

A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation

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Most eukaryotic mRNAs possess a 5' cap and a 3' poly(A) tail, both of which are required for efficient translation. In yeast and plants, binding of eIF4G to poly(A)-binding protein (PABP) was implicated in poly(A)-dependent translation. In mammals, however, there has been no evidence that eIF4G binds PABP. Using 5' rapid amplification of cDNA, we have extended the known human eIF4GI open reading frame from the N-terminus by 156 amino acids. Co-immunoprecipitation experiments showed that the extended eIF4GI binds PABP, while the N-terminally truncated original eIF4GI cannot. Deletion analysis identified a 29 amino acid sequence in the new N-terminal region as the PABP-binding site. The 29 amino acid stretch is almost identical in eIF4GI and eIF4GII, and the full-length eIF4GII also binds PABP. As previously shown for yeast, human eIF4G binds to a fragment composed of RRM1 and RRM2 of PABP. In an *in vitro* translation system, an N-terminal fragment which includes the PABP-binding site inhibits poly(A)-dependent translation, but has no effect on translation of a deadenylated mRNA. These results indicate that, in addition to a recently identified mammalian PABP-binding protein, PAIP-1, eIF4G binds PABP and probably functions in poly(A)-dependent translation in mammalian cells.

Keywords: eukaryotic translation initiation factor 4G/poly(A)-binding protein/poly(A)-dependent translation/translation initiation

Introduction

Most eukaryotic mRNAs possess a cap structure at the 5' end and a poly(A) tail structure at the 3' end. The cap structure is bound by eukaryotic initiation factor (eIF) 4F, which consists of eIF4E, eIF4A and eIF4G. eIF4E is the cap-binding subunit. eIF4A is an RNA-dependent ATPase and ATP-dependent RNA helicase, which, in combination with eIF4B, is thought to unwind the secondary structure in the 5'-untranslated region of the mRNA to facilitate ribosome binding (for reviews, see Merrick and Hershey, 1996; Sonenberg, 1996). eIF4G serves as a scaffold for eIF4E and eIF4A to coordinate their functions. eIF4G also binds eIF3, which associates with the 40S ribosomal subunit (for reviews, see Pain, 1996; Morley *et al.*, 1997; Sachs *et al.*, 1997).

The poly(A) tail is bound by the poly(A)-binding protein (PABP), which is necessary for efficient translation (for reviews, see Jacobson, 1996; Sachs *et al.*, 1997). In yeast, eIF4G was found to associate with PABP (Tarun and Sachs, 1996). The PABP-binding site was mapped to an N-terminal region proximal to the eIF4E-binding site, which mediates the poly(A) tail-dependent translation and circularization of the mRNA (Tarun and Sachs, 1996; Tarun *et al.*, 1997; Wells *et al.*, 1998). In plants, PABP was reported to bind to eIF-iso4G and eIF4B. Their interaction with PABP increased the RNA-binding activity of PABP (Le *et al.*, 1997). In mammals, a PABP-binding protein (PAIP-1) was cloned by far Western screening with PABP as a probe (Craig *et al.*, 1998). PAIP-1 bound eIF4A as well as PABP, and enhanced translation *in vivo* (Craig *et al.*, 1998). Interaction between eIF4G and PABP, however, has not been documented in mammals.

We recently have cloned a cDNA for human eIF4GII (Gradi *et al.*, 1998), a closely related functional homologue of eIF4G (hereafter called eIF4GI) (Yan *et al.*, 1992). The open reading frame (ORF) of eIF4GII was found to be N-terminally longer by 158 amino acids relative to the methionine which aligns with the first methionine of the published eIF4GI sequence (Gradi *et al.*, 1998). We suspected that the original cDNA clone of eIF4GI (Yan *et al.*, 1992) contained an intron, because the similarity of this clone to that of eIF4GII in nucleotide sequence stops abruptly at a putative splice acceptor site (Gradi *et al.*, 1998). Consequently, we performed 5' rapid amplification of cDNA ends (5' RACE), and extended the N-terminus of eIF4GI by 109 amino acids to form a segment which exhibits significant homology to the corresponding region of eIF4GII (Gradi *et al.*, 1998).

Here we show that the extended N-terminal region of human eIF4GI contains a PABP-binding site, and functions in translation in a poly(A)-dependent manner.

Results

Full-length eIF4G binds PABP

Using 5' RACE, we extended the N-terminal sequence of human eIF4GI by 109 amino acids (Gradi *et al.*, 1998) as compared with the original eIF4GI clone (Yan *et al.*, 1992). However, the new sequence did not contain an in-frame stop codon upstream of the first ATG. Consequently, to extend the 5' end of human eIF4GI further, we repeated the 5' RACE, and obtained several extended cDNAs. The first methionine in the ORF of the longest clone was assigned tentatively as the first amino acid of human eIF4GI (Figure 1). It is not completely certain that the assigned methionine is indeed the authentic initiator, since there is still no in-frame stop codon upstream of the first AUG. However, this methionine aligns with the initiator methionine in eIF4GII (Gradi *et al.*, 1998), and is thus

