Pituitary Tumor-transforming Gene Protein Associates with Ribosomal Protein S10 and a Novel Human Homologue of DnaJ in Testicular Cells*

Lin Pei‡

From the Division of Endocrinology, Cedars-Sinai Research Institute, UCLA School of Medicine, Los Angeles, California 90048

Pituitary tumor-transforming gene (PTTG) is a recently characterized proto-oncogene that is expressed specifically in adult testis. In this study, we have used in situ hybridization and developmental Northern blot assays to demonstrate that PTTG mRNA is expressed stage-specifically in spermatocytes and spermatids during rat spermatogenic cycle. We have used the yeast two-hybrid system to identify proteins that interact with PTTG in testicular cells. Two positive clones were characterized. One of the clones is a novel ribosomal protein S10, the other encodes a novel human DnaJ homologue designated HSJ2. Northern blot analysis showed that testis contains higher levels of HSJ2 mRNA than other tissues examined, and the expression pattern of HSJ2 mRNA in postnatal rat testis is similar to PTTG. S10 mRNA levels do not vary remarkably among different tissues and remains unchanged during testicular germ cell differentiation. In vitro binding assays demonstrated that both S10 and HSJ2 bind to PTTG specifically and that PTTG can be co-immunoprecipitated with S10 and HSJ2 from transfected cells. Moreover, the binding sites for both proteins were located within the C-terminal 75 amino acids of the PTTG protein. These results suggest that PTTG may play a role in spermatogenesis.

*This work was supported by National Institutes of Health Grant DK-02346 (to L. Pei). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Div. of Endocrinology, Cedars-Sinai Medical Center, 8700 Beverly Blvd., D-3086, Los Angeles, CA 90048. Tel.: 310-855-7682; Fax: 310-559-2357; E-mail: pei@cshs.org.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF080659.

The abbreviations used are: PTTG, pituitary tumor-transforming gene; SD, synthetic dropout; HA, hemagglutinin; kbp, kilobase pair(s); GST, glutathione S-transferase; TBST, Tris-buffered saline containing 0.5% Tween 20; HA, hemagglutinin; kbp, kilobase pair(s); HSJ2, homosapien DnaJ2 homologue. L. Pei, unpublished data.

This paper is available on line at http://www.jbc.org

(Received for publication, July 14, 1998, and in revised form, November 9, 1998)
dipped in NT2B emulsion, exposed for 1 to 3 weeks, developed, and stained with methyl green.

**Cloning PTTG into pAS2 DNA Binding Domain Vector**—The coding region of PTTG was synthesized by polymerase chain reaction using the following primers: 5'-CAGGATGCTACTCTGATCTTTGTTGAT-3' and 5'-CTGTTAAATATCTGCATCGTAACAAACAGG-3'.

The polymerase chain reaction product was cloned into TA vector (CLONTECH) according to manufacturer's instructions. Plasmid DNA prepared from the library and pAS2-PTTG were co-transformed into yeast strain CG-1945 (CLONTECH) using the lithium acetate method (15, 16). Approximately 10^-6 independent clones were screened. Positive clones were identified by growth on -Leu- -Trp/- His-synthetic dropout (SD) agar plates containing 5 μg 3-amino-1,2,4-triazole (3-AT). β-Galactosidase Assays—Transformants grown on -Leu/-Trp/- His/-/3 AT SD plates were screened further by colony-lift filter assay. 

For each plate of transformants, a Whatman No 5 filter was presoaked in 2.5 ml of Z buffer/5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (16.1 μg/liter NaH2PO4·7 H2O, 5.5 μg/liter Na2HPO4·H2O, 0.75 μg/liter KCl, 0.246 μg/liter MgSO4·7 H2O, 0.34 mg/ml 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside). A dry filter was placed over the surface of the plate of colonies, and after the filter was pressed down to the plate, it was lifted off and transferred to a pool of liquid nitrogen over the surface of the plate of colonies, and after the filter was oriented to the plate, it was thawed at room temperature and placed on the presoaked filter. The filters were incubated at 30 °C for 30 min to 4 h, for 10 s. The filter was thawed at room temperature and placed on the presoaked filter. The filters were incubated at 30 °C for 30 min to 4 h, and the blue colonies were identified.

