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The yeast poly(A)-binding protein Pab1p stimulates *in vitro* poly(A)-dependent and cap-dependent translation by distinct mechanisms

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Translation initiation in extracts from *Saccharomyces cerevisiae* involves the concerted action of the cap-binding protein eIF4E and the poly(A) tail-binding protein Pab1p. These two proteins bind to translation initiation factor eIF4G and are needed for the translation of capped or polyadenylated mRNA, respectively. Together, these proteins synergistically activate the translation of a capped and polyadenylated mRNA. We have discovered that excess Pab1p also stimulates the translation of capped mRNA in extracts, a phenomenon that we define as *trans*-activation. Each of the above activities of Pab1p requires its second RNA recognition motif (RRM2). We have found that RRM2 from human PABP cannot substitute functionally for yeast RRM2. Using the differences between human and yeast RRM2 sequences as a guide, we have mutagenized yeast RRM2 and discovered residues that are required for eIF4G binding and poly(A)-dependent translation but not for *trans*-activation. Similarly, other residues within RRM2 were found to be required for *trans*-activation but not for eIF4G binding or poly(A)-dependent translation. These data show that Pab1p has at least two biochemically distinct activities in translation extracts.

Keywords: poly(A)/RNA-binding protein/translation/translation initiation factors/yeast

Introduction

Eukaryotic translation initiation begins with the binding of the small ribosomal subunit to the 5′ end of mRNA near the cap structure, and scanning along the mRNA by this subunit until the translation initiation codon is identified (reviewed in Merrick and Hershey, 1996). Binding of the ribosomal subunit to the mRNA requires the concerted activities of many translation initiation factors, including the eIF4F complex. In eukaryotes, eIF4F contains the cap-binding protein eIF4E, the adaptor protein eIF4G and the RNA-stimulated ATPase eIF4A. These proteins help to create an optimal binding surface for the ribosomal subunit at the 5′ end of the mRNA (reviewed in Gingras *et al*., 1999).

Besides the addition of the cap structure to the 5′ end, the 3′ end of the majority of mRNAs is modified by the addition of a poly(A) tail (reviewed in Keller and Minvielle Sebastia, 1997). Many observations have shown that translation can be influenced by this poly(A) tail (reviewed in Jacobson, 1996; Sachs *et al*., 1997). For example, during early development, the addition of a poly(A) tail to certain maternal mRNAs can stimulate their translation, while removal of the poly(A) tail from another subset of mRNAs results in their masking from the translational machinery (Jackson and Wickens, 1997). Furthermore, mRNAs which are polyadenylated but have no 5′ cap are translated more efficiently in *in vitro* systems than non-capped, poly(A)-deficient mRNAs (Iizuka *et al*., 1994). Lastly, experiments performed both *in vivo* and *in vitro* have revealed that the cap and poly(A) tail can act synergistically to stimulate translation (Gallie, 1991; Iizuka *et al*., 1994).

It was assumed that the effect of the poly(A) tail in translation would be mediated by the poly(A)-binding protein (Pab1p), the major protein associated with poly(A) tails, as *Saccharomyces cerevisiae pab1* mutants exhibited translational defects, and suppressors of this phenotype also led to alterations in ribosomal subunits (Sachs and Davis, 1989, 1990). The development of yeast translation extracts which were sensitive to the effects of the poly(A) tail (Iizuka *et al*., 1994) allowed for the direct investigation of the role of the yeast Pab1p in translation (Tarun and Sachs, 1995). It was shown that immunodepletion or immunoneutralization of Pab1p in the extract destroyed poly(A)-dependent translation, defined as the translation of an mRNA that has a poly(A) tail but no 5′ cap. This occurs by preventing the small ribosomal subunit binding to the mRNA (Tarun and Sachs, 1995). Addition of recombinant Pab1p to the inactivated extract restored this activity. The discovery that Pab1p interacted with eIF4G suggested that Pab1p could stimulate translation initiation in a similar manner to eIF4E (Tarun and Sachs, 1996). This interaction was indeed found to be an essential requirement *in vitro* for poly(A)-dependent translation (Tarun *et al*., 1997). It was shown subsequently by atomic force microscopy that it is possible to make circular a capped, polyadenylated RNA in the presence of recombinant eIF4E, eIF4G and Pab1p, thus demonstrating a physical interaction between the two ends of mRNA (Wells *et al*., 1998). An interaction between human eIF4G and the human poly(A)-binding protein (hPABP) recently has been shown to occur, further reinforcing the importance of the poly(A)-binding protein and the poly(A) tail in translation throughout eukaryotes (Imataka *et al*., 1998; Piron *et al*., 1998).

Yeast Pab1p is an essential protein that consists of four N-terminal RNA recognition motifs (RRMs), which are highly conserved in sequence between different species, and a more divergent C-terminal domain (Adam *et al*., 1986; Sachs *et al*., 1986). The RRM is found in a number of different RNA-binding proteins. The structure of the

U1-A RRM has been solved (Nagai *et al*., 1990). It is believed that all RRMs assume a similar fold and, by alignment of different RRM sequences, it is possible to predict the boundaries of the different elements of the RRM structure (Kenan *et al*., 1991). There is a certain amount of functional redundancy between the Pab1p RRMs, as any individual RRM can be removed and the mutant protein will still support viability (Sachs *et al*., 1987; Kessler and Sachs, 1998). However, simultaneous mutations in RRM2 and RRM4 are lethal (Deardorff and Sachs, 1997). RRM2 of yeast Pab1p contains the high-affinity poly(A)-binding site, is essential for Pab1p to function in poly(A)-dependent translation and is needed for eIF4G binding (Deardorff and Sachs, 1997; Kessler and Sachs, 1998). Among the four RRMs, point mutations in RRM4 have the greatest effect on non-poly(A) RNA binding by Pab1p (Deardorff and Sachs, 1997).

In addition to playing a role in poly(A)-dependent translation, Pab1p also contributes to the synergistic activation of translation which occurs when an mRNA possesses both a cap structure and a poly(A) tail (Gallie, 1991; Tarun and Sachs, 1995). Synergy is defined as the amount of translation of a capped, polyadenylated mRNA divided by the sum of translation of a capped mRNA and that of a polyadenylated mRNA. Thus far, it is known that mutations within RRM2 of Pab1p, eIF4E or the 300 amino acids at the N-terminus of eIF4G disrupt synergy (Tarun and Sachs, 1995; Tarun *et al*., 1997; Kessler and Sachs, 1998). However, the mechanism by which synergy is induced has not been elucidated. A number of different mechanisms are possible, including an enhanced affinity of the initiation factors for each other when they are all present, or the activation of an enzymatic activity, such as the ATPase within eIF4A, when all of the factors have bound.

It is also unclear whether Pab1p plays distinct roles in poly(A)-dependent translation and in mediating synergy. For example, is an interaction between Pab1p and eIF4G required for synergy, as it is for poly(A)-dependent translation, or might the critical process involve binding of Pab1p to another factor recruited in a cap-dependent and, perhaps, eIF4G-dependent manner? It had been shown previously that addition of exogenous poly(A) to rabbit reticulocyte lysates stimulated the translation of capped, poly(A)-deficient mRNA (Jacobson and Favreau, 1983; Munroe and Jacobson, 1990). It was believed that this effect was due to unbound Pab1p binding to the poly(A) and acting *in trans* to stimulate translation. It is possible that this *trans*-activation occurs via the same mechanism as synergy. Therefore, if addition of excess Pab1p were to stimulate translation of capped mRNA, it might be possible to utilize this phenomenon to study the mechanism by which Pab1p induces synergy. Such an assay would be invaluable since it would provide a tool to study the role of Pab1p in translational synergy separately from the role of Pab1p in the stimulation of poly(A)-dependent translation.

