

The Eukaryotic Polypeptide Chain Releasing Factor (eRF3/GSPT) Carrying the Translation Termination Signal to the 3'-Poly(A) Tail of mRNA

DIRECT ASSOCIATION OF eRF3/GSPT WITH POLYADENYLATE-BINDING PROTEIN*

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The mammalian GTP-binding protein GSPT, whose carboxyl-terminal sequence is homologous to the eukaryotic elongation factor EF1 α , binds to the polypeptide chain releasing factor eRF1 to function as eRF3 in the translation termination. The amino-terminal domain of GSPT was, however, not required for the binding. Search for other GSPT-binding proteins in yeast two-hybrid screening system resulted in the identification of a cDNA encoding polyadenylate-binding protein (PABP), whose amino terminus is associating with the poly(A) tail of mRNAs presumably for their stabilization. The interaction appeared to be mediated through the carboxyl-terminal domain of PABP and the amino-terminal region of GSPT. Interestingly, multimerization of PABP with poly(A), which is ascribed to the action of its carboxyl-terminal domain, was completely inhibited by the interaction with the amino-terminal domain of GSPT. These results indicate that GSPT/eRF3 may play important roles not only in the termination of protein synthesis but also in the regulation of mRNA stability. Thus, the present study is the first report showing that GSPT/eRF3 carries the translation termination signal to 3'-poly(A) tail ubiquitously present in eukaryotic mRNAs.

The yeast *GSPT* gene, whose product is a GTP-binding protein structurally related to the translation elongation factor EF1 α , was first isolated based on the ability to complement a temperature-sensitive *gst1* mutation of *Saccharomyces cerevisiae* (1). Because DNA synthesis was substantially arrested at the nonpermissive temperature in this mutant, the *GSPT* gene product appears to play an essential role at the G₁ to S phase transition in the yeast cell cycle. On the other hand, *SUP35* was cloned by another group investigating omnipotent sup-

pressor mutant of *S. cerevisiae*, and the gene turned out to be identical to *GSPT* (2). Omnipotent suppressor is a class of nonsense suppressors that is recessive and effective toward three types of nonsense codons (3). Mutations in the *GSPT/SUP35* gene were shown to increase the level of translational ambiguity (4, 5), suggesting that this gene product may also function as a positive regulator of translational accuracy in yeast.

In eukaryotic protein synthesis, all three types of termination codons are directly recognized by a polypeptide chain releasing factor, eRF1, to release synthesized polypeptide chain from ribosome, and another releasing factor, eRF3, appears to be essentially required for the ribosomal binding of eRF1. Recently, it was reported in *S. cerevisiae* (6) and *Xenopus laevis* (7) that the product of the *GSPT/SUP35* gene forms a complex with eRF1 to function as eRF3. We previously cloned a human homologue of the yeast *GSPT* gene (8) and more recently isolated two mouse GSPT genes, the counterpart of human GSPT1 and a novel member of the GSPT gene family, GSPT2 (9). The mammalian GSPT1 and GSPT2 could also associate with eRF1 to function as eRF3, although the two GSPTs were clearly distinct from each other in terms of their amino-terminal sequences, the expression during cell cycle progression, and tissue distribution (9). It thus appears that there is a functional conservation of this family from yeast to mammals in terms of translation termination. In this report, we present evidence that GSPTs may have another function by interacting with PABP that binds to the 3'-poly(A) tail of eukaryotic mRNAs. Our present results indicate that GSPT/eRF3 may play important roles not only in the termination of protein synthesis but also in the regulation of mRNA stability.

EXPERIMENTAL PROCEDURES

Screening of Yeast Two-hybrid Library—A yeast two-hybrid screen was performed according to the standard method of Fields and Song (10) utilizing the Matchmaker Two-hybrid System (CLONTECH). A human T cell lymphoma cDNA library in the pACT vector was screened using the full-length mouse GSPT1 inserted into pGBT9 as a bait. The library and bait were cotransformed into the yeast two-hybrid strain HF7c using a standard lithium acetate-polyethylene glycol method. A total of 6.5×10^6 individual recombinant clones were screened. Positive clones grown on His⁻ medium were selected for activation of HIS3 reporter gene, and β -galactosidase assay was performed as described previously (9, 11). A series of deletion mutants of GSPTs and PABP¹ I were constructed using a PCR-based strategy. PCR products encoding amino- and carboxyl-terminal truncations of GSPTs and PABP I were subcloned into pGBT9 in-frame with the GAL4 DNA-binding domain. The two-hybrid vectors thus obtained were cotransformed into the yeast host strain SFY526. Transformed colonies were replated on tryptophan- and leucine-deficient medium.

