UNIVERSITY AT ALBANY
State University of New York

DMITRY BELOSTOTSKY
Department of Biological Sciences

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Department of Biological Sciences
University at Albany, SUNY
1400 Washington Ave.
Albany NY 12222
Phone / FAX (518) 442 4368
Email dab@albany.edu

Dr. Yves Brun

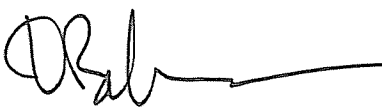
Systems Biology/Microbiology Faculty Search
Department of Biology, Indiana University
Jordan Hall 142, 1001 E 3rd Street
Bloomington, IN 47405-7005

Dear Dr. Brun,

I am applying for a faculty position at the Associate Professor level as advertised in *Science*. My research group is studying mRNA biogenesis, processing, export, turnover and translation, using two model systems, Saccharomyces cerevisiae and Arabidopsis thaliana. In Arabidopsis, we are specifically interested in the role of posttranscriptional regulatory mechanisms in reproductive development of the plant, embryo fate specification and gene imprinting, while in Saccharomyces, our focus is on the most fundamental aspects of posttranscriptional events in gene expression. These efforts are funded by NIH, NSF, USDA and BARD. Our research is question-driven, and uses a variety of cutting edge tools and concepts, such as a massively parallel genetic analysis and affinity tag-assisted proteomics. Both approaches are well matched with the goals of the ambitious expansion in life sciences at the University of Indiana, and in addition, the latter technology also nicely dovetails with the focus of the expansion in Biochemistry that is being undertaken by the IU Chemistry Department.

My decision to seek a new position is motivated by a desire to maintain and augment the considerable research momentum that we have built, which requires a more intellectually stimulating environment and a more vigorous graduate program than my present position offers. Your Department not only possesses such attributes and is widely known for its tradition of excellence in research and teaching, but it is also particularly attractive because it represents excellent match to our research interests. Being in the Department next to colleagues focusing on plant development, RNA biology and gene expression in a variety of model systems is the best possible situation for us. Conversely, our team will help catalyze the expansion in the life sciences at UI, and contribute to success of its key components, such as biocomplexity initiative and the NSF-IGERT training grant.

If you have questions or require more materials, please do not hesitate to contact me.

Sincerely, 

Dmitry Belostotsky, Ph.D.
Associate Professor of Biology

Evidence that poly(A) binding protein has an evolutionarily conserved function in facilitating mRNA biogenesis and export

JULIA A. CHEKANOVA and DMITRY A. BELOSTOTSKY
Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222, USA

ABSTRACT

Eukaryotic poly(A) binding protein (PABP) is a ubiquitous, essential cellular factor with well-characterized roles in translational initiation and mRNA turnover. In addition, there exists genetic and biochemical evidence that PABP has an important nuclear function. Expression of PABP from *Arabidopsis thaliana*, PAB3, rescues an otherwise lethal phenotype of the yeast *pab1Δ* mutant, but it neither restores the poly(A) dependent stimulation of translation, nor protects the mRNA 5′ cap from premature removal. In contrast, the plant PABP partially corrects the temporal lag that occurs prior to the entry of mRNA into the decay pathway in the yeast strains lacking Pab1p. Here, we examine the nature of this lag-correction function. We show that PABP (both PAB3 and the endogenous yeast Pab1p) act on the target mRNA via physically binding to it, to effect the lag correction. Furthermore, substituting PAB3 for the yeast Pab1p caused synthetic lethality with *rna15-2* and *gle2-1*, alleles of the genes that encode a component of the nuclear pre-mRNA cleavage factor I, and a factor associated with the nuclear pore complex, respectively. PAB3 was present physically in the nucleus in the complemented yeast strain and was able to partially restore the poly(A) tail length control during polyadenylation in vitro, in a poly(A) nuclease (PAN)-dependent manner. Importantly, PAB3 in yeast also promoted the rate of entry of mRNA into the translated pool, rescued the conditional lethality, and alleviated the mRNA export defect of the *nab2-1* mutant when overexpressed. We propose that eukaryotic PABPs have an evolutionarily conserved function in facilitating mRNA biogenesis and export.

Keywords: PABP; mRNA biogenesis; mRNA export

INTRODUCTION

Poly(A) binding protein (PABP) is highly conserved in eukaryotes, and its function is essential for viability in *Saccharomyces cerevisiae* (Sachs et al. 1987), *Aspergillis nidulans* (Marhoul and Adams 1996), *Caenorhabditis elegans* (A. Petcherski and J. Kimble, pers. comm.) and *Drosophila melanogaster* (Sigrist et al. 2000). PABP participates in at least three major post-transcriptional events, for example, mRNA biogenesis, regulation of mRNA turnover, and initiation of protein synthesis. However, the exact reasons why the PABP function is essential remain incompletely understood.

