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Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G

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Although the cap structure and the poly(A) tail are on opposite ends of the mRNA molecule, previous work has suggested that they interact to enhance translation and inhibit mRNA degradation. Here we present biochemical data that show that the proteins bound to the mRNA cap (eIF-4F) and poly(A) tail (Pab1p) are physically associated in extracts from the yeast *Saccharomyces cerevisiae*. Specifically, we find that Pab1p co-purifies and co-immunoprecipitates with the eIF-4G subunit of eIF-4F. The Pab1p binding site on the recombinant yeast eIF-4G protein Tif4632p was mapped to a 114-amino-acid region just proximal to its eIF-4E binding site. Pab1p only bound to this region when complexed to poly(A). These data support the model that the Pab1p–poly(A) tail complex on mRNA can interact with the cap structure via eIF-4G.

Keywords: cap structure/poly(A) tail/RNA binding protein/translation initiation/yeast

Introduction

Eukaryotic mRNA is post-transcriptionally modified with a cap structure at the 5′ end and a poly(A) tail at the 3′ end. A functional interaction between the two ends of mRNA in eucaryotes has been documented for several different reactions. mRNA cap ribose methylation requires the presence of a poly(A) tail in developing amphibian embryos (Kuge and Richter, 1995). In the yeast *Saccharomyces cerevisiae*, one pathway of mRNA degradation requires removal of the poly(A) tail before removal of the cap structure (reviewed in Beelman and Parker, 1995). Finally, the cap and the poly(A) tail have been observed to stimulate translation initiation synergistically *in vivo* and *in vitro* (Gallie, 1991; Iizuka *et al.*, 1994; Tarun and Sachs, 1995). Each of these observations prompted us to examine the potential interaction between the cap and the poly(A) tail binding proteins on mRNA through biochemical approaches.

The cap structure on cytoplasmic mRNA is bound by the cap binding protein complex eIF-4F. This translation initiation factor is responsible for recruiting the 40S ribosomal subunit to the mRNA via an interaction of its eIF-4G subunit with the ribosome-bound initiation factor eIF-3. eIF-4G is also responsible for recruiting RNA helicase activity to the mRNA (reviewed in Hershey *et al.*, 1996).

In the yeast *S.cerevisiae*, eIF-4F has been found to contain the essential 24 kDa cap binding protein eIF-4E and two other proteins, p20 and p150 (Goyer *et al.*, 1989; Lanker *et al.*, 1992). Purification of yeast eIF-4F is achieved through ^{7m}GDP–agarose affinity chromatography (Goyer *et al.*, 1989). A temperature-sensitive mutation in the eIF-4E gene *CDC33*, *cdc33-1*, leads to the loss of the protein's affinity for the cap structure (Altman and Trachsel, 1989). As a result, eIF-4F cannot be purified by ^{7m}GDP chromatography from extracts of cells harboring this mutation. *In vivo*, *cdc33-1* mutants exhibit a partial shutdown of cellular translation after prolonged exposure to the restrictive temperature (Altman and Trachsel, 1989).

The other two subunits of yeast eIF-4F have also been characterized. The p150 subunit of eIF-4F is encoded by the *TIF4631* gene (Goyer *et al.*, 1993) and, based on its sequence and biochemical properties, it is an eIF-4G family member. The *TIF4631* gene is not essential for cell viability, presumably because of the presence of the closely related *TIF4632* gene encoding a 104 kDa protein (Goyer *et al.*, 1993). Although Tif4632p has not been shown to co-purify with eIF-4F, its significant homology to Tif4631p and the synthetic lethality observed between *TIF4631* and *TIF4632* mutations (Goyer *et al.*, 1993) suggest that it is functionally related to Tif4631p. The p20 subunit of eIF-4F, encoded by the *CAF20* gene, is not essential for cell viability and has no known function (Lanker *et al.*, 1992). Herein, the term eIF-4G will refer to Tif4631p or Tif4632p, and the term eIF-4F to eIF-4E and its associated proteins eIF-4G and p20.

The poly(A) tail on yeast mRNA is bound by the essential poly(A) binding protein (Pab1p) (Sachs *et al.*, 1986). Pab1p is the prototype for a very large number of proteins containing a highly conserved RNA recognition motif (RRM) of ~80 amino acids. Pab1p is highly conserved in eukaryotes and has four RRMs; deleting some of these from the protein does not impair its ability to support yeast cell viability (Sachs *et al.*, 1987). Like eIF-4F, Pab1p is known to stimulate the binding of the 40S ribosomal subunit to mRNA (Tarun and Sachs, 1995). Furthermore, Pab1p has been shown functionally to protect mRNA from decapping (Caponigro and Parker, 1995). Finally, Pab1p has been shown to stimulate the degradation of the poly(A) tail by activation of a non-essential poly(A) nuclease encoded by the *PAN2* and *PAN3* genes (Boeck *et al.*, 1996). The mechanisms by which Pab1p performs each of these functions remain unknown.

Several biochemical experiments were performed in order to test the hypothesis that Pab1p and eIF-4F interact. Here we report that Pab1p co-purifies and co-immunoprecipitates with yeast eIF-4G in an RNA-dependent manner. The direct association of Pab1p with recombinant eIF-4G is also demonstrated, and the Pab1p binding site on the eIF-4G homolog Tif4632p is delineated. These data support the model that mRNA 5′ and 3′ ends can

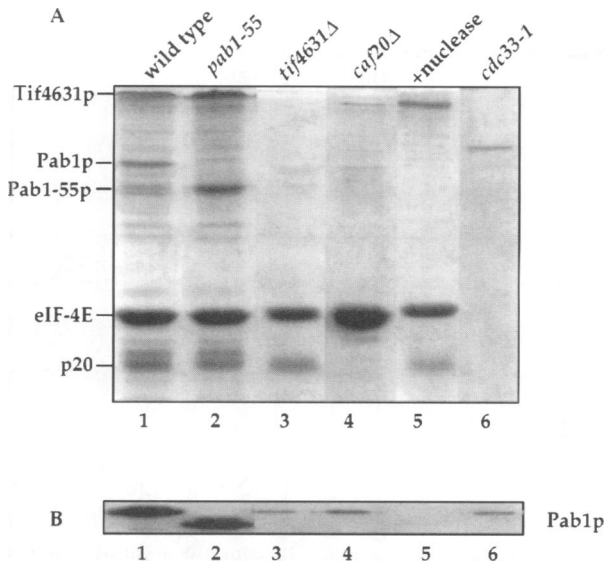

Fig. 1. Pab1p co-purifies with the cap binding protein complex eIF-4F in crude yeast extracts. (**A**) Large-scale eIF-4F preparations from the following are shown. Lane 1, wild-type extract (YAS1882); lane 2, Pab1-55p extract (YAS1883); lane 3, Tif4631p-deficient extract (YAS1889); lane 4, p20-deficient extract (YAS1910); lane 5, Pab1-55p (YAS1883) preparation treated with micrococcal nuclease before the elution step with ^{7m}GTP; and lane 6, *cdc33-1* extract (YAS1888). eIF-4F was purified by ^{7m}GDP chromatography (see Materials and methods). Coomassie blue-stained 12% SDS–PAGE gels of the preparations are shown. The locations of Tif4632p (150 kDa), Pab1p (68 kDa), Pab1-55p (55 kDa), eIF-4E (24 kDa) and p20 (20 kDa) are indicated. The 76 kDa protein in lane 6 and the proteins surrounding p20 in each of the preparations were shown by microsequencing to be the L-A Gag protein and eIF-4E proteolytic fragments, respectively (data not shown). Aliquots of each preparation containing equal amounts of eIF-4E were analyzed. (**B**) Western analysis for Pab1p. Lane assignments are as in (A), with each lane (except lane 6) containing nearly equal amounts of eIF-4E.

interact via the association of eIF-4E and Pab1p with eIF-4G.