Production of Recombinant Proteins—For production of GST-fused PABP, a full-length human PABP I cDNA was excised from yeast two-hybrid positive clone (pGAD10:PABP) and directionally subcloned into pGEX6P1 vector (Amersham Pharmacia Biotech) downstream of the glutathione *S*-transferase (GST) gene. For the GST-fused amino-terminal domain (amino acids 1–204) of GSPT2, PCR products encoding the corresponding sequences were subcloned into pGEX6P1 in-frame with the GST. The GST-fused proteins and GST alone were expressed in *Escherichia coli* HB101 following induction by 0.1 mM isopropyl-1-thio- β -D-galactopyranoside at 20 °C for 9 h. The expressed proteins were recovered from the cells and resuspended in TEDN buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40)

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¹ The abbreviations used are: PABP, polyadenylate-binding protein; PCR, polymerase chain reaction; GST, glutathione *S*-transferase.

containing protein inhibitors (1 μ g/ml of leupeptins, 1 μ g/ml of pepstatin A, 2 μ g/ml of aprotinin, and 100 μ M phenylmethylsulfonyl fluoride). Following incubation with 1 mg/ml of lysozyme at 4 °C for 30 min, the cell lysates were sonicated for 3 min on ice. The extracts were centrifuged at 100,000 \times g for 60 min, and the expressed proteins in the clear supernatant were purified using a glutathione-Sepharose column according to the manufacturer's instructions. For electrophoretic mobility shift assay, the GST portion of the expressed PABP was excised using PreScission protease (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

For production of a recombinant GSPT1, the *NcoI-XhoI* fragment of pTrcHis vector (Invitrogen, Co) was first removed and annealed with the synthetic adaptor MCS (MCS5' plus MCS3') as described below to make pTrc. The pTrc was then digested with *Bam*HI and *Pst*I and replaced by the *Bam*HI-*Pst*I portion of the pGH5, which contained the full length of human GSPT1 cDNA sequence (9). The GSPT1 expression vector (pTrc:GSPT1) thus obtained was introduced into *E. coli* strain JM109 to produce the recombinant GSPT1. The bacterial cells were cultured at 37 °C with vigorous shaking in 1 liter of an LB medium containing 0.2% glucose. After the culture reached the density of 0.4 A_{600} , GSPT1 was induced by the addition of 0.1 mM isopropyl-1-thio- β -D-galactopyranoside. After an additional 2-h culture, the cells were harvested by centrifugation at 4,000 \times g for 25 min. The cells were resuspended in TEDN and processed as described above for the preparation of the GST fusion protein. The synthetic oligonucleotides used were: MCS5'-CATGGATCCCGGGTTCGACTCTAGAGGCC and MCS3'-TCGAGGCCTCTAGAGTTCGACCCGGGATC. FLAG-tagged mouse GSPT2 and its amino-terminal deletion mutants were produced in COS-7 cells as described previously (9).

In Vitro Binding Assay—The purified GST-fused PABP or GST alone (0–10 μ g) was incubated with *E. coli* extract containing GSPT1 (approximately 10 μ g) at 4 °C for 60 min in 200 μ l of TEDN. Glutathione-Sepharose resin (20 μ l) that had been pretreated with 5% bovine serum albumin was added to the reaction mixture and further incubated at 4 °C for 60 min with gentle mixing. The resin was extensively washed three times with 500 μ l of TEDN containing the protein inhibitors and incubated with 20 μ l of 20 mM glutathione at 4 °C for 30 min in 50 mM Tris-HCl (pH 7.5). After centrifugation, 20 μ l of the supernatant was mixed with 20 μ l of 2 \times SDS-polyacrylamide sample buffer and boiled for 5 min, followed by SDS-polyacrylamide gel electrophoresis (12.5% of acrylamide). Immunoblots were performed with rabbit antisera raised against a recombinant human GSPT1, PABP, or GST as described previously (9).