PABP is an abundant protein [4 μM in Hela cells (Gorlach et al. 1994)], and the majority of PABP in the cell is cytoplasmic at steady state. PABP is the key factor responsible for the poly(A) tail stimulated pathway of translational initiation (for reviews, see Sachs 2000; Schwartz and Parker 2000). Both yeast (Tarun and Sachs 1996) and human (Imataka et al. 1998) PABPs interact with the translation initiation factor eIF4G, thereby causing circularization of the mRNA via bridging its 5′ and 3′ termini (closed loop model [Jacobson 1996]). Such an interaction could facilitate formation of the 48S translation initiation complex de novo, the 60S ribosomal subunit joining, and/or translational reinitiation (Munroe and Jacobson 1990; Tarun and Sachs 1995; Kahvejian et al. 2001; Searfoss et al. 2001). However, the mechanism of translational stimulation may be more complex than just an interaction between PABP and eIF4G, as these two phenomena could be genetically uncoupled (Kessler and Sachs 1998). Moreover, observations made in the yeast strains conditionally defective in poly(A) tail synthesis suggest the possibility that yeast PABP (Pab1p) also interacts directly with ribosomes (Proweller

Reprint requests to: Dmitry A. Belostotsky, Department of Biological Sciences, State University of New York at Albany, Albany, NY 12222, USA; e-mail: dab@albany.edu; fax: (518) 442 4368.

Abbreviations: CFI, cleavage factor I; FISH, fluorescent in situ hybridization; PABP, poly(A) binding protein; PAN, poly(A) nuclease; PAP, poly(A) polymerase; RRM, RNA recognition motif.

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and Butler 1996). In addition, recent evidence in yeast and mammalian cells links PABP to translational termination and eukaryotic release factor 3 (eRF3; Cosson et al. 2001; Uchida et al. 2002). Thus, PABP may participate in translation in multiple ways.

In addition, PABP participates in the control of the mRNA degradation in the cytoplasm, although the exact way in which PABP regulates mRNA decay may vary between species and between different transcripts. In yeast, the major mRNA degradation route occurs via deadenylation-dependent decapping (for reviews, see Schwartz and Parker 2000; Wilusz et al. 2001b). The yeast PABP, Pab1p, impedes mRNA decapping until the process of deadenylation progresses to the point when only 12–15 Å residues are left, and the last Pab1p binding site on the mRNA poly(A) tail has been eliminated. It can be envisioned that dissociation of the last molecule of Pab1p causes a significant rearrangement in the structure of the mRNP, and leads to a loss of the 3′ end–5′ end association, enabling the decapping enzyme to attack the 5′ cap. A competition between the translation initiation and mRNA decay factors plays an important role in the control of deadenylation and decapping, for example, mutations in yeast translation initiation factors promote both decapping and deadenylation rates (Schwartz and Parker 1999). Inhibition of decapping by PABP was also observed in mammalian cell extracts, although in that case, it seems to be independent of the eIF4E/eIF4G interaction with PABP (Gao et al. 2001). Moreover, the circularization of the mRNA via the eIF4E/eIF4G/Pab1p interaction in yeast extracts also accounts for only a part of the inhibitory effect of Pab1p on decapping, as a partial inhibition of decapping by Pab1p could still be observed when eIF4E was prevented from interacting with the 5′ cap (Wilusz et al. 2001a). Furthermore, a deletion of the eIF4G-interacting domain from the yeast PABP that was tethered to the mRNA in a poly(A)-independent manner did not affect its ability to inhibit mRNA decay (Coller et al. 1998).

