tetrahymena thermophila cells were grown at 30°C in 2% PPYS media to a density of 2×10^5 cells/ml. Cells were harvested by centrifugation, washed in 10 mM Tris (pH 7.5) and resuspended in 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 3 mM $CaCl_2$, 0.25 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. NP-40 was added to a concentration of 1%, and the cells were stirred for 20 min at 4°C. Whole cell extracts were prepared by sonication and cleared by centrifugation at 9000 *g* for 30 min. The total protein concentration of these extracts was 5–6 mg/ml. The extracts used for cross-linking reactions were fractionated by ammonium sulfate precipitation (52% saturation) and chromatography over DEAE-cellulose (DE52). Chromatography was carried out in 0.1 M/buffer D (0.1 M KCl, 20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM EDTA, 2 mM dithiothreitol), and the PSE-binding activity was eluted in a 0.25 M KCl step fraction. Gel filtration chromatography (Superose 6 HR 10/30 resin) was performed with protein eluted from the DE52 0.25 M KCl step fraction under conditions described previously (18).

Electrophoretic mobility shift assay

A double-stranded oligonucleotide probe containing the *Tetrahymena TER1* PSE was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The sequences of the top and bottom strands for this probe are 5′-GATCCTTAAACAGACCCAT-AAAAATTCGCT-3′ and 5′-GATCAGCGAATTTTTATGG-GTCTGTTTAAG-3′. Approximately 3 fmol of the probe was incubated with *Tetrahymena* extracts in a 25 μl reaction volume containing 40 mM KCl, 2 mM $MgCl_2$, 8 mM HEPES (pH 7.9), 0.08 mM EDTA, 8% glycerol, 2 mM dithiothreitol, 80 μg/ml poly(dI)·poly(dC) at 25°C for 30 min. For competition studies, samples contained excess unlabeled oligonucleotides mixed with the probe before the addition of protein in amounts detailed in the figure legend. DNA–protein complexes were separated on 4% polyacrylamide/Tris–acetate/EDTA gels as described previously (19).

Protein–DNA cross-linking

An internally-radiolabeled double-stranded oligonucleotide containing the *Tetrahymena TER1* PSE element was prepared as described previously (20,21). Oligonucleotides 5′-GATC-CTTAAACAGACCCATAAAAATTCGCT-3′ and 5′-AGCG-AATTTT-3′ were annealed and filled in with Klenow DNA polymerase, [α-^{32}P]dGTP, 5-bromo-2′-deoxyuridine-5′-triphosphate (Sigma), dATP and dCTP. Approximately 24 fmol of PSE probe was incubated with 30 μg of extract in a solution with a total volume of 20 μl containing 100 μg/ml of poly(dI)·poly(dC),

