Analysis of an essential requirement for the poly(A) binding protein function using cross-species complementation

Julia A. Chekanova*†, Randal J. Shaw* and Dmitry A. Belostotsky*†

Poly(A) binding protein (PABP) is an essential, well-conserved, multifunctional protein involved in translational initiation, mRNA biogenesis, and degradation [1–5]. We have used a cross-species complementation approach to address the nature of the essential requirement for PABP in yeast. The expression of Pab3p, a member of the *Arabidopsis thaliana* PABP multigene family, rescues the lethal phenotype associated with the loss of the yeast Pab1p. However, Pab3p neither protects the mRNA 5′ cap from premature removal, nor does it support poly(A)-dependent translational initiation or the synergistic enhancement of translation by the poly(A) tail and 5′ cap in yeast. However, Pab3p corrects the temporal lag prior to the entry of the mRNA into the degradation pathway characteristic of *pab1*Δ yeast strains. Furthermore, this lag correction by Pab3p requires Pan3p, a subunit of poly(A) nuclease, an enzyme involved in the mRNA 3′-end processing. Importantly, the substitution of Pab3p for the yeast Pab1p is synthetically lethal with the *PAN3* gene deletion. These results show that the function of PABP in mRNA biogenesis alone could be sufficient to support cell viability in yeast.

Addresses: *Department of Biological Sciences and †Center for Molecular Genetics, State University of New York at Albany, Albany, New York 12222, USA.

Correspondence: Dmitry A. Belostotsky
E-mail: dab@albany.edu

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Results and discussion

The roles of PABP in translational initiation, mRNA biogenesis, and degradation have been described (reviewed in [6–8]), but the exact nature of the essential requirement for PABP is not completely understood. In this study, we employed an *Arabidopsis thaliana* Pab3p that exhibits specific, high-affinity binding to poly(A) in vitro and is able to rescue the yeast *pab1*Δ mutant in vivo (see Supplementary materials and methods available with this article online; the fragment of Pab3p containing amino acids 42–660 was used in all experiments). Strong (>10-fold) poly(A)-dependent stimulation of translation, as well as synergism between the effects of the 5′ cap and the poly(A) tail, have been demonstrated in several systems [9–12]. This phenomenon is largely mediated by an interaction of PABP with the translation initiation factor eIF4G [13]. To test whether the poly(A)-dependent translation and cap/poly(A) synergism were essential for the ability of Pab3p to rescue the *pab1* null phenotype, luciferase (LUC) mRNAs with and without the 5′ cap and poly(A) tail were translated in the S30 extracts from YDB203, a strain complemented by Pab3p expressed under the control of the *GAL1* promoter and from the isogenic wild-type cells (genotypes are given in Table 1). The doubling time of YDB203 on galactose was increased by only 38% relative to the isogenic wild-type strain, and the respective PABPs accumulated to comparable levels (Figure 1c and Supplementary materials and methods). The uncapped, nonpolyadenylated LUC mRNA did not translate efficiently in either extract (Figure 1a). Adding the 5′ cap to the reporter mRNA led to a more than 20-fold enhancement of translation in both extracts. However, as opposed to the wild-type strain, the poly(A) tail did not enhance translation efficiency in the YDB203 extract over that observed with the LUC mRNA. Moreover, the synergism between the effects of the 5′ cap and the poly(A) tail, defined as a ratio of translation of the capLUCpA to the sum of translation of capLUC and LUCpA, was only marginal (~1.1-fold). In contrast, extracts from the isogenic wild-type strain showed expected cap-dependent and poly(A)-dependent translation (>20-fold each) as well as strong synergism between the cap and the poly(A) tail (16-fold). To show that the significance of these findings was not limited to in vitro extracts, the LUC transcripts were translated in electroporated spheroplasts from YDB203 and wild-type cells. In agreement with the in vitro data, Pab3p-complemented cells showed the wild-type magnitude of cap-dependent stimulation of translation but were totally unable to support poly(A)-dependent translation and showed only a marginal (<1.7-fold) cap/poly(A) synergism (Figure 1b). Thus, the rescue of the *pab1* null mutant by Pab3p was not associated with the restoration of the poly(A)-dependent translation and cap/poly(A) synergism.

