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A Novel Role of the Mammalian GSPT/eRF3 Associating with Poly(A)-binding Protein in Cap/Poly(A)-dependent Translation*

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The mammalian GSPT, which consists of amino-terminal (N) and carboxyl-terminal (C) domains, functions as the eukaryotic releasing factor 3 (eRF3) by interacting with eRF1 in translation termination. This function requires only the C-domain that is homologous to the elongation factor (EF) 1α, while the N-domain interacts with polyadenylate-binding protein (PABP), which binds the poly(A) tail of mRNA and associates with the eukaryotic initiation factor (eIF) 4G. Here we describe a novel role of GSPT in translation. We first determined an amino acid sequence required for the PABP interaction in the N-domain. Inhibition of this interaction significantly attenuated translation of capped/poly(A)-tailed mRNA not only in an *in vitro* translation system but also in living cells. There was a PABP-dependent linkage between the termination factor complex eRF1-GSPT and the initiation factor eIF4G associating with 5′ cap through eIF4E. Although the inhibition of the GSPT-PABP interaction did not affect the *de novo* formation of an 80 S ribosomal initiation complex, it appears to suppress the subsequent recycle of ribosome. These results indicate that GSPT/eRF3 plays an important role in translation cycle through the interaction with PABP, in addition to mediating the termination with eRF1.

The process of eukaryotic protein biosynthesis is divided into three steps: initiation, elongation, and termination. Among them, termination had been the least investigated aspect. However, the identification of two releasing factors, eRF1[1] (1) and eRF3 (2), provided a breakthrough in understanding the termination process. eRF1 recognizes all stop codons to release the completed polypeptide chain from the ribosome (1), and eRF3 is essential for the GTP-dependent releasing activity (2).

Mammalian eRF3 gene, *GSPT*, was first isolated based on its ability to complement a temperature-sensitive *gst1* mutant of *Saccharomyces cerevisiae* (3). At present, two distinct eRF3 genes termed *GSPT1* and *GSPT2* have been identified (4) and mapped to chromosomal band 16p13.1 (5) and Xp11.23 to p11.21 (6), respectively, in the human genome. The structural analysis revealed that both subtypes consist of an N-terminal region (~200 amino acids) and a C-terminal EF1α-like GTP-binding domain (428 amino acids). The C-domain, which interacts with eRF1, is sufficient not only for the termination reaction (2, 4, 7, 8) but also for the compensation of yeast *gst1*- growth arrest (3). In contrast, the N-domain is not required for the eRF1 binding and the termination reaction. We previously reported that the N-domain associates with PABP and inhibits its multimerization (9, 10).

PABP has two major functions: mRNA stabilization (11–14) and translation enhancement (15–20). PABP prevents mRNA from deadenylation, a late-limiting step of mRNA decay, by binding to the poly(A) tail. On the other hand, the involvement of PABP in translation enhancement is based on the finding that efficient translation requires the synergistic interplay between the 5′ cap and 3′ end-poly(A) tail of mRNA. The 5′ cap and 3′ poly(A) tail are recognized by eIF4E and PABP, respectively, and eIF4G mediates the association between them. These interactions result in the formation of a circularized mRNA (21–23), and this suggests the hypothetical machinery of efficient translation; ribosome after translation termination is recruited to the next cycle of translation initiation. However, it is noteworthy that PABP was also reported to stimulate translation of capped, nonpolyadenylated mRNA (24).

In this study, we analyzed the biological significance of the interaction between GSPT and PABP in the several steps of translation reaction. Inhibition of this interaction significantly attenuated translation of capped/poly(A)-tailed mRNA. There was a PABP-dependent linkage between eRF1-GSPT and the 5′ cap-initiation factor complex, and this linkage appeared to be responsible for the reentry of ribosome to the initiation factor complex. Thus, GSPT/eRF3 plays important roles not only in translation termination with eRF1 but also in the translation cycle through its interaction with PABP.

EXPERIMENTAL PROCEDURES

Plasmids—For production of N-terminally GST-fused GSPT2 mutants, PCR products were inserted to pGEX4T1 (Amersham Biosciences). To produce full-length GSPT2 and its deletion mutants fused with N-terminally GST and C-terminally His_6 epitope, respectively, the *Sal*I-*Not*I fragment of pGEX6P1 vector (Amersham Biosciences) was ligated with the synthetic adaptor HIS (HIS5′ plus HIS3′ as described blow) to make pGPH6. PCR products encoding the corresponding sequences (amino acid sequence 1–632, 1–204, 58–204, and 80–204) of GSPT2 were inserted to pGPH6, resulting in pGP2-full, pGP2-1, pGP2-58, and pGP2-80. To produce the amino acid 45–204 of eIF4G I fused

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[1] The abbreviations used are: eRF, eukaryotic releasing factor; EF, elongation factor; PABP, polyadenylate-binding protein; eIF, eukaryotic initiation factor; GSPT, GTP-binding protein appeared to be essential for the G_1 to S phase transition of cell cycle; aa, amino acids; HCV IRES, hepatitis C virus internal ribosome entry site; Ni-NTA, nickel-nitrilotriacetic acid; GST, glutathione *S*-transferase; RRL, rabbit reticulocyte lysate; R-luc, *Renilla* luciferase; F-luc, firefly luciferase.