Here we report that the addition of recombinant Pab1p to a yeast translation extract stimulated the translation of capped, poly(A)-deficient mRNA. RRM2 and RRM4 were required for this effect. Replacement of yeast RRM2 by human RRM2 destroyed the ability of Pab1p to stimulate cap-dependent translation and also prevented poly(A)-dependent translation and eIF-4G binding. The differences in sequence between human and yeast RRM2 were utilized to develop a series of Pab1 proteins containing various amino acid substitutions within RRM2. These mutations made it possible to determine that the regions of Pab1p needed for this *trans*-activation of capped mRNA were different from those required for Pab1p to mediate poly(A)-dependent translation. Furthermore, we discovered a mutated form of Pab1p that neither bound to eIF4G nor mediated poly(A)-dependent translation, but was capable of stimulating cap-dependent translation. These data suggest that Pab1p can stimulate translation *in vitro* by at least two mechanisms that are biochemically separable. In addition, they provide the basis for a new assay that may be useful in future studies directed at understanding translational synergy.

Results

Recombinant Pab1p can stimulate translation of capped, poly(A)-deficient mRNA

The role of Pab1p in poly(A)-dependent translation is well established, but it was unclear whether Pab1p could also act *in trans* to influence the translation of a message to which it was not bound via a poly(A) tail. In order to address this issue, recombinant Pab1p was added to a translation extract in which expression of capped luciferase (capLUC) mRNA was being measured. When increasing amounts of Pab1p were added to the translation extract, an increase in the amount of translation of the capLUC mRNA was observed (Figure 1A). The addition of 30 pmol of recombinant Pab1p, approximately equal to the amount of endogenous Pab1p present in the extract (Tarun and Sachs, 1995), resulted in a 5-fold stimulation of cap-dependent translation. We will refer to this effect as *trans*-activation. As the mRNA was not polyadenylated, the effect of Pab1p on its expression cannot be due to its binding *in cis* to a poly(A) tail. In addition, a mutated version of Pab1p, Pab1-6p, which exhibits a decreased affinity for poly(A) (Deardorff and Sachs, 1997), was competent for *trans*-activation (data not shown). Another mutated version of Pab1p, Pab1-8p, in which non-specific RNA binding has been reduced significantly (Deardorff and Sachs, 1997), was also capable of *trans*-activation (data not shown). This suggests that Pab1p is not binding non-specifically within the body of the mRNA, but is indeed acting *in trans*. It is unclear whether the excess Pab1p is acting as a free protein, or as a complex with the endogenous poly(A) in the extract. It is also unclear, as yet, whether the phenomenon of *trans*-activation is restricted to the stimulation of translation of capLUC mRNA or whether it would also be observed for other capped, poly(A)-deficient mRNAs.

In order to determine which regions of Pab1p are required for *trans*-activation, a series of deletion mutants of Pab1p were utilized in which each of the four RRMs and the C-terminal domain have been deleted in turn (Kessler and Sachs, 1998). The recombinant mutant proteins were added to translation extracts and their effects on the translation of capLUC mRNA were examined as above. Deletion of either RRM2 or RRM4 abolished the ability of Pab1p to stimulate cap-dependent translation (Figure 1B). When RRM1,

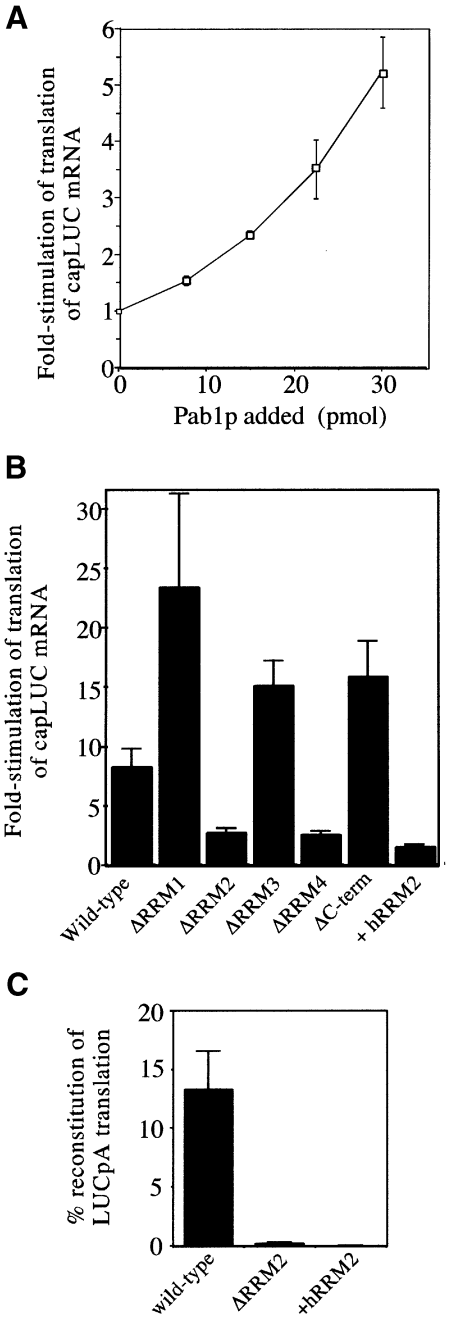

Fig. 1. Stimulation of cap- and poly(A)-dependent *in vitro* translation by Pab1p. (**A**) Pab1p can stimulate cap-dependent translation. Wild-type translation extracts were incubated with between 0 and 30 pmol of recombinant Pab1p, and then the translation of capLUC mRNA was measured. The fold stimulation of translation of capLUC mRNA was calculated. (**B**) RRM2 and 4 of Pab1p are required for stimulation of cap-dependent translation. Translation extracts were incubated with 30 pmol of each of the indicated Pab1p mutants, and translation of capLUC mRNA was then measured. (**C**) The RRM2 of human PABP cannot function in yeast Pab1p to reconstitute poly(A)-dependent translation. Pab1p-immunoneutralized yeast translation extracts were incubated with between 0 and 22.5 pmol of the indicated recombinant Pab1p, and then the translation of LUCpA mRNA was measured. The percentage translational activity of non-neutralized extracts achieved upon addition of 15 pmol of Pab1p is shown. ΔRRM2, Pab1p lacking RRM2; +hRRM2, Pab1p containing hPABP RRM2.

RRM3 or the C-terminal domain were deleted, the stimulatory activity of Pab1p increased. The underlying cause of this effect remains unknown. The above data suggest that the stimulation of cap-dependent translation by Pab1p requires RRM2 and RRM4.

RRM2 of human PABP cannot mediate poly(A)-dependent translation or stimulate cap-dependent translation

Treatment of a yeast translation extract with a monoclonal antibody to Pab1p destroys the ability of the extract to perform poly(A)-dependent translation (Tarun and Sachs, 1995). This activity can be restored by the addition of recombinant Pab1p but not by the addition of Pab1p lacking RRM2 (Kessler and Sachs, 1998). Thus, these immunoneutralized extracts can be used to study the ability of different mutated forms of Pab1p to mediate poly(A)-dependent translation. The lack of a correlation between the ability of mutated Pab1 proteins to bind to the antibody and their ability to reconstitute poly(A)-dependent translation previously has addressed the possibility that reconstitution could result from a competitive displacement of the endogenous Pab1p from the antibody (Kessler and Sachs, 1998).

It was of interest to determine whether RRM2 from other eukaryotic poly(A)-binding proteins also possessed the ability to perform poly(A)-dependent translation and *trans*-activation within yeast translation extracts. We chose to study RRM2 from hPABP since we had found that the hPABP open reading frame could functionally replace yeast Pab1p when it was expressed on a multicopy plasmid (data not shown). In order to focus specifically on the RRM2 of hPABP, a chimeric protein was made consisting of RRM2 from hPABP with RRMs 1, 3 and 4 and the C-terminal domain derived from yeast Pab1p. The ability of this recombinant hybrid protein to restore poly(A)-dependent translation in an immunoneutralized yeast extract was examined. Wild-type Pab1p gave 14% reconstitution. This value is lower than that previously reported (Tarun and Sachs, 1995) as the degree of possible reconstitution varies between different extract preparations. No reconstitution of poly(A)-dependent translation was seen upon addition of either Pab1-ΔRRM2p or the hybrid protein, Pab1–hRRM2p (Figure 1C). This would suggest that human RRM2 cannot substitute functionally for yeast RRM2 with respect to its involvement in poly(A)-dependent translation.