Results

Pab1p co-purifies with eIF-4F

The co-purification of Pab1p with the eIF-4F was examined directly. To do this, the polypeptide composition of large-scale preparations of eIF-4F prepared by ^{7m}GDP chromatography was analyzed. After modifying a published eIF-4F purification procedure (Goyer *et al.*, 1989) to enhance the recovery of the eIF-4G (Tif4631p) subunit, we found that a new 68 kDa protein appeared in the complex (Figure 1A, lane 1). We confirmed that this protein was Pab1p in two separate experiments. First, eIF-4F purified from yeast extracts containing a 55 kDa truncated form of Pab1p [Pab1-55p (Sachs *et al.*, 1987)] contained a new protein at 55 kDa and no longer contained one at 68 kDa (Figure 1A, lane 2). Second, monoclonal antibodies to Pab1p (Anderson *et al.*, 1993) recognized both the 68 and 55 kDa proteins (Figure 1B, lanes 1 and 2), and quantitative Western blots showed that the observed signal with this antibody was consistent with the amount of Pab1p or Pab1–55p present in the preparations (data not shown). The low amounts of other polypeptides found in these preparations are presumably proteolytic fragments of Tif4631p because they vary in amounts between preparations and are nearly absent in preparations lacking the Tif4631 protein (Figure 1A, lane 3). Pab1p's purification in these experiments was not due to its binding directly to the ^{7m}GDP–resin because it was not purified from extracts containing the mutant Cdc33-1p (Figure 1A and B, lane 6), and because recombinant Pab1p did not bind to the resin (data not shown). The co-purification of Pab1p with eIF-4F suggests that they interact *in vivo*.

The amount of Tif4631p and Pab1p in these preparations, as judged by Coomassie blue staining, appears to be nearly stoichiometric. In support of the hypothesis that equal amounts of Tif4631p and Pab1p are in the preparation, we find that when different preparations are compared with each other, the abundance of both proteins relative to the amount of eIF-4E in the preparation varies by ~50%, and the degree of variation is nearly equal for Pab1p and Tif4631p. We note that the majority (>95%) of cellular Pab1p is not purified in these experiments. We assume this is because the ^{7m}GDP–resin is capturing the small percentage of Pab1p which is engaged in the initiation process at the time the extracts are prepared.

The failure of earlier studies to identify Pab1p in eIF-4F preparations (Goyer *et al.*, 1989) probably resulted from a combination of factors. These include the method by which the yeast cells were lysed (bead beating versus hand agitation) and the absence of detergent in the preparation. In addition, we find that Pab1p is visualized most easily in eIF-4F preparations from L-A-deficient strains, since the 76 kDa Gag protein from the L-A particle is also purified by ^{7m}GDP chromatography (Blanc *et al.*, 1994) and masks the presence of Pab1p on SDS–polyacrylamide gels.

eIF-4G and RNA are required for Pab1p's co-purification with eIF-4F from crude extracts

The above data support the general model that Pab1p interacts with eIF-4F. To determine which polypeptide in this complex was needed for Pab1p's association, the co-purification of Pab1p with eIF-4F from large-scale preparations of extracts deficient in either Tif4631p or p20 was examined. Because Tif4632p is not visible by Coomassie blue staining of these preparations, any Pab1p which is associated with it would not contribute greatly to the total amount of Pab1p in the preparation. This allowed us to examine the effects of removing the eIF-4G homolog Tif4631p on Pab1p co-purification without having to create yeast strains missing both eIF-4G homologs. The low abundance of Tif4632p in these preparations also made it unnecessary to investigate the effects of removing it on the co-purification of Pab1p with eIF-4F.

As seen in Figure 1 (lane 3), eIF-4F prepared from a yeast extract deficient in Tif4631p yielded near normal levels of the eIF-4E and the p20 proteins, but no visible Pab1p. A Western blot of this preparation showed that Pab1p yields were decreased at least 10-fold (Figure 1B, lane 3). The residual amount of Pab1p found in this eluate could reflect the co-purification of Pab1p with low amounts of Tif4632p in the preparation. Because eIF-4F prepared from extracts deficient in p20 contained low amounts of Tif4631p and Pab1p (Figure1A and B, lane 4), it could not be determined whether a p20 deficiency led directly to a loss of Pab1p binding to the eIF-4F complex, or

whether a p20 deficiency led to destabilization of Tif4631p binding, and therefore, of Pab1p binding.

Pab1p's co-purification with eIF-4F also required an RNA component because treatment of the material bound to the resin with micrococcal nuclease resulted in the removal of Pab1p from it (Figure 1A and B, lane 5) (discussed in more detail below). Together with the above data, these data suggest that the large-scale purification of Pab1p with eIF-4F from crude yeast extracts requires the Tif4631p subunit and an RNA component.

Tif4632p co-purifies with eIF-4E

It was necessary to verify that Tif4632p interacts with eIF-4E because no previous biochemical characterization of Tif4632p has been performed. Although Tif4632p would be expected to be present in eIF-4F preparations due to its structural and genetic similarities with Tif4631p, it was not detected in Coomassie blue-stained SDS–PAGE gels of eIF-4F complexes from large-scale purifications described above (Figure 1) and previously (Goyer *et al.*, 1989).

In order to visualize both eIF-4G homologs in small-scale preparations of eIF-4F by Western blotting, Tif4631p and Tif4632p were epitope tagged at their N-termini with a c-Myc or an influenza virus hemagglutinin (HA) peptide, respectively. The genes encoding these tagged proteins were then introduced on centromeric vectors into yeast cells containing chromosomal deletions of both *TIF4631* and *TIF4632* to yield strain YAS1959. These epitope-tagged proteins were shown to be functional *in vivo* because each allowed growth of a yeast strain harboring deletions of both *TIF4631* and *TIF4632* genes (data not shown).

The eIF-4F complex from YAS1959 extracts was isolated by ^{7m}GDP affinity chromatography. As a control for the binding specificity of Tif4632p to the ^{7m}GDP–resin, an eIF-4F preparation from a yeast extract (YAS1970) harboring the *cdc33-1* and *tif4632Δ* mutations and an epitope-tagged *TIF4632* on a centromeric plasmid was also examined. This extract served as a control because no eIF-4E-associated proteins in this extract are expected to be retained on the resin, due to the inability of Cdc33-1p to bind to the resin. After binding each of the extracts to the ^{7m}GDP–resin, and washing the resin with both the binding buffer and buffer containing GDP, the bound proteins were eluted from the resin with ^{7m}GTP. Eluates were resolved by SDS–PAGE, and then visualized by Western analyses using monoclonal antibodies recognizing either the epitope-tagged Tif4631p or Tif4632p.

As shown in Figure 2A, approximately equal amounts of Tif4632p were present in both *CDC33* (lane 1) and *cdc33-1* (lane 2) extracts. However, only eIF-4F preparations from the *CDC33* extracts contained Tif4632p in the ^{7m}GTP eluate (compare lanes 3 and 4). As expected, tagged Tif4631p was also present in *CDC33* eIF-4F preparations (data not shown), and <5% of eIF-4E was recovered in eluates from the *cdc33-1* preparation (data not shown).

In order to confirm further that Tif4632p binds to eIF-4E, recombinant eIF-4E (a generous gift from J.McCarthy) was incubated with immobilized recombinant Tif4631p or Tif4632p fused to a glutathione *S*-transferase (GST) protein. After extensive washing of the immobilized

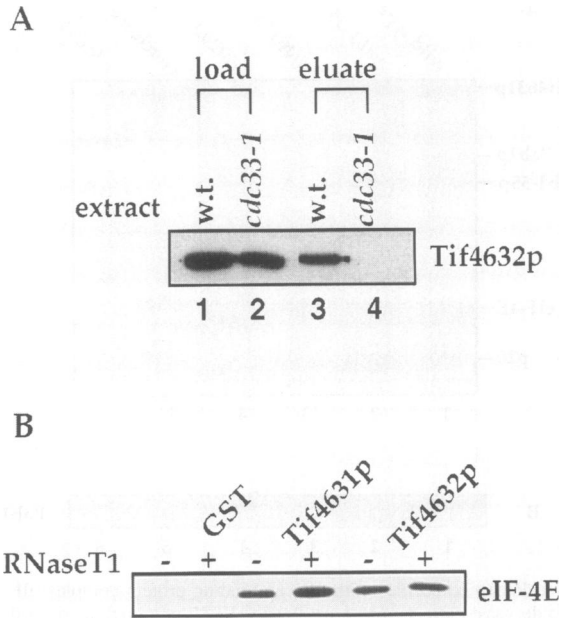

Fig. 2. Tif4632p is an eIF-4E-associated protein. (**A**) Small-scale purification of eIF-4F from crude extracts. Western blots of equal amounts of crude extracts (load) from *CDC33* (w.t.) (YAS1959) or *cdc33-1* (YAS1970) strains, or of eIF-4F preparations (eluate) from these extracts are shown. Samples (2% of load or 5% of eluate) were resolved by SDS–PAGE, and HA-tagged Tif4632p was visualized by Western analysis. (**B**) eIF-4E associates with recombinant Tif4631p and Tif4632p. The indicated GST fusion proteins immobilized on glutathione resin were incubated with recombinant eIF-4E. Half of each sample was treated with RNase T1, and then all samples were washed extensively before eIF-4E was eluted and then visualized by Western analysis.