The blue colonies were characterized further by quantitative liquid β-galactosidase assays. A single colony was used to inoculate a 5 ml liquid medium, and the culture was incubated at 30 °C overnight. 2 ml of the overnight culture was inoculated into 8 ml yeast/peptone/glucose liquid medium, and the culture was incubated at 30 °C overnight. 2 ml of Z buffer. After recording the time, transferred to a fresh tube, frozen in liquid nitrogen, and thawed at 37 °C. 0.7 ml of Z buffer was added to the tube. After recording the time, 0.16 ml of O-nitrophenyl-β-D-galactopyranoside was added and the tube was incubated at 30 °C. After the yellow color developed, 0.4 ml of 1 M Na2CO3 was added to the reaction, and the elapsed time was recorded. After removing cell debris by centrifugation, the A600 was measured. The β-galactosidase units were calculated as the following: β-gal units = V × 300 × A600 × 10^-6, where t = elapsed time (in min) of incubation, V = 0.1 ml concentration factor, and A600 is of 1 ml of culture.

**Characterization of Positive Clones**—Yeast plasmid DNA was isolated using Yeastmaker yeast plasmid isolation kit (CLONTECH) according to manufacturer's protocol. Plasmid isolated from yeast was used to transform HB101 Escherichia coli cell using electroporation. Cells were grown on M9 agar plates containing 50 μg/ml ampicillin. Plasmid DNA was isolated from Leu̇, AmṗHB101 transformants using a standard plasmid mini-prep procedure. Positive clones were divided into groups based on restriction enzyme digestion patterns. The insert of each independent positive clone was sequenced using Gal4 AD sequencing primer (CLONTECH) and Sequenase (U. S. Biochemical Corp.). A homology search was performed using BLAST system. The inserts of several positive clones containing cDNAs of interest were excised from pAC2 vector by BglII digest and were cloned at the BamHI site into the pBCMV eukaryotic expression vector (Stratagen) for further analysis.

**In Vitro Transcription and Translation**—The above-characterized cDNAs were transcribed from T3 promoter and translated in reticulocyte lysate using TNT-coupled reticulocyte lysate system (Promega). A typical reaction contains 25 μl of rabbit reticulocyte lysate, 2 μl of reaction buffer, 20 μM amino acid mixture, 1 μg of DNA template, 40 units of ribonuclease inhibitor, 10 units of T7 RNA polymerase, and 1 μl of Transcend420 Biotin-lysyl-tRNA (Promega) in a total volume of 50 μl. The reactions were carried out at 30 °C for 1 h.

**Construction of GST-Mycamer S-Transferase (GST)-PTTG Fusion Protein and in Vitro Binding**—To construct GST-PTTG, plasmid TA-PTTG was digested with EcoRI, and the insert was subcloned in frame at the EcoRI site of pGEX-4T-1 (Amersham Pharmacia Biotech). Expression of GST fusion protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 90 min. Cells were centrifuged, and the resulting pellet was resuspended in a sonication buffer containing 150 mM KCl, 40 mM HEPES, pH 7.9, 0.5 mM EDTA, 5 mM MgCl2, 1.0 mM dithiothreitol, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. Cells were lysed by sonication. Cell debris was removed by centrifugation, and the supernatant was added to Sepharose 4B beads (Amersham Pharmacia Biotech). The in vitro binding assay was performed as follows: 20 μl of the in vitro translated protein in the supernatant was incubated with beads containing 200 ng of GST or GST-PTTG fusion protein in the sonication buffer for 90 min at 4 °C. Complexes were washed extensively with the sonication buffer, boiled in loading buffer, and separated on 10% SDS-polyacrylamide gels. Gels were transferred to nylon membranes and blocked by incubation with Tris-buffered saline containing 0.5% Tween 20 (TBSS). The membranes were incubated with Streptavidin-H-radiish peroxidase conjugate in TBST for 45 min, washed 4 times with TBST and 3 times with TBS. The membranes were then incubated with the chemiluminescent substrate mixture for 1 min and exposed to Kodak x-ray film for 20 min.