with GST at the N terminus, a PCR product was inserted to pGEX6P1 to make pGEX4GI/aa 45–204. To express PABP in mammalian cells, human PABP I cDNA was inserted to pFlag-CMV-2 (Eastman Kodak Co.) to make pFlagPABP. PCR products encoding the N-domain or C-domain of GSPT2 were also inserted to pFlag-CMV-2 to produce pFlagGSPT2N and pFlagGSPT2C, respectively. To construct pcDNA3/GSPT2/aa 1–204-$(His)_6$, GSPT2/aa 1–204-$(His)_6$ cDNA excised from pGP2-1 was inserted to pcDNA3 (Invitrogen). pcDNA3/GSPT2/aa 19–204-$(His)_6$, aa 36–204-$(His)_6$, aa 58–204-$(His)_6$, aa 80–204-$(His)_6$, and aa 1–204:65–71A-$(His)_6$ were created from pcDNA3/GSPT2/aa 1–204-$(His)_6$ by the Kunkel method. The plasmid to express N-terminally FLAG-tagged GSPT2 and eRF1 was previously described (4). To construct a luciferase reporter gene, the *Bgl*II-*Nco*I fragment of pGL3 control vector (Promega) was ligated with the synthetic adaptor T7 (T75′ plus T73′ as described below), which encodes T7 promoter to make pGL3:T7. The *Xba*I-*Bam*HI fragment of the pGL3:T7 was then ligated with the synthetic adaptor pA55 (pA5′ plus pA3′ as described below) to make pGL3:T7-pA. To construct pUC18-T7-R-luc-HCV IRES-F-luc, T7 promoter, *Renilla* luciferase (R-luc), hepatitis C virus internal ribosome entry site (HCV IRES), and firefly luciferase (F-luc) were placed in this order in the multicloning site of pUC18. The synthetic oligonucleotides used were: HIS5′-TCG ACC ATC ATC ATC ATC ATC ATT GAG C, HIS3′-GGC CGC TCA ATG ATG ATG ATG ATG ATG G, pT75′-GAT CTT AAT ACG ACT CAC TAT AGG CCT AAG CTT GTC GAC, pT73′-CAT GGT CGA CAA GCT TAG GCC TAT AGT GAG TCG TAT TAA, pA5′-CTA $GA_{55}G$, and pA3′-GAT CCT_{55}.

Production of Recombinant Proteins—Proteins were induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h in *Escherichia coli* JM109. The cells were resuspended in buffer A consisting of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 2 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml of leupeptin. After incubation with 1 mg/ml lysozyme at 4 °C for 30 min, the cell lysate was sonicated for 3 min on ice. The supernatant after centrifugation at 100,000 × *g* for 60 min was subjected to glutathione-Sepharose 4B (Amersham Biosciences) and/or Ni-NTA-agarose (Qiagen). If necessary, GST was removed using PreScission™ Protease (Amersham Biosciences). The purified proteins were dialyzed against buffer B consisting of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1% Nonidet P-40. PABP I was purified as described previously (10).

Cell Culture, DNA Transfection, and in Vivo Translation Assay—COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum and maintained at 37 °C in 5% CO_2. Transfections were performed with Lipofectin (Invitrogen).

HeLa cells that had been transfected with pcDNA3/GSPT2 mutants and a reporter pUC18-T7-R-luc-HCV IRES-F-luc were incubated for 40 h and infected with vaccinia virus vTF-3 (25) for 4 h. Dual luciferase activities were measured using Stop & Glo luciferase assay system (Promega).

In Vitro Binding Assay—Recombinant GST-fused proteins were incubated with glutathione-Sepharose 4B for 30 min at 4 °C. After removal of the unbound fraction, the resin was mixed with recombinant PABP in buffer B and further incubated at 4 °C for 30 min. The resin was washed with buffer B and incubated with synthetic peptides or recombinant proteins at 4 °C for 60 min. After washing with buffer B, proteins were eluted from the resin with SDS-polyacrylamide sample buffer and subjected to SDS-PAGE and immunoblot analysis.

Immunoprecipitation and Ni-NTA Pull-down Assay—The transfected cells were lysed in buffer C consisting of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, 10 μg/ml boiled RNase A, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, and 2 μg/ml of leupeptin. After centrifugation at 15,000 × *g* for 20 min, the lysate was incubated at 4 °C for 30 min with anti-FLAG IgG-agarose (Sigma) or Ni-NTA-agarose, and then the resin was washed with buffer C. As the need arose, recombinant proteins or synthetic peptides were added, and the resin was further incubated at 4 °C for 60 min. After washing with buffer C, proteins retained in the resin were subjected to SDS-PAGE and immunoblot analysis. Immunoprecipitation from nuclease-treated rabbit reticulocyte lysate (RRL, Promega) was performed in the same manner as described above using an anti-GSPT polyclonal antibody and protein A-agarose 4B (Amersham Biosciences).

In Vitro Translation Assay—Luciferase mRNAs containing poly(A) tail or not were synthesized with T7 RNA polymerase after linearization of pGL3:T7-pA with *Bam*HI or *Xba*I, respectively. When capped mRNAs were synthesized, m^7GpppG (Stratagene) was used. *In vitro* translation reaction was performed as described below. Nuclease-treated RRL (10 μl) was reconstituted with 10 μl of a buffer consisting of 10 mM Hepes-KOH (pH 7.5), 142 mM KCl, 1.32 mM $MgCl_2$, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 20 μM each of complete amino acid mixture (Promega), 1.6 units/μl RNasin (Promega), 5 μg/ml of luciferase mRNA, and the indicated amounts of synthetic peptides or recombinant proteins and further incubated at 30 °C for 60 min or for the indicated times. Luciferase activity was measured using Bright-Glo luciferase assay regent (Promega).