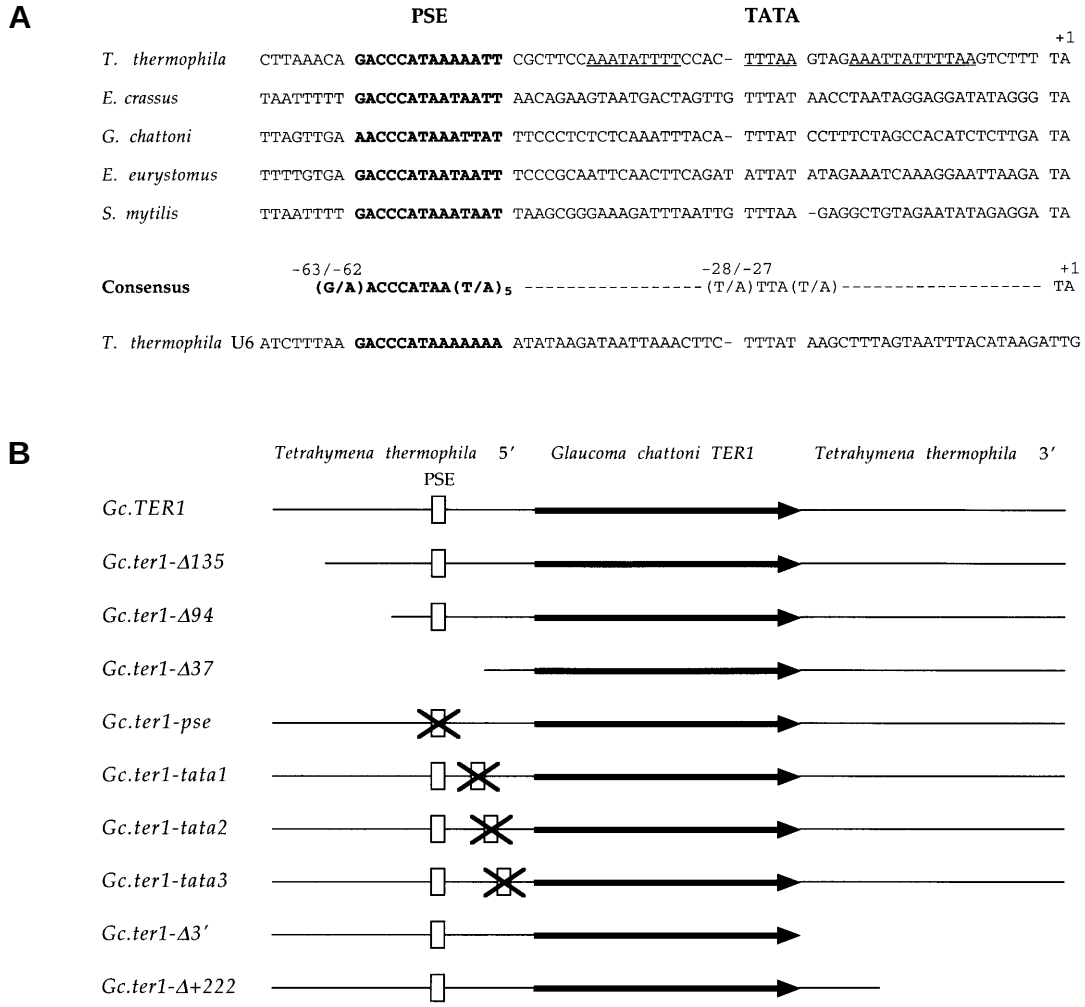

Figure 1. *TER1* 5′ flanking region in various ciliated protozoa. (**A**) Comparison of ciliate *TER1* gene and *T.thermophila* U6 snRNA gene proximal region sequences. The conserved PSE is shown in bold type. In addition, A/T-runs that were disrupted in various plasmids (tata1, tata2, tata3, respectively, from left to right) are underlined in the top line. (**B**) Diagram of chimeric *TER1* genes used for *Tetrahymena* transformation. All inserts were subcloned into the polylinker of prD4-1 prior to electroporation of *Tetrahymena* cells.

2.5 mM $MgCl_2$, 40 mM KCl, 8 mM HEPES (pH 7.9), 8% glycerol, 0.08 mM EDTA, 2.5 mM dithiothreitol. Some mixtures contained unlabeled oligonucleotide as detailed in the figure legend. Samples were incubated at 30°C for 30 min. Irradiation conditions, nuclease treatment and electrophoresis on a 10% polyacrylamide gel were performed as described previously (21).

RESULTS

Identification of 5′-flanking elements within the *T.thermophila* TER1 promoter

A comparison of the proximal 5′ flanking regions of several ciliate telomerase RNA subunit genes and the *Tetrahymena thermophila* U6 snRNA gene is shown in Figure 1A. A highly conserved sequence that could function as a PSE is centered ~55 bp upstream of the nucleotide encoding the 5′-end of these RNAs (12,13). Furthermore, a conserved (T/A)TTA(T/A) sequence is located ~25 bp from the 5′-end. Several runs of A/T exist between the PSE and initiation site that could serve as TATA boxes (underlined in Fig. 1A). To examine the function of the 5′ sequences, we prepared *TER1* gene plasmids that were truncated in the flanking regions or specifically mutated within the putative PSE or TATA boxes (Fig. 1B). The parent *TER1* was a chimeric gene containing the telomerase RNA transcribed region from *G.chattoni* and flanking regions from the *T.thermophila* gene. Previous work demonstrated that the *G.chattoni* telomerase RNA was expressed in transformed *Tetrahymena* cells (15). Hence, we used the *G.chattoni* telomerase RNA as a reporter for assessing the relative level of transcription from each construct.