The ability of the hybrid protein to stimulate cap-dependent translation was also examined. Addition of the recombinant Pab1–hRRM2p had no effect on translation of the capLUC mRNA (Figure 1B). Therefore, human RRM2 is also incapable of acting in place of yeast RRM2 in the stimulation of cap-dependent translation.

Translational properties of extracts derived from pab1 mutants

Yeast Pab1p and hPABP are highly conserved in sequence throughout the region containing the four RRMs. However, it has been demonstrated above that key differences must exist between the RRM2 sequences of these proteins since human RRM2 fails to function in place of yeast RRM2 in both poly(A)-dependent translation and *trans*-activation. We decided to introduce the non-conserved amino acids of human RRM2 systematically into yeast RRM2 in order to identify those amino acids in yeast Pab1p that are required for these functions. This analysis

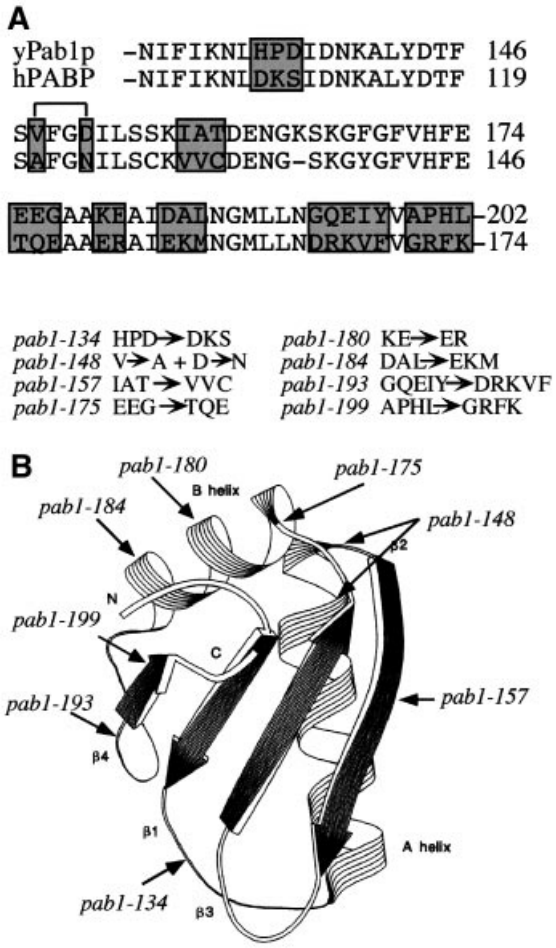

Fig. 2. Mutagenesis of Pab1p RRM2. (**A**) Alignment of yeast Pab1p and human PABP RRM2. The majority of amino acids in RRM2 are conserved between yeast and human. The regions chosen for mutagenesis are highlighted and outlined. The mutagenesis performed and names of the mutant alleles are shown beneath the alignment. (**B**) Positions of the RRM2 mutations relative to the structure of the U1-A RRM. The alignment of RRM sequences from Kenan *et al.* (1991) was used to place the mutations onto the U1-A RRM structure (Nagai *et al*., 1990).

was designed to reveal whether the involvement of Pab1p in poly(A)-dependent translation and in the stimulation of cap-dependent translation requires different residues within RRM2.

Figure 2A shows an alignment of yeast and human RRM2. Eight blocks of amino acids were chosen for mutagenesis. The human residues were introduced into the yeast protein. The changes which are relatively conservative were not investigated. The likely positions of these mutations were determined using the structure of the U1-A RRM (Nagai *et al*., 1990) as a template and the RRM alignments of Kenan *et al*. (1991) (Figure 2B). It can be seen that the mutations were concentrated in the upper portion of the RRM. The two central β-strands, which contain the RNP1 and RNP2 motifs, were not mutated at all. These regions are completely conserved between the yeast and human proteins.

The mutant *pab1* alleles on a yeast centromeric plasmid were introduced into a yeast strain deleted for *PAB1* in the genome (see Materials and methods). All eight mutant genes gave viable strains. These strains all grew at nearly the same rate as the wild-type strain in YPD at 30°C, with doubling times of 1.6–1.8 h. The viability of these cells is not surprising since deletion of RRM2 within Pab1p only results in a moderate slow-growth phenotype (Kessler and Sachs, 1998).

Translation extracts were prepared from each of the mutant strains by liquid nitrogen lysis. In this new method, lysis is performed by crushing the yeast in liquid nitrogen using a pestle and mortar. After lysis, the extracts are thawed on ice. As a result, heating of the extract, which may occur during the original bead-beating method of extract preparation, is avoided. The liquid nitrogen lysis method appeared to give extracts which were more translationally active and which showed a higher and more reproducible degree of synergy.

The ability of the mutant extracts to translate luciferase mRNA containing a cap, a poly(A) tail, both or neither (capLUC, LUCpA, capLUCpA and LUC, respectively) was assayed, and these results were compared with those obtained for wild-type and *pab1*-ΔRRM2 extracts. The most striking changes in the extracts derived from the point mutants were the effects on poly(A)-dependent translation (Figure 3; Table I). Three of the point mutant extracts showed reduced levels of translation of the LUCpA mRNA in comparison with the translation of capLUC mRNA. In *pab1-148*, the translation of LUCpA mRNA was somewhat reduced; in *pab1-180*, this reduction was more severe; while in *pab1-184*, there was virtually no translation of LUCpA mRNA. The failure of the *pab1-184* extract to exhibit poly(A)-dependent translation was a direct consequence of the mutation in Pab1p, rather than an indirect effect upon another translation initiation factor, because addition of recombinant Pab1p to the extract restored poly(A)-dependent translation (data not shown).

The ratio of the capLUCpA mRNA translation to the sum of capLUC mRNA and LUCpA mRNA translation gives the amount of synergy in the extract. With the exception of the *pab1-184* mutant extract, all of the extracts showed 4.5- to 8.5-fold synergy (Figure 3). The *pab1-184* mutation gave the most severe phenotype, probably because it completely prevents poly(A)-dependent translation, and we assume that a minimum amount of this activity is required in order to observe synergy (see Discussion). The characteristics of this extract were identical to those observed in the extract containing Pab1p-ΔRRM2, where there is no synergy and no poly(A)-dependent translation. The *pab1-184* mutation thus appears to have affected a key functional site within RRM2 of Pab1p. This will be analyzed further in the following sections.