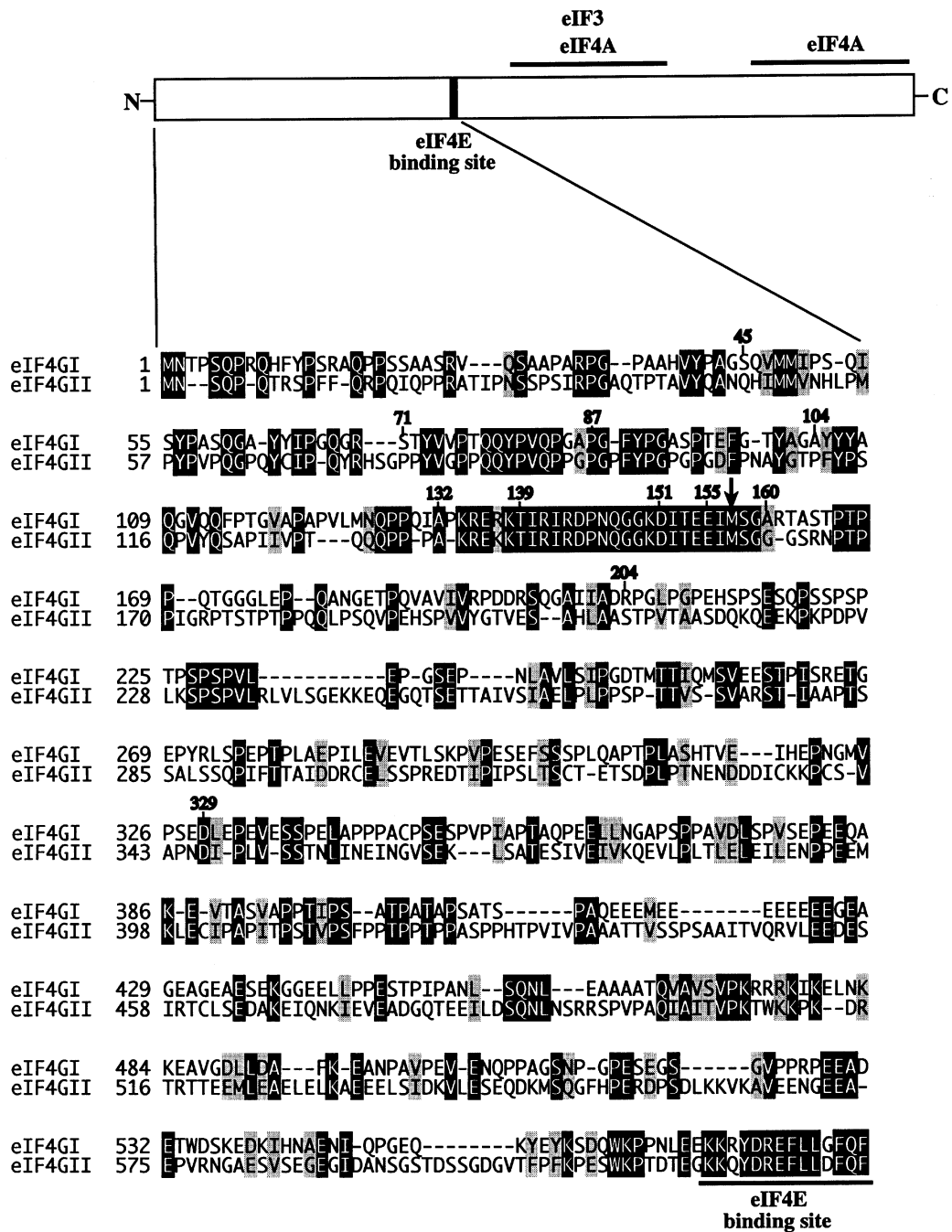


Fig. 1. Alignment of amino acid sequences of the N-terminal region of human eIF4GI and eIF4GII. The pattern-induced multi-sequence alignment program (Smith and Smith, 1992) was used to align amino acid sequences of the N-terminal region of eIF4GI (Yan *et al.*, 1992; Imataka and Sonenberg, 1997; this study) and eIF4GII (Gradi *et al.*, 1998). Identical amino acids are boxed and conservative substitutions are shaded. The first methionine of the original eIF4GI (Yan *et al.*, 1992) is indicated by an arrow. Numbers above amino acids represent positions of truncations used in the text. A schematic representation of the binding sites of eIF4E (Mader *et al.*, 1995), eIF4A and eIF3 (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997) is shown above the alignment.

likely to be the initiator methionine of eIF4GI. The new ORF of eIF4GI (Figure 1) is 156 amino acids longer than that of Yan *et al.* (1992). The N-terminally extended sequence was fused to the original cDNA clone (1404 amino acids) (Yan *et al.*, 1992) to construct a cDNA encoding an ORF of 1560 amino acids (accession No. AF104913) with a predicted molecular mass of 171 kDa. The resulting clone is referred to as the extended eIF4GI throughout this manuscript.

As the original clone of human eIF4GI (Yan *et al.*,

1992) did not exhibit any binding activity to PABP using several methods, including the yeast two-hybrid system (Craig *et al.*, 1998), co-immunoprecipitation or *in vitro* binding assays with recombinant proteins (H.Imataka, unpublished data), we wished to determine whether the extended eIF4GI could interact with PABP. The original eIF4GI (Yan *et al.*, 1992) and the extended eIF4GI were tagged with the hemagglutinin (HA) epitope, and expressed in HeLa cells. Cytoplasmic extracts were immunoprecipitated with an anti-HA antibody, and the

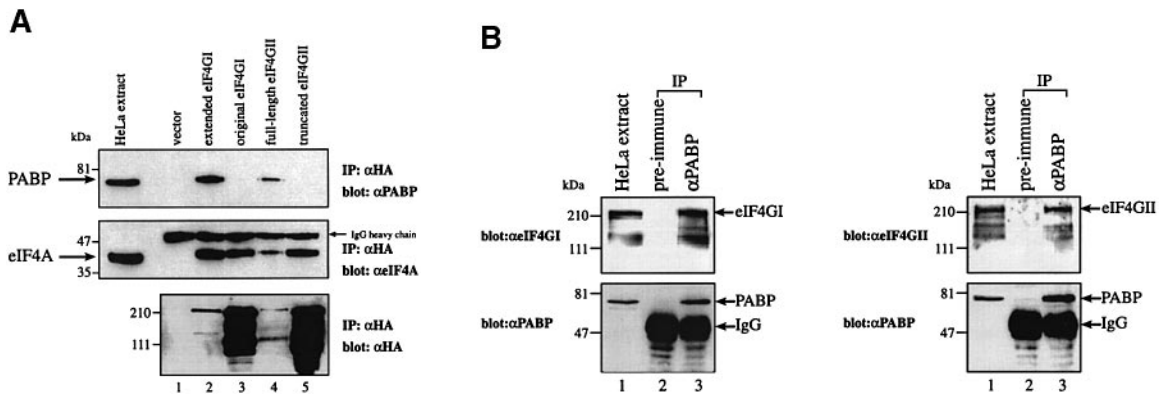


Fig. 2. Co-immunoprecipitation of eIF4GI and eIF4GII with PABP. **(A)** HeLa cells infected with vTF7-3 were transfected with vector (pcDNA3-HA) alone (lane 1) or the vector expressing an HA-tagged protein as indicated in each lane (lanes 2–5). Proteins were immunoprecipitated with anti-HA antibody and immunoprecipitates were resolved by SDS–10% PAGE. Western blotting was performed with anti-PABP (upper panel), anti-eIF4A (middle panel) or anti-HA antibody (lower panel). HeLa cell extract (40 μ g of protein) was loaded to the left of lane 1. **(B)** Extracts from uninfected and non-transfected HeLa cells were incubated with pre-immune serum (lane 2) or anti-PABP serum (lane 3). Following precipitation with protein G–Sepharose, bound proteins were resolved by SDS–8% PAGE. Western blotting was performed with anti-eIF4GI (left upper panel), anti-eIF4GII (right upper panel) or anti-PABP antibody (lower panels). One-fiftieth of the extracts used for immunoprecipitation was loaded in lane 1.

immunoprecipitated proteins were subjected to Western blotting with anti-PABP, anti-eIF4A or anti-HA antibodies. Much smaller amounts of the HA-extended eIF4GI than those of the original eIF4GI were detected in the immunoprecipitates. [Figure 2A, lower panel; compare lanes 2 and 3; the expression of HA-extended eIF4GI in the whole cell is one-fifth of that of the original eIF4GI, for unknown reasons. In addition, the extended eIF4GI could not be extracted with the buffer used (see Materials and methods) as efficiently as the original eIF4GI, probably because the extended N-terminal region of eIF4GI is rich in hydrophobic amino acids (Figure 1), which causes the protein to sediment with the cell debris. As a consequence, to visualize the extended product, the X-ray film was overexposed.] In spite of this difference, PABP was co-precipitated efficiently with the extended eIF4GI (upper panel, lane 2), while no PABP was precipitated with the original eIF4GI (lane 3) or from control extracts (lane 1). Both versions of eIF4GI bound eIF4A (middle panel, lanes 2 and 3), as the binding sites for eIF4A are contained in both (Imataka and Sonenberg, 1997). Similar results were obtained for eIF4GII. Full-length eIF4GII bound PABP (upper panel, lane 4), while an N-terminally truncated form of eIF4GII, which corresponds to the original eIF4GI in the amino acid alignment, failed to bind PABP (lane 5). eIF4A was associated with both forms of eIF4GII (middle panel, lanes 4 and 5). We expressed the same proteins in 293T cells, and confirmed the specific interaction of the HA-extended eIF4GI and endogenous PABP (data not shown). These results clearly show that the full-length eIF4GI and eIF4GII are able to bind PABP, and the N-terminal region which is missing from the original eIF4GI is essential for PABP binding.