Gel Mobility Shift Assay—The assay was performed as described previously (15). Indicated amounts (up to 10 pmol) of the purified PABP was first incubated with or without the GST-fused amino-terminal domain (amino acids 1–204) of GSPT2 (40 pmol) in TEDN at 4 °C for 30 min. The mixture was then incubated with 5'-³²P-labeled poly(A) RNA (23-mers; 1 nM) at 4 °C for 30 min in a buffer (total volume, 20 μ l) consisting of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 0.01% acetylated bovine serum albumin, 5.3 mM EDTA, 0.01% Nonidet P-40, and 100 mM β -mercaptoethanol. Half of the reaction mixture was electrophoresed at 20 V/cm in 6% polyacrylamide gels (60:1; 1 \times TBE and 0.1% Triton X-100) under nondenaturing conditions at 4 °C for 60 min. The radioactivity retained on the gel was visualized with a Fuji BAS 2000 bioimaging analyzer.

RESULTS AND DISCUSSION

No Involvement of the Amino-terminal Domain of GSPT in the Binding to eRF1—In the previous study (9), we reported that mammalian GSPT1 and GSPT2 could directly interact with eRF1 in the assays of yeast two-hybrid screening and *in vitro* binding. Because the GSPT family consist of a conserved carboxyl-terminal region homologous to EF1 α GTP-binding protein and an extra nonhomologous amino-terminal region, we further investigated which domain is responsible for the association with eRF1. A FLAG-tagged full-length GSPT2 (amino acids 1–632) and its amino-terminal deletion mutant (amino acids 138–632) were produced in COS-7 cells, and the cell lysates were immunoprecipitated with an anti-FLAG antibody. As shown in Fig. 1, a 54-kDa eRF1 protein of COS-7 cells was observed in both of the two precipitated fractions, suggesting that the amino-terminal region of GSPT2 is not involved in the binding to eRF1. Essentially the same results were obtained if GSPT1 was used instead of GSPT2 (data not shown).

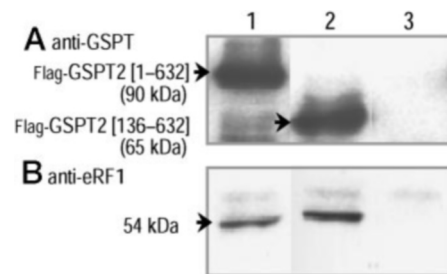


FIG. 1. Association of amino-terminal deleted GSPT2 with eRF1. Expression vectors for FLAG-tagged full-length GSPT2 (amino acids 1–632; lane 1) and its amino-terminal deletion mutant (amino acids 136–632; lane 2) or the vector alone (lane 3) were transfected into COS-7 cells, and the cell extracts were immunoprecipitated with an anti-FLAG monoclonal antibody (9). The precipitated proteins were subjected to immunoblot analysis with anti-GSPT1 (A) and anti-eRF1 (B) antibodies.

Search for Other GSPT-binding Proteins: Association with Poly(A)-binding Proteins via the Amino-terminal Domain of GSPT—To investigate possible function of the unique amino-terminal region of GSPT and other molecules interacting with the GSPT family, we first screened a human T cell lymphoma cDNA library with GSPT1 as a bait in yeast two-hybrid assay system. The initial screening (6.5 \times 10⁶ independent clones) resulted in the identification of 10 positive clones. Sequencing analyses revealed that all clones encoded PABP I (12). Positive interaction with PABP was also observed with GSPT2 more effectively under the present conditions (data not shown). As shown in Fig. 2A, PABP I contains four RNA-binding domains in tandem repeat at its amino-terminal side; these domains bind to the 3'-poly(A) tail of mRNAs probably for their stabilization and/or translocation from nuclei to cytoplasm (for review see Refs. 13 and 14). The carboxyl-terminal domain of PABP is suggested to contribute in multiple, regularly spaced organization of the RNA-binding protein on the poly(A) tail (15).