Effects of mutagenesis of RRM2 on in vitro reconstitution of poly(A)-dependent translation

In order to examine the behavior of the mutant Pab1 proteins in the *in vitro* assays for Pab1p activity, recombinant versions of these proteins were purified. The ability of the recombinant proteins to restore poly(A)-dependent translation in an immunoneutralized extract was examined. Three of the mutated proteins (Pab1-134p, Pab1-193p and Pab1-199p) reconstituted poly(A)-dependent translation to the same extent as the wild-type

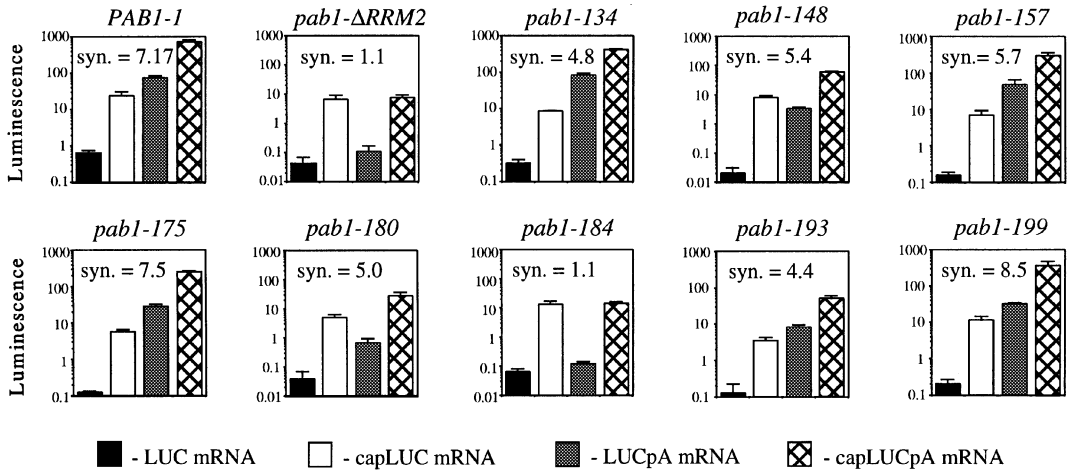

Fig. 3. Characterization of translation extracts containing the mutated *pab1* alleles. Translation extracts were prepared by liquid nitrogen lysis from yeast strains containing the indicated *pab1* gene as the sole source of Pab1p. The ability of these extracts to translate capLUCpA, capLUC, LUCpA and LUC mRNAs was then measured. The synergy in each extract was calculated by dividing the value for translation of the capLUCpA mRNA by the sum of the values for translation of capLUC and LUCpA mRNAs.

Table I. Summary of data for Pab1p point mutants

Pab1p protein	Ratio of LUCpA to capLUC translation[a]	Synergy in translation extract	*In vitro* reconstitution of poly(A)-dependent translation[b]	Fold stimulation of cap-dependent translation[c]	eIF4G binding
Pab1p	3.13	7.17	13.27 ± 3.32	8.3 ± 1.59	yes
Pab1-ΔRRM2p	0.02	1.1	0.02 ± 0.1	1.44 ± 0.27	no
Pab1-134p	9.4	4.8	10.04 ± 2.75	1.56 ± 0.62	yes
Pab1-148p	0.42	5.4	2.63 ± 1.33	4.52 ± 0.61	yes
Pab1-157p	6.6	5.7	2.37 ± 0.66	1.76 ± 0.62	yes
Pab1-175p	5.0	7.5	1.76 ± 1.62	1.87 ± 0.13	yes
Pab1-180p	0.13	5.0	0.00 ± 0.28	1.92 ± 0.17	no
Pab1-184p	0.01	1.1	0.00 ± 0.09	4.42 ± 0.51	no
Pab1-193p	2.3	4.4	13.56 ± 1.34	5.47 ± 0.7	yes
Pab1-199p	2.8	8.5	7.532 ± 1.23	15.13 ± 3.98	yes

[a]The ratio of LUCpA to capLUC translation is given to eliminate variations in the absolute activities of the extracts.
[b]Percentage reconstitution of LUCpA translation/15 pmol of Pab1p added.
[c]Fold stimulation of cap-dependent translation is calculated relative to the amount of capLUC translation seen with no added Pab1p.

protein (Figure 4; Table I). Pab1-148p, Pab1-157p and Pab1-175p also stimulated poly(A)-dependent translation, but to a lesser degree than the wild-type protein. As poly(A)-dependent translation was not reduced in the translation extracts derived from the *pab1-157* and *pab1-175* strains (Figure 3), we cannot conclude that these proteins are defective for poly(A)-dependent translation. Pab1-148p was reduced for poly(A)-dependent translation in *pab1-148* extracts (Figure 3) and in the *in vitro* reconstitution assay (Figure 4) and, therefore, probably lacks residues that contribute to poly(A)-dependent translation.

Pab1-180p and 1-184p were incapable of reconstituting poly(A)-dependent translation in the immunoneutralized extract (Figure 4; Table I). Translation of LUCpA mRNA was also affected in the extracts from both *pab1-180* and *pab1-184* strains (Figure 3). In the *pab1-180* extract, there was slightly more measurable poly(A)-dependent translation than in the *pab1-184* extract, suggesting that Pab1-180p may have a small degree of activity in its native context. Pab1-184p, however, was completely inactive for poly(A)-dependent translation both in the extracts and when added as a recombinant protein. This suggests

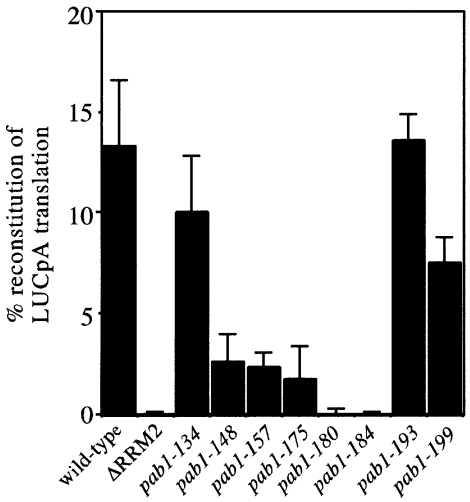

Fig. 4. Ability of the mutated Pab1 proteins to reconstitute poly(A)-dependent translation. Recombinant Pab1 proteins (0–22.5 pmol) were incubated with Pab1p-immunoneutralized translation extracts which had been nuclease treated, and translation of LUCpA mRNA was then measured. The percentage of the activity measured in non-neutralized extracts is shown.

that Pab1-184p, and possibly Pab1-180p, lacks essential residues for stimulating poly(A)-dependent translation.

Poly(A)-dependent translation and Pab1p stimulation of cap-dependent translation have different functional requirements

The ability of the recombinant mutated Pab1 proteins to stimulate cap-dependent translation was also investigated. Addition of these proteins to translation extracts in which the translation of capLUC mRNA was being measured gave a very different pattern of results to that observed for the reconstitution of poly(A)-dependent translation. Four of the mutated proteins (Pab1-148p, Pab1-184p, Pab1-193p and Pab1-199p) stimulated capLUC mRNA translation, while the remaining proteins (Pab1-134p, Pab1-157p, Pab1-175p and Pab1-180p) were unable to do so (Figure 5A; Table I). The amount of stimulation of translation shown is for the highest amount of Pab1p used. For those proteins showing no stimulation, lower concentrations of protein also had no effect (data not shown).

These data show that there is no direct correlation between the ability of the mutated proteins to support poly(A)-dependent translation and their ability to stimulate cap-dependent translation. Notably, Pab1-184p, which did not allow for poly(A)-dependent translation, did stimulate cap-dependent translation. In addition, it appears to be possible to disrupt the ability of Pab1p to stimulate cap-dependent translation without affecting its ability to reconstitute poly(A)-dependent translation. Specifically, three of the mutated proteins (Pab1-134p, Pab1-157p and Pab1-175p) restored poly(A)-dependent translation but did not stimulate cap-dependent translation. Particularly striking is Pab1-134p, which restored poly(A)-dependent translation to the same extent as wild-type Pab1p in the *in vitro* reconstitution assay but had no effect on cap-dependent translation. Therefore, we conclude that these two activities of Pab1p in the *in vitro* translation assay are separable and likely to be mechanistically distinct.