To determine whether endogenous eIF4GI and eIF4GII are associated with PABP *in vivo*, cytoplasmic extracts from HeLa cells were used for immunoprecipitation with anti-PABP serum. Both eIF4GI and eIF4GII co-immunoprecipitated with anti-PABP serum (Figure 2B, lane 3), while a pre-immune serum failed to precipitate either PABP or eIF4G (lane 2). About 5% of eIF4GI and eIF4GII in the extract was immunoprecipitated with anti-PABP serum, as determined by laser densitometry. These results

indicate that endogenous eIF4GI and eIF4GII are associated with PABP in mammalian cells. We could not perform the reciprocal experiment with an antibody to eIF4G, as the titre of our antibodies was not sufficiently high for immunoprecipitation.

PABP-binding site in eIF4GI

To localize the PABP-binding site in the extended human eIF4GI, fragments of eIF4GI were N-terminally tagged with glutathione *S*-transferase (GST) and co-expressed with PABP-HA (C-terminally HA-tagged human PABP) in HeLa cells. A portion of the cell extract was subjected to Western blotting with anti-GST antibody to confirm the expression of the eIF4GI fragments (Figure 3A, B and C, upper panels). The rest of the extract was immunoprecipitated with anti-HA antibody, and immunoprecipitates were used for Western blotting with anti-GST antibody (Figure 3A, B and C, middle panels) or anti-HA antibody (Figure 3A, B and C, lower panels) to determine PABP binding. eIF4GI(1–329) bound PABP to a significant extent (Figure 3A, lane 1), while chloramphenicol acetyltransferase (CAT), which serves as a negative control, did not bind (Figure 3A, lane 6). Progressive N-terminal truncations extending to amino acid 131 led to a graded decrease in the amount of eIF4GI fragments co-precipitated with PABP (Figure 3A, lanes 2–5 and B, lanes 1–3). The N-terminal boundary for PABP binding resides between amino acids 132 and 139, because eIF4GI(132–329) bound PABP, but eIF4GI(139–329) did not (Figure 3B, compare lanes 3 and 4). Similar immunoprecipitation experiments were performed to determine the C-terminal boundary of the PABP-binding site. While eIF4GI(45–160) bound PABP (Figure 3C, lane 3) to the same extent as eIF4GI(45–329) (lane 1), the fragment eIF4GI(45–155) failed to bind PABP (lane 4). Thus, the C-terminal boundary for PABP binding resides between amino acids 155 and 160. The sequence (amino acids 132–160) of eIF4GI is almost identical to the corresponding sequence (amino acids 135–162) of eIF4GII, of which only two amino acids are different, but represent conservative changes, R to K and A to G (see Figure 1).