The absence of poly(A)-dependent translation and cap/poly(A) synergism in the Pab3p-complemented yeast strain could be caused by the inability to interact with the yeast eIF4G. This would be expected based on several amino acid differences between Pab3p and Pab1p in the region of the RRM II domain known to be critical for the

Table 1

***Saccharomyces cerevisiae* strains used in this study.**

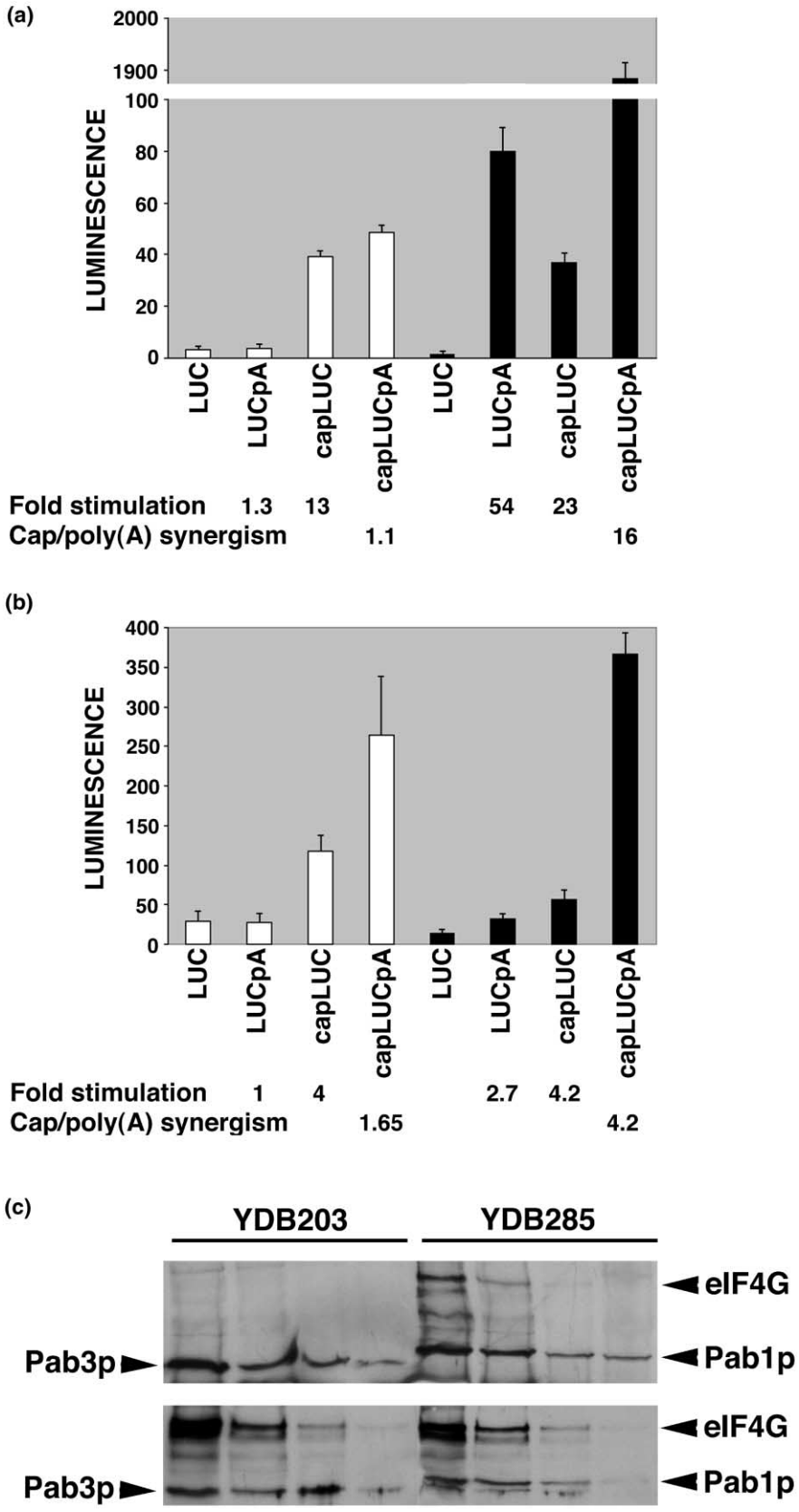
Strain	Genotype	Plasmid	Source
YAS319	*ade2, his3, leu2, trp1, ura3, pab1::HIS3*	pAS77 (*URA3/CEN/PAB1*)	A. Sachs
YAS1881	*ade2, his3, leu2, trp1, ura3, pab1::HIS3, cdc33-1*	pAS77 (*URA3/CEN/PAB1*)	A. Sachs
YDB203	*ade2, his3, leu2, trp1, ura3, pab1::HIS3*	pDB419 (*TRP1/CEN/pGAL1-PAB3*)	this work
YDB218-5	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3, pan3::TRP*		this work
YDB221	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3*	pDB463 (*TRP1*/2μ/*pADHI-PAB3*)	this work
YDB225	*ade2, his3, leu2, trp1, ura3, pab1::HIS3*	pDB463 + pRP590	this work
YDB236	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3, pan3::TRP*	pDB488 (*G418*^{r}/2μ/*pADHI-PAB3*)	this work
YDB239	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3, pan3::TRP*	pDB489 (*G418*^{r}/*CEN/PAB1*)	this work
YDB267	*ade2, his3, leu2, trp1, ura3, pab1::HIS3, pan3::TRP1*	pAS77 (*URA3/CEN/PAB1*)	this work
YDB285	*ade2, his3, leu2, trp1, ura3, pab1::HIS3*	pDB533 (*TRP1/CEN*/peptide-tagged *PAB1*)	this work
YRP840	*trp1, ura3, leu2, his4, cup1::LEU2pm*		R. Parker
YRP843	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3*		R. Parker
YRP881	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3*		R. Parker