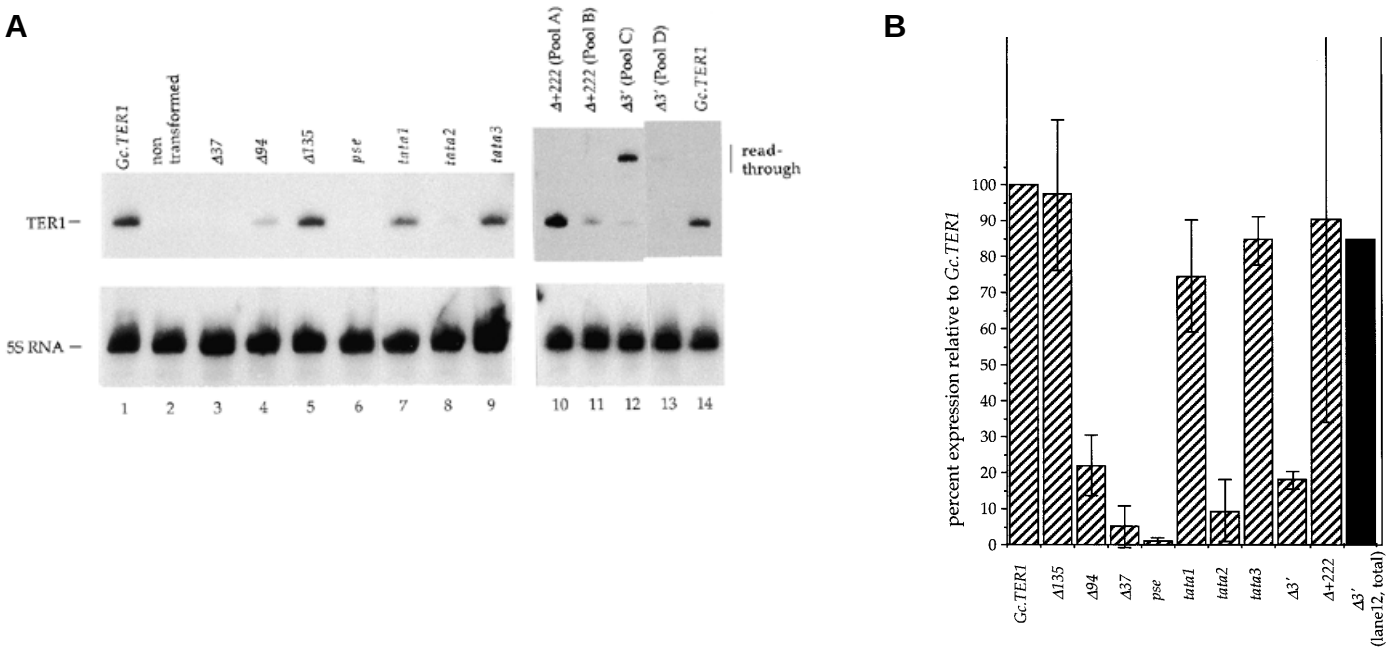

Figure 2. Expression of *TER1* in transformed *Tetrahymena* cells. (**A**) Typical results showing RNA levels on northern blots. The top panels correspond to *TER1* expression from the chimeric gene plasmids using a *G.chattoni TER1* probe. The bottom panels show levels of 5S RNA on the same blot used as a normalization standard. (**B**) Quantitation of transformation experiments. RNA levels on northern blots were quantified with a Fujix BAS2000 PhosphorImager (Fuji). After background subtraction, the level of TER1 RNA for each sample was normalized to the 5S RNA band intensity in that lane and compared with the signal from the *Gc.TER1* plasmid included in each experiment. All plasmids were tested in at least three separate transformation experiments, except for *Gc.ter1-Δ94* and *Gc.ter1-Δ+222*, which were tested twice. The height of each column represents the average value, and the height from the midpoint of the error bar shows one standard deviation from the mean. The solid black bar labeled 'Δ3′ (lane 12, total)' represents the sum of all RNAs in lane 12 that hybridize to the *GcTER1* probe relative to the amount of expression from cells containing the *Gc.TER1* plasmid (lane 14).