**Immunoprecipitation**—S10 and HS2 were solubilized in pCMV eukaryotic expression vector with HA tag: pCMV-S10 and pCMV-HS2 were transiently transfected into COS-7 cells either alone or together with pCMV-PTTG. 48 h post transfection, whole cell extracts were made from transfected cells. Cells were lysed in NET-N buffer containing 50 mM Tris, pH 7.6, 5 mM EDTA, 0.3 mM NaCl, 1 mM diethiothreitol, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin, and centrifuged at 30,000 × g for 1 h at 4 °C. The supernatant was stored at −80 °C. 1 mg of cell extract was incubated with anti-HA (chloroform Mannheim) for 2 h at 4 °C. Immune complexes were then precipitated with protein A/G-Sepharose at 4 °C for 1 h. The precipitate was collected by centrifugation and washed extensively in NET-N buffer. Protein complexes were separated by SDS-polyacrylamide gel electrophoresis and Western blotted using 1:5000 dilution of the anti-PTTG polyclonal antibody.

**Mutagenesis**—The terminal deletion mutant of pAS2-PTTG was generated using ExSite polymerase chain reaction-based site-directed mutagenesis kit (Stratagen) according to manufacturer’s instructions. The upper strand primers were

1. 5'-TTAGATGGGGAAAATGGGAGGTTTCAACGCGCA-3',
2. 5'-GTCACACAGGCTTCTGAAAACGCACTGAA-3',
3. 5'-TCTACTAAGACACAAAGCGTCGCTCTGCT-3',
4. 5'-AGTTTTTGACTGGTGAGGAGGACAGATC-3', and
5. 5'-AAGGGCTGCACTGGACCCCCTTTCCCCT-3'.

The lower strand primer was: 5'-CATCCTGAAGCAGGGATC- CGGCCCTCAGGCT-5'.

**Northern Blot Analysis**—The human RNA Master Blot and Multi-tissue blot were purchased from CLONTECH. Total RNA was isolated from the testes of rats of various ages using RNAeasy RNA isolation kit (Qiagen). RNA was reverse transcribed using Superscript II (Invitrogen), and the insert was digested with either EcoRI site or BamHI site into the pBCMV eukaryotic expression vector (Stratagen) for further analysis. Hybridization was performed in ExpressHyb hybridization solution (CLONTECH) at 65 °C overnight for RNA Master Blot and total RNA blots or for 1 h for Multi-tissue blot. The membrane was washed three times in 2× SSC and 0.05% SDS at 65 °C, followed by two washes in 0.1× SSC and 0.1% SDS at 55 °C. Each wash was 20 min. The membrane was exposed to x-ray film for 3 to 24 h. It was then stripped by boiling for 10 min in 0.5% SDS and was hybridized to the second probe as described above.

**Southern Blotting**—DNA was extracted from human lymphocytes. Cells were incubated in lysis buffer (100 mM NaCl, 25 mM EDTA, 1% SDS, and 100 μg/ml proteinase K) at 50 °C overnight. Protein-contaminating agarose was removed by phenol/chloroform extractions, and DNA was precipitated with ethanol and resuspended in Tris-EDTA. 10 μg of DNA was digested with either BamHI, EcoRI, HindIII, or PstI at 37 °C overnight. The DNA was separated on 0.8% agarose gel. The gel was denatured and neutralized, and the DNA was transferred to nylon membrane. The membrane was probed with the HS2 cDNA using standard Southern blot hybridization and washing conditions. The membrane was exposed to x-ray film for 48 h before developing.