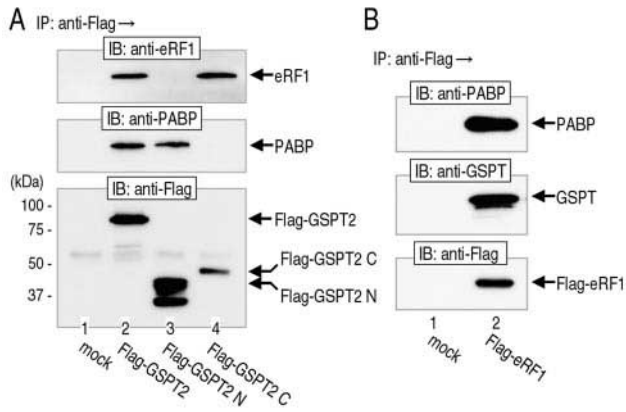

FIG. 1. **The N-domain of GSPT2 interacts with PABP in living cells, and GSPT mediates the interaction between eRF1 and PABP.** *A*, a control vector (*lane 1*) or plasmids that can express N-terminally FLAG-tagged GSPT2 (*lane 2*), FLAG-GSPT2 N domain (*lane 3*), and FLAG-GSPT2 C domain (*lane 4*) was introduced into COS-7 cells, and cell extracts were subjected to immunoprecipitation assay using an anti-FLAG antibody. SDS-PAGE and immunoblot analysis with anti-eRF1 (*upper*), anti-PABP (*middle*), and anti-FLAG (*lower*) antibodies were performed. *B*, a control vector (*lane 1*) or a plasmid that can express N-terminally FLAG-tagged eRF1 (*lane 2*) was introduced into COS-7 cells, and cell extracts were subjected to immunoprecipitation as described in *A*. SDS-PAGE and immunoblot analysis with anti-PABP (*upper*), anti-GSPT (*middle*), and anti-FLAG (*lower*) antibodies were performed.

Assay for the Formation of an 80 S Ribosomal Initiation Complex—Globin mRNA (Invitrogen) was 3′-^{32}P-labeled using T4 RNA ligase and 5′-^{32}P-labeled pCp. For the formation of a *de novo* 80 S ribosomal initiation complex, the nuclease-treated RRL (26 μl) was reconstituted with 14 μl of a buffer consisting of 6.5 mM Hepes-KOH (pH 7.5), 65 mM KCl, 0.65 mM $MgCl_2$, 65 μM EDTA, 4.5 mM β-mercaptoethanol, 28 μM each complete amino acid mixture, 2.3 units/μl RNasin, 25 ng of 3′-^{32}P-labeled globin mRNA, and 0.14 mM cycloheximide. The reaction mixture was incubated at 30 °C for 15 min, and aliquots (20 μl) were analyzed on 5 ml of 15–30% (w/v) linear sucrose gradient. After centrifugation at 160,000 × *g* for 45 min, fractions were collected using a piston-gradient fractionator (Biocomp), and the radioactivity of each fraction was measured by a liquid scintillation counter.

RESULTS

Interaction between GSPT and PABP in Intact Cells—We have previously shown that the N-domain of GSPT interacts with PABP in a yeast two-hybrid assay and *in vitro* binding experiments (10). To investigate the significance of the interaction in intact cells, N-terminally FLAG-tagged GSPT2 was expressed in COS-7 cells, and the cell extract was subjected to immunoprecipitation using an anti-FLAG antibody. Endogenous PABP co-precipitated with FLAG-GSPT2 (Fig. 1*A*, *lane 2*). Co-immunoprecipitation of PABP was also observed if GSPT1 was expressed in the cells (data not shown). To check if this interaction is dependent on the N-domain rather than the C-domain of GSPT, either of the two domains was produced in COS-7 cells. PABP co-immunoprecipitated with the N-domain (*lane 3*), whereas eRF1 co-precipitated with the C-domain (*lane 4*). To investigate whether these three factors are in the same complex, an immunoprecipitation experiment was performed using extracts from COS-7 cells expressing N-terminally FLAG-tagged eRF1. Endogenous GSPT and PABP co-immunoprecipitated with FLAG-eRF1 (Fig. 1*B*). These interactions

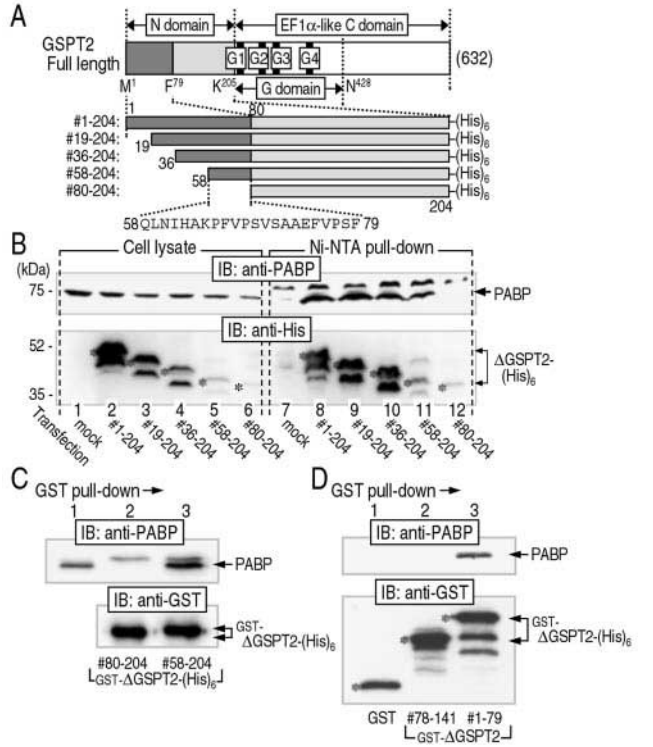

FIG. 2. **Identification of the site critical for PABP-binding in the N-domain of GSPT2.** *A*, the GSPT family consists of a N-domain and an EF1α-like C-domain. The C-domain contains four GTP-binding motifs (*G1–G4*). Series of N-domain deletion mutants fused with a His_6 tag were constructed. *B*, the deletion mutants (*A*) were produced in COS-7 cells, and a pull-down experiment was performed. *Asterisks* indicate the position of the GSPT2 mutants. *C*, recombinant PABP and deletion mutants of N-domain (*lane 2*, GSPT2/aa 80–204 and *lane 3*, GSPT2/aa 50–204) fused with an N-terminal GST and a C-terminal His_6 tag were incubated with glutathione-Sepharose. Proteins that associated with the resin were analyzed by SDS-PAGE and immunoblot with anti-PABP (*upper*) and anti-GST (*lower*) antibodies. *Lane 1* shows the purified PABP used in the pull-down assay. *D*, PABP and deletion mutants of N-domain (*lane 2*, GSPT2/aa 78–141 and *lane 3*, GSPT2/aa 1–79) fused with N-terminal GST were incubated with glutathione-Sepharose. SDS-PAGE and immunoblot analysis were done as described above. As a control, GST was used (*lane 1*).