yeast Pab1p-eIF4G interaction in vitro [14]. To address this question directly, an association of Pab3p with the yeast eIF4G in vivo was measured in a quantitative immunoprecipitation experiment with an antibody against the C-terminal peptide of Pab3p. As a control, the yeast Pab1p was C-terminally tagged with the same peptide used to raise the anti-Pab3p antibody (strain YDB285). Little, if any, eIF4G was found in association with Pab3p, compared with the peptide-tagged Pab1p (Figure 1c, top panel). In addition, neither purified recombinant yeast eIF4GI nor eIF4GII interacted with Pab3p in vitro (data not shown).

Another important role of PABP in yeast cells is to regulate mRNA turnover. One manifestation of this function is a PABP-dependent delay of mRNA decapping until the poly(A) tails are shortened down to ~12 A residues ([2], Figure 2a). Presumably, at that point, the last molecule of PABP dissociates from the mRNA 3′ end, the 3′-5′ association is disrupted, and the 5′ cap becomes susceptible to the decapping enzyme. This cap-protective effect of PABP is most easily visualized using reporter mRNAs containing a poly(G) insert in the 3′ UTR, such as *PGK1pG* or *MFA2pG* [2]. The poly(G) tract forms a stable structure that halts the 5′-3′ exonuclease, thereby "trapping" the pG → 3′ products that arise following decapping. The pG → 3′ products are trimmed to the 5′ junction of the poly(G) tract and normally have short (≤12 As) poly(A) tails because decapping and subsequent 5′ → 3′ decay occur only after poly(A) shortening. However, in *pab1*Δ strains, decapping takes place without prior deadenylation, resulting in pG → 3′ products with broad a distribution of poly(A) tail lengths. It was hypothesized that protection from premature decapping and poly(A)-dependent enhancement of translational initiation are the two activities that make PABP an essential protein [2]. This model predicts that the alleles of PABP capable of supporting cell viability can be either deficient in poly(A)-dependent translation or in the regulation of decapping, but not in both. To test if mRNA decapping in the Pab3p-complemented strain occurred after deadenylation or prior to it, an *MFA2pG* reporter was introduced into YDB203, and the poly(A) tails of the full-length *MFA2pG* mRNA and its pG → 3′ products were examined. Although the poly(A) tails' length distribution in the YDB203 was somewhat shifted toward shorter tails compared to the wild-type strain (Figure 2b), it was broad, indicating that decapping in YDB203 cells took place prior to the completion of deadenylation.