**RESULTS**

**Cellular Localization of PTTG mRNA in Testis**—Because testis is composed of multiple cell types, including somatic Sertoli and Leydig cells, as well as proliferating and differentiating germ cells, it is important to determine in which cell type and in which differentiation stage during the spermatogenic cycle PTTG is expressed. In situ hybridization was performed.
were exposed to hybridized to antisense (A). In contrast, there was no hybridization signal to the sense signal was again detected in spermatocytes and spermatids (Fig. 1). Strong hybridization strengths could be seen between tubule cross-sections, indicative of spermatogenic stage-specific expression. The hybridization signal was detected. Fig. 1A shows two cross-sections probed with the sense probe. No hybridization signals over spermatocytes and spermatids appear as dark grains. Panel D, high magnification, bright-field view of a stage 14 tubule, probe with sense control. As shown in Fig. 1D, when the antisense PTTG probe was hybridized to adult testis sections, a whole range of signal strengths could be seen between tubule cross-sections, indicative of spermatogenic stage-specific expression. Fig. 1D compares two tubules at early (stage 3 or 4, left) or late (stage 12 or 13, right) stage of the spermatogenic cycle for comparison of PTTG mRNA expression levels. Panel E, high magnification, bright-field view of a stage 14 tubule; strong hybridization signals over spermatocytes and spermatids appear as dark grains. Panel F, high magnification, bright-field view of a stage 14 tubule, probe with sense control.

Identification of the cycle of the seminiferous epithelium was determined by parameters such as the number of layers of the tubules at various stages of the cycle at higher magnification. Fig. 1 shows a group of seminiferous tubules at various stages of the cycle at higher magnification. As shown in Fig. 1A, the antisense PTTG probe hybridized to adult rat testis sections, a wide range of signal strengths could be seen between tubule cross-sections, indicative of spermatogenic stage-specific expression. Identification of the cycle of the seminiferous epithelium was determined by parameters such as the number of layers of spermatids, their position in the epithelium, and their nuclear shape. Fig. 1D compares two tubules at early (stage 3 or 4, left) or late (stage 12 or 13, right) stage of the cycle. The tubule in the later stage expressed PTTG mRNA to much higher levels than the tubules at the early stage (Fig. 1D). The hybridization signals were strongest over the cell layers representing spermatocytes and spermatids (Fig. 1D). Fig. 1E shows a stage 14 tubule hybridized to PTTG antisense probe. Strong hybridization signals were again detected in spermatocytes and spermatids. In contrast, there was no hybridization signal to the sense probe in a tubule of the same differentiation stage (Fig. 1F).

To confirm these results, developmental Northern blot analysis was performed using testis RNA from rat of various ages. As shown in Fig. 2, PTTG mRNA was barely detectable in the testis of 7-day-old rat. At this time, testis contained somatic cells and spermatogonia cells only (17). By day 14, when leptotene spermatocytes appeared (17), low level PTTG expression was detected. Increasing amount of PTTG mRNA was detected on day 21 when zygotene spermatocytes are present (17). By day 28, PTTG mRNA levels increased dramatically and reached the expression levels in adult animals. At this stage, both pachytene spermatocytes and spermatids are present in rat testis. These results indicate that PTTG mRNA is expressed in both spermatocytes and spermatids.

**Screening PTTG Interactive Proteins Using Yeast Two-hybrid System**—Stage and cell type-specific expression of PTTG suggests that it may play a role in spermatogenesis. Because the amino acid sequence of PTTG does not provide any functional information, we sought to identify cDNAs that encode proteins able to interact with PTTG using a yeast two-hybrid screening strategy. The entire coding region of the rat PTTG was cloned into a Gal4 DNA binding domain vector pAS2 (18). This hybrid plasmid and plasmid from a human testis cDNA library constructed in the activation domain vector were co-transformed into the yeast host strand CG-1945. The transformants were plated on minimal medium lacking Leu, Trp, and His to select for those containing both types of plasmids (i.e., Leu\(^{+}\), Trp\(^{+}\)) and that also express interacting hybrid proteins (His\(^{+}\)). Primary His\(^{+}\) transformants were then tested for expression of the second reporter gene, LacZ, using a filter assay for \(\beta\)-galactosidase activity (19). Approximately 10\(^{6}\) transformants were plated under selection. Of the transformants plated on SD/−His/−Leu/−Trp/+5

**TABLE I**

| Clone number | \(\beta\)-gal units |
|--------------|---------------------|
| 1            | 12.0 ± 0.4          |
| 2            | 12.7 ± 0.6          |
| 3            | 29.4 ± 1.1          |
| 4            | 30.9 ± 2.3          |
| 5            | 23.8 ± 1.0          |
| 6            | 17.7 ± 0.7          |
| 7            | 39.2 ± 3.5          |
| 8            | 31.5 ± 2.9          |
| 9            | 19.2 ± 0.8          |
| 10           | 28.8 ± 1.6          |