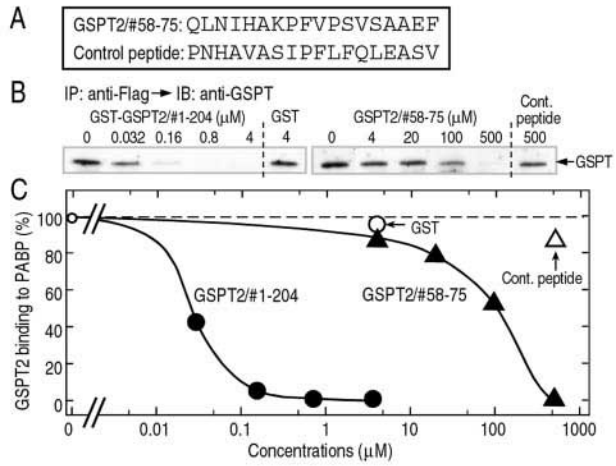

FIG. 3. **Effects of the whole N-domain and the PABP-binding peptide of GSPT2 on the GSPT-PABP interaction.** *A*, a synthetic peptide corresponding to the PABP-binding sequence of GSPT2 (aa 58–75) is illustrated in single-letter codes (*upper*). A scrambled peptide consisting of the same amino acid composition (*lower*, *control peptide*) was used in the control experiments. *B* and *C*, FLAG-tagged PABP was produced in COS-7 cells, and the cell extracts were immunoprecipitated (*IP*) with anti-FLAG IgG-agarose. The resin containing FLAG-tagged PABP and endogenous GSPT was incubated with the GST-fused whole N-domain (*left* in *B* and *closed circles* in *C*) or the synthetic peptide (*right* in *B* and *closed triangles* in *C*) at the indicated concentrations. GSPT that associated with the resin was analyzed by SDS-PAGE and immunoblot (*IB*) with anti-GSPT antibody. GST alone (4 μM, *open circle*) or the control peptide (500 μM, *open triangle*) was also used in this assay. *C*, the results in *B* are shown as the functions of the concentrations of competitors.

appear to be independent of RNA tethering because cell extracts had been treated with RNase. Thus, these experiments show that GSPT associates with PABP and eRF1 via its N-domain and C-domain, respectively, in living cells, and consequently GSPT mediates the association between eRF1 and PABP.

Identification of the Site Critical for PABP Binding in the N-domain of GSPT—To identify a PABP-binding sequence in the N-domain of GSPT, a co-precipitation assay was performed using COS-7 cells expressing deletion mutants of the N-domain (Fig. 2*A*). As shown in Fig. 2*B*, deletion mutants starting from amino acid positions 1, 19, 36, and 58 interacted with PABP (*lanes 7–11*), while a mutant starting from the 80th amino acid (aa 80–204) failed to associate with PABP (*lane 12*). Since the expression of this mutant (aa 80–204) was rather low in COS-7 cells, we performed a binding assay using recombinant proteins. GSPT2/aa 58–204 associated with PABP, but aa 80–204 did not (Fig. 2*C*). Furthermore, GSPT2/aa 1–79 but not aa 78–141 was sufficient for the PABP binding (Fig. 2*D*). Thus, the amino acid sequence 58–79 of GSPT2 (see Fig. 2*A*) was identified as a critical region for PABP binding. This sequence is conserved well between GSPT1 and GSPT2 in mice and humans (4).

The significance of the identified sequence was further investigated using a synthetic peptide corresponding to amino acid 58–75 (Fig. 3*A*). As shown in Fig. 3, *B* and *C*, the GSPT-PABP interaction was progressively inhibited with increasing amounts of the whole N-domain or the synthetic peptide but not with GST or a control peptide consisting of the same amino acid composition in a scrambled order (see Fig. 3*A*). The complete inhibition of the interaction by the synthetic peptide supports the notion that the sequence aa 58–75 of GSPT2 constitutes a critical site for the PABP-binding. However, since the half-maximum inhibition by the synthetic peptide was observed at about 100 μM, which is almost three orders of magnitude higher than that of the whole N-domain (Fig. 3*C*), we cannot exclude the possibility that other regions might also be involved in the interaction.