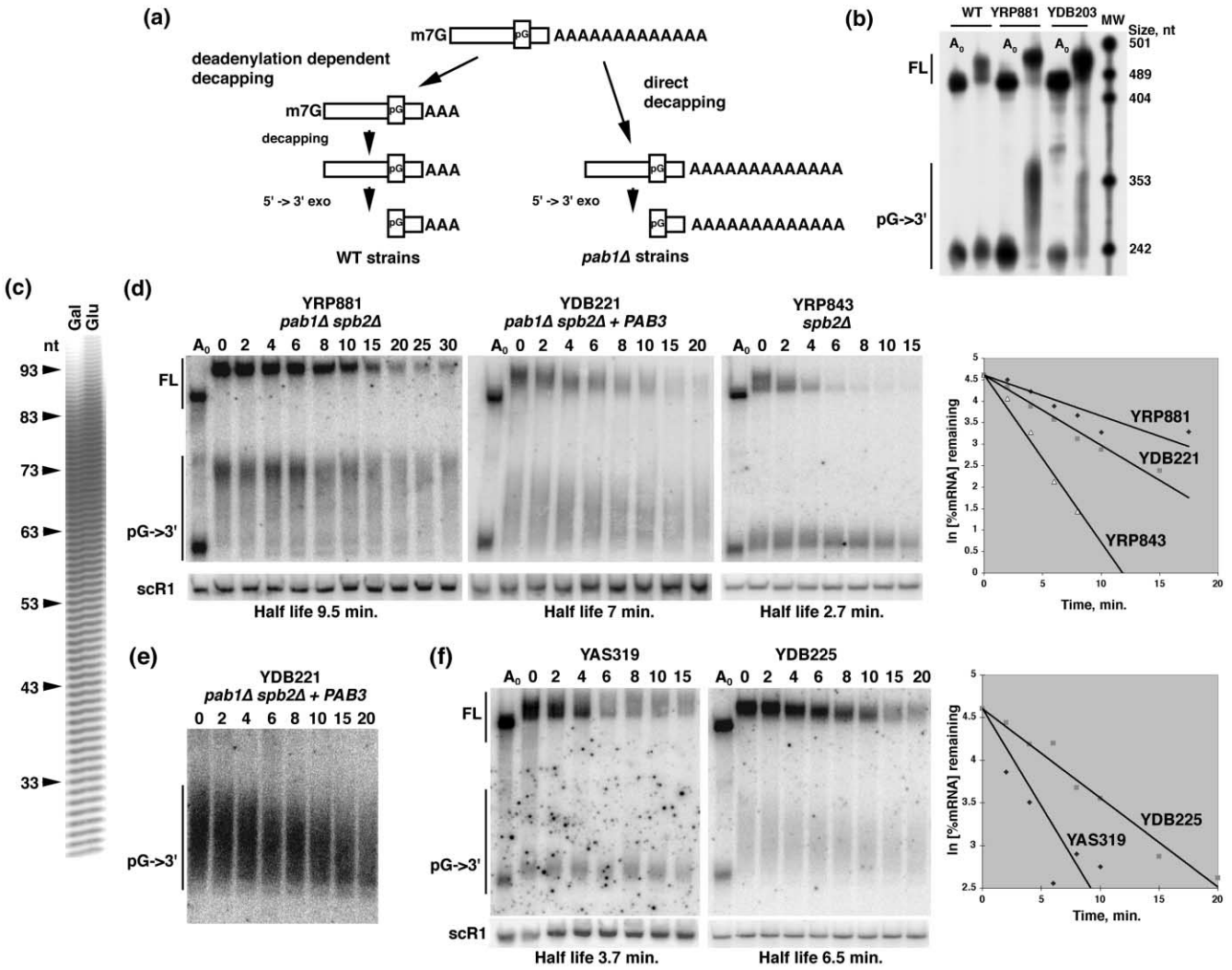
That *Arabidopsis* Pab3p restores the viability of the PABP-deficient yeast but performs neither of the two functions proposed to explain the essential requirement for PABP in the cell prompted us to seek other consequences of its expression in yeast. A slight reduction in poly(A) tails' length of the pG → 3′ products in YDB203 compared to the *pab1*Δ, *spb2*Δ strain (Figure 2b) could have been a consequence of Pab3p-promoted partial poly(A) shortening that occurred prior to, concurrently, or after decapping. To extend this observation to other mRNAs, YDB203 was shifted from galactose to glucose to repress Pab3p expression, and the length distribution of poly(A) tails on the total mRNA population was examined. A modest, but highly reproducible, increase in the poly(A) tails' length was observed upon the depletion of Pab3p (Figure 2c). We have shown previously that such elongation of the poly(A) tails is not a general consequence of growth arrest [15]. Therefore, while being unable to prevent premature 5′ cap removal in yeast, Pab3p can partially activate poly(A) tail shortening. To gain further insight into its role in mRNA metabolism in yeast, we examined decay kinetics of the *MFA2pG* mRNA in the presence or absence of Pab3p, as well as of the yeast Pab1p, in the *pab1*Δ, *spb2*Δ strain background. *spb2*Δ is a bypass suppressor mutation that causes a loss of the 60S ribosomal protein RPL39 [16] and does not affect mRNA turnover directly [2]. The suppressor phenotype is probably due to underaccumulation of the ribosomal 60S subunits [16], which, thus, are

Figure 1



The rescue of the *pab1* null phenotype by Pab3p does not require poly(A)-dependent translation, poly(A) tail/cap synergism, or interaction with eIF4G. **(a)** S30 translation extracts from YDB203 (white bars) or YAS319 (dark bars) were programmed with 50 ng of luciferase mRNA, containing no cap, no poly(A) (LUC), cap only (capLUC), poly(A) only (LUCpA), and both cap and poly(A) (capLUCpA). Luciferase synthesis was measured by a luminescence assay. The values that are shown represent an average of three independent experiments, and bars denote standard deviations. Fold stimulation by cap and poly(A) tail, as well as cap/poly(A) synergism are given below each panel. All translation assays were within the linear range, and chemical and functional stabilities of all LUC mRNA species in S30 extracts were nearly identical. **(b)** Translation of the same set of four luciferase mRNAs in electroporated spheroplasts prepared from YDB203 (white bars) or YAS319 (dark bars). **(c)** Subsaturating amounts of antibody were used to immunoprecipitate eIF4G complexed with the respective PABP in the YDB203 and YDB285. A 3-fold dilution series of the 25% of the total immunocomplexes (top) or 5% of the supernatants (bottom) obtained in the immunoprecipitation of the YDB203 and YDB285 extracts using the antibody against the C-terminal peptide of Pab3p were analyzed by Western blotting with antibodies against eIF4G and the same antibodies that were used for immunoprecipitation. Both eIF4GI and II, which comigrate on the gel shown, are detected by the antibody against eIF4G used here.