For further characterization of the interaction between GSPT and PABP, the varying lengths of GSPT2 and PABP were cloned downstream of the GAL4-binding (pGBT9 vector) and activation (pGAD424 vector) domains, respectively, and the resulting plasmids were transformed into the *S. cerevisiae* SFY526 cells. As shown in Fig. 2B, we observed strong β -galactosidase activity with the full-length GSPT2 (amino acids 1–632) and PABP in the yeast two-hybrid assay. When 135 amino acids were deleted from the amino terminus of GSPT2 (amino acids 136–632), the interaction was abolished. In sharp contrast, β -galactosidase activity was markedly enhanced if the carboxyl-terminal EF1 α -like domain was deleted from GSPT2 (amino acids 1–204).

Because PABP has the two-domain structure, we further analyzed which domain is important for the binding to GSPT2. The interaction with GSPT2 appeared to be mediated through the carboxyl-terminal side (amino acids 369–633) of PABP rather than its amino-terminal RNA-binding domain (amino acids 1–368). Such difference observed between the two regions of PABP was also ascertained if the amino-terminal region of GSPT2 (amino acids 1–204) was used instead of the full-length GSPT2 (amino acids 1–632). Thus, the interaction appeared to be mediated through the carboxyl-terminal domain of PABP and the amino-terminal region of GSPT.

Direct Interaction between GSPT and Poly(A)-binding Protein—The direct interaction between GSPT and PABP was further confirmed by an *in vitro* binding assay with recombinant proteins. *E. coli* cell lysates containing a constant amount of GSPT1 were incubated with increasing amounts of purified GST-fused PABP or GST alone, and glutathione-Sepharose resin was added to the reaction mixture. Proteins bound to the

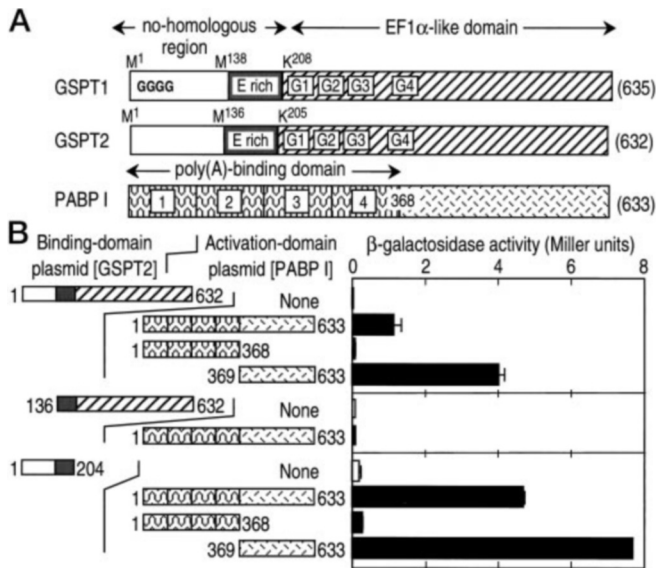


FIG. 2. Schematic representation of the primary structure of mouse GSPTs and PABP I and analysis of the interaction between GSPT2 and PABP in the yeast two-hybrid system. *A*, the GSPT family consists of two regions characterized as an amino-terminal nonhomologous region (approximately 200 amino acids) and a conserved EF1 α -like domain (428 amino acids). The EF1 α -like domain contains four GTP-binding motifs (G1–G4). A Glu-rich (E-rich) region is present between the amino-terminal region and EF1 α -like domain, and a Gly repeat structure (GGGG) is only present in GSPT1. PABP I consists of two regions, four RNA-binding domains (1–4) in tandem repeat at the amino-terminal side and the carboxyl-terminal portion. *B*, a series of the amino- and carboxyl-terminal deletion mutants of mouse GSPT2 and human PABP I were inserted into pGBT9 and pGAD424 vectors, respectively, and transformed into SFY526 cells. β -Galactosidase activity of the cell lysate was measured as described under “Experimental Procedures.” Values shown are the means \pm S.D. of triplicate determinations.

resin were separated on a SDS-polyacrylamide gel and immunoblotted with anti-GSPT1, anti-PABP, and anti-GST antibodies. As shown in Fig. 3, there were increasing amounts of 90-kDa GSPT1 in the resin when the increasing amounts of GST-PABP were added to the reaction mixture (lanes 2–4). Such interaction was not observed upon incubation with GST (lanes 5–7). Essentially the same results were obtained if GSPT2 was used instead of GSPT1 (data not shown).