**FIG. 2.** PTTG mRNA expression in postnatal rat testis. 20 \(\mu\)g of total RNA isolated from testes of rats of various ages was separated on agarose gel, blotted, and probed with PTTG (panel A) or actin (panel B). The age of the rat is indicated on the top of each lane. Hybridization signals are indicated by arrows.
mm 3AT medium, about 2000 grew into colonies within 3 to 5 days. Seventy of the His blue colonies were also blue when screened for their ability to produce β-galactosidase using the filter lift assay. These His blue colonies were considered positive in the initial screen and were used for additional studies.

To further test whether the phenotype observed in the original screen was reproducible and dependent on the pAS2-PTTG hybrid, plasmid DNA from each of the seventy clones was used to transform CG-1945 either alone or with pAS2-PTTG. Transformants were assayed for β-gal activity, and those showing activity only in the presence of pAS2-PTTG were considered positive. Of the 70 initial isolates, 10 of the recovered plasmids induced the expression of lacZ only in the presence of pAS2-PTTG, and their relative β-activity was shown in Table I.

Characterization of Positive Clones Dependent on PTTG Hybrid Expression—Sequence analysis of the 10 positive clones revealed that eight clones showed no significant homology to known protein sequences reported to GenBank. One cDNA insert (y9) encoded a protein with a predicted molecular mass of 19 kDa and was identical to human S10 ribosomal protein (20). Another positive clone (y3) contained an open reading frame of 217 amino acids (Fig. 3A) and was 62 and 47% identical to MSJ-1 (mouse DnaJ) (21) and HSJ1 (homosapiens DnaJ 1) (22), respectively. The highest identity shared by the three proteins was at the N termini, where the 82 amino acids were 70% identical (Fig. 3B). This similarity was shared by several other DnaJ proteins, including the E. coli DnaJ (23, 24) and yeast, homologous of DnaJ, SCJ1(25), YDJ1 (26), and SIS1 (27). The similarity between y3 and other DnaJ proteins suggested that y3 is a new member of the human DnaJ family of proteins, and therefore was designated HSJ2 (homosapiens DnaJ 2).

Tissue Distribution of S10 and HSJ2—A human RNA master blot containing mRNA from 50 human tissues was probed with the S10 or HSJ2 cDNA probe. Both S10 and HSJ2 mRNAs were detected in all the tissues examined, although the expression level of HSJ2 mRNA varies among different tissues (data not shown). To determine the transcript size of S10 and HSJ2, Northern blot analysis was performed on selected tissues. The HSJ2 cDNA detected two bands of about 2.8 and 1.7 kb (Fig. 4A). The highest level of expression was detected in testis for the 1.7-kb transcript (normalized to loading control, Fig. 4C). Other tissues, including spleen, intestine, ovary, thymus, prostate, and leukocytes showed much lower level of HSJ2 mRNA expression (Fig. 4A). The S10 probe detected a single transcript of about 600 base pairs, and the expression levels of this transcript were similar in the tissues examined (Fig. 4B).

Developmental Regulation of HSJ2 and S10 Expression—To determine whether HSJ2 and S10 expression undergo developmental regulation, we have used postnatal rat testis as a model. RNAs were isolated from testes of postnatal rats of various ages and used in Northern blot analysis. As shown in Fig. 5A, very low level HSJ2 mRNA was detected at day 7. Increasing levels of HSJ2 mRNA were detected on day 14 and 21. By day 28, there was a more than 10-fold increase in HSJ2 expression (Fig. 5A) (after normalizing to actin control, Fig. 5C). There was no further increase in HSJ2 mRNA levels in adult rat testis (Fig. 5A). S10 mRNA, on the other hand, was expressed to similar levels from postnatal day 7 to adult (Fig. 5B). These results indicate that although HSJ2 expression undergoes developmental regulation, S10 is constitutively
expressed.