To determine whether the 29 amino acid segment (132–

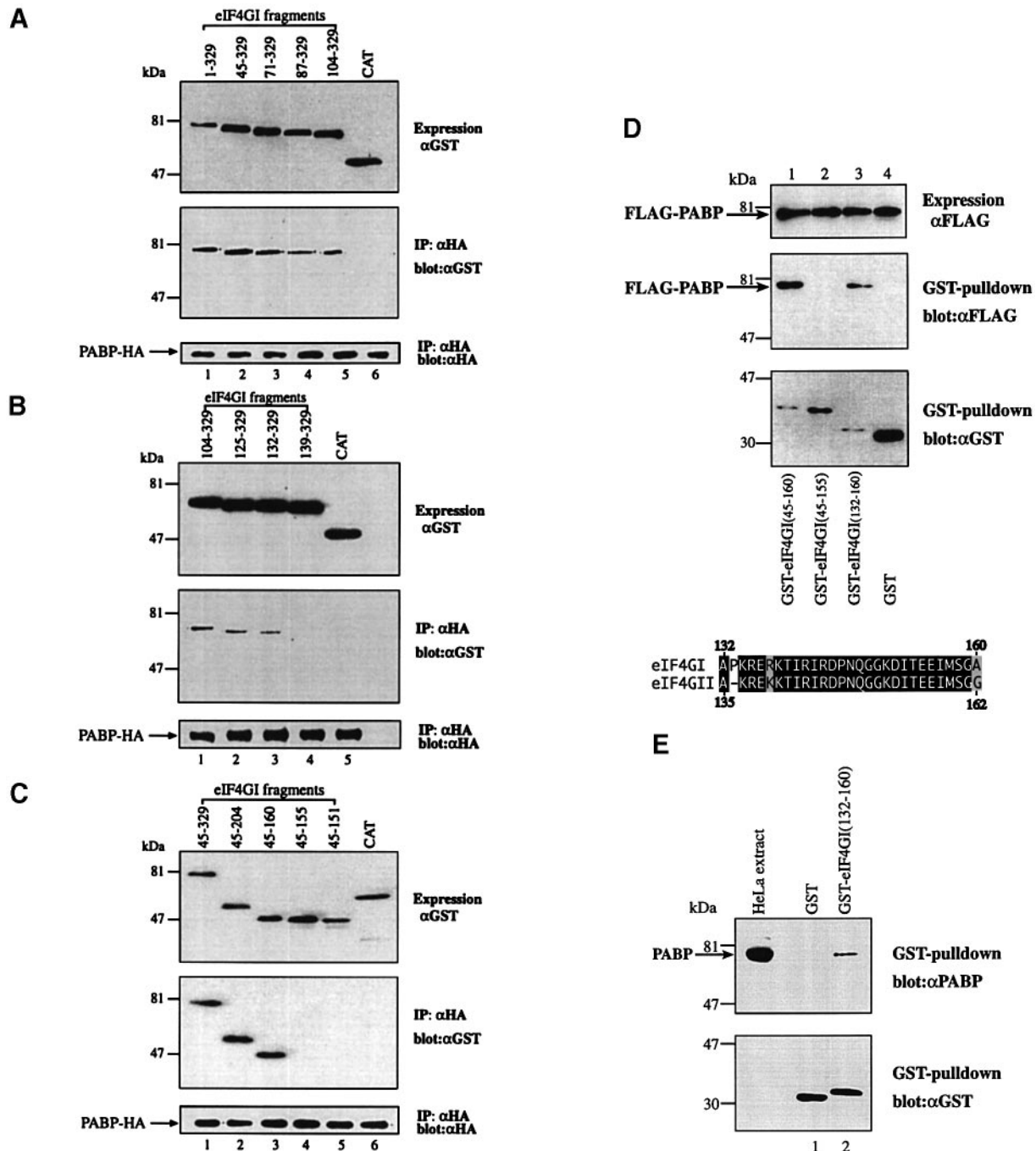


Fig. 3. Localization of the PABP-binding site in human eIF4GI. **(A)** N-terminal deletions. HeLa cells infected with vTF7-3 were co-transfected with pcDNA3-PABP-HA and pcDNA3-GST-eIF4GI fragments indicated in each lane (lanes 1–5) or pcDNA3-GST-CAT (lane 6). One-tenth of the cell extract was used for Western blotting with anti-GST antibody (upper panel). The remaining extract was used for immunoprecipitation with anti-HA antibody. Immunoprecipitates were resolved by SDS–10% PAGE. Western blotting was performed with anti-GST (middle panel) or anti-HA antibody (lower panel). **(B)** N-terminal boundary. Experiments were as in (A), with plasmids expressing GST fusion proteins indicated in each lane. **(C)** C-terminal boundary. Experiments were as in (A), with plasmids expressing GST fusion proteins indicated in each lane. **(D)** eIF4GI(132–160) is the PABP-binding site. HeLa cells infected with vTF7-3 were co-transfected with pcDNA3-FLAG-PABP and pcDNA3-GST-eIF4GI fragments indicated in each lane (lanes 1–3) or pcDNA3-GST (lane 4). One-tenth of the cell extract was used for Western blotting with anti-FLAG antibody (upper panel), and the remaining extract was mixed with glutathione–Sepharose beads. Bound proteins were eluted with reduced glutathione, and were resolved by SDS–12.5% PAGE. Western blotting was performed with anti-FLAG (middle panel) or anti-GST antibody (lower panel). The amino acid sequence of the PABP-binding site in human eIF4GI and the corresponding region of human eIF4GII are aligned at the bottom of the figure. Identical amino acids are boxed and conservative substitutions are shaded. **(E)** eIF4GI(132–160) binds endogenous PABP. GST (lane 1) or GST–eIF4GI(132–160) (lane 2) was expressed as in (D), but without co-expression of FLAG-PABP. The cell extract was mixed with glutathione–Sepharose beads. Bound proteins eluted with reduced glutathione were subjected to Western blotting with anti-PABP (upper panel) or anti-GST antibody (lower panel). HeLa cell extract (40 μg protein) was loaded to the left of lane 1.

160) of eIF4GI alone has the capacity to bind PABP, a GST fusion protein, GST–eIF4GI(132–160), was co-expressed with FLAG-PABP in HeLa cells. A portion of

the cell extract was subjected to Western blotting with anti-FLAG antibody to confirm the expression of FLAG-PABP (Figure 3D, upper panel). The rest of the extract

was incubated with glutathione–Sepharose beads, and bound proteins were used for Western blotting with anti-FLAG (middle panel) or anti-GST antibody (lower panel). As expected, FLAG-PABP was co-precipitated with GST–eIF4GI(45–160), but not with GST–eIF4GI(45–155) (Figure 3D, compare lanes 1 and 2). While GST alone did not interact with FLAG-PABP (lane 4), the GST–eIF4GI(132–160) amino acid fusion protein bound FLAG-PABP (lane 3). To examine whether the 29 amino acid segment binds to the endogenous PABP, GST or GST–eIF4GI(132–160) was expressed in HeLa cells. The cell extract was incubated with glutathione–Sepharose beads, and the bound proteins were used for Western blotting with anti-PABP (Figure 3E, upper panel) or anti-GST (lower panel). The endogenous PABP was co-precipitated with GST–eIF4GI(132–160), but not with GST (Figure 3E, compare lanes 1 and 2). Based on these results, we conclude that the 29 amino acid segment (132–160) of eIF4GI contains the core PABP-binding site. Although we did not delimit the PABP-binding domain further, it is likely that it constitutes the smallest binding motif, because of the high conservation between amino acids 132–139 of eIF4GI and 135–162 of eIF4GII (Figure 3D). The region (amino acids 45–131) of eIF4GI might increase the binding affinity of the binding domain for PABP by stabilizing the structure. Interestingly, there is no significant homology in the amino acid sequence between the PABP-binding sites of human and yeast eIF4G (Tarun *et al.*, 1997). This will be addressed in the Discussion.

eIF4GI-binding site in PABP

PABP consists of four highly evolutionarily conserved RNA recognition motifs (RRMs) and a less conserved C-terminal region (Sachs *et al.*, 1986; Burd *et al.*, 1991) (Figure 4A). In the yeast PABP, a region composed of RRM1 and RRM2 is essential for interaction with eIF4G (Kessler and Sachs, 1998). As the sequence of the PABP-binding site in eIF4G is not conserved between yeast and human, we wished to determine the binding site of human eIF4GI in human PABP. Initially, two fragments of PABP, an N-terminal region, PABP(N), which contains the four RRMs, and a C-terminal region, PABP(C), which is devoid of the RRMs, were FLAG tagged, and co-expressed with HA–eIF4GI(1–329) in HeLa cells. Subsequently, fragments encompassing the separate RRMs and various combinations of the RRMs were also generated. The constructs are shown schematically in Figure 4A. A portion of the extract was subjected to Western blotting with anti-FLAG antibody to confirm the expression of the PABP fragments (Figure 4B and C, upper panels). The rest of the extract was immunoprecipitated with anti-HA antibody, and immunoprecipitates were used for Western blotting with anti-FLAG (Figure 4B and C, middle panels) or anti-HA antibody (Figure 4B and C, lower panels) to determine which fragment of PABP bound eIF4GI. The N-terminal fragment composed of RRMs 1, 2, 3 and 4 clearly bound eIF4GI(1–329) (Figure 4B, lane 1), while the C-terminal region of PABP (lane 2) and eIF4E, which served as a negative control (lane 3), failed to bind, indicating that human eIF4GI binds to the N-terminal RRM region of PABP. To delimit the eIF4GI-binding site in the N-terminal region, pairs of RRMs (RRMs 1–2, RRMs 2–3 or RRMs 3–4) or single RRMs (RRM1 or RRM2) were fused to

the C-terminal region of PABP (Figure 4A) and tested for binding to eIF4GI. RRMs 1–2–C (Figure 4C, lane 2) bound eIF4GI(1–329) to the same extent as full-length PABP (lane 1), while RRMs 2–3–C bound eIF4GI with a considerably decreased affinity (lane 3). Other PABP fragments and luciferase (negative control) did not bind (lanes 4–7). Finally, to examine whether RRMs 1–2 is able to bind eIF4GI(132–160), GST or GST–eIF4GI(132–160) was co-expressed with FLAG-RRMs 1–2 in HeLa cells. A portion of the cell extract was subjected to Western blotting with anti-FLAG antibody to confirm the expression of FLAG-RRMs 1–2 (Figure 4D, upper panel). The rest of the extract was incubated with glutathione–Sepharose beads, and bound proteins were used for Western blotting with anti-FLAG (middle panel) or anti-GST antibody (lower panel). FLAG-RRMs 1–2 was able to interact with GST–eIF4GI(132–160) (lane 2), but not with GST alone (lane 1). These results demonstrate that, similarly to the yeast PABP–eIF4G interaction (Kessler and Sachs, 1998), a region composed of RRM1 and RRM2 contains the eIF4G-binding site. As RRMs 2–3 showed a relatively weak but significant affinity for eIF4GI, RRM2 might be the most important region for binding eIF4GI, as was shown for yeast (Kessler and Sachs, 1998). However, RRM2 alone exhibited little binding affinity for eIF4GI (Figure 4C, lane 6). Presumably, a combination of the first two RRMs is necessary for an appropriate structural conformation to facilitate eIF4G binding. We failed to detect endogenous eIF4Gs co-immunoprecipitated with PABP(RRMs 1–2–C)–HA or with PABP–HA, probably because endogenous PABP is much more abundant than endogenous eIF4G (see Discussion). Thus, eIF4G is most likely saturated with endogenous PABP, and the expressed PABP could not replace the endogenous PABP efficiently for eIF4G-binding.