Inhibition of Multimerization of PABP with Poly(A) by the Amino-terminal Domain of GSPT—We next investigated the functional significance of the interaction between GSPT and PABP. Previous studies indicated that the carboxyl-terminal domain of PABP confers multimerization activity, which can be measured by gel mobility shift assay (15). As shown in Fig. 4, 5'- 32 P-labeled, synthetic poly(A) RNA bound to PABP, resulting in the formation of the retarded band. There was a progressive reduction of the complex mobility indicative of more than one copy of PABP on the labeled poly(A) as the amount of the RNA-binding protein was increased (lanes 2–5). However, in the presence of the amino-terminal domain of GSPT (lanes 6–10), a discrete transition rather than a gradual reduction of the complex mobility was observed. It thus appeared that only one complex forms at saturating concentrations, although its mobility was slower than that of a single complex of poly(A)-PABP probably due to the association of GSPT amino-terminal domain (compare lane 8 with lane 2). These results indicate that PABP associated with GSPT in its carboxyl terminus could no longer interact with another PABP to form a multiple, regularly spaced complex on the poly(A) tail of mRNAs.

Possible Functions of GSPT/eRF3 in Eukaryotic Translation System—The precise mechanism by which eRF1 and GSPT/

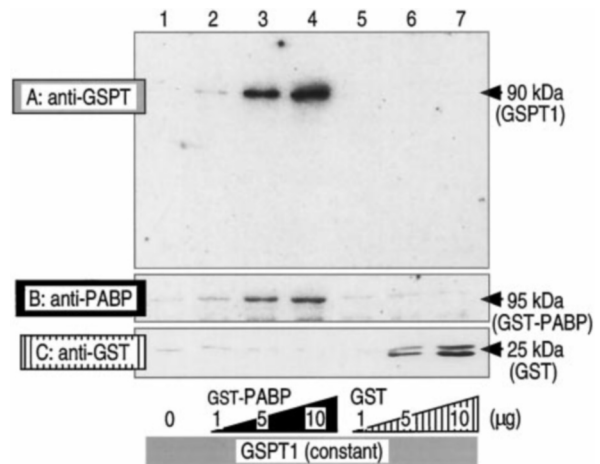


FIG. 3. *In vitro* assay for the interaction between GSPT1 and PABP. Crude *E. coli* extract containing approximately 10 μ g of GSPT1 was incubated with the indicated amounts of purified GST-fused PABP or GST in TEDN, and then glutathione-Sepharose beads were added to the mixture. Proteins bound to the beads were separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) and immunoblotted with anti-GSPT1 (*A*), anti-PABP (*B*), and anti-GST (*C*) antibodies.

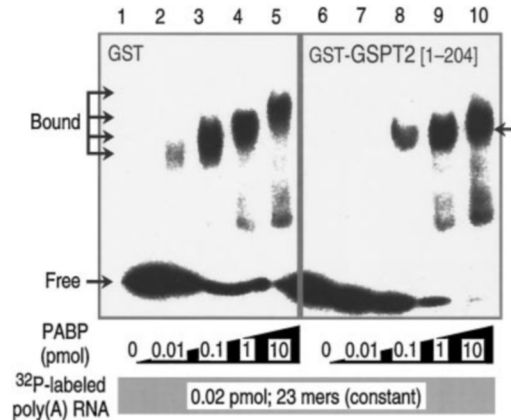


FIG. 4. Effect of amino-terminal region of GSPT2 on interaction between PABP and poly(A) mRNA. The indicated amounts of purified PABP (up to 10 pmol) was incubated with GST-fused amino-terminal domain (amino acids 1–204) of GSPT2 or GST alone (40 pmol) in TEDN. The mixture was further incubated with 0.02 pmol (23-mers) of 32 P-labeled poly(A) RNA and subjected to gel shift assay as described under “Experimental Procedures.”