Genomic Analysis of HSJ2—As an initial step to characterize HSJ2 gene structure, human genomic DNA analysis was performed. As shown in Fig. 6, the HSJ2 probe detected two bands in human genomic DNA digested with BamHI, EcoRI, HindIII, and PstI. Because these enzymes do not cut within the cDNA, these results suggest that either the sites for these enzymes are present within the introns of the gene or there are two different HSJ2 genes if the enzymes do not cut within the genes.

Both S10 Ribosomal Protein and HSJ2 Bind PTTG in Vitro—To confirm and extend the protein interaction data obtained in yeast, PTTG was expressed as a GST fusion protein in E. coli (28). To test the ability of S10 and HSJ2 proteins to bind PTTG in vitro, S10 and HSJ2 was in vitro transcribed and translated in the presence of Transcent™ Biotin-lysyl-tRNA (Promega). After translation, GST-PTTG immobilized on glutathione-Sepharose was added. The beads were washed extensively, and the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. After electroblotting, the bound, biotinylated protein was visualized by binding to streptavidine-horseradish peroxidase, followed by chemiluminescent detection. As shown in Fig. 7, both S10 and HSJ2 were retained on the glutathione beads when they were incubated with GST-PTTG, whereas neither protein was retained when incubated with GST alone. These results indicated that S10 and HSJ2 protein were retained on the matrix through the PTTG segment of the fusion protein.

Both S10 and HSJ2 Proteins Can Be Coimmunoprecipitated with PTTG Protein—To further characterize the association of PTTG protein with S10 and HSJ2 proteins, we examined whether PTTG protein could be coimmunoprecipitated from transfected cells. Cos-7 cells were transiently transfected with HA-tagged HSJ2 and S10 expression vectors, either alone or together with PTTG expression plasmid. Cells were lysed, and the resulting extracts were immunoprecipitated with anti-HA monoclonal antibody (29). The immunoprecipitates were then resolved by SDS-polyacrylamide gel electrophoresis, and the gel was immunoblotted and probed with an antibody specific for the N-terminal 17 amino acids of PTTG (1). A band corresponding to PTTG was seen only in cells that were cotransfected with both S10 and PTTG or HSJ2 and PTTG expression vectors (Fig. 8). These results indicated that PTTG could form a complex with either S10 or HSJ2.

The C Terminus of PTTG Protein Is Required for Binding of S10 and HSJ2—To determine the region of PTTG protein involved in interaction with S10 and HSJ2, sequential N-terminal deletion mutants (Fig. 9A) of PTTG were subcloned into

---

**Fig. 4.** Transcript size of HSJ2 and S10: Northern blot analysis. HSJ2 probe (A), S10 probe (B), actin probe (C) poly(A)+ RNA from eight different human tissues (indicated at the top of the figure) were used for Northern blot analysis. Molecular mass markers are shown on the side. The exposure time was 24 h.

**Fig. 5.** HSJ2 and S10 mRNA expression in postnatal rat testis. 20 µg of total RNA isolated from testes of rats of various ages was separated on agarose gel, blotted, and probed with HSJ2 (panel A), S10 (panel B), or actin (panel C). The age of the rat is indicated on the top of each lane. Hybridization signals are indicated by arrows.

**Fig. 6.** Genomic Southern blot analysis of HSJ2. 10 µg of human genomic DNA was digested with the indicated restriction enzyme, separated on agarose gel, blotted, and probed with HSJ2 cDNA. The hybridization signals are indicated by asterisks, and the molecular weight markers are indicated on the side of the gel.

---

**E. coli** (28). To test the ability of S10 and HSJ2 proteins to bind PTTG in vitro, S10 and HSJ2 was in vitro transcribed and translated in the presence of Transcent™ Biotin-lysyl-tRNA (Promega). After translation, GST-PTTG immobilized on glutathione-Sepharose was added. The beads were washed extensively, and the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. After electroblotting, the bound, biotinylated protein was visualized by binding to streptavidine-horseradish peroxidase, followed by chemiluminescent detection. As shown in Fig. 7, both S10 and HSJ2 were retained on the glutathione beads when they were incubated with GST-PTTG, whereas neither protein was retained when incubated with GST alone. These results indicated that S10 and HSJ2 protein were retained on the matrix through the PTTG segment of the fusion protein.