Functional analysis of the eIF4GI N-terminal region

To determine whether the PABP binding to the N-terminal region of eIF4G is functionally significant, a recombinant N-terminal region (amino acids 1–204) of eIF4GI, which contains the PABP-binding site, was prepared as a GST fusion protein, GST–eIF4GI(1–204). As a control, we also prepared GST–eIF4GI(1–204:mut), in which amino acids 134–138, KRERK, in the PABP-binding site were converted to alanines. To determine the binding of these eIF4GI fragments to PABP, they were mixed with PABP(RRMs 1–4)–His protein, and precipitated with glutathione–Sepharose beads. Bound proteins were subjected to Western blotting with anti-GST (Figure 5A, upper panel) or anti-His antibody (lower panel). While PABP(RRMs 1–4)–His failed to associate with GST (Figure 5A, lower panel, lane 1) or GST–eIF4GI(1–204:mut) (lane 3), GST–eIF4GI(1–204) interacted with PABP(RRMs 1–4)–His to a significant extent (lane 2). This binding assay was performed without addition of poly(A) RNA. In similar binding experiments using yeast eIF4G and PABP, the presence of poly(A) RNA was essential for the interaction between these proteins (Tarun and Sachs, 1996).

Next, we wished to examine whether the N-terminal region of eIF4GI containing the PABP-binding site is able to act as an inhibitor of poly(A)-dependent translation. A rabbit reticulocyte lysate was mixed with GST, GST–eIF4G(1–204), GST–eIF4GI(1–204:mut) or buffer alone,

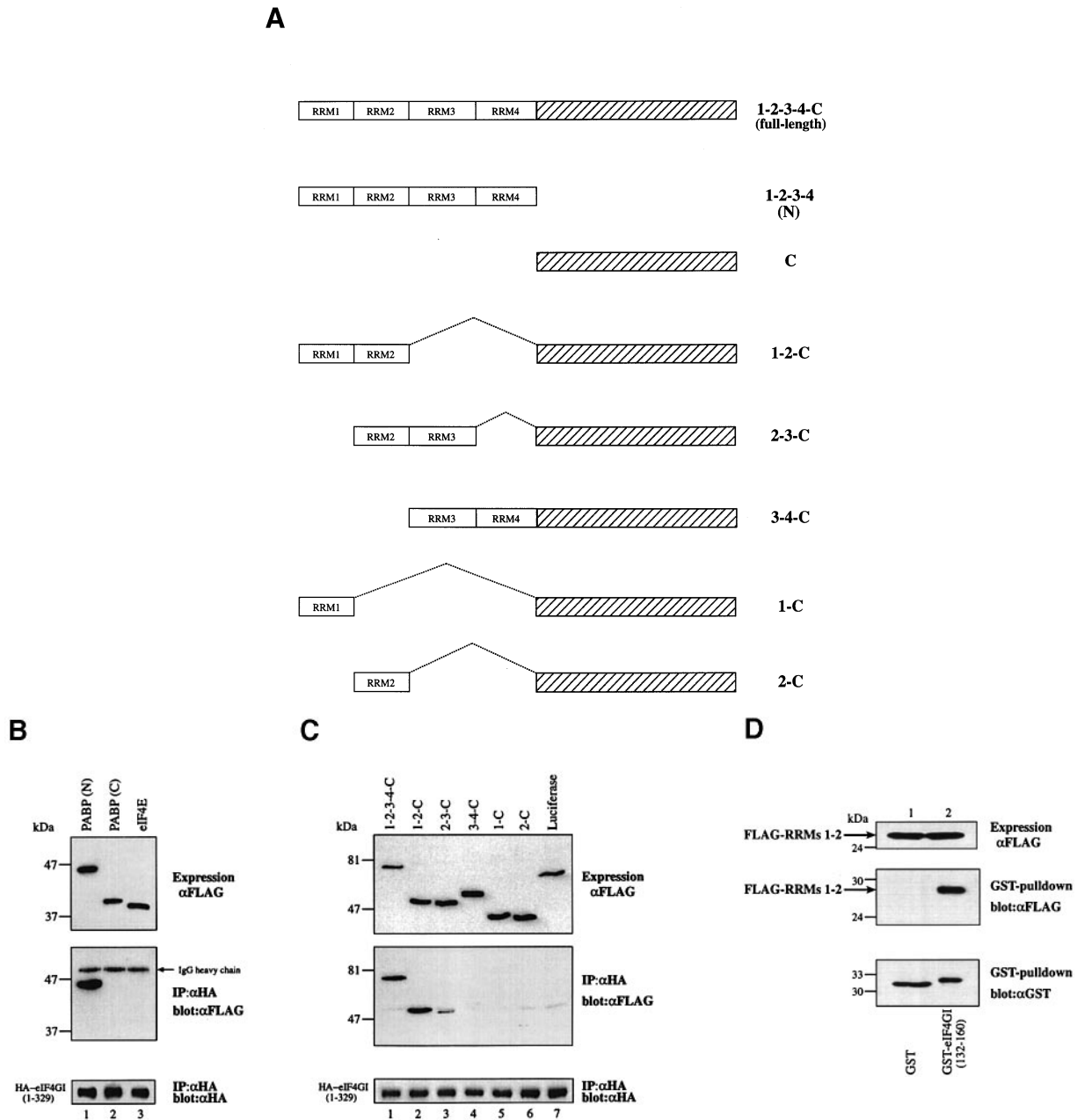


Fig. 4. Localization of the eIF4GI-binding site in PABP. (A) Schematic representation of PABP mutants examined in (B) and (C). (B) eIF4GI binds the N-terminal region (RRMs 1–4) of PABP. HeLa cells infected with vTF7-3 were co-transfected with pcDNA3-HA-eIF4G(1–329) and pcDNA3-FLAG-PABP(RRMs 1–4) (lane 1), -PABP(C) (lane 2) or -eIF4E (lane 3). One-twentieth of the cell extract was used for Western blotting with anti-FLAG antibody (upper panel). The remaining extract was used for immunoprecipitation with anti-HA antibody. Immunoprecipitates were resolved by SDS–10% PAGE. Western blotting was performed with anti-FLAG (middle panel) or anti-HA antibody (lower panel). (C) eIF4GI binds RRM1–2. Experiments were as in (A), with plasmids expressing FLAG-tagged proteins indicated in each lane. (D) RRM1–2 binds eIF4GI(132–160). GST (lane 1) or GST-eIF4GI(132–160) (lane 2) was co-expressed with FLAG-RRMs 1–2 as in Figure 3D. One-tenth of the cell extract was used for Western blotting with anti-FLAG antibody (upper panel), and the remaining extract was mixed with glutathione–Sepharose beads. Bound proteins eluted with reduced glutathione were subjected to Western blotting with anti-FLAG (middle panel) or anti-GST antibody (lower panel).

and programmed with capped luciferase RNA (capLUC) or capped and poly(A)-tailed luciferase RNA (capLUCpA) for *in vitro* translation followed by monitoring of luciferase activity. None of the recombinant proteins exhibited any effects on translation of capLUC (Figure 5B, lanes 1–4). As observed by others (Grossi de Sa *et al.*, 1988; Munroe and Jacobson, 1990), the presence of a poly(A) tail increased translation of the mRNA in the rabbit reticulocyte lysate by ~2-fold (the average of four experiments with a standard error of 10%; Figure 5B, compare lanes