eRF3 promote translation termination when the A site of the ribosome is occupied by a termination codon has not been fully elucidated. As shown in Fig. 5, one suggested model would be comparable with the elongation system; eRF1 may structurally mimic the stem of an aminoacyl-tRNA, whereas GSPT/eRF3 may mimic the function of EF1 α , which carries GTP-dependently the aminoacyl-tRNA to the A site. This model may be consistent with the present finding that the association with eRF1 required only the carboxyl-terminal domain of GSPT/eRF3, which covers almost the full length of EF1 α (Fig. 1). Thus, the extra amino-terminal region (approximately 200 amino acid residues) of GSPT/eRF3 appeared to be responsible for other functions. In the present report, we identified that one of the possible functions is to associate with the carboxyl-terminal domain of PABP (Figs. 2 and 3). Furthermore, the amino-terminal domain of GSPT/eRF3 could inhibit the formation of multimeric PABP-PABP interactions on poly(A) RNA (Fig. 4).

These results led us to speculate that the extra region of amino-terminal GSPT/eRF3 may consequently destabilize the

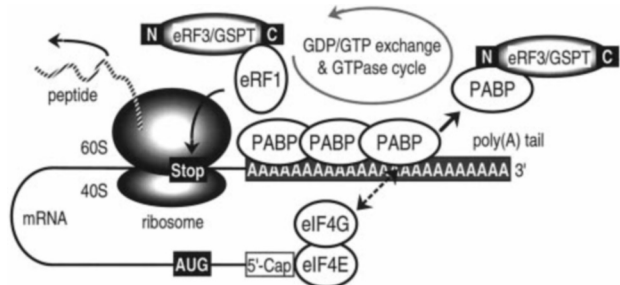


FIG. 5. **A proposed model for the functions of eRF3/GSPT in eukaryotic protein synthesis.** When the A site of ribosome is occupied by a stop codon, eRF3/GSPT carries eRF1 for the translation termination via its carboxyl-terminal EF1 α -like domain. eRF3/GSPT also associates with PABP via its amino-terminal region for the destabilization of mRNA and/or the initiation of new translation cycle. For details, see "Possible Functions of GSPT/eRF3 in Eukaryotic Translation System."

association of PABP with 3'-poly(A) tail, leading to the degradation by ribonuclease(s) of the mRNA recruited for the translation. In other words, a signal from each translation termination cycle in the ribosomal A site would be transferred to the 3'-poly(A) tail of mRNA as a degradation signal via the interaction between GSPT/eRF3 and PABP. This idea may be supported by the findings that repetitive translation shortens the 3'-poly(A) tail of mRNA and that PABP can extend the lifetime of mRNA (13, 14, 16, 17). In this relation, Czaplinski *et al.* (18) have quite recently reported another similar function of eRF1/eRF3. They demonstrated that the product of the *UPF1* gene, which is a RNA-dependent ATPase containing RNA helicase activity, interacts with the releasing factors in a nonsense mutation-mediated mRNA decay pathway. The interaction of *UPF1* gene product with eRF1/eRF3 appears to enhance the termination and degrade the aberrant mRNAs.

Moreover, it has recently been reported that the poly(A)-binding domains of PABP can associate with the translation initiation factor eIF4G, which binds to the 5'-cap structure of

mRNA through eIF4E to promote the translation initiation (for review see Refs. 19 and 20). Thus, the signal from the translation termination cycle might also regulate the new initiation step via the eRF3/PABP/eIF4G signaling cascade. It will be very interesting to determine how the GDP-GTP exchange reaction and/or the GTPase cycle are involved in the multi-functions of GSPT/eRF3. In any event, the results presented here have identified GSPT/eRF3 as a regulator of PABP interacting with the 3'-poly(A) tail of mRNAs, suggesting that it may also play an important role in the degradation of mRNAs and/or the regulation of translation efficiency mediated through the initiation factor(s). Clearly, further experimentation is required to test this fundamental hypothesis.

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