Figure 2

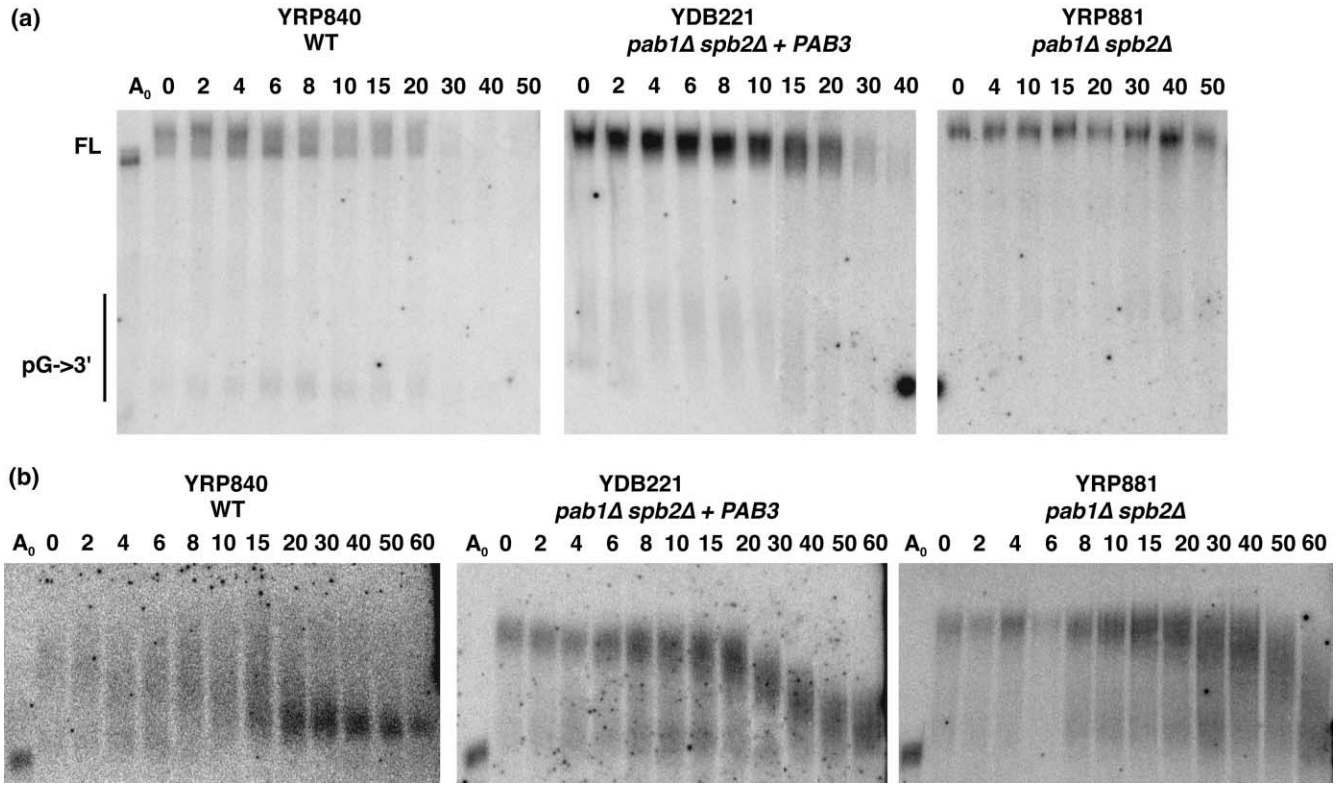


Pab3p promotes *MFA2pG* mRNA deadenylation and decay in yeast but does not prevent mRNA decapping prior to deadenylation. **(a)** A diagram of the deadenylation-dependent decapping pathway of mRNA decay in the wild-type and in *pab1*Δ yeast strains, as revealed by examining poly(G)-containing reporter transcripts. **(b)** Northern blot analysis of the *MFA2pG* mRNA in the wild-type strain YRP840 (WT), *pab1*Δ, *spb2*Δ strain YRP881, and the Pab3p-complemented strain YDB203. MW, molecular weight marker. The full-length mRNA and the pG → 3′ product are indicated. Samples loaded in the lanes marked A_o were treated with RNaseH/oligo(dT) to remove poly(A) tails. **(c)** Poly(A) tail length analysis of the total cellular mRNA from galactose grown YDB203 or YDB203 shifted to glucose for 8 hr. Total mRNA was 3′ end-labeled by ligating [^{32}P]pCp, nonpoly(A) segments removed by RNases A and T1, and poly(A) tails were visualized on a sequencing gel. **(d)** An analysis of the *MFA2pG* mRNA decay in the *pab1*Δ, *spb2*Δ strain (YRP881), the *PAB1 spb2*Δ strain (YRP843), and the *pab1*Δ, *spb2*Δ strain expressing *Arabidopsis* Pab3p (YDB221). Time points after the transcriptional repression are shown above the lanes. The lane labeled A_o contains a 0 min sample that has been treated with RNase H and oligo(dT) to remove the poly(A) tail **(e)** A deliberately overloaded gel showing that, in YDB221, the poly(A) tails on the pG → 3′ products continue to shorten with time. Pixel density profiles of this gel are shown in the Supplementary material (Figure S3). **(f)** An analysis of the *MFA2pG* mRNA decay by transcriptional chase from the steady state in the Pab3p-complemented strain (YDB225) and in the isogenic wild-type strain (YAS319). The decay curves shown in (d) and (f) were built from Phosphorimager-quantitated hybridization signals after normalizing to the levels of the scR1 RNA (lower panels in [d] and [f]).

less able to sequester the 40S subunits in empty 80S couples. The resulting excess of free 40S subunits might allow more efficient translation, indirectly compensating for the loss of PABP.