The data for the translation extracts prepared from the different *pab1* mutant strains suggest that endogenous Pab1p may also affect cap-dependent translation. An increase in the ratio of LUCpA mRNA to capLUC mRNA translation was observed in extracts from three of the strains (*pab1-134*, *pab1-157* and *pab1-175*) (Figure 3; Table I). As it is very difficult to compare absolute values between different extracts, it is not possible to say whether these strains have elevated poly(A)-dependent translation or reduced cap-dependent translation. However, the recombinant Pab1-134p, Pab1-157p and Pab1-175p proteins were incapable of stimulating cap-dependent translation (Figure 5; Table I) and had either normal or slightly reduced abilities to restore poly(A)-dependent translation (Figure 4; Table I). Thus, it is more likely that the increase in the ratio of LUCpA mRNA to capLUC mRNA translation in the extracts is due to a reduction in cap-dependent translation. These results are consistent with the previous observation that there is a mild inhibition of cap-dependent translation in Pab1p-immunoneutralized extracts (Tarun and Sachs, 1995).

Identification of amino acids essential for eIF4G binding by Pab1p

In order for Pab1p to support poly(A)-dependent translation, an interaction between Pab1p and the translation initiation factor eIF4G is required. Is this interaction also required for Pab1p stimulation of cap-dependent translation? Binding of eIF4G by Pab1p is known to occur through RRM2 (Kessler and Sachs, 1998). If RRM2 of hPABP is incapable of binding to yeast eIF4G, then eIF4G binding should be affected in one or more of the Pab1p point mutants described above.

To measure Pab1p binding to eIF4G, recombinant Pab1p is incubated, in the presence of poly(A), with 115 amino acid Pab1p-binding fragments of either eIF4G1 or eIF4G2 fused at their N-termini to glutathione *S*-transferase (GST) (Tarun and Sachs, 1996; Tarun *et al*., 1997). eIF4G1 and eIF4G2 are the two yeast isoforms of eIF4G and are encoded by the functionally redundant *TIF4631* and *TIF4632* genes, respectively (Goyer *et al*., 1993). The

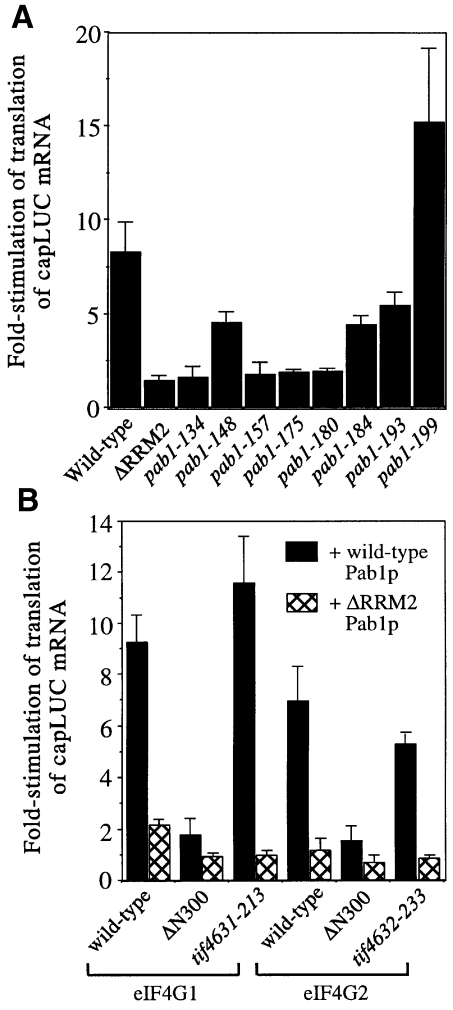

Fig. 5. Analysis of the requirements for Pab1p stimulation of cap-dependent translation. (**A**) Ability of the mutated Pab1 proteins to stimulate cap-dependent translation. The fold stimulation of cap-dependent translation was measured upon addition of 30 pmol of recombinant protein to aliquots of wild-type translation extracts. (**B**) eIF4G is required for stimulation of cap-dependent translation by Pab1p. Translation extracts were prepared from yeast strains containing either eIF4G1 (YAS2069) or eIF4G2 (YAS1981), eIF4G1-ΔN300 (YAS2071) or eIF4G2-ΔN300 (YAS1984), which are proteins lacking their N-terminal 300 amino acids, and eIF4G1-213 (YAS2075) or eIF4G2-233 (YAS2001), which are proteins with reduced Pab1p binding (Tarun *et al*., 1997). Aliquots of these extracts were incubated with 30 pmol of either wild-type or Pab1-ΔRRM2p, and capLUC mRNA translation was then measured.

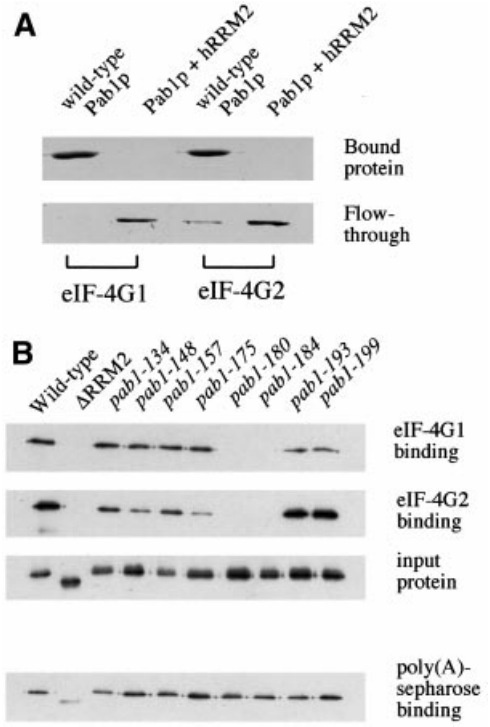

Fig. 6. Identification of amino acids within Pab1p involved in eIF4G binding. (**A**) Human PABP RRM2 cannot bind to yeast eIF4G. A 150 pmol aliquot of either recombinant wild-type Pab1p or the hybrid Pab1p containing human RRM2 was incubated with the immobilized Pab1p-binding fragments of eIF4G1 and eIF4G2, and poly(A). Proteins remaining associated with the eIF4G proteins (upper panel) or not binding to the eIF4G proteins (lower panel) were resolved by SDS–PAGE and detected by Coomassie Brilliant Blue staining. (**B**) Ability of the Pab1p point mutants to bind eIF4G. Each of the indicated Pab1 proteins were incubated with immobilized eIF4G1 or eIF4G2, and poly(A). The upper two panels show binding of the mutants to eIF4G1 and eIF4G2. Aliquots of each binding reaction (input protein) were also analyzed to ensure that equal amounts of the Pab1 proteins were used. In the bottom panel, the ability of the mutated Pab1 proteins to bind to poly(A)-Sepharose is shown (see Materials and methods). Bound proteins were resolved by SDS–PAGE and detected by Western blotting with a Pab1p polyclonal antibody.

ability of Pab1–hRRM2p to bind to eIF4G was measured using this assay. While wild-type Pab1p bound well to the eIF4G Pab1p-binding fragments, the Pab1–hRRM2p did not bind to either eIF4G1 or eIF4G2 (Figure 6A). These data show that RRM2 of human PABP is incapable of interacting with yeast eIF4G.

The ability of the mutated yeast Pab1p proteins to bind eIF4G was then analyzed using the *in vitro* binding assay. The mutated proteins fell into three categories (Figure 6B; Table I). Two of the proteins (Pab1-193p and Pab1-199p) bound with wild-type affinity to both eIF4G1 and eIF4G2. Others (Pab1-134p, Pab1-148p, Pab1-157p and Pab1-175p) had a wild-type affinity for eIF4G1 but a somewhat reduced affinity for eIF4G2. Pab1-180p and Pab1-184p bound to neither eIF4G1 nor eIF4G2.