The role of PABP in deadenylation is complex. PABP acts as a deadenylation inhibitor in mammalian cell extracts (Bernstein et al. 1989; Ford et al. 1997) and when overexpressed in *Xenopus* oocytes (Wormington et al. 1996). In the case of the α-globin mRNA, PABP interacts with the αCP complex that binds to the 3′-UTR of this transcript, and slows the rate of its deadenylation (Wang et al. 1999). A more complex picture arises in the case of the *c-fos* mCRD (major coding region determinant), a sequence element that specifies rapid deadenylation and decay of the *c-fos* transcript in a manner that is dependent on translation (Grosset et al. 2000). The mCRD binding complex interacts with PABP, thus bridging the mCRD and the poly(A) tail. This interaction prevents deadenylation prior to the initial round(s) of translation, but promotes it after the complex has been displaced by the ribosomes traversing the mCRD, possibly because the removal of mCRD binding complex also promotes dissociation of PABP from the poly(A) tail, making it vulnerable to exonucleolytic attack (Grosset et al. 2000). Yeast Pab1p also inhibits deadenylation in vitro (Wilusz et al. 2001a). On the other hand, Pab1p is also required for the proper rate of deadenylation in yeast in vivo (Caponigro and Parker 1995). A possible resolution of this paradox can be envisioned if Pab1p actually promotes the entry of the mRNA into the decay pathway, rather than accelerates deadenylation per se. Yeast strains lacking Pab1p (but viable due to bypass suppressor mutations) exhibit a temporal lag before mRNA decay commences, which likely reflects a role of Pab1p in efficient mRNA biogenesis (Caponigro and Parker 1995).

In this study, we provide evidence supporting and extending the view that PABP is important for the mRNA biogenesis. Using cross-species complementation of the yeast *pab1* null mutant by the *Arabidopsis* PAB3 cDNA, we have shown previously that rescue of viability of the yeast *pab1*Δ mutant required neither the restoration of poly(A)-dependent translation nor the protection of the 5′ cap from premature removal (Chekanova et al. 2001). However, plant PABP significantly reduced or eliminated the lag prior to mRNA decay in yeast (Chekanova et al. 2001). In this work, this system is further exploited to examine the nature of the lag prior to mRNA decay in *pab1*Δ cells and its correction by PABP. We have found that the *Arabidopsis* PAB3 accelerated the entry of the mRNA into the degradation pathway, as well as its entry into the translated pool when expressed in yeast. PAB3 was also able to partially restore the poly(A) tail length control during polyadenylation reaction, in the PAN-dependent manner. Furthermore, a fraction of PAB3 was physically present in the nucleus in the complemented yeast strain. The substitution of the plant PAB3 for the endogenous yeast Pab1p caused synthetic lethality with *rna15-2*, an allele of the gene encoding the Rna15p subunit of the pre-mRNA cleavage factor CFIA, and with *gle2-1*, a mutant allele of the gene encoding the NPC-associated protein. Finally, overexpression of PAB3 in yeast rescued the cold sensitivity and alleviated the mRNA export block of the *nab2-1* mutant strain, which is defective in one of the shuttling hnRNP proteins required for mRNA export. We propose that eukaryotic PABPs have an evolutionarily conserved function in facilitating mRNA biogenesis and export.

RESULTS

PAB3 and Pab1p act directly on the target mRNA to correct the lag prior to the entry of mRNA into the decay pathway in yeast

To ascertain that PABPs (the heterologous *Arabidopsis* PAB3, as well as the endogenous yeast Pab1p) reduce or eliminate the lag prior to the entry of the mRNA into the degradation pathway directly (that is, by physically binding to the target mRNAs) a yeast strain was constructed that

simultaneously expressed two nearly identical *MFA2*-based reporter mRNAs that differed mainly in the presence or absence of the poly(A) tail—the binding site for the PABP. The first reporter was the same *GAL1* promoter-driven *MFA2pG* mRNA (Caponigro and Parker 1995) that we used previously in the transcriptional pulse-chase analysis in the presence or absence of PAB3 in yeast (Chekanova et al. 2001). The second *MFA2*-based reporter (*MFA2-MS2-RZ*) contained the ribozyme derived from the hepatitis δ virus in place of the normal polyadenylation signal. This latter construct gives rise to a transcript that undergoes autocatalytic processing in vivo in yeast cells, resulting in nonadenylated mRNA (Quadt et al. 1995; Coller et al. 1998). This construct was also expressed from the *GAL1* promoter. Other differences between these two reporter transcripts, namely the presence of the poly(G) tract in the 3′-UTR of the first reporter, and the MS2 protein binding sites in the second reporter, have been demonstrated previously to have no effect on the mRNA turnover properties of the respective transcripts (Decker and Parker 1993; Coller et al. 1998).