Both S10 and HSJ2 Proteins Can Be Coimmunoprecipitated with PTTG Protein—To further characterize the association of PTTG protein with S10 and HSJ2 proteins, we examined whether PTTG protein could be coimmunoprecipitated from transfected cells. Cos-7 cells were transiently transfected with HA-tagged HSJ2 and S10 expression vectors, either alone or together with PTTG expression plasmid. Cells were lysed, and the resulting extracts were immunoprecipitated with anti-HA monoclonal antibody (29). The immunoprecipitates were then resolved by SDS-polyacrylamide gel electrophoresis, and the gel was immunoblotted and probed with an antibody specific for the N-terminal 17 amino acids of PTTG (1). A band corresponding to PTTG was seen only in cells that were cotransfected with both S10 and PTTG or HSJ2 and PTTG expression vectors (Fig. 8). These results indicated that PTTG could form a complex with either S10 or HSJ2.

The C Terminus of PTTG Protein Is Required for Binding of S10 and HSJ2—To determine the region of PTTG protein involved in interaction with S10 and HSJ2, sequential N-terminal deletion mutants (Fig. 9A) of PTTG were subcloned into
pAS2 and used to co-transform yeast CG-1945 with the Gal4 activation domain-S10 or HSJ2 fusion plasmid. The resulting transformants were then assayed for β-gal activity. As shown in Fig. 9B, transformants resulting from co-transformation of Gal4-S10 or Gal4-HSJ2 with PTTG mutants containing deletions up to 123 N-terminal amino acids (i.e., mutant 1 to 4) grew on SD−Trp−Leu−His+/+3AT plates and showed β-gal activity similar to transformants of the wild type PTTG. When the N-terminal deletion was made to amino acid 153 (mutant 5), however, there was no growth on SD−Trp−Leu−His+/+3AT plates, suggesting that this PTTG mutant was unable to bind to S10 and HSJ2. These results suggested that both S10 and HSJ2 bind to PTTG within the 75 amino acids at the C terminus and that amino acid residues 124–153 play an essential role for PTTG interaction with these proteins.

**DISCUSSION**

Previous studies indicated that PTTG is specifically expressed in adult rat testis (1). In this study, the precise cellular location of PTTG mRNA has been determined. Our results show that PTTG messenger is predominantly expressed in spermatocytes and spermatids and in a stage-specific manner during the rat spermatogenic cycle. This expression pattern of PTTG is similar to that of proto-oncogene c-kit, in that both transcripts are present in germ cells. However, expression of c-kit mRNA starts in spermatogonia and continues into spermatocytes (30). c-kit is known to play a role in supporting spermatogonia proliferation and survival. Spermatogonia proliferate to replace themselves and differentiate to give rise to primary spermatocytes in which DNA synthesis is carried out to double chromosomes, after which they enter the first meiotic prophase. Expression of PTTG mRNA in nonproliferating spermatocytes and spermatids suggests that it may play a role in survival and differentiation of these germ cells.

Because the primary structure of PTTG protein does not provide functional information, we have used the yeast two-hybrid system to identify proteins that interact with PTTG in testicular cells. We have used the rat PTTG to screen a human library, because the human PTTG cDNA was not available. Because of the species difference, it is possible that PTTG-interactive proteins that do not share high homology between human and rat may not be detected using this screen. However, using this method, we identified and characterized two proteins capable of interacting with PTTG. We demonstrated that both proteins were able to bind to PTTG in vitro and in vivo and that the C-terminal 75 amino acids of PTTG protein were required for this interaction.