The interaction between Pab1p and GST–eIF4G is known to be dependent on the binding of Pab1p to poly(A) (Tarun and Sachs, 1996). It is possible that the absence of binding of Pab1-180p and Pab1-184p to eIF4G was due to an inability to bind poly(A). In order to test this possibility, the ability of the recombinant proteins to bind to poly(A)-Sepharose was examined (Kessler and Sachs, 1998). Each of the mutated proteins bound to the poly(A)-Sepharose to nearly the same degree as wild-type Pab1p (Figure 6B, bottom panel). The Pab1-ΔRRM2 protein bound less well to the poly(A)-Sepharose, which was expected since RRM2 contains the high-affinity poly(A)-binding site for Pab1p (Deardorff and Sachs, 1997; Kessler and Sachs, 1998). Thus, poly(A) binding is not severely affected in the mutated proteins and therefore cannot be the cause underlying the above observations. These data show that mutations within Pab1-180p and Pab1-184p prevent eIF4G binding *in vitro*.

Stimulation of cap-dependent translation does not correlate with ability to bind eIF4G in vitro

The above results suggested that an inability of Pab1p to bind eIF4G does not necessarily correlate with an inability to stimulate cap-dependent translation. Specifically, Pab1-184p did not bind eIF4G but did stimulate cap-dependent translation. In order to confirm this lack of correlation, extracts from a series of eIF4G mutants that interact poorly with Pab1p and do not exhibit significant poly(A)-dependent translation were analyzed for their ability to be *trans*-activated by Pab1p (Tarun *et al*., 1997). Deletion of the first 300 amino acids of either eIF4G1 or eIF4G2 removes the Pab1p-binding site, while mutation of a conserved motif within this binding site (*tif4631-213* and *tif4632-233*) also disrupts Pab1p binding (Tarun *et al*., 1997).

Pab1p stimulated cap-dependent translation within extracts containing only wild-type eIF4G1 or eIF4G2 (Figure 5B). As expected, the Pab1-ΔRRM2 protein had no stimulatory effect. Extracts prepared from *tif4631-213* and *tif4632-233* strains still exhibited *trans*-activation of capLUC mRNA translation by Pab1p (Figure 5B). This observation confirms the conclusions drawn above that a normal interaction between Pab1p and eIF4G is not essential for Pab1p to stimulate cap-dependent translation. However, deletion of the first 300 amino acids of either eIF4G1 or eIF4G2 destroyed the ability of Pab1p to stimulate cap-dependent translation. This suggests that eIF4G is still required for *trans*-activation by Pab1p for reasons other than Pab1p binding. The need for eIF4G in *trans*-activation also provides good evidence that this process is occurring at the initiation step of translation.

Discussion

In this study, we have shown that Pab1p is capable of acting *in trans* to stimulate cap-dependent translation. We have analyzed which amino acids in Pab1p are required for poly(A)-dependent translation and stimulation of cap-dependent translation, and have found that the two activities do not have the same requirements. In addition, we have identified amino acids which are needed for Pab1p binding to eIF4G. While poly(A)-dependent translation has an absolute requirement for eIF4G binding, this is not true for the stimulation of cap-dependent translation by Pab1p. These data suggest that Pab1p is utilizing different mechanisms to support poly(A)-dependent translation and to stimulate cap-dependent translation.

How might Pab1p be functioning differently to stimulate cap-dependent translation? Translation initiation depends on the formation of a complex comprising

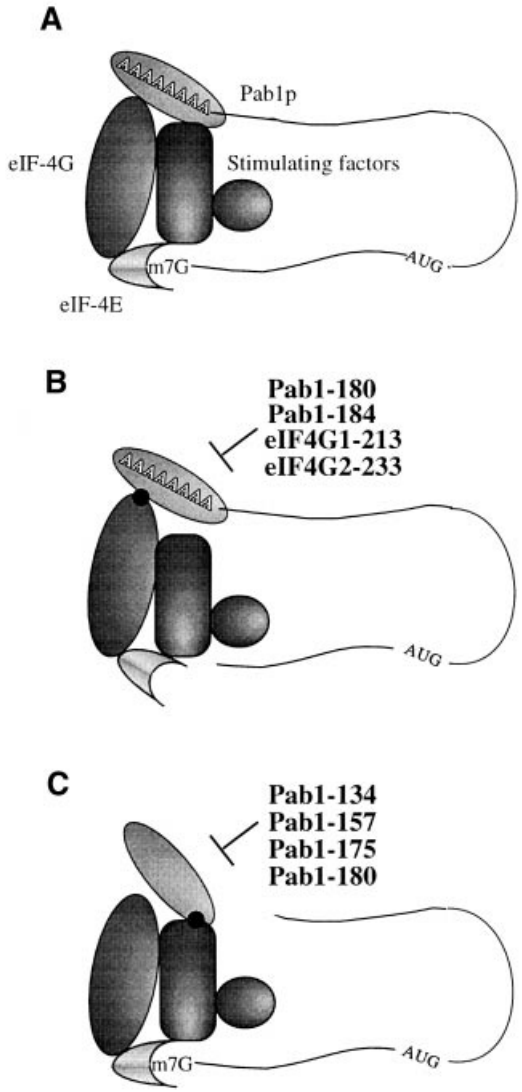

Fig. 7. Models depicting interactions of Pab1p with other translation initiation factors on various mRNA substrates. (**A**) Capped and polyadenylated mRNA. In the presence of both a cap and a poly(A) tail, eIF4E, eIF4G and Pab1p act cooperatively to stimulate translation initiation. Pab1p makes multiple contacts within the initiation complex. (**B**) Polyadenylated mRNA. In the absence of a cap structure, eIF4G is only recruited to mRNA by its interaction with Pab1p. With the mutated proteins Pab1-180p, Pab1-184p, eIF4G1-213p and eIF4G2-233p, this interaction is reduced and, therefore, these proteins are unable to support significant poly(A)-dependent translation. The interaction between Pab1p and the additional stimulatory factor(s) has been omitted from this diagram since it may depend on binding of eIF4E to the cap structure. (**C**) Capped mRNA. Pab1p may *trans*-activate cap-dependent translation by interacting with the stimulating factor(s) that is recruited to the mRNA in an eIF4G- and possibly eIF4E-dependent manner. The mutations in Pab1-134p, Pab1-157p, Pab1-175p and Pab1-180p may reduce this interaction, thereby leading to their inability to perform *trans*-activation.

multiple initiation factors (Figure 7A). In poly(A)-dependent translation, an interaction between Pab1p and eIF4G is vital because Pab1p represents the sole means of recruitment of eIF4G to the mRNA (Figure 7B). With a capped mRNA, however, eIF4G can be recruited via its interaction with the cap-binding protein eIF4E, perhaps lessening the importance of an interaction between Pab1p and eIF4G (Figure 7C). The effect of Pab1p on cap-dependent translation does seem to be at the level of translation initiation, however, as deletion of the first 300 amino acids of eIF4G destroyed the ability of an extract to be stimulated by Pab1p. Therefore, there is a requirement for eIF4G in order for Pab1p to exert its activity, but not necessarily for an interaction between Pab1p and eIF4G. Pab1p could be functioning by forming other contacts within the translation initiation complex (Figure 7C).

It seems likely that the influence of Pab1p on cap-dependent translation *in vitro* is related to its role in synergy. One possible model for synergy is that it is caused by the formation of a more stable initiation complex when both eIF4E and Pab1p are present. This could be due to the binding of eIF4E and/or Pab1p to factors other than eIF4G. In Pab1p stimulation of cap-dependent translation, some of the additional interactions hypothesized to occur in the synergistic complex could be formed by Pab1p binding *in trans* to the complex at the 5′ end of the mRNA (Figure 7C). Thus, the mutated proteins that are unable to stimulate cap-dependent translation might also be expected to show reduced levels of synergy in the translation extracts. However, with the exception of *pab1-184*, significant synergy was observed in extracts from all the mutant strains. It is possible that the *pab1-134*, *pab1-157*, *pab1-175* and *pab1-180* mutations do affect interactions which contribute to synergy but, as a result of the translational advantages of Pab1p being bound to the poly(A) tail, which include gaining the ability to bind to eIF4G (Figure 7A), the loss of these interactions is not destabilizing enough to prevent synergy. In contrast, when Pab1p is acting *in trans* to stimulate cap-dependent translation, Pab1p is not bound to poly(A) and these other interactions become critical for its association with the initiation complex (Figure 7C).