Because of the lethality associated with the *pab1*Δ mutation, the rate of decay of these two reporters was analyzed in the *spb2*Δ suppressor background, expressing either yeast Pab1p, *Arabidopsis* PAB3, or no PABP (strains YDB246, YDB256, and YRP881, respectively; genotypes of the yeast strains used in this work are given in Table 1), by use of a transcriptional shutoff of the *GAL1* promoter. The *spb2*Δ mutation is a bypass suppressor of *pab1*Δ, which causes a loss of the 60S ribosomal subunit protein RPL39 (Sachs and Davis 1989), but does not have any direct effect on mRNA turnover (Caponigro and Parker 1995). The suppressor phenotype is probably due to the resulting underaccumulation of the ribosomal 60S subunits, which are thus less able to sequester the 40S subunits into empty 80S couples (Sachs and Davis 1989). The resulting excess of the free 40S subunits (which are normally limiting for initiation) may

TABLE 1. Genotypes of the yeast strains used in this work

Strain	Relevant genotype	Reference
YRP881	*trp1 ura3 leu2 his4 cup1::LEU2pm spb2::URA3 pab1::URA3*	Caponigro and Parker (1995)
YDB203	*ade2 his3 leu2 trp1 ura3 pab1::HIS3, pDB419 (TRP1/CEN/pGAL1-PAB3)*	Chekanova et al. (2001)
YDB220	*PDB464 (TRP1/CEN/PAB1)* in *YRP881*	This work
YDB221	*trp1 ura3 leu2 his4 cup1::LEU2pm spb2::URA3, pab1::URA3, pDB463 (TRP1/2μ/pADHI-PAB3)*	Chekanova et al. (2001)
YDB227	*pMM2-6* in *YRP881*	This work
YDB236-8	*trp1 ura3 leu2 his4 cup1::LEU2pm spb2::URA3 pab1::URA3 pan3::TRP, pDB488 (G418/2μ/pADHI-PAB3)*	Chekanova et al. (2001)
YDB246	*pMM2-6* and *pDB490* in *YRP881*	This work
YDB256	*pMM2-6* and *pDB493* in *YRP881*	This work
YJA223	*his3 leu2 trp1 ura3 nab2::LEU2 TRP1::nab2-21*	Hector et al. (2002)
YRH201C	*ade2 his3 leu2 trp1 ura3 can1 nab2::HIS3 pGAL::NAB2*	Hector et al. (2002)
ACY429-1038	*trp1 ura3 leu2 his3 NAB2::HIS3, pAC1038 (LEU2/CEN/nab2-1)*	Green et al. (2002)
BJ5464	*ura3 trp1 leu2 his3 pep4::HIS3 prbD1.6R can1*	Jones (1991)
rna14-1W	*ura3 trp1 ade2 leu2 his3 rna14-1*	Amrani et al. (1997)
rna15-2W	*ura3 trp1 ade2 leu2 his3 rna15-2*	Amrani et al. (1997)
SWY1022	*ade2 ade3 ura3 his3 leu2 trp1 can1-100 nup100::HIS3*	Murphy et al. (1996)
SWY1024	*ade2 ade3 ura3 his3 leu2 lys2 can1-100 nup100::HIS3*	Murphy et al. (1996)
YCH130	*ade2 ade3 ura3 his3 leu2 lys2 can1-100*	Murphy et al. (1996)
W303α	*ade2 ade3 ura3 his3 leu2 trp1 can1-100*	Murphy et al. (1996)
SWY1136	*ade2 ura3 his3 leu2 trp1 can1-100 gle2-1*	Murphy et al. (1996)
mtr2-9	*ura3 ade2 leu2 his3 trp1 mtr2::HIS3, pRS315/mtr2-9*	Santos-Roza et al. (1998)
MTR2	*ura3 ade2 leu2 his3 trp1 mtr2::HIS3, pRS316/MTR2*	Santos-Roza et al. (1998)
mex67-5	*ade2 his3 leu2 trp1 ura3 mex67::HIS, pUN100/mex67-5*	Segref et al. (1997)
MEX67	*ade2 his3 leu2 trp1 ura3 mex67::HIS3, pUN100/MEX67*	Segref et al. (1997)
FY23	*ura3 leu2 trp1*	Winston et al. (1995)
FY86	*his3 leu2 ura3*	Winston et al. (1995)
CCY282	*ura3 leu2 trp1 rat2-1*	Heath et al. (1995)
OLY101	*his3 leu2 ura3 rat3-1*	Li et al. (1995)
LGY101	*his3 leu2 ura3 rat7-1*	Gorsch et al. (1995)
TDY105	*trp1 leu2 ura3 nup145-10*	Dockendorff et al. (1997); Snay-Hodge et al. (1998)
CSY550	*trp1 leu2 ura3 rat8-2*	Snay-Hodge et al. (1998)

Mutant and corresponding isogenic wild-type strains used in synthetic lethal tests are separated into the *bottom* portion of the table and are shaded in gray.

allow the cell to translate mRNA more efficiently, thereby indirectly compensating for the loss of Pab1p. In support of this view, certain mutations that exacerbate the 40S subunit deficit are partially suppressed by decreasing the 60S subunit levels (Finley et al. 1989). In addition, the binding of the charged tRNA to the ribosome A site, as well as the k_{cat} of the peptidyltransferase in *spb2*Δ mutant are increased by 40%–70% relative to the wild-type ribosomes (Dresios et al. 2000, 2001) at the expense of translational fidelity.