proteins, the bound eIF-4E was eluted, resolved by SDS–PAGE and visualized by Western analysis. As shown in Figure 2B, eIF-4E bound to both columns, was specific for Tif4631p or Tif4632p and was not dependent on RNA (compare ± RNase T1).

These data support the hypothesis that, like Tif4631p, the eIF-4G homolog Tif4632p is also an eIF-4E-associated protein in yeast. The failure to detect large amounts of Tif4632p in large-scale eIF-4F preparations could be due to differences in the amounts of Tif4631p and Tif4632p in soluble yeast extracts, or to the dissociation of Tif4632p from eIF-4E during the extended wash times in the large-scale purifications.

Pab1p co-immunoprecipitates with eIF-4G and eIF-4E from crude extracts

The interactions between Pab1p, eIF-4G, and eIF-4E described above were also observed in co-immunoprecipitation experiments. Crude extracts with (YAS1959) or without (YAS538) N-terminally tagged (myc)Tif4631p and (HA)Tif4632p proteins were incubated with protein A–Sepharose beads coated with either monoclonal antibodies to Pab1p, the c-myc epitope or the influenza virus HA epitope. After several washes with detergent-containing buffer, the proteins in the immunocomplexes were eluted from the beads, resolved by SDS–PAGE and visualized by Western analysis. As shown in Figure 3, immunoprecipitation of either Tif4632p or Tif4631p resulted in the co-immunoprecipitation of Pab1p and eIF-4E (Figure 3, lanes 2 and 4). The absence of Tif4632p in

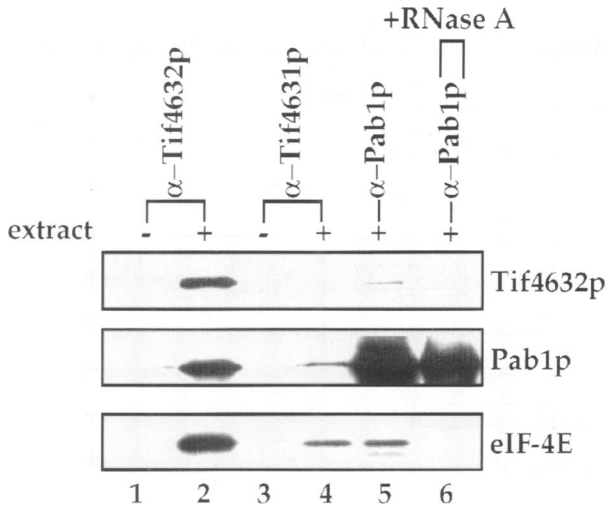

Fig. 3. Pab1p co-immunoprecipitates with eIF-4E, Tif4631p and Tif4632p. Extracts with (+) (YAS1959) or without (–) (YAS538) epitope-tagged myc-Tif4631p and HA-Tif4632p were incubated with protein A–Sepharose beads coated with the antibody listed at the top of each lane. After treating one-half of the Pab1p immunoprecipitate with RNase A, all samples were fractionated by SDS–PAGE and subject to Western analysis with the antibodies listed to the right of each panel.

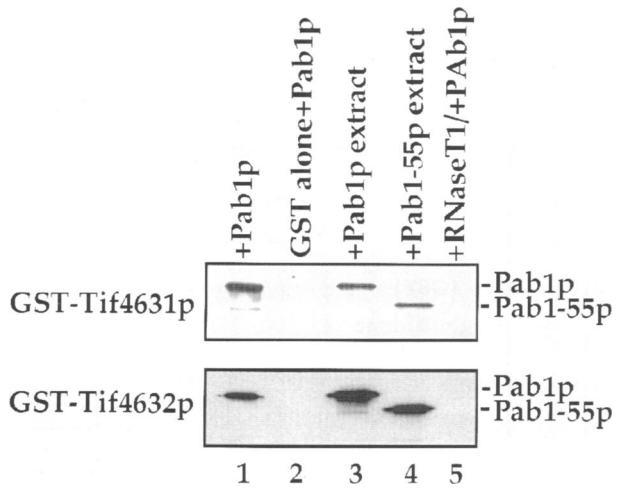

Fig. 4. Pab1p binds to recombinant GST–Tif4631p and GST–Tif4632p. Eluates from the GST–Tif4631p and GST–Tif4632p resins are shown. Recombinant fusion proteins (indicated to the left of each panel) were immobilized on glutathione columns and incubated with either recombinant Pab1p or crude yeast extracts from a wild-type strain (YAS1882) or a *pab1-55* mutant (YAS1883). Alternatively, the GST fusion proteins were treated with RNase T1 before incubation with the recombinant Pab1p. Pab1p was detected in the eluted samples by Western analysis.

Tif4631p immunoprecipitates (Figure 3, lane 4) suggests that these two proteins are in physically distinct particles. Immunoprecipitation of Pab1p also resulted in the co-immunoprecipitation of Tif4632p and eIF-4E (Figure 3, lane 5), and pre-treatment of the Pab1p immunocomplex with RNase before its elution resulted in the loss of interaction between Pab1p and the other proteins (Figure 3, lane 5 versus lane 6). Because RNase A can cleave poly(A) tracts under the low salt conditions used in our assay, we cannot conclude whether the disruption of the Pab1p-immunoprecipitated complex was due to loss of poly(A) tails or to loss of other RNA sequences. Due to the very weak Western signal of myc-tagged Tif4631p in these immunoprecipitates, we also cannot determine if the Pab1p immunocomplexes contain Tif4631p.

These data are consistent with the conclusions derived from the co-purification studies described above that Tif4631p interacts with Pab1p. They also show that Tif4632p interacts with Pab1p in crude yeast extracts, a result that could not be obtained using the large-scale eIF-4F preparations since Tif4632p is not visible in them. Finally, these data show that Pab1p interacts with eIF-4E and Tif4632p in an RNA-dependent manner.

Reconstitution of Pab1p binding to recombinant eIF-4G

To investigate further the interactions between the eIF-4G homologs Tif4631p and Tif4632p and Pab1p, and to understand in more detail the RNA requirement for this interaction, recombinant Tif4631p or Tif4632p fused to GST (GST–Tif4631p or GST–Tif4632p, respectively) was immobilized on a glutathione column. Recombinant Pab1p (Sachs *et al.*, 1987) bound to the immobilized GST fusion proteins (Figure 4A, lane 1) and did not bind to immobilized GST alone (Figure 4A, lane 2). Almost 75% of the input Pab1p (15 μg) was bound to the GST fusion proteins under these conditions. Furthermore, when either immobilized GST–Tif4631p or GST–Tif4632p was incubated with yeast extracts containing either a full-length or a truncated version of Pab1p, either the full-length 68 kDa Pab1p or a 55 kDa truncated version of Pab1p was purified (Figure 4A, lanes 3–4). These last experiments show that recombinant Pab1p does not bind to either the GST–Tif4631p or GST–Tif4632p resins through non-specific interactions, since these resins are capable of binding to Pab1p in crude yeast extracts. Pab1p binding to GST–Tif4631p or GST–Tif4632p also required an RNA component because pre-treatment of the immobilized GST fusion proteins with RNase T1 abolished the binding of recombinant Pab1p to them (Figure 4A, lane 5).