Glaucoma telomerase RNA was detected by northern blot analyses for various chimeric genes introduced into *Tetrahymena* cells on a high copy number plasmid (Fig. 2A). These blots were stripped and reprobed for 5S RNA in order to normalize for differential RNA recovery or gel loading. Results from several experiments were quantitated and displayed relative to the level of *Glaucoma* telomerase RNA expressed from the parent vector (*Gc.TER1*; Fig. 2B). Deletion of 5′ flanking sequences to position –135 had no effect on promoter activity. However, further deletion to position –94 resulted in nearly 80% reduction in expression. Telomerase RNA expression from the dl-37 template was extremely low. In most experiments no TER1 RNA was detected from this template (e.g. Fig. 2A).

A putative PSE is located between positions –94 and –38, and deletion of this segment virtually eliminated telomerase RNA gene expression. Therefore, we constructed a plasmid containing multiple mutations in this element (*Gc.ter1-pse*; Fig. 1B). No *TER1* expression was detected from the *pse* chimeric gene, identifying the PSE as an essential promoter element (Fig. 2). In addition, we examined whether the conserved (A/T)TTA(A/T) sequence found at –25 in ciliate telomerase RNA genes was important for transcription. We disrupted this sequence (*tata2*) as well as other possible TATA boxes in the near 5′ flanking region of the *Tetrahymena* gene (*tata1*, *tata3*; Fig. 1B). Only the conserved A/T-sequence at –25 exerted a significant role on *TER1* expression (*tata2*; Fig. 2).

We also examined whether the 3′ flanking region is important for expression of the *TER1* gene by constructing a plasmid with an exact truncation of that region. Only ~20% of wild-type level RNA was expressed from the *Gc.ter1-Δ3′* chimeric gene (Fig. 2). However, several longer transcripts could be detected, and in one experiment the amount of longer transcript was substantial, with the sum of hybridizing transcripts totalling 85% of the wild-type level (Fig. 2, lane 12). When a substantial portion of the 3′-flanking region was deleted (*Gc.ter1-Δ+222*), expression was reduced only modestly (Fig. 2, lanes 10 and 11). Therefore, we believe that the longer transcripts expressed from the *Gc.ter1-Δ3′* plasmid are likely to be read-through products and originate from a defect in transcription termination (Discussion).

The relative copy number of introduced DNA from several transformant pools was analyzed on Southern blots. No significant difference in the amount of DNA hybridizing to the *Glaucoma* probe was found among various transformants, including those with high *TER1* expression levels (*Gc.TER1*, *tata3*), low expression levels (*Δ37*, *tata2*), or moderate expression levels (*Δ3′*) (data not shown). Therefore, the different expression levels from the chimeric telomerase RNA genes were not a

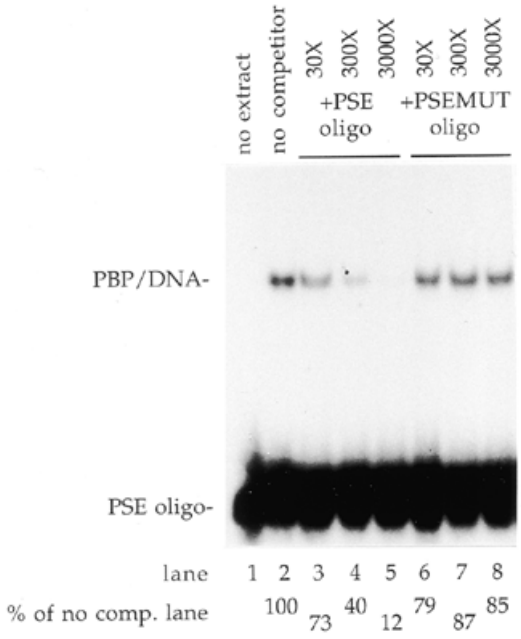

Figure 3. Identification of PBP in a *Tetrahymena* extract. A double-stranded oligonucleotide probe containing the *T.thermophila TER1* PSE was radiolabeled, incubated with protein and analyzed by electrophoretic mobility shift assay as detailed in Materials and Methods. Approximately 3 fmol of probe was used for each sample. The samples used for lanes 2–8 contained 15 μg of protein. Unlabeled oligonucleotide was added prior to the extract protein in the fold-molar excess denoted above each lane (30X = 90 fmol, 300X = 900 fmol, 3000X = 9000 fmol). The PSEMUT oligonucleotide contained the same mutations as the disrupted PSE promoter (*Gc.ter1-pse*) that are noted in Materials and Methods. Radioactivity in the PBP/DNA complex band from each lane was quantitated using a phosphorimager and compared to the level when no competitor oligonucleotide was added (lane 2).