The decay rates of the *MFA2pG* and *MFA2-MS2-RZ* mRNAs in the absence of PABP in the cell were virtually indistinguishable (Fig. 1, $T_{1/2}$ = 7 min in both cases). Introduction of either the Pab1p-expressing construct or PAB3-expressing construct resulted in selective acceleration of the decay of the *MFA2pG* mRNA, but not of the *MFA2-MS2-RZ* mRNA. Because we have shown previously that PAB3 accelerates mRNA decay in yeast chiefly via acceleration of the rate of entry of the mRNA into the decay pathway, rather than via activating the mRNA decay per se, these data indicate that the endogenous yeast Pab1p, as well as the plant PAB3 expressed in yeast (Chekanova et al. 2001), must act on the target mRNAs directly to be able to correct the lag prior to the onset of the mRNA decay that is observed in the PABP-deficient strains. In other words, these PABP proteins promoted the rate of entry into the decay pathway of only those mRNA to which they were able to bind physically. Two possible explanations for this result are: (1) that the *MFA2-MS2-RZ* mRNA that could not bind PABP was normally exported from the nucleus, but was somehow shielded from the cytoplasmic decay; or (2) that its dwelling time in the nucleus was longer than in the case of the *MFA2pG* mRNA. We favor the latter explanation, because the mRNAs that cannot bind PABP are usually less stable, rather than more stable (Bernstein et al. 1989; Wormington et al. 1996; Ford et al. 1997; Wang et al. 1999; Grosset et al. 2000), and because the results of a recent FISH study using an analogous ribozyme-containing construct in yeast, strongly suggest that its export from the nucleus is compromised (Libri et al. 2002).

Lagging mRNA population retains an intact 5′ cap

In an effort to shed additional light on the mechanistic nature of the temporal lag prior to the entry of mRNA into the decay pathway in the *pab1*Δ strains, we examined the 5′ cap status of transcripts experiencing the lag. This was prompted by a recent discovery that some of the decapping and the 5′–3′ degradation steps of the major mRNA decay pathway in yeast occur in the discrete cytoplasmic processing bodies (P bodies; Sheth and Parker 2003). Importantly, mRNA decay intermediates that lack the 5′ cap appear to be concentrated in P bodies, whereas the intact full-length mRNAs are distributed throughout the cytoplasm (Sheth and Parker 2003). This raises the possibility that transcripts experiencing the lag were retained in the P bodies, but were somehow resistant to further decay. To assess the cap status of the mRNAs experiencing the lag, the RNA samples from the transcriptional pulse-chase experiment conducted in the YRP881 (*pab1*Δ *spb2*Δ) strain were subjected to the exhaustive treatment by recombinant Xrn1p in vitro. Xrn1p is a Mg^{++}-requiring enzyme that readily degrades uncapped RNA, but is inactive toward RNA containing the 5′ cap structure. Degradation of the endogenous 7S rRNA precursor, which is naturally uncapped, served as an internal control for the completeness of the Xrn1p digestion. As shown in Figure 2, *MFA2pG* mRNA experiencing the lag prior to its decay was completely resistant to the Xrn1p activity. These data demonstrate that the lagging population of mRNA retains its 5′ cap, and thus rule out the possibility that the observed lag is due to a sequestration of the messenger RNA and shielding it from the 5′–3′ exonucleolytic degradation after it has undergone decapping.

Substitution of the PAB3 for the yeast Pab1p causes synthetic lethality in combination with the *rna15-2* and *gle2-1*

A genetic approach was also used to try to gain further insight into the nature of the lag. We have demonstrated previously that PAB3 is no longer capable of supporting cell viability in yeast in the absence of the *PAN3* gene, which encodes a subunit of the poly(A) nuclease (PAN) that is indispensable for the catalytic function of this enzyme. This situation is essentially equivalent to a synthetic lethal interaction, if one assumes that the substitution of the plant PAB3 for the endogenous yeast Pab1p is equivalent to a partial loss of function of Pab1p. This assumption is justified, because the plant PAB3 cannot perform two of the known functions of the yeast Pab1p, that is, it neither protects the mRNA 5′ cap from the premature removal in yeast, nor interacts with the yeast eIF4G. As a consequence, it is incapable of supporting the poly(A)-dependent stimulation of translation, as well as the synergy in translational stimulation that is normally observed between the effects of the 5′ cap and the poly(A) tail in yeast cells. In addition, PAB3 corrects the temporal lag prior to the entry of mRNA into the decay pathway in the *pab1*Δ strains partially, rather than perfectly (Chekanova et al. 2001).