These data indicate the presence of an RNA-binding site on Tif4631p and Tif4632p that can bind to bacterial RNA, and possibly the presence of RNA sequences within bacterial RNA that are bound efficiently by Pab1p. Each of the above experiments supports the hypotheses derived from the experiments illustrated in Figures 1 and 3 that Pab1p interacts with eIF-4G in an RNA-dependent manner, and that Pab1p interacts with eIF-4E via eIF-4G.

The Pab1p binding site on Tif4632p resides within a 114-amino-acid region proximal to the eIF-4E binding site

A detailed analysis of Tif4632p was pursued in order to understand in more detail the RNA requirement for Pab1p binding and to delineate where Pab1p bound to this protein. An indirect Pab1p binding assay was developed for these studies (Figure 5A). In this assay, [^{32}P]poly(A) is saturated with excess Pab1p, and then added to the glutathione resin containing the fusion protein. Following extensive washing of the resin, the amount of retained [^{32}P]poly(A) is determined by scintillation counting. This assay has the advantage of not measuring the Pab1p that could be associated with the glutathione resin through

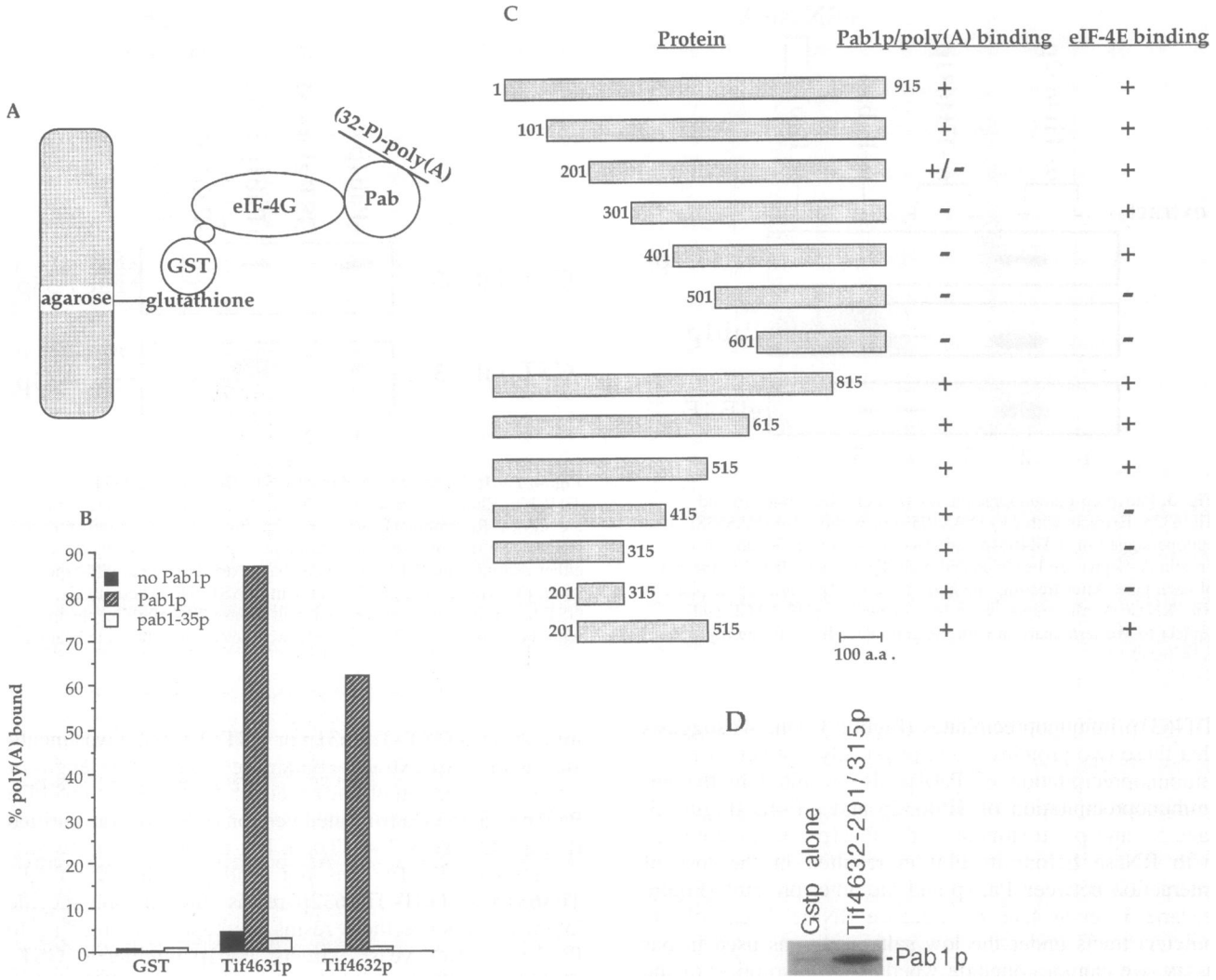

Fig. 5. Delineation of the Pab1p binding site of Tif4632p. (**A**) Schematic diagram of the indirect Pab1p binding assay. (**B**) Pab1p can bind simultaneously to poly(A) and Tif4631p or Tif4632p. [^{32}P]Poly(A) pre-bound to an excess of the indicated Pab1ps or to no Pab1p was incubated with the indicated GST fusion proteins attached to glutathione resin. The amount of [^{32}P]poly(A) remaining associated with the resin was determined by scintillation counting. (**C**) The Pab1p binding site on Tif4632p is between amino acids 201 and 315. GST fusion proteins containing the indicated regions of Tif4632p were immobilized on glutathione resin and analyzed for their ability to bind the [^{32}P]poly(A)–Pab1p complex as depicted in (A), or eIF-4E as described in Figure 2B. For [^{32}P]poly(A), a (+) indicates >60% binding, a (±) between 10 and 60%, and a (–) <10% binding. For eIF-4E binding, a (+) indicates binding, while a (–) indicates no visible Western signal. (**D**) Tif4632-201/315p binds to Pab1p in crude yeast extracts. The immobilized GST fusion protein Tif4632-201/315p or GST alone was incubated with crude extract from wild-type yeast and then washed extensively. Bound proteins were eluted in SDS and resolved by 10% SDS–PAGE. Pab1p was visualized by Western analysis.

tethering by the eIF-4G-bound bacterial RNA to Pab1p's poly(A) binding site.

An example of this assay using full-length Tif4631p or Tif4632p and either Pab1p or a truncated version of Pab1p, Pab1-35p (Sachs *et al.*, 1987), is shown in Figure 5B. Pab1-35p contains only one RNA-binding domain and therefore serves as an example of a protein that cannot bind simultaneously to both resin-associated RNA and [^{32}P]poly(A). While [^{32}P]poly(A) is unable to bind to the resin by itself, the addition of Pab1p and [^{32}P]poly(A) allows near total retention of RNA on the resin. In contrast, Pab1-35p was unable to promote binding of the [^{32}P]poly(A) to the resin. Prior treatment of the resins with RNase T1 did not destroy the ability of Pab1p to stimulate the binding of [^{32}P]poly(A) to the resin, although the degree of stimulation was much smaller since the resin also bound [^{32}P]poly(A) in the absence of Pab1p (data not shown). These data indicate that Pab1p does not interact with the full-length Tif4631p or Tif4632p fusion proteins through a tethering interaction of RNA with Pab1p's poly(A) binding site, but instead suggest that a specific surface of Pab1p is interacting with them. They do not rule out completely the possibility that Pab1p is tethered to the resin via a second RNA-binding site. However, previous work argues strongly against the possibility that Pab1p can remain bound to two different RNA molecules (Sachs *et al.*, 1987), and data presented in Figure 6C also argue against this possibility.

The failure of Pab1-35p to associate with Tif4632p, even though it can bind to poly(A), indicates that it lacks the surface needed to interact with Tif4632p via protein–protein interactions. These data suggest that the surface of Pab1p which interacts with Tif4632p may be located in the N-terminal region of the protein. Future work will be needed to define this region accurately.