In this experiment, the *MFA2pG* reporter mRNA was driven by the *GAL1* promoter, while Pab3p was expressed constitutively. Upon the addition of glucose, transcription from the *GAL1* promoter stopped, and degradation of the *MFA2pG* mRNA was followed by Northern analysis. Two major consequences of the expression of Pab3p in the *pab1*Δ, *spb2*Δ strain were observed (Figure 2d). First,

Figure 3



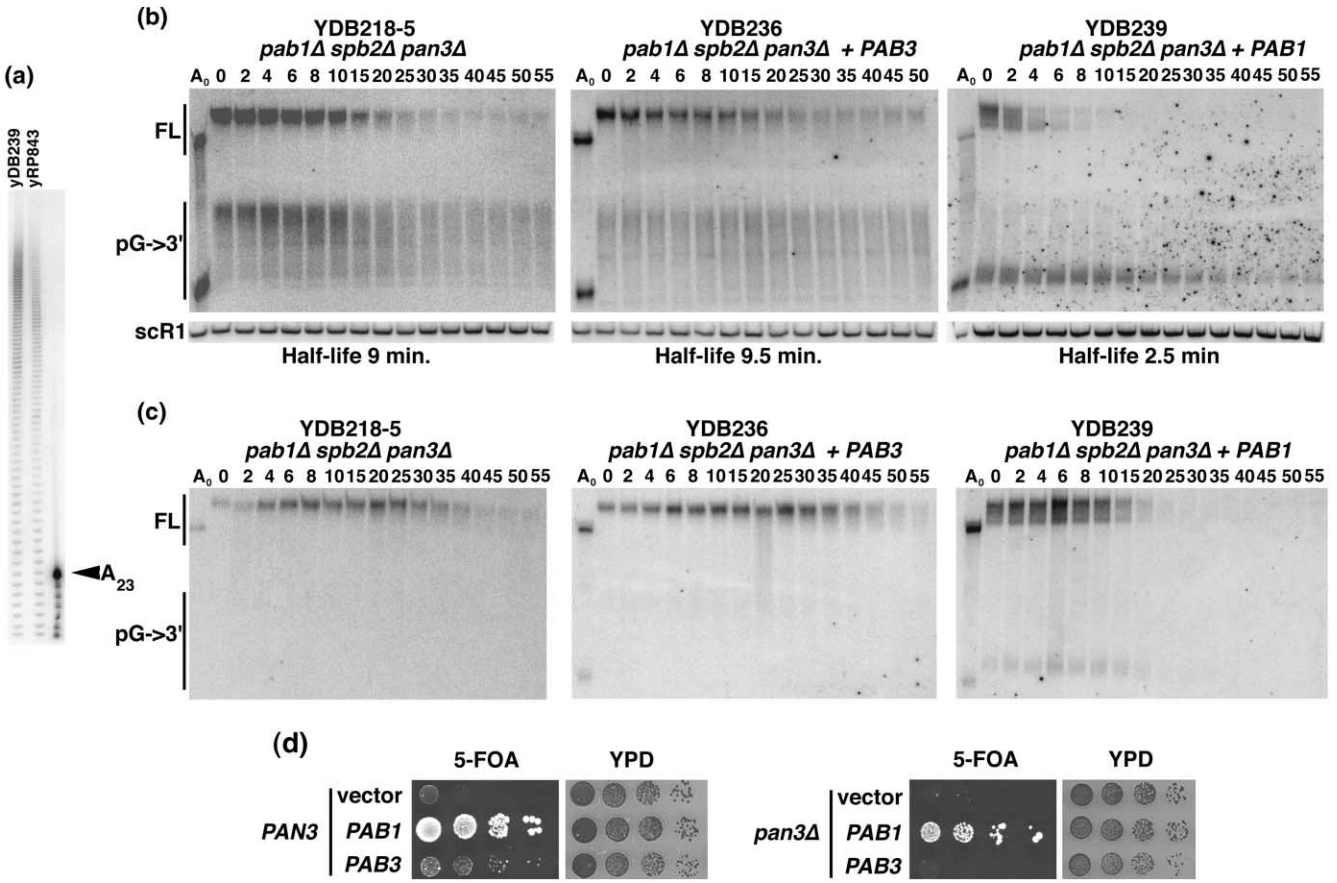
Pab3p corrects the lag prior to the onset of *MFA2pG* mRNA decay in yeast. Transcriptional pulse-chase analysis in the wild-type strain (YDB840), the *pab1*Δ, *spb2*Δ strain (YRP881), and the *pab1*Δ, *spb2*Δ strain expressing *Arabidopsis* Pab3p (YDB221) of the **(a)** *MFA2pG* mRNA and the **(b)** *PGK1pG* mRNA. Full-length (FL) and pG → 3′ products of *MFA2pG* mRNA are indicated. To improve the resolution of poly(A) tail lenghts of the large (1.4 kb) *PGK1pG* transcript, each mRNA sample shown in (b) was treated with RNase H and the oligonucleotide oRP70 (CGGATAAGAAAGCAACACCTGG), complementary to the sequence 9–30 nucleotides 5′ to the *PGK1* stop codon.