1 and 5). The functional half-life (Gallie, 1991; Tarun and Sachs, 1995) for capLUC and capLUCpA was 14 ± 2 and 15 ± 2 min (the mean \pm the standard error of three independent experiments), respectively, indicating that the stimulation by the poly(A) tail was not attributable to a difference in mRNA stability. When luciferase RNAs (capLUC and capLUCpA) and CAT RNAs (capCAT and capCATpA) were translated in the presence of [35 S]methionine, the poly(A) tail increased incorporation of the radioactivity into the translated products by ~2-fold for

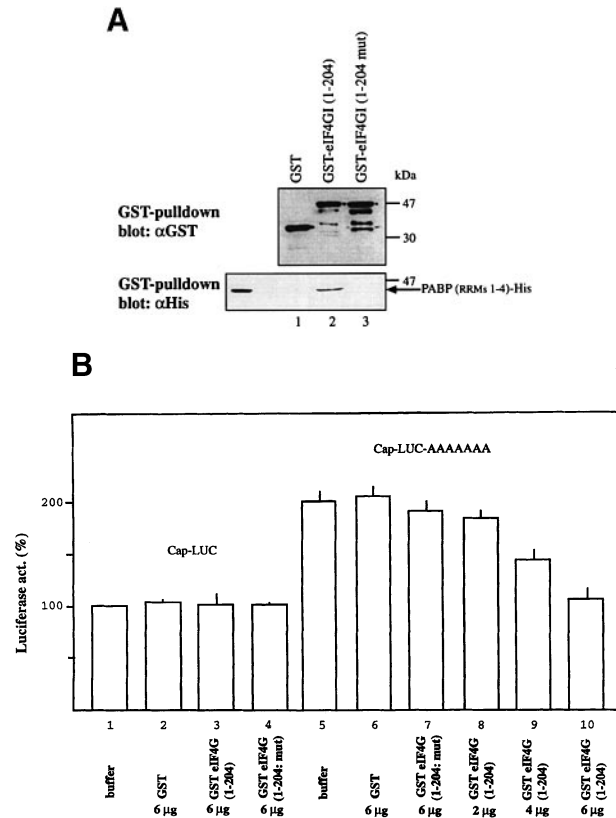


Fig. 5. Functional analysis of the N-terminal region of eIF4GI. (A) *In vitro* binding of the N-terminal sequence of eIF4GI to PABP. PABP(RRMs 1-4)-His was incubated with GST (lane 1), GST-eIF4GI(1-204) (lane 2) or GST-eIF4GI(1-204:mut) (lane 3) immobilized on glutathione-Sepharose beads. Bound proteins were eluted with reduced glutathione, and resolved by SDS-12.5% PAGE. Western blotting was performed with anti-GST (upper panel) or anti-His (lower panel) antibody. One-fifth of the input PABP(RRMs 1-4)-His was loaded to the left of lane 1. (B) Effects of the N-terminal region on poly(A)-dependent translation. Buffer (lanes 1 and 5) (1 μ l), GST (lanes 2 and 6), GST-eIF4G(1-204) (lanes 3, 8, 9 and 10) or GST-eIF4G(1-204:mut) (lanes 4 and 7) (2, 4 or 6 μ g) (1 μ l) was added to a rabbit reticulocyte lysate (10 μ l). After incubation on ice 30 min, the lysate was programmed with capLUC (lanes 1-4) or capLUCpA RNA (lanes 5-10) (1 μ l, 60 ng). The translation reaction mixture was incubated at 30°C for 30 min. Luciferase activity was measured using a luminometer. The luciferase activity of the lysate programmed with capLUC in the presence of buffer alone (lane 1) was set at 100%. Error bars denote the standard error of four independent experiments.

both luciferase and CAT (data not shown), indicating that the observed effect is independent of a reporter mRNA. Moreover, translation of capLUCpA was inhibited more strongly (77%) by incubation with poly(A) (10 ng/ μ l) than with poly(C) (40% inhibition at 10 ng/ μ l), while translation of capLUC was inhibited with poly(A) and poly(C) to the same extent (50%, at 10 ng/ μ l) (data not shown). These observations validate the use of the rabbit reticulocyte lysate and luciferase mRNA for the functional analysis. The translational enhancement by the poly(A) tail was decreased proportionally by increasing amounts of GST-eIF4G(1-204) (2-6 μ g) (lanes 8, 9 and 10). Addition of GST alone showed no effect on the poly(A)-dependent translation (lane 6). The effect of GST-eIF4G(1-204) on translation of the poly(A) RNA is explained by the disruption of the interaction between eIF4G and PABP, because GST-eIF4G(1-204:mut), which

failed to bind PABP, decreased translation of capLUCpA only slightly (10%) (lane 7). We quantified the amount of PABP in the reticulocyte lysate to be 0.2 μ g/10 μ l. Considering the molecular masses of PABP (70 kDa) and GST-eIF4GI(1-204) (47 kDa), 6 μ g of GST-eIF4GI(1-204), which was required to abrogate the effect of the poly(A) tail (compare lanes 8-10), corresponds to a 45-fold molar excess over PABP. Thus, to suppress poly(A) tail-dependent translation, a large excess of GST-eIF4GI(1-204) over endogenous PABP is required. The GST portion of GST-eIF4GI(1-204) might hinder this fusion protein from gaining access to PABP which is associated with the full-length eIF4Gs. We attempted to neutralize the effect of GST-eIF4GI(1-204) (6 μ g) by adding recombinant PABP (0.2-1.4 μ g) to the lysate, but failed to restore translation, because the excess GST-eIF4GI(1-204) in the system should readily neutralize exogenously added PABP. These functional assays suggest that the PABP-eIF4G interaction is required for the poly(A)-dependent translation, although an experiment using full-length recombinant eIF4G, which could not be obtained, is required to prove this. Taken together, our results show that the N-terminal region of human eIF4GI binds PABP, and probably functions to mediate the translational enhancement by the poly(A) tail.

Discussion

We have shown that human eIF4G interacts with PABP in a functionally significant manner. PAIP-1, a recently identified mammalian PABP-binding protein, binds eIF4A, and stimulates translation (Craig *et al.*, 1998). Thus, mammalian cells possess dual systems, PABP-PAIP-1 and PABP-eIF4G, to effect poly(A)-dependent translation. Irrespective of whether the binding sites of eIF4G and PAIP-1 in PABP are overlapping, both systems could operate in a non-competitive manner *in vivo*, since PABP is as abundant as eIF4A (Görlach *et al.*, 1994), while eIF4G is six times less abundant than eIF4A (Duncan *et al.*, 1987), and PAIP-1 appears to be present at 6-fold lower amounts than PABP (A.Craig, unpublished observations). As these values of protein concentrations are applicable only to HeLa cells, it would be of interest to quantify the amounts of these proteins in different cell lines, and to determine whether PABP-PAIP-1 or PABP-eIF4G interactions vary among cells or tissues. It is conceivable that eIF4Gs and PAIP-1 might cooperate in translation by binding to PABP molecules associated with the same RNA.