Involvement of the Interaction between the N-domain of GSPT and PABP in Cap/Poly(A)-dependent Translation—PABP was reported to regulate translation in a cap/poly(A)-dependent manner by mediating the interaction between the cap-binding complex eIF4F and the poly(A) tail of mRNA (21–23). To investigate whether the interaction between GSPT and PABP is involved in cap/poly(A)-dependent translation, we utilized nuclease-treated RRL as a cell-free translation system. It was previously reported that the synergistic stimulation by cap and poly(A) was observed in the RRL system with partial removal of ribosome and the associated initiation factors (26, 27). However, such a synergistic stimulation was observed only by changing the concentrations of $MgCl_2$ and KCl (Fig. 4*A*). In this system, cap-dependent translation was markedly stimulated by the simultaneous presence of the poly(A) tail, while mRNA containing only the poly(A) tail had little activity. The N-domain of GSPT2 fused to GST markedly inhibited the cap/poly(A)-dependent translation (Fig. 4*B*, *closed triangles*). Interestingly, the N-domain was also capable of inhibiting translation of capped and non-poly(A)-tailed mRNA (*closed circles*). In contrast, GST alone had little effect on any of the mRNAs (*open*

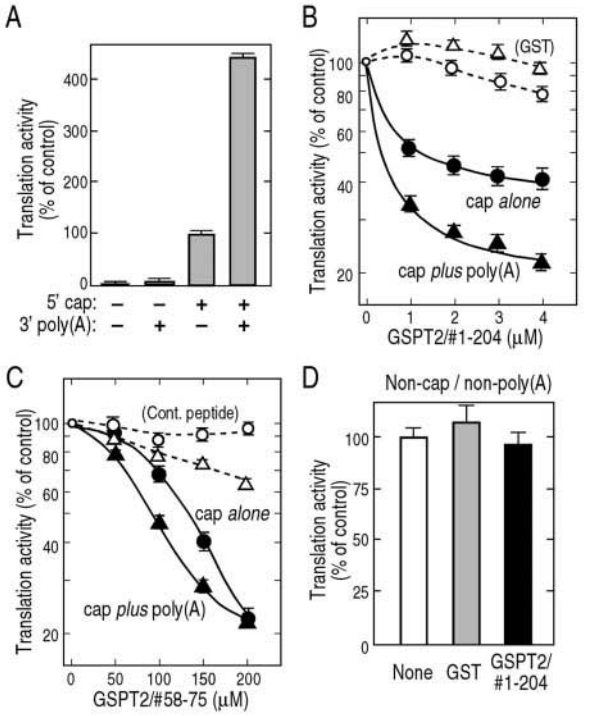

FIG. 4. **Involvement of the GSPT-PABP interaction in cap/poly(A)-dependent translation.** *A*, luciferase mRNAs (50 ng) were added to an *in vitro*-translation mixture. Luciferase activity is shown as a percentage of the value obtained with the only capped mRNA. *B* and *C*, luciferase mRNAs (50 ng) containing cap plus poly(A) (*closed triangles*) or cap alone (*closed circles*) were used in the translation assay in the presence of the indicated concentrations of the GST-fused N-domain of GSPT2 (*B*, aa 1–204) or the synthetic peptide (*C*, aa 58–75). As control experiments, GST (*B*) or the control peptide (*C*) was also used. Luciferase activity is shown as a percentage of the value obtained without the competitors. *D*, luciferase mRNA (250 ng) containing neither cap nor poly(A) was used in the translation assay in the presence of 4 μM GST-fused N-domain of GSPT2 or GST. At this time, the background luminescence with no RNA was almost about 25% of the value when buffer alone was used, and the illustrated value does not contain the background luminescence. Luciferase activity is shown as a percentage of the value obtained in the addition of buffer alone.

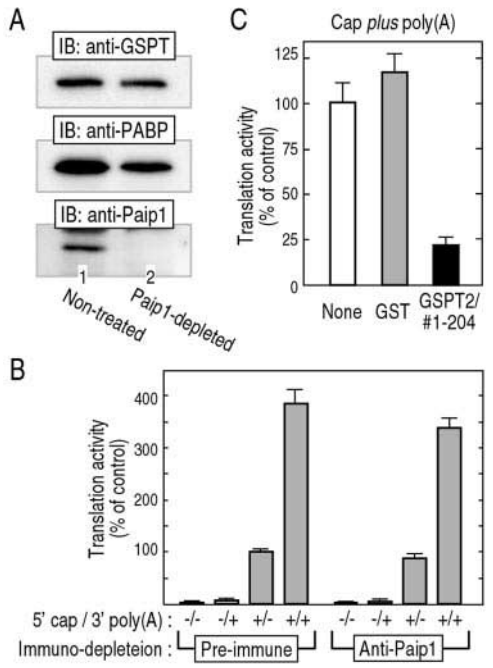

FIG. 5. **No involvement of Paip1 in the inhibition of cap/poly(A)-dependent translation by GSPT N-domain.** *A*, RRL was immunodepleted (*ID*) using anti-Paip1 antibodies. Untreated RRL (*lane 1*) and immunodepleted RRL (*lanes 2*) were analyzed by SDS-PAGE and immunoblot analysis (*IB*) with anti-GSPT (*upper*), anti-PABP (*middle*), and anti-Paip1 (*lower*) antibodies. *B*, an *in vitro* translation assay was performed using Paip1-immunodepleted RRL and luciferase mRNAs (50 ng) As a control, preimmune IgG was used for immunodepletion. Luciferase activity is shown as a percentage of the value obtained with the only capped mRNA in a control experiment. *C*, Paip1-immunodepleted RRL was used in an *in vitro* translation assay with luciferase mRNA (50 ng) containing both cap and poly(A) in the presence of 2 μM GST or GST-fused N-domain of GSPT2. Luciferase activity is shown as a percentage of the value obtained in the addition of buffer alone.

circles and *triangles*). The effect of the synthetic peptides was also investigated. The synthetic peptide aa 58–75 inhibited translation not only of capped/poly(A)-tailed mRNA but also of capped mRNA (Fig. 4*C*). In accordance with the results in Fig. 3, the concentration of the synthetic peptide required for translation inhibition was much higher than that of the whole N-domain of GSPT2. Thus, both the GSPT-PABP interaction and the cap/poly(A)-dependent translation are inhibited by the synthetic peptide aa 58–75 and the whole N-domain in a similar concentration-dependent manner. In addition, the results presented here suggest that the interaction between GSPT and PABP may also be involved in poly(A)-independent translation (Fig. 4, *B* and *C*). Although the exact mechanism is still unclear, it is noteworthy that PABP was reported to stimulate cap/poly(A)-dependent and poly(A)-independent translation by distinct mechanisms (24).