Pab3p promoted an increase in the rate of *MFA2pG* mRNA degradation, from a half-life of ∼10 min in the *pab1*Δ, *spb2*Δ strain to ∼7 min in the presence of Pab3p. Second, at least some of the Pab3p-dependent deadenylation occurred after, rather than prior to, decapping, since, in contrast to the *pab1*Δ, *spb2*Δ strain without Pab3p, the poly(A) tails on the pG → 3′ products in the Pab3p-expressing strain continued to shorten throughout the time course (Figures 2e and S3 in the Supplemental material). An alternative explanation would be that this effect is due to a rapid replacement of the population of pG → 3′ products by a new population of pG → 3′ products derived from the full-length *MFA2pG* mRNA that undergoes continuous poly(A) shortening itself. However, since the half-life of the pG → 3′ products (∼15 min, [17]) is considerably longer than the time intervals between the samples analyzed here, this explanation can be ruled out.

To further substantiate the *MFA2pG* mRNA half-life measurements, its decay was analyzed in the YDB203 (*SPB2*) strain, into which the *MFA2pG* construct was introduced on a plasmid (strain YDB225, Figure 2f) as well as in the isogenic wild-type strain YAS319. The resulting values were very similar to the ones observed in the presence of the *spb2*Δ suppressor mutation (6.5 min in the presence of Pab3p, and 3.7 min in the presence of Pab1p).

One consequence of the absence of PABP in yeast is a temporal lag before the mRNA becomes a substrate to deadenylation and decay, which was proposed to reflect some additional role played by the yeast Pab1p in mRNP maturation [2]. To address the possibility that Pab3p accelerated the *MFA2pG* mRNA decay by eliminating or reducing this lag, transcriptional pulse-chase analysis was employed [18]. Transcription of the *GAL1* promoter-driven reporter mRNA was induced for 9.5 min and then immediately repressed ("pulse"), and the fate of the newly synthesized mRNA pool was then followed by Northern analysis ("chase"). In agreement with published observations [2], *MFA2pG* mRNA in the *pab1*Δ, *spb2*Δ strain persisted in intact form for up to 50 min (YRP881, Figure 3a), after which it slowly degraded (data not

Figure 4



Pan3p function is essential for the ability of Pab3p to eliminate the lag prior to the onset of *MFA2pG* mRNA decay and for the rescue of the lethal phenotype of the *pab1*Δ mutant. **(a)** A poly(A) tail length assay of the total cellular mRNA showing that the disruption of the *PAN3* gene used in the subsequent mRNA analysis led to expected poly(A) tail elongation in the *PAB1* genetic background (compare the *pan3*Δ strain yDB239 to near-isogenic *PAN3* strain yRP843). **(b)** Transcriptional chase from steady state and **(c)** transcriptional pulse-chase in the *pab1*Δ, *spb2*Δ, *pan3*Δ strain (YDB218-5) and *pab1*Δ, *spb2*Δ, *pan3*Δ strain expressing the yeast Pab1p (YDB239) or *Arabidopsis* Pab3p (YDB236). **(d)** Pab3p expression restores the viability of the *pab1*Δ mutant containing the wild-type *PAN3* gene, but not in the presence of the *pan3*Δ mutation. Yeast strain YAS319, which contains its only copy of the *PAB1* gene on a *URA3* plasmid, or its *pan3*Δ derivative (YDB267) were transformed with the plasmid encoding either the wild-type yeast Pab1p (pDB489), *Arabidopsis* Pab3p (pDB488), or with vector alone (pDB491). The ability of the respective constructs to support cell viability in *pan3*Δ and *PAN3* genetic backgrounds was tested by allowing the transformants to grow without selection for a *URA3*/*PAB1* plasmid for 3 days and then plating them onto 5-FOA medium, which selects against Ura^+ cells.