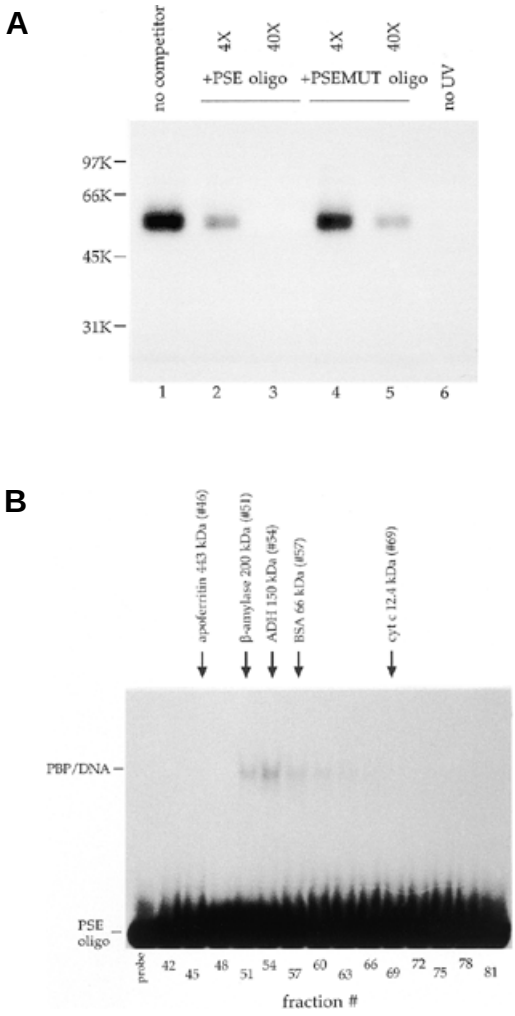

Figure 4. Approximate size of *Tetrahymena* PBP. (**A**) Ultraviolet light-mediated cross-linking to identify a DNA binding polypeptide(s). An internally radiolabeled double-stranded oligonucleotide containing the *Tetrahymena TER1* PSE was prepared, incubated with protein, irradiated with ultraviolet light, treated with nucleases, and electrophoresed as described in Materials and Methods. The mobilities of protein markers electrophoresed on the same gel are noted on the left. The sample loaded in lane 6 was not irradiated, but otherwise treated the same as the sample electrophoresed in lane 1. For the samples loaded in lanes 2–5, unlabeled double-stranded oligonucleotides were added in the fold-molar excess amounts relative to the radiolabeled probe as indicated above each lane prior to incubation with protein (4X = 100 fmol, 40X = 1000 fmol). (**B**) Estimation of molecular mass of native PBP by gel filtration chromatography. *Tetrahymena* whole cell extract fractionated by DEAE-cellulose chromatography was loaded on a calibrated Superose 6 HR10/30 column. The elution positions of marker proteins are indicated at the top, with the peak fraction number shown in parentheses. Not shown is the elution position of thyroglobulin (669 kDa) at fraction #37. Fractions were analyzed for PSE-binding activity by electrophoretic mobility shift assay as in Figure 3. Single fractions from 49–63 were analyzed on another gel, and the peak of PBP activity eluted in fraction #53 (data not shown).

consequence of difference in gene dosage for the various transformants.

Characterization of a transcription factor that binds to the PSE element

To further investigate the role of the essential PSE element in the transcription of the telomerase RNA subunit gene, we searched for protein(s) that specifically recognize this DNA sequence. Using a whole cell extract prepared from vegetatively-growing *Tetrahymena* cells, a single protein–DNA complex was observed by gel mobility shift analysis with the PSE oligonucleotide (Fig. 3, lane 2). Addition of competitor oligonucleotides demonstrated sequence specificity of this interaction, since an excess of the unlabeled wild-type oligonucleotide competed complex formation (Fig. 3, lanes 3–5), while addition of a mutant oligonucleotide containing the PSE sequence from the *Gc.ter1-pse* plasmid (Fig. 1B) did not compete (Fig. 3, lanes 6–8).