Previous studies reported that the C-domain of GSPT is sufficient for translation termination (2, 4, 7, 8). However, it is possible that the translation inhibition observed here might be the result of inhibition of translation termination or elongation. Therefore, we investigated whether the inhibition of the GSPT-PABP interaction may affect translation termination or elongation by using an uncapped/non-poly(A)-tailed mRNA. If the termination step is inhibited, luminescence would diminish because luciferase has no activity when it is not released from ribosome (28), and if the elongation step is inhibited, luminescence would also diminish. As shown in Fig. 4*D*, the N-domain of GSPT2 had no inhibitory effect on the cap/poly(A)-independent translation, which is in sharp contrast to the results obtained with capped/poly(A)-tailed mRNA. These results suggest that the GSPT-PABP interaction is not involved in translation termination or elongation.

No Involvement of Paip1 in the Inhibition of Cap/Poly(A)-dependent Translation by the N-domain of GSPT—In addition to GSPT, two other proteins that interact with PABP have been reported. One is Paip1 identified as a translation activator (29), and the other is Paip2 identified as a translation repressor (19, 20). The PABP-binding sites, which were recently reported in Paip1 and Paip2 (19, 20, 30), are similar to that of GSPT2 identified in this study. Thus, it is possible that the translation inhibition by the N-domain of GSPT2 may have resulted from the inhibition of the Paip1-PABP interaction. To examine this possibility, we used RRL immunodepleted of Paip1 by anti-Paip1 antibodies. As shown in Fig. 5*A*, Paip1 was completely depleted, but PABP and GSPT were little affected. Under these conditions, significant effect was not observed in cap/poly(A) synergy (Fig. 5*B*), and the N-domain of GSPT2 had still inhibitory effect on translation (Fig. 5*C*). These results indicate that the inhibitory effects of the N-domain of GSPT are independent of Paip1.

The N-domain of GSPT Inhibits Cap/Poly(A)-dependent Translation in Living Cells—To confirm that the GSPT-PABP interaction is indeed involved in cap/poly(A)-dependent translation in living cells, we examined the effect of overproducing

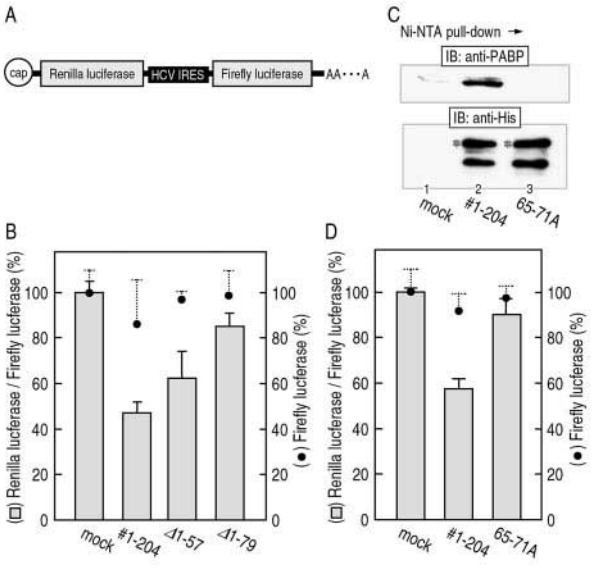

FIG. 6. **The N-domain of GSPT inhibits cap/poly(A)-dependent translation in living cells.** *A*, a reporter mRNA expressing *Renilla* and firefly luciferases from the bicistronic construct pUC18-T7-R-luc-HCV IRES-F-luc was illustrated. *B* and *D*, HeLa cells that had been transfected with pcDNA3/GSPT2 mutants and the reporter plasmid were infected with vaccinia virus to express T7 RNA polymerase. The cells were assayed for dual luciferase activities. Results are averages of three independent assays with standard deviations from the means as percentages of the value obtained with pcDNA3. The ratios of *Renilla* luciferase/firefly luciferase are illustrated with *bars*, and *closed circles* show the firefly luciferase activity. *C*, this experiment was performed as described in Fig. 2*B*. *Asterisks* indicate the position of the GSPT2 mutants.

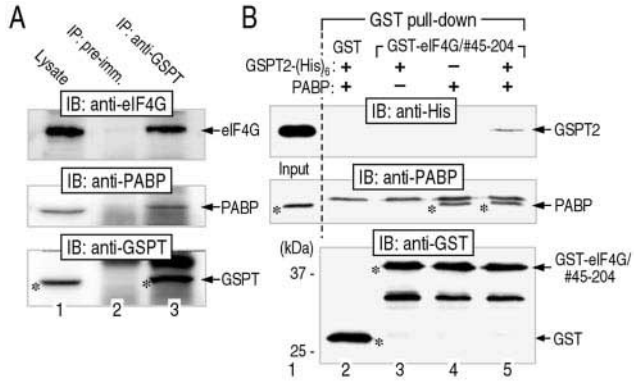

FIG. 7. **GSPT and eIF4G forms the complex mediated through PABP.** *A*, RRL was incubated with the anti-GSPT antibody (*lane 3*) or preimmune serum (*lane 2*) immobilized to protein A-agarose. Proteins that associated with the resins (*lanes 2* and *3*) and the lysate (*lane 1*) were analyzed by SDS-PAGE and immunoblot with anti-eIF4G (*upper*), anti-PABP (*middle*), and anti-GSPT (*lower*) antibodies. *Asterisks* indicate the position of GSPT. *B*, GST-fused eIF4G/aa 45–204 (*lanes 3–5*) or GST (*lane 2*) was immobilized to glutathione-Sepharose and incubated with the purified PABP and/or GSPT2-$(His)_6$. Proteins that associated with the resin (*lanes 2–5*) were resolved by SDS-PAGE and immunoblotted with anti-His (*upper*), anti-PABP (*middle*), and anti-GST (*lower*) antibodies. *Lane 1* shows the purified GSPT2-$(His)_6$ and PABP (marked by *asterisks*) used in the pull-down assay.