A truncation analysis of Tif4632p was performed in

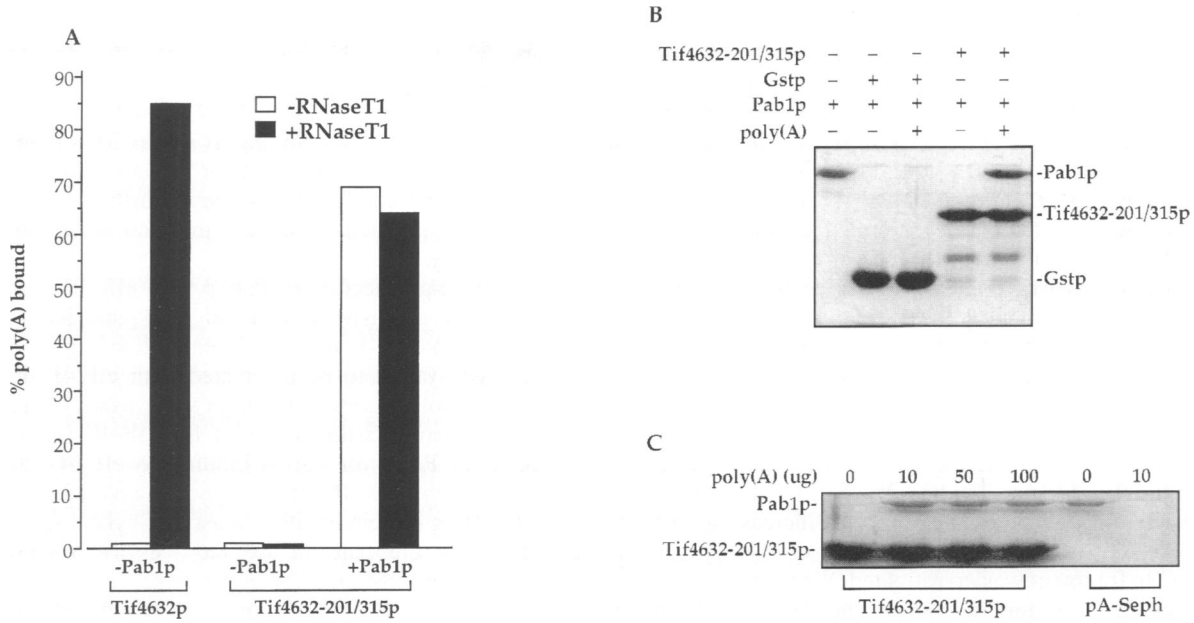

Fig. 6. Poly(A) stimulates the binding of Pab1p to the Pab1p binding domain of Tif4632p. (**A**) Tif4632-201/315p does not bind to [^{32}P]poly(A) without Pab1p. The indicated GST fusion protein was immobilized on glutathione resin, treated with RNase T1 as indicated, and then incubated with [^{32}P]poly(A) with or without Pab1p. The percentage of input [^{32}P]poly(A) retained on the resin is shown. (**B**) Pab1p does not bind to Tif4632-201/315p without poly(A). The GST fusion protein containing amino acids 201–315 of Tif4632p or GST alone was immobilized on glutathione resin, treated with RNase T1, and then incubated with the listed components. Following extensive washing of the resin, proteins were eluted in SDS, resolved by SDS–PAGE and visualized by Coomassie blue staining. The positions of the different proteins are indicated. (**C**) Excess poly(A) does not inhibit Pab1p's association with Tif4632-201/315p. Tif4632-201/315p was immobilized on glutathione resin, treated with RNase T1, and then incubated with the indicated amount of poly(A) and 10 μg of Pab1p. Alternatively, poly(A)–Sepharose was mixed with the indicated amounts of poly(A) and 10 μg of Pab1p. Following pelleting and washing of the the resin, bound proteins were eluted in SDS sample buffer, resolved by SDS–PAGE, and visualized by Coomassie blue staining. The positions of the different proteins are indicated.

order to identify the region of Tif4632p that interacts with Pab1p. Tif4632p was studied in detail since translation extracts from yeast lacking this protein have scorable deficiencies (S.Z.Tarun,Jr and A.B.Sachs, in preparation). In these experiments, Tif4632p fragments were fused to GST and immobilized on the glutathione column. Pab1p binding to these fragments was analyzed using the indirect Pab1p binding assay outlined in Figure 5A. As shown in Figure 5C, all Tif4632p fragments containing amino acids 201–315 were capable of binding to the [^{32}P]poly(A)–Pab1p complex. A GST fusion protein containing only amino acids 201–315 (Tif4632-201/315p) was also capable of binding to the [^{32}P]poly(A)–Pab1p complex. In contrast, [^{32}P]poly(A) did not bind to Tif4632-201/315p in the absence of Pab1p (see Figure 6A). These data support the hypothesis that the 114-amino-acid region between residues 201 and 315 in Tif4632p binds specifically to Pab1p. This region is ~100 amino acids proximal to the proposed eIF-4E binding site on Tif4632p (see Mader *et al.*, 1995 and below).

Binding of the Tif4632p fragments to recombinant eIF-4E was also investigated (Figure 5C). The eIF-4E binding site on Tif4632p was mapped to between residues 400 and 515. To confirm this, a fragment containing amino acids 201–515 was shown to bind both Pab1p and eIF-4E (Figure 5C). These data are in agreement with a recently published report on the probable location of the eIF-4E binding site on Tif4632p (Mader *et al.*, 1995), and they highlight the separation of protein interaction domains on Tif4632p.

The ability of Tif4632-201/315p to bind to Pab1p in a crude yeast extract was investigated to confirm the specificity of the interaction. As shown in Figure 5D, Pab1p was purified specifically when the GST–Tif4632p fusion protein but not the GST protein alone was incubated with the crude extract. These data further support the hypothesis that amino acids 201–315 of Tif4632p provide a specific binding surface for Pab1p.

Pab1p requires poly(A) to bind to Tif4632-201/315p

A key feature of the interaction between Tif4631p or Tif4632p and Pab1p is its requirement for RNA. Our data using the indirect [^{32}P]poly(A) binding assay (see Figure 5), and our finding that large excesses of poly(A) in solution do not inhibit Pab1p binding to full-length Tif4631p or Tif4632p (data not shown), are both consistent with the hypothesis that Pab1p is not tethered to these proteins via an RNA molecule. In order to show that RNA tethering could not explain the interaction between Tif4632-201/315p and Pab1p, we investigated whether this fragment could bind to poly(A) in the absence of Pab1p and whether excess poly(A) inhibited its interaction with Pab1p.

RNase T1-treated Tif4632-201/315p did not bind to free [^{32}P]poly(A) (Figure 6A). This is in contrast to the full-length Tif4632p, which bound nearly 85% of the poly(A) after nuclease treatment (Figure 6A). These data show that under the conditions of this assay, Tif4631-201/315p does not bind to poly(A). We next analyzed Pab1p binding to Tif4632-201/315p using the indirect [^{32}P]-poly(A) binding assay (Figure 6A). The [^{32}P]poly(A)–Pab1p complex bound efficiently to Tif4632-201/315p. Because no [^{32}P]poly(A) was bound in the absence of

Pab1p, the [^{32}P]poly(A) binding must result from an interaction between Pab1p and Tif4632-201/315p.

Pab1p was found only to associate with the resin in the presence of poly(A) (Figure 6B). In this experiment, Pab1p in the absence or presence of poly(A) was incubated with immobilized Tif4632-201/315p. Following washing of the resin, the amount of retained Pab1p was determined by Coomassie blue staining of the eluted sample. Pab1p only bound to the resin in the presence of poly(A). These data indicate that Pab1p must be bound to poly(A) in order to interact with Tif4632-201/315p via protein–protein interactions. Future studies will need to address what function, if any, the RNA-binding sites on full-length Tif4631p or Tif4632p serve in modulating this Pab1p association.