Ultraviolet light-mediated protein–DNA cross-linking was used to determine the molecular weight of a polypeptide(s) for *Tetrahymena* PSE-binding protein (PBP). A closely-migrating doublet of ~50–60 kDa was detected by SDS–PAGE (Fig. 4A, lane 1). We do not know whether the doublet corresponds to two different polypeptides, the same polypeptide that has been differentially modified, or a single polypeptide that has been partially degraded. The UV cross-linking assay captured a

sequence-specific interaction because addition of unlabeled wild-type oligonucleotide efficiently competed away the radiolabeled bands, while addition of the PSEMUT oligonucleotide reduced the signal much less (Fig. 4A; compare lanes 2 and 3 with lanes 4 and 5). The overall mass of *Tetrahymena* PBP was estimated by gel filtration (Fig. 4B). PSE-binding activity peaked at fraction 53 from this column, indicating an approximate molecular mass of 160 kDa for this complex. In combination, the UV cross-linking and gel filtration results indicate that *Tetrahymena* PBP is a multi-subunit complex. Although the large size predicted by gel filtration chromatography could reflect a non-globular shape of the protein, PBP complexes from other organisms contain multiple subunits (22–24), and our data suggests that *Tetrahymena* PBP is a multi-subunit complex also. Further information about the exact composition of PBP awaits purification of this protein.

DISCUSSION

During macronuclear development in *Tetrahymena*, telomerase activity and telomerase RNA levels are coincidentally increased. In order to understand this regulatory event, we have undertaken a study of the transcription of the gene that encodes the RNA subunit. We identified at least two discrete elements, the PSE at –55 and an A/T-rich element at –25, which constitute the promoter of the *T.thermophila TER1* gene. Therefore, this promoter appears to be very similar to those for the pol III genes that encode vertebrate U6/7SK snRNAs. Given the essential role of the PSE, we sought to identify a *Tetrahymena* protein that bound this sequence. We detected a protein, probably containing multiple subunits, that binds specifically to this element, which is likely to be an important factor for transcription of the *TER1* gene.

The PSE is conserved in telomerase RNA subunit genes among ciliated protozoa, all of which are likely to be transcribed by RNA pol III. However, it is not specific to this class of genes because a very similar sequence is found at the same position in *Tetrahymena* U6 snRNA genes (Fig. 1A) as well as those that encode U1, U2, U3 and U5 snRNAs (25). Indeed, we find that *Tetrahymena* U1 and U2 PSEs are effective competitors for binding of PBP to the *TER1* PSE (A.Domitrovich and G.Kunkel, unpublished results). The human PSE-binding protein, known as $SNAP_c$ or PTF, is required for transcription of the U6 snRNA gene by pol III and the U1 snRNA gene by pol II (22,23). HeLa cell $SNAP_c$ contains five different subunits ranging in size between 190 and 19 kDa (26). Furthermore, a multiple-subunit PSE-binding protein with a total mass of ~375 kDa has been identified from *Drosophila* (24). We suspect that the *Tetrahymena* PBP may be a homolog of the human and *Drosophila* proteins. Similar to the human and *Drosophila* versions, *Tetrahymena* PBP appears to contain multiple subunits, but it is somewhat smaller.

In addition to the essential PSE, our data indicate that the conserved A/T-rich segment at –25 is important for transcription, albeit to a lesser extent. Two other A/T-rich segments in the near 5′-flanking region were not important for *Tetrahymena TER1* expression when mutated individually. However, it is possible that one or both of these sequences act as non-optimal TATA boxes, resulting in the observed low level transcription in the absence of a functional –25 sequence element.

Our experiments uncovered another region upstream of the PSE that is a positive control element for *Tetrahymena TER1* transcription. Deletion of the segment between –135 and –94 resulted in a 5-fold decrease in expression. This region is extremely A/T-rich [almost 90%; (27)]. We were unable to detect any specific *Tetrahymena* protein that binds to this sequence (data not shown), nor could we identify any sequence similarity to the upstream region of other ciliate *TER1* genes. Therefore it is unknown whether this region contains a transcriptional control element that is a binding site for a sequence-specific factor or adopts a particular structure that stimulates transcription (28). It is possible that the *Tetrahymena TER1* gene promoter is composed of separable proximal and distal regions such as found in vertebrate U6 snRNA gene promoters (29–31). Vertebrate U6 distal regions act in enhancer-like fashion and contain at least two elements that bind distinct transcriptional activator proteins (19,21). One such activator protein, Oct-1, stabilizes formation of transcriptional initiation complexes on the human U6 promoter, at least in part, by facilitating the binding of $SNAP_c$ to the PSE (32,33).