The finding that *pan3*Δ mutation is synthetically lethal with the substitution of PAB3 for Pab1p prompted us to conduct a series of binary synthetic lethal tests between the substitution of PAB3 for Pab1p and mutations in various factors involved in mRNP biogenesis, processing, and export, including *rna14-1*, *rna15-2*, *hrp1-3*, *nup100*Δ, *nup145-10*, *rat2-1*, *rat3-1*, *rat7-1*, *rat8-2*, *mex67-5*, *mtr2-9*, and *gle2-1* (see Table 1 and Materials and Methods). Two additional synthetic lethal interactions were found among the 12 mutants tested. Importantly, we have observed no correlation between the degree of the growth defect in the various mutants tested and their tendency to exhibit syn-

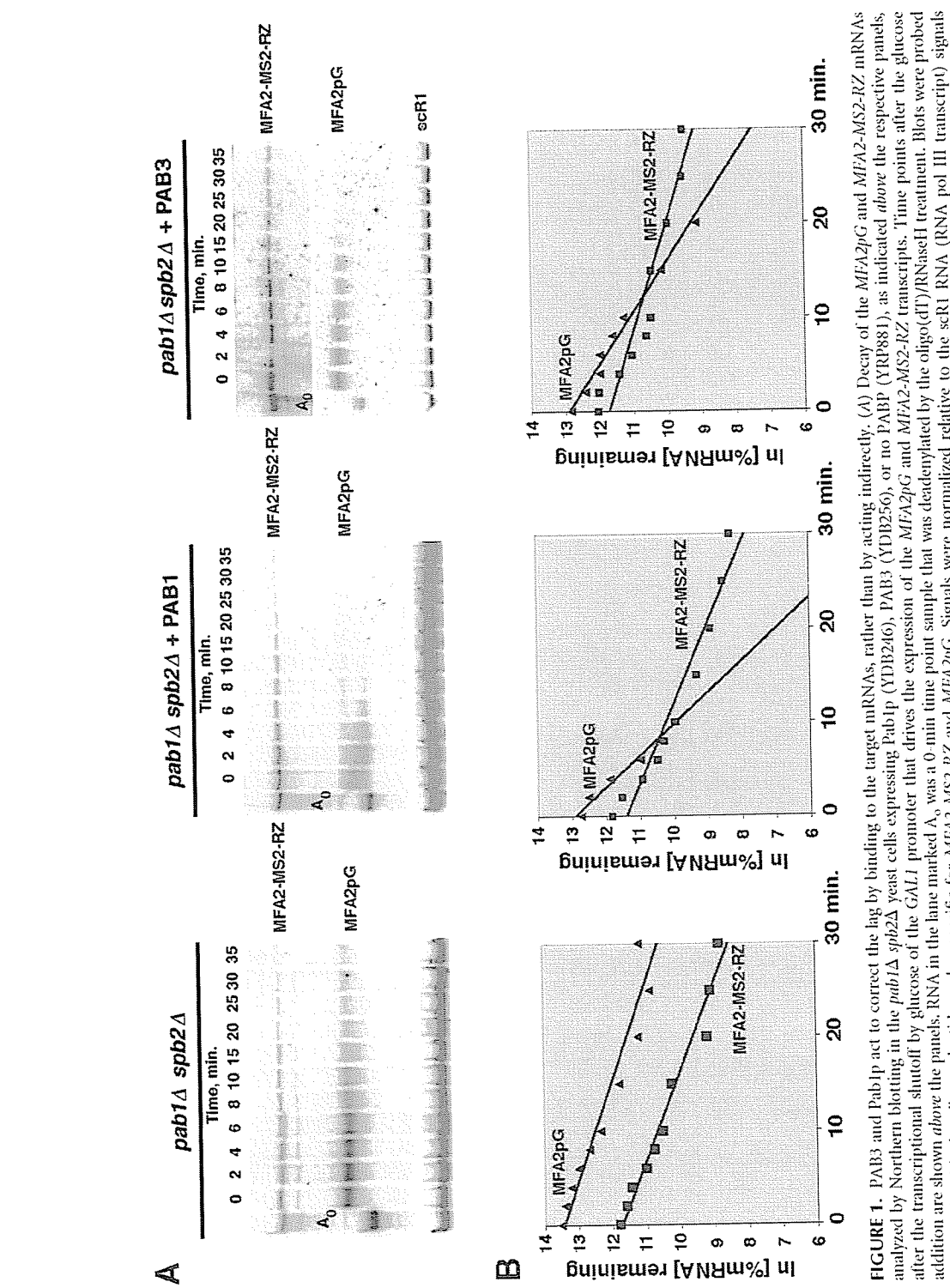

FIGURE 1. PAB3 and Pab1p act to correct the lag by binding to the target mRNAs, rather than by acting indirectly. (*A*) Decay of the *MFA2pG* and *MFA2-MS2-RZ* mRNAs analyzed by Northern blotting in the *pab1Δ spb2Δ* yeast cells expressing Pab1p (YDB246), PAB3 (YDB256), or no PABP (YRP881), as indicated *above* the respective panels, after the transcriptional shutoff by glucose of the *GAL1* promoter that drives the expression of the *MFA2pG* and *MFA2-MS2-RZ* transcripts. Time points after the glucose addition are shown *above* the panels. RNA in the lane marked A_0 was a 0-min time point sample that was deadenylated by the oligo(dT)/RNaseH treatment. Blots were probed sequentially with the oligonucleotide probes specific for *MFA2-MS2-RZ* and *MFA2pG*. Signals were normalized relative to the scR1 RNA (RNA pol III transcript) signals (*bottom*). (*B*) Quantitation of the decay profiles shown in *A*.

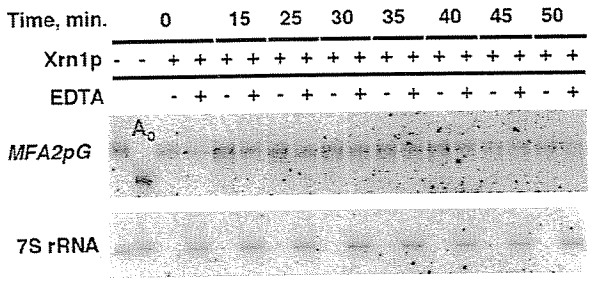