In order to rule out the possibility that poly(A) was tethering the Pab1p to Tif4632-201/315p in the experiment described in Figure 6B, the ability of increasing amounts of poly(A) to inhibit the association of Pab1p with the Tif4632p fragment was investigated. We note that although the results of Figure 6A show the Tif4632p fragment does not bind poly(A), it was important to provide an independent test for this possibility. In these experiments, Pab1p was first mixed with 10, 50 or 100 μg of poly(A), which represents an 8-, 40- or 80-fold excess of poly(A) binding sites (see Materials and methods), and was then added to nuclease-treated Tif4632p fragment. As shown in Figure 6C, the large excesses of poly(A) did not significantly inhibit Pab1p's association with the Tif4632p fragment. That sufficient poly(A) was present to saturate the input Pab1p was shown by the ability of the 8-fold excess (10 μg) of poly(A) to prevent Pab1p's association with poly(A)–Sepharose (Figure 6C). The inability of excess poly(A) to compete for Pab1p binding to the immobilized Tif4632-201/315p is not predicted from the tethering model. As a result, both this experiment and those described in Figure 6A and B support the hypothesis that Pab1p, when bound to poly(A), interacts with the Tif4632p fragment through protein–protein contacts.

Discussion

Based on the above experiments, we conclude that Pab1p can interact with eIF-4G. The observations that Pab1p co-purifies and co-immunoprecipitates with eIF-4G provide independent support for this conclusion. Our delineation of the Pab1p binding site on Tif4632p between amino acids 201 and 315, and our finding that Pab1p binds to this region only when complexed to poly(A) supports the hypothesis that poly(A) serves as a positive regulator of Pab1p binding to eIF-4G. Based on these data, we hypothesize that mRNA could be circularized in eucaryotic cells through Pab1p's interaction with eIF-4E via eIF-4G.

The RNA requirement for Pab1p's interaction with Tif4632p is complex since both of these proteins can bind to RNA. We have simplified this complexity by analyzing the interaction between Pab1p and a fragment of Tif4632p that does not bind RNA in our assay. Because the data in Figures 5 and 6 are derived from studies using only one RNA-binding protein (i.e. Pab1p), Pab1p's interaction with Tif4632-201/315p cannot be explained by a simple tethering via a shared RNA molecule. While our experiments show that Pab1p only interacts with eIF-4G when bound to RNA, they do not address the functional significance of the RNA-binding site on eIF-4G. One possibility is that this binding site exposes the Pab1p-binding region on eIF-4G. In this model, RNA would act as a positive regulator for eIF-4G binding to Pab1p. This model would predict that only the eIF-4G–RNA complex would bind to the Pab1p–poly(A) complex. Further work will be necessary in order to address this and other possibilities adequately.

The co-purification of Pab1p with eIF-4F is surprising given the extensive amount of work that has been performed on eIF-4F. It is possible that Pab1p previously has not been found to be associated with eIF-4F due to the presence of RNases in the high salt washes of ribosomes, the common starting material for eIF-4F isolation. The need for RNA for Pab1p binding to eIF-4G could also explain why eIF-4G may not be discovered as a Pab1p-interacting protein in either yeast two-hybrid screens using Pab1p as the bait, or by far Western analysis using radiolabeled Pab1p as the probe. A previous report (Gallie and Tanguay, 1994) has come to more general conclusions about the interactions between the poly(A) tail and eIF-4F through studies on how poly(A) inhibits the *in vitro* translation of various mRNAs.

The target of Pab1p binding on eIF-4F appears to be the eIF-4G protein. An extensive amount of work on this protein has been reported recently (Lamphear *et al.*, 1995; Mader *et al.*, 1995; Ohlmann *et al.*, 1996). By combining these results and the data presented here, a map of binding sites on yeast Tif4632p can be made. The N-terminal half of this protein contains the Pab1p and eIF-4E binding sites, while the C-terminal half contains both the eIF-3 and putative RNA-binding sites. The sequence of the eIF-4A binding site found at the very end of the C-terminus of higher cell eIF-4G proteins (Lamphear *et al.*, 1995) is not found in the yeast Tif4631p or Tif4632p sequences (A.B.Sachs, personal observation) and, therefore, an eIF-4A binding site on yeast eIF-4G is not considered. Future work will determine whether there is a Pab1p binding site on higher cell eIF-4G polypeptides.

The biological significance of Pab1p's interaction with eIF-4G can only be speculated upon from these data. One obvious role of this interaction would be to mediate the poly(A) tail's stimulation of mRNA translation (Tarun and Sachs, 1995). We are testing this hypothesis by developing *in vitro* translation assays that depend on eIF-4G, and developing appropriate yeast strains suitable for eIF-4G genetic analysis. The interaction of Pab1p and eIF-4F could also have other ramifications for RNA metabolism. For instance, mRNA deadenylation could stimulate mRNA decapping (Caponigro and Parker, 1995) because of the dissolution of the interaction between the Pab1p and eIF-4F. Similarly, the requirement for mRNA polyadenylation for cap ribose methylation in developing amphibian embryos (Kuge and Richter, 1995) could also be understood with our results. It is possible that the interaction of an embryo-specific Pabp with proteins bound to the cap structure leads to the recruitment of the methylase in a manner similar to the way in which Pab1p recruits the poly(A) nuclease (Boeck *et al.*, 1996).

The direct evidence presented here that yeast mRNA poly(A) tail and cap binding proteins interact also suggests that mRNA could be circularized in the cell. The creation

Table I. Yeast strains[a]

YAS 1882	α *pab1::HIS3 mak10::URA3 ade2 his3 leu2 trp1 ura3* p*PAB1LEU2CEN* (pAS419)
YAS 1883	α *pab1::HIS3 mak10::URA3 ade2 his3 leu2 trp1 ura3* p*pab1-55LEU2CEN* (pAS427)
YAS 1888	α *cdc33-1 pep4::HIS3 ade2 his3 leu2 trp1 ura3*
YAS 1889	α *tif4631::LEU2 pep4::HIS3 mak10::URA3 ade2 his3 leu2 trp1 ura3*
YAS 1910	**a** *caf20::URA3 pep4::HIS3 mak10::ura3 ade2 his3 leu2 trp1 ura3*
YAS 1959	α *tif4631::LEU2 tif4632::ura3 pep4::HIS3 mak10::URA3 ade2 his3 leu2 trp1 ura3* p(*MYC*)*TIF4631URA3CEN* (pAS488) p(*HA*)*TIF4632TRP1CEN* (pAS486)
YAS1970	α *cdc33-1tif4632::ura3 ade2 his3 leu2 trp1 ura3* p(*HA*)*TIF4632TRP1CEN* (pAS486)
YAS 306	α *ade2 his3 leu2 trp1 ura3*
YAS 307	**a** *ade2 his3 leu2 trp1 ura3*
YAS 538	α *pep4::HIS3 ade2 his3 leu2 trp1 ura3*
YCG312	α/a *tif4631::LEU2/+ tif4632::URA3/-+ leu2-3,112 ura3-52 his4-917d*
CB101	**a** *cdc33-1 leu1 ura3 trp1*
T149	α *caf20::URA3 his3 leu2 trp1*

[a]All strains are were made isogenic by utilizing progeny from at least three backcrosses to the parent strains YAS 306, 307 or 538. The starting strain for the indicated *PAB1* deletions has been described (Sachs *et al.*, 1987). The starting strains for the *cdc33-1*, *caf20::URA3*, *tif4631::LEU2* and the *tif4632::URA3* mutations were CB101 (Brenner *et al.*, 1988), T149 (Lanker *et al.*, 1992) and YCG312 (Goyer *et al.*, 1993), respectively. The *mak10::URA3* disruption was created by transforming yeast with a *Xho*I–*Not*I fragment of plasmid p406 (Widner and Wickner, 1993), selecting for Ura prototrophy and analyzing the resulting transformants for their content of L-A RNA. *tif4632::ura3* and *mak10::ura3* were isolated as spontaneous revertants on 5-fluoroorotic acid plates (Guthrie and Fink, 1991).

of circular mRNA could allow stable RNA structures or RNA–protein complexes in the 3′-untranslated region of an mRNA to regulate events at the 5′ end of the mRNA without interfering with the scanning of the ribosome. Furthermore, the regulation of circular mRNA formation in the cytoplasm of cells could be an unexplored control point in the post-transcriptional control of gene expression. Future work will be needed to explore each of these possibilities.