Expression from the *Gc.ter1-Δ3′* plasmid is likely to be impaired in transcriptional termination. The wild-type gene contains an extensive run of eight Ts at the putative pol III termination site. In the *Gc.ter1-Δ3′* plasmid only four Ts remain, followed by plasmid polylinker sequence. Termination by RNA pol III varies, and is dependent on the length of the T-run and other nucleotides immediately flanking the T-run (34). Longer transcripts were detected from cells transformed with the *Gc.ter1-Δ3′* plasmid that are likely to be read-through products. Several T-runs ranging in length from 4 to 8 nt exist in the plasmid vector sequence downstream of the inserted gene. Read-through RNAs would have predicted sizes of 241 (4 Ts), 302 (4 Ts), 314 (5 Ts) and 351 nt (8 Ts). By comparison of the mobilities of DNA markers electrophoresed on the same gel, we calculate the size of the major read-through transcript to be 315 nt, a size which is consistent with that expected from termination at the 5 nt T-run, the first potential termination sequence encountered by the polymerase with a T-run longer than 4. The stabilities of these longer transcripts relative to the normal-length transcript are unknown, which compromises our ability to rigorously quantitate their expression levels. Indeed, whereas the expression levels of the normal-length transcript from a given plasmid remained relatively constant in different experiments, the amount of longer transcripts from *Gc.ter1-Δ3′* fluctuated widely. Alternatively, a downstream modulatory element could be necessary whose absence in the *Gc.ter1-Δ3′* plasmid results in reduced and/or aberrant expression. For example, the *S.cerevisiae* U6 snRNA gene contains a downstream B block element that is essential *in vivo*, but is dispensable *in vitro* (35–37). However, such a downstream element must be located very close to the terminator since deletion of most of the 3′-flanking region (*Gc.ter1-Δ+222*) had little effect on expression from this gene.

Our experiments did not address whether there are intragenic control elements in ciliate *TER1* genes. However, we could not identify any obvious A or B block elements that typify tRNA gene intragenic motifs within the transcribed sequence. Therefore we do not consider it likely that the *Tetrahymena TER1* gene contains both an internal B block element and upstream PSE and TATA motifs such as found in the *Xenopus laevis* selenocysteine tRNA gene (38).

Little is known about the transcription of other telomerase RNA subunit genes. The human and mouse genes contain potential TATA and CCAAT boxes, but their functional roles have yet to be proven (9,10). Other upstream elements may contribute to the promoter activity that has been detected in these genes (10). In contrast to the ciliate *TER1* and vertebrate snRNA gene promoters, the 5′ flanking regions of the human and mouse telomerase RNA subunit genes lack a PSE.

Interestingly, while the telomerase RNA subunit gene is transcribed by pol III in ciliated protozoa, it is almost certainly transcribed by pol II in mammals and yeast. The human and mouse genes appear to have pol II mRNA-like promoters, and *S.cerevisiae* telomerase RNA (TLC1) contains long U-runs that would correspond to pol III terminators and therefore exclude synthesis by this RNA polymerase (39). The polymerase specificity of most small RNA genes is phylogenetically conserved. For example, U6 snRNA gene transcription is maintained by pol III in all organisms even though the promoter structure varies considerably between yeasts and mammals (40). It will be especially interesting to compare the telomerase RNA gene promoters of non-mammalian metazoan organisms to those of ciliate *TER1*. Use of very different types of promoters to drive synthesis of telomerase RNA genes between ciliates and mammals could reflect distinct pathways of telomerase regulation.

ACKNOWLEDGEMENTS

We thank Tim Shrode for construction of the *Gc.ter1-pse* plasmid and Eric Greene for assistance with the calibrated gel filtration column. This research was supported by the Texas Advanced Research Program under Grant No. 999902-036.

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