One of the cDNA clones isolated from this screen encodes a novel human homologue of the bacterial heat-shock protein DnaJ (HSJ2). HSJ2 shares highest sequence similarity with the previously characterized MSJ-1 (21) and HSJ1 protein (22). Like these proteins, the similarity between HSJ2 and *E. coli* DnaJ is restricted to the N-terminal region of the molecule. MSJ-1 was shown to have two transcripts of 1.2 and 1 kb that are specifically expressed in testis (21). HSJ1 has two alternatively spliced transcripts of 2 and 3 kb, which are preferentially expressed in neurons (22). In contrast, HSJ2 mRNA is more widely expressed, although testis shows the highest level of expression. They are also two HSJ2 transcripts (1.7 and 2.8 kb) that may be the result of alternative splicing, use of alternative promoters, or polyadenylation sites. Like MSJ-1, HSJ2 expression undergoes developmental regulation in testis. However, although MSJ-1 expression is restricted in spermatids (21), HSJ2 is expressed in germ cells in the earlier stages of differentiation pathway as well as in spermatids. The developmental expression pattern of HSJ2 in postnatal rat is similar to PTTG mRNA expression profile. Both transcripts show very low lev-
els expression on postnatal day 7, with increasing levels of expression on days 14 and 21 and dramatic increase on day 28. It is likely that HSJ2 mRNA is expressed in similar germ cells where PTTG messenger is found. Future in situ hybridization studies of HSJ2 in rat testis will confirm these results.

HSJ2 also shows high sequence similarity (59%) to the yeast DnaJ homologue, SIS1 protein (27). The SIS1 gene is essential for viability. In the absence of normal SIS1 function, the poly-some levels decrease, and 80S ribosomes accumulate to high levels (13). It is believed that SIS1 functions to dissociate 80S ribosome into 40S and 60S subunits. SIS1 was shown to be associated with 40S ribosomal subunits and the smaller polysomes (13). This evidence indicates that SIS1 is required for the normal initiation of translation (13).

Another protein that interacts with PTTG protein is the human ribosomal large subunit protein S10 (20). The mammalian ribosomal is a complex structure assembled from approximately 80 proteins and four ribosomal RNA molecules (31). The ribosomal proteins are thought to facilitate the folding and maintenance of an optimal configuration of the rRNAs (32), perhaps in this way conferring speed and accuracy on protein synthesis. Recently, the involvement of the ribosomal proteins in cell proliferation (33–37), differentiation (38), and apoptosis (39) has been demonstrated. It has been show that S10 protein can be cross-linked to eukaryotic initiation factor 3 (40), an observation suggesting that S10 protein forms part of the domain involved in binding of the initiation factor to the 40S subunit at the start of the translation (40). The binding of PTTG protein to S10 protein suggests that PTTG protein may associate with ribosomes through this interaction.

What is the significance of the association of PTTG with HSJ2 and S10 proteins? Both S10 and SIS1 proteins have been shown to be associated with 40S ribosomal subunit and are required for the initiation of translation (13, 40). Although the association of HSJ2 with ribosome is unknown at the present, because DnaJ has been shown to mediate the dissociation of several protein complexes, it is possible that HSJ2 might be able to mediate the dissociation of a specific protein complex of the translation. Interestingly, PTTG also interacts with S10, a ribosome protein. One possibility is that PTTG is targeted to the ribosome through interaction with S10 protein, and association with HSJ2 results in dissociation of PTTG-S10 complex and separates it from the ribosome. These hypotheses will be tested in future studies.

In summary, we demonstrated in this study that PTTG is expressed in a stage-specific manner in spermatocytes and spermatids during the spermatogenic cycle. Through interaction with ribosomal protein S10 and a novel human homologue of DnaJ protein, PTTG may play a role in male germ cell differentiation.

Acknowledgments—I would like to thank Dr. Amiya P. Sinha Hikim (Harbor-UCLA Medical Center) for advice on testis morphology and identifying stages of the rat spermatogenic cycle and Dr. John S. Adams (Cedars-Sinai Medical Center) for critical reading of the manuscript.

REFERENCES
1. Pei, L., and Melmed, S. (1997) Mol. Endocrinol. 11, 433–441
2. Pei, L. (1998) J. Biol. Chem. 273, 5219–5225
3. Fields, S., and Song, O. (1989) Nature 340, 245–246
4. Chien, C. T., Bartel, P. L., Steiner, L., and Field, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9578–9582
5. Dalton, S., and Treisman, R. (1992) Cell 68, 597–612
6. Yang, X., Hubbard, J. E. A., and Carlson, M. (1992) Science 257, 680–682
7. Rothman, J. E. (1989) Cell 59, 591–601
8. Craig, R. A., Gambill, B. D., and Nelson, R. J. (1993) Microbiol. Rev. 57, 402–412
9. Hartl, P. U. (1996) Nature 381, 571–580