shown). The key finding was that, in the *pab1*Δ, *spb2*Δ + Pab3p strain (YDB221), *MFA2pG* mRNA was virtually completely degraded after 30 min. Importantly, deadenylation of *MFA2pG* mRNA in the presence of Pab3p commenced immediately following transcriptional repression. In order to extend this observation to other yeast mRNAs, we examined the *PGK1pG* transcript, which is much more stable than *MFA2pG* but is also subject to a lag prior to deadenylation and decay in the *pab1*Δ, *spb2*Δ strain. A pulse-chase analysis of the *PGK1pG* mRNA showed that Pab3p substantially accelerated the entry of this transcript into the decay pathway as well (Figure 3b); although, in this case, the lag was reduced but not completely eliminated. Thus, the lag prior to the entry of both unstable (*MFA2pG*) and stable (*PGK1pG*) mRNA into the decay pathway was either completely or partially corrected by *Arabidopsis* Pab3p in yeast.

As part of a systematic search for factors that might be involved in the Pab3p-dependent lag correction, we constructed a strain YDB218-5 (*pab1*Δ, *spb2*Δ, *pan3*Δ) lacking both Pab1p and Pan3p, a subunit of poly(A) nuclease (PAN) that is involved in mRNA 3′-end maturation (Figure 4a). PAN promotes a rapid poly(A) trimming that, in vivo, appears to be closely linked to the mRNA 3′-end processing [19]. The examination of the *MFA2pG* mRNA decay in this genetic background has led to two main observations. First, the Pab3p-dependent deadenylation

did not take place in the *pan3*Δ strain (Figure 4b). In contrast, rapid deadenylation and degradation of *MFA2pG* mRNA in the presence of Pab1p occurred normally, despite the absence of Pan3p. Second, Pab3p was not able to promote *MFA2pG* mRNA decay in the absence of Pan3p: *MFA2pG* mRNA half-life was 9–9.5 min in the *pab1*Δ, *spb2*Δ, *pan3*Δ strain in the presence or absence of Pab3p (Figure 4b), very close to the half-life of 9.5 min observed in the *pab1*Δ, *spb2*Δ, *PAN3* strain YRP881 (Figure 2d). Western blot analysis showed that Pab3p accumulated to similar levels in *pan3*Δ and *PAN3* strains (data not shown). Moreover, the pulse-chase analysis in the YDB218-5 strain transformed with the plasmid encoding Pab3p, yeast Pab1p, or vector alone showed that, while the lag in the *pan3*Δ background was eliminated in presence of Pab1p, it was not affected by Pab3p (Figures 4c and 3a). Therefore, the ability of Pab3p to accelerate the entry of the mRNA into the decay pathway required Pan3p. We explain this difference in the Pan3p requirement by proposing that Pan3p functionally interacts with PABP in the course of mRNP biogenesis, and the inability of Pab3p to promote deadenylation and decay in the absence of Pan3p reflects the fact that it requires either Pan3p itself or other factors that are assembled into mRNP in a Pan3p-dependent manner in order to act upon the mRNP substrate. In contrast, Pab1p may interact with additional partners in the 3′ mRNP domain that are not well recognized by a heterologous Pab3p, making it less sensitive to the absence of Pan3p. Therefore, in the *pab1*Δ, *spb2*Δ, *pan3*Δ + Pab1p strain, the subsequent mRNA transactions, including deadenylation and decay, are virtually normal.

That the correction of the lag prior to the entry of the mRNA into the decay pathway is the only known Pab1p activity that *Arabidopsis* Pab3p can perform in yeast cells suggests that it reflects some aspect of the essential function of PABP in the cell. The finding that the Pab3p-dependent acceleration of mRNA entry into the decay pathway requires Pan3p has led to a testable prediction that substituting *Arabidopsis* Pab3p for the yeast Pab1p in the *pan3*Δ background would not allow cell viability. We found that substitution of the plant *PAB3* for the yeast *PAB1* gene is indeed synthetically lethal with the *pan3*Δ mutation (Figure 4d). This observation provides genetic evidence that the role of PABP in the mRNP biogenesis is critical for subsequent mRNA transactions and cell viability.

Our findings extend earlier observations of the involvement of PABP in mRNA biogenesis [2, 20–23]. The novel aspect of this work is that the cross-species complementation approach has allowed us to reveal that it is neither poly(A)-dependent translation nor protection of the mRNA 5′ cap from premature removal, but rather the role of PABP in mRNA biogenesis, that is the basis of the rescue of the *pab1*Δ lethality by *Arabidopsis* Pab3p. This set of functional properties of Pab3p distinguishes it from the previously characterized PABP variants and leads us to conclude that the function of PABP in mRNA biogenesis makes a significant contribution to the essential role of this protein in yeast cell viability. Further inquiry into the nature of the lag prior to mRNA entry into the decay pathway should prove fruitful in mechanistic dissection of the role of PABP in mRNA biogenesis.

Supplementary material

Supplementary material including additional Results and discussion and methodological detail is available at http://images.cellpress.com/supmat/supmatin.htm.

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