Previous attempts to detect an association of human eIF4G and PABP have failed (Craig *et al.*, 1998; H.Imataka, unpublished data), because the original truncated cDNA for eIF4GI (Yan *et al.*, 1992) was used for the binding experiments. Results of co-immunoprecipitation of endogenous PABP and eIF4G were also negative, because experiments were done without knowledge of the binding sites. The use of an antibody directed against a sequence in the region which overlaps a protein-binding site would inhibit the interaction of the binding protein. In our hands, an antibody directed against an N-terminal sequence of PABP could not precipitate endogenous eIF4GI (H.Imataka, unpublished data), presumably because the binding site of eIF4GI in PABP resides in the

N-terminus of the protein (Figure 4). The strength of the antibody is also an important factor for immunoprecipitation; we failed to co-immunoprecipitate eIF4G with a monoclonal antibody directed against a C-terminal region of PABP (Craig *et al.*, 1998). The polyclonal antiserum used for co-immunoprecipitation of endogenous PABP in this study, which is directed against a C-terminal region (amino acids 462–633) of PABP, should recognize more than one epitope of PABP. It is conceivable that eIF4G–PABP interaction is intrinsically weak, because we failed to detect PABP in eIF4F by Western blotting, while eIF4E, eIF4G and eIF4A were readily detected (H.Imataka, unpublished data). Probably, PABP dissociated from eIF4F during the purification.

The amino acid sequences of the PABP-binding site in eIF4G are not conserved between yeast (Tarun *et al.*, 1997) and human (this study). Also, the sequences of eIF4G from various species, including those of *Drosophila* (Hernández *et al.*, 1998) and plants (Allen *et al.*, 1992), do not show significant homology in their N-termini. This is rather surprising, because the eIF4G-binding site in human and yeast PABP resides in a region comprising RRM-1 and RRM-2, whose amino acid sequence is evolutionarily conserved (Sachs *et al.*, 1986; Burd *et al.*, 1991). Furthermore, another important motif in eIF4G, the eIF4E-binding site, is highly conserved between human and yeast (Mader *et al.*, 1995). The difference in amino acid sequences of the PABP-binding site between the two species may account for the difference in the RNA requirement for the interaction between eIF4G and PABP. Poly(A) RNA is absolutely required for the interaction between eIF4G and PABP in yeast (Tarun and Sachs, 1996). In contrast, RNA does not seem to be necessary for human eIF4G–PABP interaction; association of human eIF4G and PABP was readily detectable without addition of poly(A) RNA, and treatment of proteins with micrococcal endonuclease did not decrease the binding affinity (H.Imataka, unpublished observation). The possibility, however, that a short RNA which remains even after extensive digestion with the nuclease may be sufficient for human eIF4G to bind PABP cannot be excluded.

How does the association of eIF4G with PABP support poly(A)-dependent translation? eIF4G serves as a scaffold for other translation factors to coordinate their functions. The interaction between eIF4G and PABP brings about circularization of the mRNA. Indeed, such circularization has been observed by atomic force microscopy using recombinant yeast eIF4G, eIF4E and PABP (Wells *et al.*, 1998). The circularization could enhance translation by shunting terminating ribosomes directly to the 5' end of the mRNA. Another mechanism of translational enhancement by the poly(A) tail proposed by Preiss and Hentze (1998) is that the poly(A) tail acts as 'a translation promoter' by increasing the concentration of eIF4G on the mRNA. eIF4G is required for both cap-dependent and cap-independent translation (Belsham and Sonenberg, 1996; Morley *et al.*, 1997; Sachs *et al.*, 1997). One mechanism by which eIF4G functions in both cap-dependent and cap-independent ribosome binding is to bind the mRNA, presumably through the RRM-like sequence in the middle domain of the protein (Goyer *et al.*, 1993). The RNA-binding activity of the middle domain of eIF4G is sufficient to promote cap-independent translation, as

this domain was shown to interact tightly with a specific sequence in the internal ribosome entry site of the encephalomyocarditis virus RNA (Pestova *et al.*, 1996). For cap-dependent translation, the binding of the middle domain to the mRNA is apparently not sufficient to activate translation, and interaction of eIF4E with the cap structure plays a crucial role. It is also possible that the interaction of PABP with eIF4G could increase ribosome-binding rates by enhancing the association of the mRNA with eIF4G. eIF4G might undergo a conformational change when it binds PABP so that the mRNA can be more accessible to eIF4G. In this regard, binding of eIF4E to eIF4G renders eIF4G more susceptible to proteases, suggesting that the structure of eIF4G changes upon eIF4E binding (Haghighat *et al.*, 1996; Ohlmann *et al.*, 1997). Detailed studies of eIF4G structure in the presence or absence of binding factors are necessary to understand the mechanism of the poly(A)-dependent translation.

Materials and methods

Plasmids

To express proteins in mammalian cells for immunoprecipitation, the HA, FLAG (Sigma) or GST sequence was inserted into the multi-cloning site of pcDNA3 (Invitrogen) to construct pcDNA3-HA, pcDNA3-FLAG or pcDNA3-GST. To extend the 5' sequence of eIF4G, 5' RACE was performed as described (Gradi *et al.*, 1998). The 5' RACE product was fused to the original eIF4G (Yan *et al.*, 1992) to construct the extended eIF4G. The full-length eIF4G cDNA was inserted in pcDNA3-HA to construct pcDNA3-HA-extended eIF4G. The full-length eIF4GII (Gradi *et al.*, 1998) was inserted in pcDNA3-HA to construct pcDNA3-HA-full-length eIF4GII. To avoid confusion, the published constructs, pcDNA3-HA-eIF4G (Imataka and Sonenberg, 1997; Imataka *et al.*, 1997) and pcDNA3-HA-eIF4GII (Gradi *et al.*, 1998) were called pcDNA3-HA-original eIF4G and pcDNA3-HA-truncated eIF4GII, respectively, herein. pcDNA3-HA-original eIF4G and pcDNA3-HA-truncated eIF4GII had been constructed using an *EcoRI* site (Gradi *et al.*, 1998) to express HA-eIF4G(142–1560) and HA-eIF4GII(144–1585) (Figure 1), respectively. To delete amino acids 1–44 and 330–1560 from eIF4G, restriction enzyme *BamHI* and *BglIII* sites were utilized, respectively. Other truncation mutants and point mutants (amino acids 134–138 into alanine residues) of eIF4G were obtained by PCR with *Pfu* DNA polymerase. All the clones were confirmed by sequencing. We also used *Pfu* DNA polymerase for all PCRs described below.

For construction of pcDNA3-FLAG-PABP, a PCR-amplified cDNA encoding PABP (amino acids 1–33) was ligated into pcDNA3-FLAG with a cDNA fragment encoding PABP (34–633) to express FLAG-tagged full-length PABP (1–633) (Grange *et al.*, 1987). For construction of pcDNA3-PABP-HA, a cDNA encoding PABP (amino acids 1–594) was ligated into pcDNA3 with a PCR-amplified cDNA encoding PABP (595–633) followed by the HA-amino acid sequences. cDNAs encoding RRM1–4 of PABP (amino acids 1–376) and the remaining C-terminal part (377–633) were PCR-amplified, and ligated into pcDNA3-FLAG to construct pcDNA3-FLAG-PABP(RRMs 1–4) and pcDNA3-PABP(C). cDNAs encoding RRM1 (amino acids 1–90), RRM2 (91–179), RRM1–2 (1–179) and RRM2–3 (91–279) of PABP were PCR-amplified, and fused to the C-terminal part (amino acids 377–633) in pcDNA3-FLAG to construct pcDNA3-FLAG-PABP(RRM 1–C), pcDNA3-FLAG-PABP(RRM 2–C), pcDNA3-FLAG-PABP(RRMs 1–2–C) and pcDNA3-FLAG-PABP(RRMs 2–3–C), respectively. pcDNA3-FLAG-PABP(RRMs 3–4–C) was constructed by ligating a cDNA encoding PABP (234–633) and a PCR-amplified cDNA encoding PABP (180–233) in pcDNA3-FLAG.