FIGURE 2. Assay of the 5′ cap status of the *MFA2pG* mRNA during the lag in the YRP881 (*pab1Δ spb2Δ*) strain. Total RNA samples corresponding to the time points of the transcriptional pulse-chase experiment indicated on the *top* of the figure were treated with the recombinant 5′–3′ exonuclease Xrn1p in the presence or absence of the EDTA, as indicated *above* the lanes. RNA in the lane marked A_0 was deadenylated by the oligo(dT)/RNaseH treatment. Sequential Northern blot hybridizations with the *MFA2pG* (*top*) and 7S rRNA precursor (*bottom*) specific probes are shown.

thetic lethality in combination with the substitution of PAB3 for Pab1p. One of the synthetic lethal interactions was with the *rna15-2* allele, and the other with *gle2-1*. Rna15p is a component of CFIA, a complex of four polypeptides that is required for the pre-mRNA cleavage and polyadenylation (Gross and Moore 2001). The notion of the evolutionarily conserved functional link between the PABP and Rna15p is also corroborated by the earlier finding that the yeast Pab1p suppresses the *rna15-2* allele when overexpressed, and that it interacts physically with Rna15p in the pulldown experiments in vitro (Amrani et al. 1997). Gle2p is an NPC-associated protein. The *gle2-1* mutation leads to an arrest of mRNA export at nonpermissive temperature, as well as to herniation and clustering of NPCs (Murphy et al. 1996). At the permissive temperature, ~25% of *gle2-1* cells exhibit accumulation of the poly(A)$^+$ RNA in the nucleus, but no ultrastructural aberrations are evident (Murphy et al. 1996). Furthermore, it was proposed recently that Gle2p may act to help deliver an export complex to NPC (Blevins et al. 2003). Taken together with the biochemical data presented below, these findings lead to a view that PAB3 participates in the nuclear functions of Pab1p in yeast. Specifically, the genetic interactions with *rna15-2* and *gle2-1* may suggest the links between the evolutionarily conserved nuclear function of PABP and early and/or late nuclear steps of mRNA biogenesis.