the N-domain of GSPT2 on translation by monitoring the synthesis of R-luc and F-luc from the bicistronic construct T7-R-luc-HCV IRES-F-luc (Fig. 6*A*). We used HCV IRES because it functions in eIF4G- (31) and a poly(A) tail- (20, 32) independent manners. By means of this bicistronic mRNA, efficiencies of cap/poly(A)-dependent translation (R-luc activity) and HCV IRES-dependent translation (F-luc activity as an internal control for both transfection efficiency and the amount of the reporter mRNA) can be measured at the same time. The overexpression of the N-domain of GSPT2 caused a marked decrease in the ratio of R-luc/F-luc (Fig. 6*B*), indicating that cap/poly(A)-dependent translation was inhibited by the N-domain. Moreover, the mutant lacking the amino acids 1–57 (Δ1–57), which can interact with PABP (Fig. 2), still has the inhibitory effect. Such inhibition was, however, reduced in the mutant lacking the amino acids 1–79 (Δ1–79), which cannot associate with PABP (Fig. 2*B*). To confirm this result, we constructed a mutant of aa 1–204 whose amino acids 65–71 are all converted to alanine (Ala-65–Ala71). This mutant could not interact with PABP any more (Fig. 6*C*) and had a lesser inhibitory effect on cap/poly(A)-dependent translation than the original N-domain (Fig. 6*D*). Taken together, these results further substantiate the idea that the GSPT-PABP interaction is involved in cap/poly(A)-dependent translation in living cells. However, the results that both of the mutants, Δ1–79 and Ala-65–Ala71, still exhibited just a little inhibitory activity are consistent with the idea that besides the region 58–79, the N-domain (aa 1–204) of GSPT2 contains an additional sequence(s) responsible for PABP binding as suggested by the results in Figs. 3 and 4.

PABP Mediates the Interaction between GSPT and eIF4G—Since the GSPT-PABP interaction is involved in cap/poly(A)-dependent translation, we examined the possibility that GSPT could associate with the translation initiation factor. To this end, an immunoprecipitation assay was performed against nuclease-treated RRL. As shown in Fig. 7*A*, PABP and eIF4G were co-immunoprecipitated with GSPT by anti-GSPT antibodies. This complex was also detected when cell extracts from COS-7 cells were used instead of RRL (data not shown). To substantiate these findings, we examined the interaction using recombinant proteins. PABP and the N-terminally GST-fused eIF4G/aa 45–204, which is sufficient for the PABP binding (23), were mixed with GSPT2-$(His)_6$ and subjected to a glutathione-Sepharose pull-down assay. As shown in Fig. 7*B*, the interaction between GST-eIF4G/aa 45–204 and PABP was observed both in the presence and absence of GSPT2 (*lanes 4* and *5*). However, the association between eIF4G/aa 45–204 and GSPT2 was observed only in the presence of PABP (compare *lane 5* with *3*), indicating that PABP mediates the association. These results provide a possibility that GSPT may be involved in a translation initiation step, in addition to termination.

The Interaction between GSPT and PABP Is Involved in the Multiple Rounds of Translation but Not in the de Novo Formation of an 80 S Ribosomal Initiation Complex—We next examined the involvement of the GSPT-PABP interaction in translation initiation. The final output of the translation initiation process was examined by monitoring the formation of an 80 S ribosomal initiation complex. 3′-^{32}P-labeled globin mRNA was incubated with a nuclease-treated RRL in the presence of cycloheximide, and the complex formation was monitored by a sucrose-density gradient analysis. As shown in Fig. 8*A*, labeled mRNAs were shifted at a position corresponding to the 80 S ribosomal initiation complex. The complex formation was, however, little affected by the addition of the N-domain of GSPT2, suggesting that the GSPT-PABP interaction does not function in the *de novo* formation of an 80 S initiation complex.

To further elucidate the role of GSPT-PABP interaction in translation reaction, we next analyzed the effect of the N-domain of GSPT2 on the kinetics of luciferase production in RRL. Regardless of the presence of the N-domain, production of luciferase was observed after an absolute lag time of about 8 min (Fig. 8), which was also not affected by the increasing amount of mRNA or preincubation of the reaction mixture before the addition of mRNA (data not shown). Since luciferase becomes active after its release from ribosome (28), the lag

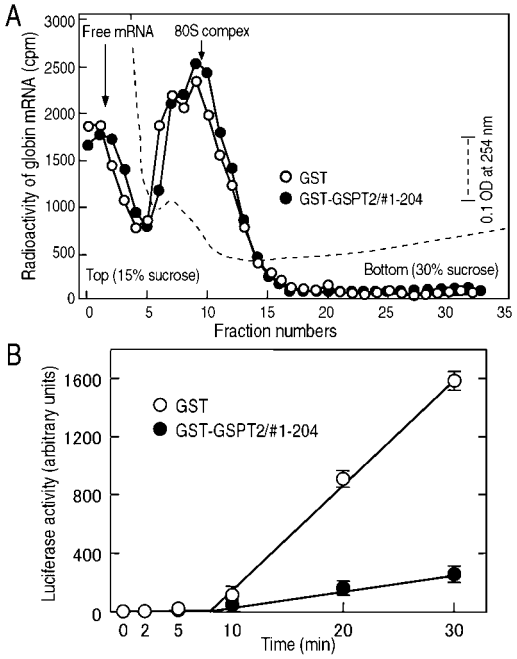

FIG. 8. **The GSPT-PABP interaction functions in the multiple rounds of translation.** *A*, 3′-^{32}P-labeled globin mRNA was incubated with the nuclease-treated RRL (40 μl) and cycloheximide (50 μM) at 30 °C for 15 min in the presence of 4 μM GST-GSPT2/aa 1–204-$(His)_6$ (*closed circles*) or GST alone (*open circles*). 20-μl aliquot of the mixture was analyzed on 5 ml of 15–30% linear sucrose gradient. *B*, a luciferase mRNA (50 ng) containing cap plus poly(A) was used in the translation assay in the presence of 4 μM GST (*open circles*) or the GST-fused N-domain of GSPT2 (*closed circles*).