A precedent for the above pattern has been observed with the eIF4G1-213 and eIF4G2-233 proteins, which bind poorly to Pab1p. Although extracts containing these proteins are severely reduced in their ability to perform poly(A)-dependent translation, they still exhibit synergy (Tarun *et al.*, 1997). Extracts from the *pab1-180* mutant described here also behave in a similar manner in that they exhibit very little poly(A)-dependent translation but show significant levels of synergy. In *pab1-184* extracts, however, where poly(A)-dependent translation is even more severely affected, no synergy is observed. In this case, the drastic effect of the mutation on poly(A)-dependent translation and eIF4G binding may not be overcome by the other interactions within the synergistic complex.

We have shown previously that the simultaneous association of eIF4E and Pab1p with eIF4G can lead to mRNA circularization (Wells *et al.*, 1998). Our demonstration here that Pab1p can work *in trans* to stimulate capped mRNA translation could suggest that circularization of mRNA is not a prerequisite for Pab1p to act upon the 5′ end of the mRNA. However, our data do not address whether Pab1p is more effective when bound to the mRNA it is activating. They also do not address other possible functions of mRNA circularization that depend upon Pab1p. These include the possibility that re-initiation of translation is stimulated by circularization, and that mRNA instability is induced upon disruption of the circle. There-

fore, while our demonstration of *trans*-activation does provide important information about Pab1p functions in *in vitro* translation extracts, it does not provide insight into why circularization could be occurring.

In the course of this study, we have identified two mutated forms of Pab1p that do not bind to either isoform of eIF4G. Referring to Figure 2B, it can be seen that the mutations within *pab1-180* and *pab1-184* lie within helix B of the RRM, and are predicted to be separated by one turn of the helix. These amino acids would appear to be accessible for interaction with another factor such as eIF4G, and may well constitute the core of the eIF4G-binding site on Pab1p. The identification of these amino acids should assist in the interpretation of any structural data for Pab1p obtained in the future.

It has been demonstrated here that human Pab1p RRM2 cannot support poly(A) translation, stimulate cap-dependent translation or bind yeast eIF4G. In light of the recent finding that human Pab1p can bind human eIF4G (Imataka *et al*., 1998; Piron *et al*., 1998), these observations might appear somewhat unexpected. However, an alignment of yeast and human eIF4G sequences does not reveal a region in the human protein with a high degree of homology to the yeast Pab1p-binding site. Perhaps the characteristics of this binding site have diverged sufficiently so that an interaction between hPABP and yeast eIF4G is not possible. Nonetheless, our identification of amino acids in Pab1p which are involved in eIF4G binding may facilitate the identification of the eIF4G binding site in hPABP.

We have identified a novel activity of Pab1p in *in vitro* translation, i.e. an ability to stimulate cap-dependent translation, and have shown that this activity is distinct from the role of Pab1p in poly(A)-dependent translation. In the future, we hope to determine whether *trans*-activation is related to the synergistic stimulation of translation observed in the presence of both a cap and poly(A) tail. These studies will include attempts to identify mutations in other translation initiation factors that destroy *trans*-activation, and analyses to determine if they also destroy synergy. This approach will also help to identify additional targets of Pab1p within the translation initiation complex. In combination with further characterization of the involvement of RRM4 of Pab1p in *trans*-activation, all of these studies should result in additional information about the importance of *trans*-activation and its relationship to other events occuring during translation *in vitro* and *in vivo*.

Materials and methods

In vitro translation

Unless stated otherwise, translation extracts were prepared by the bead-beating method, as previously described (Iizuka *et al*., 1994; Tarun and Sachs, 1995). Preparation of extracts by the liquid nitrogen lysis method was performed as follows. YPD (1.6 l) was inoculated with the appropriate yeast strain and grown to an OD_{600} of 1.5. The culture was harvested by centrifugation for 15 min at 4000 *g* in a Sorvall H6000A rotor. Cells were resuspended in buffer A (30 mM HEPES pH 7.4, 100 mM KOAc, 2 mM MgOAC, 2 mM dithiothreitol) to a final volume of 175 ml and centrifuged for 10 min at 4000 *g* in a Sorvall GSA rotor. The cell pellet was resuspended in buffer A to a final volume of 50 ml and centrifuged for 5 min in a clinical centrifuge. The resulting cell pellet was weighed and resuspended in 1/10 volume of buffer A. The suspension was frozen by dripping directly into liquid nitrogen. Lysis was then performed by crushing the frozen yeast with a pestle and mortar in the presence of liquid nitrogen. Crushing was continued until a fine paste was obtained. This paste was transferred to a 50 ml Falcon tube and allowed to thaw on ice. After centrifugation of the lysate for 5 min in the clinical centrifuge, the supernatant was transferred to 1.5 ml microfuge tubes and centrifuged for 6 min at 39 000 *g* in a Sorvall SS-34 rotor. The supernatant from this step was centrifuged once more for 6 min at 39 000 *g*. The supernatant from this spin was passed over a 2.5×8 cm G-25 Superfine column (Pharmacia) equilibrated in buffer A and subsequently treated as for the bead-beating method. All the above centrifugation steps were performed at 4°C.

All mRNAs used in the *in vitro* translation assays were transcribed *in vitro* according to the protocol described in Tarun and Sachs (1995). Translation assays were performed a minimum of three times and average values taken.

For the reconstitution of poly(A)-dependent translation, 100 µl aliquots of extract were incubated with 60 U of micrococcal nuclease (Pharmacia) for 5 min at 26°C. The nuclease reaction was quenched by the addition of 0.5 M EGTA to a final concentration of 2 mM. Immunoneutralization of the extract was performed by the addition of an appropriate dilution of the Pab1p monoclonal antibody IG1 (Anderson *et al*., 1993) and incubation for 10 min on ice. This dilution was calculated for each extract so that the minimum amount required to give efficient neutralization was added. An appropriate aliquot of nuclease-treated extract was excluded from antibody treatment. An 8.5 µl aliquot of the immunoneutralized extract was then incubated for 10 min on ice with 7.5, 15 and 22.5 pmol of the appropriate recombinant Pab1p in 2 µl. Then 4.5 µl of a reaction mixture containing the translation buffer and 9 ng of LUCpA RNA were added, the reactions incubated for 30 min at 26°C and stopped by quick freezing in liquid nitrogen. A 10 µl aliquot of each reaction was added to 50 µl of luciferase substrate (Promega) and the amount of luminescence generated was measured in a TD-20e luminometer (Turner). Reconstitution of poly(A)-dependent translation was calculated as a percentage of the translation measured in the non-neutralized extract with no Pab1p added. The percentage reconstitutions for each different protein were plotted as a function of Pab1p concentration and then the slope of the graph was calculated to give percentage reconstitution/15 pmol of recombinant Pab1p added.

The assay to measure stimulation of cap-dependent translation was performed in the following manner. Aliquots (100 µl) of extract were again treated with micrococcal nuclease as above and 7.5 µl of nucleased extract were incubated with various amounts (0–30 pmol) of recombinant Pab1p. After addition of 4.5 µl of a reaction mixture containing the translation buffer and 9 ng of capLUC mRNA, the reactions were treated as above. The fold stimulation of translation of capLUC mRNA upon addition of recombinant Pab1p was calculated relative to the value observed when no Pab1p was added.

In order to characterize the extracts derived from the Pab1p point mutants, 0.5 M EGTA was added to 100 µl aliquots of translation extract to a final concentration of 2 mM. To 7.5 µl of treated extract, 7.5 µl of reaction mixture were added, containing translation buffer and 20 ng of either LUC, capLUC, LUCpA or capLUCpA RNA. Again, the translation reactions were then treated as above.