For bacterial expression of proteins, cDNAs encoding eIF4G(1–204) and eIF4G(1–204: 134–138A), called eIF4G(1–204:mut) in the text, were cloned in pGEX2T (Pharmacia) to generate pGEX-eIF4G(1–204) and pGEX-eIF4G(1–204: 134–138A). To obtain pET3b-PABP(RRMs 1–4)-His, a PCR-amplified cDNA encoding PABP (amino acids 1–376) followed by six histidines, was cloned in pET3b (Novagen).

For synthesis of luciferase RNA, luciferase cDNA was inserted downstream of the T7 RNA polymerase promoter sequence of pSP72 (Promega) to generate pSP72-LUC. A poly(A) stretch (85 deoxyadenines)

from p97 cDNA (Imataka *et al.*, 1997) was inserted downstream of the luciferase sequence of pSP72-LUC to construct pSP72-LUC-A.

Antibodies

A C-terminal portion (amino acids 462–633) of PABP was expressed as a GST fusion protein in BL-21 and purified on glutathione–Sepharose beads (Pharmacia). The GST–PABP (amino acids 462–633) was injected into a rabbit to produce anti-PABP antiserum. Anti-eIF4GI and anti-eIF4GII antibodies were as described (Gradi *et al.*, 1998). Anti-eIF4A and anti-GST antibodies were kind gifts from H.Trachsel and J.Dostie, respectively. Anti-HA, anti-His and anti-FLAG antibodies were purchased from Babco, QIAGEN and Sigma, respectively.

Co-precipitation of proteins from cell extracts

HeLa cells (6 cm dish) were infected with vaccinia virus vTF7-3 (Fuerst *et al.*, 1986), and then transfected with the plasmids expressing proteins indicated in the figures using Lipofectin (Gibco-BRL). Twenty hours later, cells were lysed in 0.4 ml of buffer A [100 mM KCl, 0.1 mM EDTA, 20 mM HEPES–KOH pH 7.6, 0.4% NP-40, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation, the supernatant was mixed with anti-HA antibody (16B12, Babco) immobilized on protein G–Sepharose (10 µl), and incubated in the presence of RNase A (100 µg/ml) for 5 h in the cold room. After washing with buffer A (0.4 ml, three times), immunoprecipitates were collected by centrifugation and proteins were dissolved in Laemmli buffer. The sample was boiled, and proteins were resolved by SDS–10 or 12.5% PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore) for Western blotting. Protein bands were visualized on an X-ray film by the enhanced chemiluminescence detection system. For co-immunoprecipitation from uninfected and non-transfected HeLa extracts, HeLa cells (10 cm dish) were lysed in 0.8 ml of buffer A. The cell extract was incubated in the presence of RNase A (100 µg/ml) in the cold room for 3 h with pre-immune serum (50 µl) or anti-PABP serum (10 µl) which had been pre-incubated with protein G–Sepharose (25 µl). After washing with buffer A (0.8 ml, three times), bound proteins were dissolved in Laemmli buffer.

To co-precipitate FLAG-PABP with GST fusion proteins, cell extracts expressing FLAG-PABP and a GST fusion protein were incubated with glutathione–Sepharose beads (15 µl) (Pharmacia) for 4 h in the cold room. After washing with buffer A (0.4 ml, three times), bound proteins were eluted with a buffer (40 µl) [30 mM reduced glutathione, 50 mM Tris–HCl (pH 7.5), 100 mM KCl].

Recombinant proteins

To express GST–eIF4G(1–204) and GST–eIF4G(1–204:mut) in bacteria, *Escherichia coli* BL-21 was transformed with pGEX–eIF4G(1–204) or pGEX–eIF4G(1–204:mut), respectively. The transformed cells were grown in Luria broth (LB) containing ampicillin (100 µg/ml) until optical density (OD) at 600 nm reached 0.8–1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM, and cells were cultured for 2 h. After addition of PMSF (0.5 mM), cells were harvested, suspended in 30 ml of buffer B (100 mM KCl, 20 mM HEPES–KOH pH 7.5, 10% glycerol, 0.1 mM EDTA, 1 mM DTT) supplemented with a cocktail of protease inhibitors (Boehringer Mannheim), and lysed by sonication. After addition of Triton X-100 (0.5%), cellular debris was removed by centrifugation at 30 000 r.p.m. for 40 min in the Ti-60 rotor (Beckman). The supernatant was mixed with glutathione–Sepharose beads (250 µl) (Pharmacia) for 1 h in the cold room. Unbound proteins were removed by passing the beads through a Poly-Prep chromatography column (Bio-Rad), followed by washing with buffer B (15 ml) containing Triton X-100 (0.5%), and then with buffer B (15 ml). Bound proteins were eluted with a buffer [30 mM reduced glutathione, 50 mM Tris–HCl (pH 7.5), 100 mM KCl].

To express PABP(RRMs 1–4)–His in bacteria, BL-21 (DE3) was transformed with pET3b-PABP(RRMs 1–4)–His. Bacterial cell extracts were prepared as described above but in buffer C (100 mM KCl, 20 mM HEPES–KOH pH 7.5, 10% glycerol), and mixed with Ni-NTA agarose beads (Qiagen) (250 µl) for 1 h in the cold room. Unbound proteins were removed by passing the beads through the Poly-Prep chromatography column, followed by washing with buffer C (15 ml) containing Triton X-100 (0.5%), and then with buffer C (15 ml) containing 5 mM imidazole. Bound proteins were eluted by increasing the concentration of imidazole (50–500 mM gradient) in buffer C.

All purified proteins were dialysed against buffer D (50 mM potassium acetate, 20 mM Tris–HCl pH 7.5, 10% glycerol, 0.1 mM EDTA, 1 mM DTT), and stored at –80°C.

In vitro binding assay

Recombinant GST fusion proteins (100 µg) were incubated with glutathione–Sepharose beads (15 µl) for 30 min on ice. After removal of the unbound fraction, PABP(RRMs 1–4)–His (5 µg) was mixed with the beads in a binding buffer (100 mM KCl, 20 mM Tris–HCl pH 7.5, 2.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol) for 10 min on ice. After washing with the binding buffer (250 µl, three times), bound proteins were eluted by incubation with buffer (40 µl) (30 mM reduced glutathione, 50 mM Tris–HCl pH 7.5, 100 mM KCl).

In vitro translation

Capped luciferase mRNA (capLUC) and capped, poly(A)-tailed luciferase mRNA (capLUCpA) were synthesized from pSP72-LUC and pSP72-LUC-A, respectively, using a kit (mMESSAGEmMACHINE, Ambion). A rabbit reticulocyte lysate (10 µl) (Promega) which had not experienced repeated freezing and thawing was mixed with GST fusion protein (2–6 µg, 1 µl) or buffer D (1 µl) in the presence of RNasin (20 U) (Promega) and 20 amino acids. After incubation on ice for 30 min, the lysate was programmed with capLUC or capLUCpA RNA (1 µl, 60 ng). The translation reaction mixture was incubated at 30°C for 30 min. Luciferase activity was measured using a luminometer (BIOORBIT).

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Note added in proof

After submission of this paper, Piron *et al.* (*EMBO J.* 1998, **17**, 5811–5821) reported that PABP interacts with eIF4f, and that this interaction is prevented by the rotavirus NSP3 protein.