means time required for completion of the first round of translation. Thus, consistent with the results in Fig. 8*A*, the first round indexed by the lag time was not affected by the N-domain of GSPT2. In contrast, the production of luciferase after the time lag, which is indicative of the subsequent rounds of translation, was markedly inhibited by the addition of the N-domain. These results indicate that the interaction between GSPT and PABP functions in the translation cycle, possibly the recycle of ribosome to the initiation factor complex rather than the initial formation of 80S complex.

DISCUSSION

GSPT Interacts with PABP through a Site in Its N-domain—We previously presented evidence that GSPT interacts with PABP in *in vitro* experiments (9). This conclusion was confirmed and further extended by our present experiments. First, the interaction between the N-domain of GSPT and PABP was observed with cell extracts (Figs. 1, 2*B*, and 7*A*) and with purified proteins (Figs. 2*C* and 7*B*). Moreover, we identified a possible PABP-binding sequence in the N-domain (Figs. 2 and 3). The GSPT2-PABP interaction is mediated at least through the amino acid sequence aa 58–75 of GSPT2, since the synthetic peptide completely inhibited the association (Fig. 3).

In addition to GSPT, Paip1 and Paip2 have been reported to interact with PABP. PABP-binding sites in Paip1 and Paip2 are similar to the sequence aa 58–75 of GSPT2, and this motif is important for their interactions with the C-terminal domain of PABP (19, 20, 30). Thus, GSPT may compete with Paips for PABP binding. However, the relationship between GSPT and Paips may not be so simple, since Paips interact with both the N- and C-terminal regions of PABP (19, 20, 29, 30, 33). In contrast, GSPT interacts only with the C-terminal site (10). A rabbit reticulocyte lysate, which we used in this study, has a much smaller amount of Paips than a rabbit liver lysate when

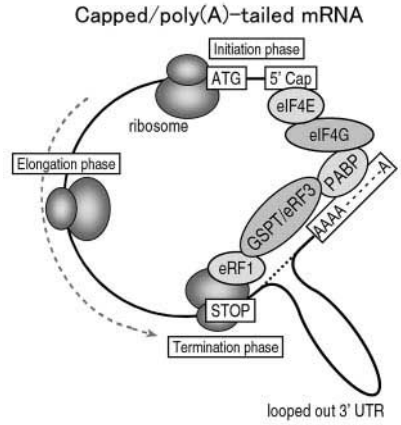

FIG. 9. **Possible roles of GSPT/eRF3 in eukaryotic translation system.** UTR, untranslated region. For details, see under "A Novel Role of GSPT/eRF3 in the Eukaryotic Translation System."

compared with the amount of PABP (data not shown). Thus, it is possible that Paips may be the factors modifying the function of PABP on the requirement of each tissue.

A Novel Role of GSPT/eRF3 in the Eukaryotic Translation System—It is generally believed that the function of GSPT was solely to facilitate the release of completed peptide chains from ribosome as a GTP-dependent stimulator of eRF1. However, the present study reveals that GSPT associates with eIF4G through PABP (Fig. 7) and that the GSPT-PABP interaction is involved in the multiple rounds of translation (Figs. 4, 6, and 8). The synergistic enhancement of translation by cap and poly(A) is explained by the circularization of mRNA, which is mediated through a complex consisting of poly(A)-PABP-eIF4F-cap (21–23). This fact suggests the hypothetical model that a translation-terminating ribosome may be recruited to the next translation initiation. However, this idea is unsatisfactory since translation is terminated at stop codons that are not always close to the poly(A) tail of mRNA. Therefore, some factors are likely to mediate the physical coupling between the terminating ribosome on the stop codon and the poly(A) tail. The fact that GSPT interacts with eRF1 and PABP at the same time (Fig. 1*B*) suggests that GSPT may be the bridging protein to connect the stop codon with the poly(A) tail. In this hypothesis, a 3′-untranslated region, which locates between a stop codon and a poly(A) tail, could be looped out, and the terminating ribosome could be passed to the 5′ cap structure through the novel protein bridge consisting of eRF1, GSPT, PABP, and eIF4F (Fig. 9).

In addition to the role of PABP in translation, several lines of evidence suggest that PABP might affect translation in a poly(A)-independent manner (18, 20, 34, 35). Furthermore, this function appears to be independent of its binding to eIF4G (24). The results presented here suggest that the GSPT-PABP interaction may also be involved in poly(A)-independent translation (Fig. 4, *B* and *C*), though the exact mechanism is still unclear.

It is well established that PABP has another function; it prevents mRNA degradation by protecting the poly(A) tail. In general, mRNA degradation, an important aspect of gene expression, is a strictly regulated process that is often linked to translation (12, 36, 37), and translation-dependent deadenylation is an important step of this mechanism in which PABP is probably involved. Moreover, several reports show that GSPT is involved in nonsense-mediated decay, a mechanism by which mRNAs containing a premature termination codon are rapidly degraded (38, 39). These mechanisms are not well understood, but it is conceivable that they are linked to the translation

termination. Further studies on GSPT/eRF3 should be important for the understandings of not only translation machinery but also mRNA-decay mechanism.

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