Materials and methods

Purification of yeast eIF-4F

Approximately 8 g of yeast cell paste from the indicated strain (Table I) was harvested from OD_{600} = 2–3 cultures in YPD, washed once and then resuspended in 12 ml of buffer A [100 mM K acetate, 2 mM Mg acetate, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 7 mM BME, 30 mM HEPES, pH 7.5] in a 50 ml Falcon tube. Following the addition of 48 g of 0.5 mm glass beads (Biospec Products), the yeast were lysed by five cycles of 1 min shaking (shoulder to hip, two cycles/s) and 1 min incubation in ice-water. Following clarification of the lysate by two 5 min spins at 30 000 *g* in an SS34 rotor, ~10 ml of supernatant was loaded by gravity onto a 0.5 ml ^{7m}GDP–agarose resin (1 cm × 1 cm) prepared as described in Edery *et al.* (1988) and pre-equilibrated in buffer A. The column was then washed with 50 ml of buffer B (100 mM KCl, 0.2 mM EDTA, 0.01% Triton X-100, 0.5 mM PMSF, 7 mM BME, 20 mM HEPES, pH 7.4), and with 15 ml of buffer B + 0.1 mM GDP. eIF-4F was eluted in five 250 μl washes with buffer B + 0.1 mM ^{7m}GTP, with peak fractions usually appearing between 0.25 and 1.0 ml. For micrococcal nuclease treatment, columns were washed in 2 ml of buffer B without Triton X-100 after the 50 ml buffer B wash step. Buffer B (1.5 ml) containing 150 U of micrococcal nuclease (Pharmacia) and 1.5 mM $CaCl_2$ was run into the column and incubated for 30 min. Nuclease treatment was terminated by beginning the 15 ml GDP wash step. All procedures were performed at 4°C. Approximately 50–100% of two pooled peak fractions were resolved by SDS–PAGE in each of the lanes shown in Figure 1. Nearly equal amounts of eIF-4E were loaded intentionally in each lane to allow for a direct comparison of the relative amounts of the other proteins present in the preparation. Volumes equal to that of the wild-type extract were loaded for the eIF-4F preparation from the *cdc33-1* extract (Figure 1, lane 6). Extracts from strains lacking the *MAK10* gene are used in order to remove the L-A dsRNA from the cells (Widner and Wickner, 1993).

For small-scale preparations of eIF-4F as shown in Figure 2A, ~0.5 g of frozen cells were resuspended in 300 μl of buffer A. Two 333 μl aliquots of this suspension were added to two 0.5 ml microcentrifuge tubes containing 0.8 g of glass beads. Following a 1 min high speed vortex, the extract was collected by punching a hole in the bottom of the tube, resting it inside a 2.0 ml microcentrifuge tube and spinning it for 1 min in the microcentrifuge. The pooled extracts were then cleared for 15 min in the microcentrifuge, and 300 μl of each extract was incubated with 50 μl of ^{7m}GDP–Sepharose in 200 μl of buffer A by rocking at 4°C for 2 h. Following five 1 ml washes with buffer B and five 1 ml washes with 1 min incubations with buffer B + 0.1 mM GDP, eIF-4E-associated proteins were eluted in 100 μl of buffer B + 0.1 mM ^{7m}GTP. Approximately 20 μl of the eluted samples or 5 μl of the other samples were mixed with gel loading buffer and resolved on 10% SDS–PAGE gels.

Table II. Bacterial strains[a]

BAS2027	GST–Tif4631 (pAS466)
BAS2030	GST–Tif4632 (pAS476)
BAS2089	GST–Tif4632-ΔN100 (OAS213/196)
BAS2090	GST–Tif4632-ΔN200 (OAS214/196)
BAS2091	GST–Tif4632-ΔN300 (OAS215/196)
BAS2092	GST–Tif4632-ΔN400 (OAS216/196)
BAS2093	GST–Tif4632-ΔN500 (OAS217/196)
BAS2094	GST–Tif4632-ΔN600 (OAS218/196)
BAS2095	GST–Tif4632-ΔC100 (OAS219/195)
BAS2096	GST–Tif4632-ΔC200 (OAS220/195)
BAS2097	GST–Tif4632-ΔC300 (OAS221/195)
BAS2098	GST–Tif4632-ΔC400 (OAS222/195)
BAS2099	GST–Tif4632-ΔC500 (OAS223/195)
BAS3000	GST–Tif4632-ΔC600 (OAS224/195)
BAS3024	GST–Tif4632-aa201–315
BAS3026	GST–Tif4632-aa201–515

[a]Bacterial strain BL21 was transformed with the indicated plasmids or plasmid pAS476 containing a *Bam*HI–*Eco*RI-digested PCR product from an amplification of *TF4632* using the indicated oligonucleotides (OAS). ΔN and ΔC refer to N- and C-terminal truncations, respectively.

Immunological techniques

For the Western analysis shown in Figure 1B, aliquots from each of the eIF-4F preparations were resolved by 10% SDS–PAGE and subjected to Western analysis as described below. The amount of preparation loaded in each lane was adjusted so that each sample had approximately equal amounts of eIF-4E. For the *cdc33-1* sample, volumes equal to that of the wild-type sample were loaded.

For the experiments described in Figure 3, mini-extracts (50 μl) prepared as described above were adjusted to 0.1% Triton X-100 and 0.01% SDS and then incubated for 2 h by rocking at 4°C with protein A–Sepharose (50 μl of a 20% suspension) (Santa Cruz Biotechnology) that had been pre-bound to either 1 μl of ascites fluid containing the Pab1p 1G1 (Anderson *et al.*, 1993), c-myc 9E10 or influenza virus HA 12CA5 monoclonal antibodies and resuspended in phosphate-buffered

Table III. Oligonucleotides

OAS192	CGC GGA TCC ACA GAC GAA ACT GCT CAA CCG ACA CAA TCT
OAS193	CCG GAA TTC TTA CTC TTC GTC ATC ACT TTC TCC CAT TAA
OAS195	CGC GGA TCC ACT GAC CAA AGA GGT CCA CCG CCC CCA CAC
OAS196	CCG GAA TTC TTA ATC ACT GTC CCC ATC GTT ATT CAT TAA
OAS197	CCA TCG ATG GCT CAT TGT ATA TGT TAC TTA GT
OAS198	CGG GAT CCC ATA TGT GCC TAC AAT TGA TCT ATT GTT C
OAS199	CGG GAT CCG AAT TCA AGA TTT TCT TTT AGA AAC GAT AA
OAS200	GCT CTA GAA TGT CTT GTG GTT TCA GAG AAA GT
OAS206	TAT GTA CCC ATA CGA CGT CCC AGA CTA CGC TA
OAS207	GAT CTA GCG TAG TCT GGG ACG TCG TAT GGG TAC A
OAS208	TAT GGA ACA AAA GCT TAT TTC TGA AGA AGA CTT GA
OAS209	GAT CTC AAG TCT TCT TCA GAA ATA AGC TTT TGT TCC A
OAS213	CGC GGA TCC TTG CCA GGT ATG CAA TGG CCA GCT
OAS214	CGC GGA TCC GCT AAT GAA GCA GTT AAA GAT ACG
OAS215	CGC GGA TCC ACT CCA CAA CAT GTA ACT GGA AGT
OAS216	CGC GGA TCC TTT GCC TAT CCG GAA AAT GTT GAA
OAS217	CGC GGA TCC AGA AGA TCT AAT AGA GGA TAT ACC
OAS218	CGC GGA TCC GAT GGC GAA ACA TTG AAA ATA GTT
OAS219	CCG GAA TTC TTA AGC GCT GTT CCA ATG CTT AAT TTC
OAS220	CCG GAA TTC TTA ACA ATA TAA GTA CCC TAT GAA ACG
OAS221	CCG GAA TTC TTA CTT GTG GAA AAT TTG TTC AAT AAC
OAS222	CCG GAA TTC TTA TTT TTC GCG ATC TTT TCT GGA GGT
OAS223	CCG GAA TTC TTA CTT GTA TTT GAT ATC AGG CCT TTC
OAS224	CCG GAA TTC TTA AAA AGT CAC GGA TTT AGT TAC ACT