Recombinant DNA and protein methods

To make the hybrid construct containing human RRM2 with yeast RRMs 1, 3 and 4 and the C-terminal domain, human Pab1p RRM2 was amplified by PCR using the primers OLO1 and OLO2 (Table II), which introduce a *Bam*HI site at the 5′ end of the amplified fragment and a *Cla*I site at the 3′ end. The amplified fragment was digested with *Bam*HI and *Cla*I while DNA from BAS3059 (*PAB1-1* in the vector pET11d) was digested with either *Bam*HI and *Kpn*I or *Cla*I and *Kpn*I. The *PAB1-1* allele had been constructed in a manner which introduced *Bam*HI and *Cla*I sites on either side of RRM2 (Deardorff and Sachs, 1997). A three-way ligation was then performed using the digested human RRM2 fragment, the 6.5 kb fragment from the *Bam*HI–*Kpn*I digest (RRM1 of Pab1p together with the majority of the pET11d sequence) and the 1.5 kb fragment from the *Cla*I–*Kpn*I digest (Pab1p RRMs 3 and 4 and the C-terminal domain together with the remainder of the pETlld sequence). This resulted in replacement of yeast RRM2 by the human RRM2 sequence.

To synthesize the Pab1p RRM2 point mutant constructs, PCR mutagenesis using the megaprimer methodology (Barettino *et al*., 1993) was performed using Vent polymerase (New England Biolabs), DNA from BAS3059 as the template, one of the mutagenesis primers and the downstream primer OLO4 (Table II), which binds within the RRM4 sequence of Pab1p. After gel purification of these primary PCR products,

Table II. Oligonucleotides used in this study

Oligo	Mutant	Sequence	Restriction enzyme analysis[a]
OLO1		5′-GCTAGGATCCGGCAACATATTCATTAAA-3′	
OLO2		5′-TATCGCATCGATTCAGCTTCTCGTTCTTT-3′	
OLO3		5′-CGTAGAGGATCGAGATCTCG-3′	
OLO4		5′-GTCATCAACGCTGTCATCTA-3′	
OLO5	Pab1-134	5′-TTTATCAAGAACTTCGACAAGAGTATTGACAACAAGG-3′	gains a *Taq*I site
OLO6	Pab1-148	5′-TGACACTTTCTCTGCGTTTGGTAACATCTTGTCCAGC-3′	loses a *Hph*I site
OLO7	Pab1-157	5′-TCTTGTCCAGCAAGGTGGTCTGCGACGAAAACGGAA-3′	gains a *Bst*XI site
OLO8	Pab1-175	5′-TTGTTCACTTCGAAACCCAGGAGGCTGCCAAGGAAGC-3′	gains a *Bst*NI site
OLO9	Pab1-180	5′-AAGAAGGTGCTGCCGAACGCGCTATTGATGCTTT-3′	gains a *Bst*UI site
OLO10	Pab1-184	5′-AAGGAAGCTATTGAGAAGATGAATGGTATGCTG-3′	loses a *Sfa*NI site
OLO11	Pab1-193	5′-TATGCTGTTGAACGATCGAAAAGTTTTTGTTGCTCCTCACT-3′	gains a *Pvu*I site
OLO12	Pab1-199	5′-AGAAATTTATGTTGGTCGTTTTAAATCCAGAAAGGAAC-3′	gains a *Dra*I site

[a]These mutations introduce the listed changes into the restriction enzyme digest pattern of RRM2.

Table III. Strains used in this study

Protein	Bacterial strain containing the expression construct[a]	Bacterial strain containing the yeast construct[a]	Yeast strain[b]
Pab1-1	BAS3059	BAS3072	YAS2261
Pab1-ΔRRM1[c]	BAS3221		
Pab1-ΔRRM2	BAS3222	BAS3228	YAS2236
Pab1-ΔRRM3	BAS3223		
Pab1-ΔRRM4	BAS3224		
Pab1-ΔCterm	BAS3325		
Pab1–hRRM2	BAS3457		
Pab1-134	BAS3441	BAS3442	YAS2467
Pab1-148	BAS3443	BAS3444	YAS2468
Pab1-157	BAS3445	BAS3446	YAS2469
Pab1-175	BAS3447	BAS3448	YAS2470
Pab1-180	BAS3449	BAS3450	YAS2471
Pab1-184	BAS3451	BAS3452	YAS2472
Pab1-193	BAS3453	BAS3454	YAS2473
Pab1-199	BAS3455	BAS3456	YAS2474

[a]The bacterial strain is DH5α.
[b]Unless otherwise stated, the genotype of the strain is α *pab1::HIS3 ade2 his3 leu2 trp1 ura3.*
[c]A full description of the Pab1 proteins lacking individual RRMs can be found in Kessler and Sachs (1998).

they were used as the downstream primer in a second PCR with OLO3 (Table II), which binds within the pET11d sequence, as the upstream primer. DNA from BAS3224, which contains the Pab1p sequence deleted for RRM4, was used as the template. The products from the second round of PCR were digested with *Nco*I and *Cla*I giving fragments containing RRMs 1 and 2. These fragments were ligated between the *Nco*I and *Cla*I sites of BAS3072 DNA which contains the *PAB1-1* allele in a *TRP1CEN4* vector. The mutagenesis primers were designed to introduce or eliminate a restriction site (Table II). Therefore, the mutated constructs could be selected on the basis of the appropriate restriction analysis. Once the correct constructs in the *TRP1CEN4* vector had been obtained, the RRM2 sequence from each mutant was subcloned into *PAB1-1* in pET11d using the flanking *Bam*HI and *Cla*I sites as for synthesis of the hybrid construct above.

Recombinant proteins were prepared for each of the mutant alleles using the pET11d expression constructs expressed in strain BL21(DE3). The proteins were purified by nickel–agarose chromatography following the method described in Deardorff and Sachs (1997).

Yeast methods

The *TRP1CEN4* constructs containing the mutant Pab1p alleles were transformed into YAS2031, which contains the plasmid p*PAB1URA3CEN* and lacks genomic *PAB1* (Table III). Transformants were selected on YM plates. These were then streaked onto YM plates containing 1 mg/ml 5-fluoro-orotic acid, which only allows growth of transformants that have lost the p*PAB1URA3CEN* plasmid (Boeke *et al*., 1987). The growth rates of the yeast strains derived in this manner were measured in YPD at 30°C.

In vitro binding assays with the recombinant Pab1p proteins

The ability of the different forms of recombinant Pab1p to bind eIF4G was examined according to the method described in Kessler and Sachs (1998). GST–eIF4G1 (amino acids 187–299) from bacterial strain BAS3035 and GST–eIF4G2 (amino acids 201–315) from bacterial strain BAS3024 were used in these assays; 10 μg of recombinant Pab1p were used for each binding assay. After denaturation in Laemmli loading buffer, either 15 μl of the sample were loaded onto a 10% SDS–PAGE gel for analysis by Coomassie staining or 15 μl of a 1/100 dilution were used for Western analysis. Western blots were performed as stated in Tarun and Sachs (1996) using a polyclonal antibody to Pab1p.

To assess the ability of the recombinant Pab1p proteins to bind to poly(A)-Sepharose, 90 mg of the latter (Pharmacia) were hydrated in 1 ml of 1.0 M NaCl for 5 min at room temperature. The resin was washed with 1 ml of 1.0 M NaCl, followed by five washes with PBS-TS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.1% Triton X-100, 0.01% SDS). The resin was suspended in a volume of 250 μl of PBS-TS and 25 μl of this suspension added to 100 μl of PBS-TS containing 5 μg of recombinant Pab1p. After incubation of the reaction for 1 h at 4°C, the resin was washed three times with PBS-TS. Each sample was resuspended in 25 μl of 2× Laemmli loading buffer and analyzed by SDS–PAGE and Western blotting as above.

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