Table IV. Plasmids

pAS466	*Bam*HI–*Eco*RI fragment of pAS483 into *Bam*HI–*Eco*RI of pGEX2T (Pharmacia Biochemicals). Produces GST–Tif4631p.
pAS476	*Bam*HI–*Eco*RI fragment of pAS480 into *Bam*HI–*Eco*RI of pGEX2T. Produces GST–Tif4632p.
pAS477	*TIF4632* 5′ UTR (PCR product of OAS197/198) as *Cla*I–*Bam*HI fragment into Bluescript KS (Stratagene) between *Cla*I and *Bam*HI
pAS478	*TIF4632* 3′ UTR (PCR product of OAS199/200) as *Bam*HI–*Xba*I fragment into *Bam*HI–*Xba*I of pAS477. Yields a bacterial vector with 5′ and 3′ UTRs of *TIF4632* containing *Nde*I, *Bam*HI and *Eco*RI sites between them.
pAS480	*TIF4632* open reading frame as *Bam*HI–*Eco*RI fragment (PCR product of OAS195/196) into pAS478 between *Bam*HI and *Eco*RI. Yields *TIF4632* gene as a *Cla*I–*Xba*I cassette with a unique *Nde*I site at codon 1.
pAS483	*TIF463*I open reading frame as *Bam*HI–*Eco*RI fragment (PCR product of OAS192/193) into pAS 478 between *Bam*HI and *Eco*RI. Yields *TIF463*I gene between *TIF4632* 5′ and 3′ UTRs as an *Cla*I–*Xba*I cassette with an *Nde*I site at codon 1.
pAS482	*Nde*I–*Bam*HI-digested pAS480 ligated to influenza virus HA tag oligos OAS 206/207
pAS484	*Nde*I–*Bam*HI-digested pAS483 ligated to c-myc tag oligos OAS 208/209
pAS486	*Cla*I–*Xba*I-digested pUN15 (Elledge and Davis, 1988) with *Cla*I–*Xba*I *TIF4632* fragment from pAS482
pAS488	*Cla*I–*Xba*I-digested pUN55 (Elledge and Davis, 1988) with *Cla*I–*Xba*I *TIF463*I fragment from pAS484

saline (PBS) with 0.1% Triton X-100 and 0.01% SDS (PBS + detergents). Following four 1 ml washes with PBS + detergents, one-half of the 1G1 immunoprecipitate was incubated in 50 µl of PBS + detergents + 20 µg of RNase A at 26°C for 5 min. All samples were then washed three more times in 1 ml of PBS + detergent. Bound proteins were eluted from the protein A–Sepharose by heating at 95°C in 45 µl of 2× Laemmli gel loading buffer. Approximately 15 µl of sample was resolved on 7.8% polyacrylamide (all but eIF-4E Westerns) or 12% polyacrylamide (eIF-4E Westerns) minigels by SDS–PAGE. Following transfer in glycine transfer buffer (Maniatis *et al.*, 1982) for 45 min onto Hybond ECL nitrocellulose (Amersham), the immobilized samples were incubated for several hours in PBS + detergent + 5% non-fat dry milk, and then for several hours to overnight in the same solution with either the Pab1p, 12CA5 or 9E10 monoclonal antibodies (diluted 1:5000) or rabbit polyclonal anti-eIF4E antibodies (the kind gift of J.E.McCarthy) diluted 1:2500. The blots were washed three times for 15 min each time in PBS + detergent, incubated with horseradish peroxidase-coupled secondary antibody to mouse or rabbit (Amersham) for 1 h, washed again three times for 15 min in PBS + detergent, and then developed using the ECL chemiluminescent system (Amersham).

For all experiments in this study, multiple dilutions of protein samples were analyzed by Western analysis to ensure the accuracy of determination of the Pab1p levels. In general, ~0.5% of each sample gave linear Western signals. Either Pab1p monoclonal or polyclonal (Sachs *et al.*, 1986) antibodies were used in the Pabp Westerns.

Recombinant Tif4631p and Tif4632p studies

Bacterial strain BL21 containing the GST fusion proteins (Tables II, III and IV) were induced at OD_{600} = 0.5 for 3–4 h with 0.5 mM IPTG, harvested, washed once in buffer C (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3), and quick-frozen in liquid nitrogen in 1/100 the original growth volume of buffer C. Following the thawing of 2 ml aliquots, cell suspensions were adjusted to 0.1% Triton X-100 final and lysed gently by brief sonication in ice-water. Lysates were clarified by centrifugation at 10 000 *g* for 5 min and then bound to 100 µl of 50% suspension of glutathione–Sepharose 4B resin (Pharmacia) pre-equilibrated in buffer CT (buffer C + 0.1% Triton X-100). Following three washes with 200 µl of buffer CT, the fresh resin was used in the listed experiments.

For the experiments in Figure 4, 10 µl of resin bound to the different proteins suspended in 200 µl of buffer CT suspension was transferred to a fresh microfuge tube and incubated for 60 min by gentle rocking at 4°C with either 15 µg of recombinant Pab1p (Sachs *et al.*, 1987) or 500 µl (~7.5 mg/ml) of S30 extract prepared as in Tarun and Sachs (1995). (Each 10 µl aliquot of resin contained ~3 µg of full-length fusion protein. However, the large number of smaller products made it difficult to quantify the total amount of fusion protein in each sample.) The resin was washed five times in 200 µl of buffer CT, and the bound proteins were eluted in 50 µl of SDS–PAGE loading buffer. For RNase T1 treatment, ~20 µl of GST resin (50% slurry) bound to the indicated proteins was incubated at 26°C for 30 min with 10 000 U of RNase T1 (Sigma) in 100 µl of buffer C. RNase T1 was removed by five 200 µl washes with buffer CT. For the experiment described in Figure 6D, 10 µl of resin-bound Tif4632-201/315p or GST was incubated with crude extract and processed as described above.

For the experiments described in Figure 2B and Figure 5C, ~2 µg of recombinant eIF-4E was used (a kind gift from J.McCarthy), and binding and elution were carried out in a manner identical to that used for Pab1p in Figure 4, lane 1. This recombinant protein was prepared from a bacteria overexpressing yeast eIF-4E following a published procedure

(Edery *et al.*, 1988) and shown to partially rescue the translational deficiency of a *cdc33-1* translation extract (data not shown).

The [^{32}P]poly(A) used in experiments shown in Figures 5 and 6 was synthesized by using yeast poly(A) polymerase (USB), oligo(A)$_{12}$ primer and [α-^{32}P]ATP ([^{32}P]ATP:ATP ratio of 1:200). Free nucleotides were removed by spin-column chromatography, and ~50 000 c.p.m. of polymer was or was not mixed with 10 μg of the indicated Pab1ps and then added to 10 μl of the indicated resins in a final volume of 200 μl of buffer CT. Following binding for 1 h at 4°C, the resins were washed five times with 1 ml of buffer CT, and the remaining associated [^{32}P]poly(A) was quantified by direct scintillation counting of the resin.

For the experiments described in Figure 6B and 6C, 10, 50 or 100 μg of dialyzed poly(A) (average length >200 nucleotides) (Sigma) with or without 10 μg of Pab1p was incubated with 20 μl of RNase T1-treated resin (50% slurry) containing Tif4632-201/315p (~10 μg of fusion protein) in a final volume of 200 μl for 60 min with gentle rocking at 4°C. Following five 1 ml washes with buffer CT, bound proteins were eluted in SDS sample buffer and then detected by Coomassie blue staining after resolution on 10% SDS–PAGE gels. Eight μl of poly(A)–Sepharose beads (50% slurry) (Pharmacia) containing 3.7 μg of poly(A) was incubated with Pab1p and the indicated amounts of poly(A) (Figure 6C) and processed identically to the resin containing Tif4632-201/315p. Pab1p binds to 25 adenine residues (Sachs *et al.*, 1987). Therefore 100 μg of poly(A) (303 nmol of adenine) contains at least 12 nmol of Pab1p binding sites. This represents an 80-fold excess of Pab1p binding sites when 10 μg (154 pmol) of Pab1p are used in the reaction. All chromatographic procedures were performed at 4°C.

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