PAB3 enters the yeast cell nucleus and partially rescues the poly(A) tail length control during polyadenylation in vitro

A fraction of the endogenous yeast Pab1p has been shown previously to localize in the nucleus and associate with CFIA (Minvielle-Sebastia et al. 1997). Genetic links between the PAB3 function in yeast and nuclear proteins (above) imply that PAB3 may also be present physically in the nucleus of the yeast cell. To address this possibility, subcellular fractionation of the PAB3-complemented YDB203 cells (*pab1Δ spb2Δ* + PAB3) was undertaken. Cells from the YDB203 strain were converted to spheroplasts, lysed, fractionated by Percoll gradient centrifugation, and the nuclear and the cytoplasm-enriched fractions probed with the PAB3-specific antibodies. The data (Fig. 3) demonstrate that a fraction of PAB3 is present in the nucleus in the PAB3-complemented yeast strain. Densitometric scanning and quantitation of the immunoblots suggest that at least 7% of the total cellular PAB3 in yeast is nuclear at steady state, although this figure is likely to be an underestimate, because the subcellular fractionation protocol causes partial loss of the cytoplasmic material, as well as contamination of the cytoplasmic fraction with the content of the lysed nuclei (as can be seen from the distribution of the Nsp1p signal, Fig. 3). On the other hand, the extent of contamination of the nuclear fraction with cytoplasmic material was estimated by probing for an abundant cytoplasmic protein Pgk1p (Fig. 3) and for eIF4G (data not shown). No detectable cytoplasmic contamination was observed.

The genetic link between PAB3 function in yeast and the endogenous Rna15p, a subunit of the cleavage factor I, suggests that PAB3 may be able to function in cleavage and/or polyadenylation of pre-mRNA in yeast. To test this possibility, we assayed cleavage, polyadenylation, as well as a coupled cleavage/polyadenylation of the *GAL7* pre-mRNA-derived artificial substrate in extracts prepared from the YDB220 (*pab1Δ spb2Δ* + Pab1p), YRP881 (*pab1Δ spb2Δ*) and YDB221 (*pab1Δ spb2Δ* + PAB3) cells. We saw no evidence of any influence of PAB3 on pre-mRNA cleavage (data not shown). This was expected, because the yeast Pab1p also has no effect on the pre-mRNA cleavage step of the 3′ end processing (Amrani et al. 1997; Kessler et al. 1997; Minvielle-Sebastia et al. 1997; Brown and Sachs 1998). Therefore, all subsequent assays were performed using the precleaved substrate. In the extracts YDB220 (*pab1Δ spb2Δ* + Pab1p; Fig. 1B, left), as well as in the wild-type extracts (data not shown), the precleaved substrate was extended by ~90 Å residues, which closely parallels the extent of polyadenylation observed in vivo. In contrast, in the *pab1Δ spb2Δ* cells (YRP881), control of the extent of polyadenylation was lost, resulting in the synthesis of the abnormally long poly(A) tails (~150 Å residues). This phenomenon has been observed previously in extracts prepared from the Pab1p-deficient, as well as PAN-deficient cells (Fig. 1B; Amrani et al. 1997; Kessler et al. 1997; Minvielle-Sebastia et al. 1997; Brown and Sachs 1998). Importantly, in extracts prepared from the YDB221 (*pab1Δ spb2Δ* + PAB3) cells, control of the extent of polyadenylation was partially restored, resulting in the maximum of the distribution of the poly(A) synthesized at the 30-min time point of ~120 As.

Because in the previous studies PAN function has been shown to be important for proper control of the polyade-

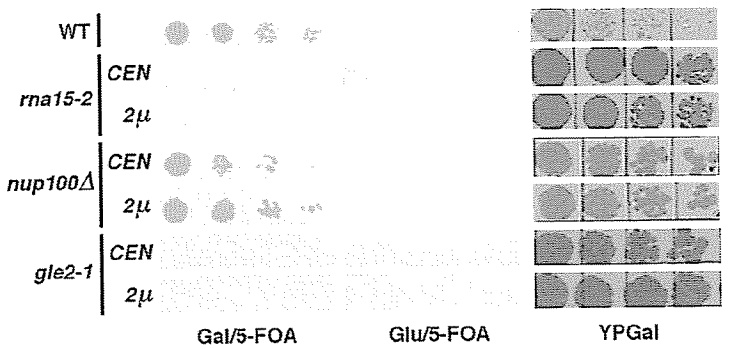

FIGURE 3. Example of the plasmid shuffle test for the synthetic lethality between the substitution of PAB3 for Pab1p and mutations in various factors involved in mRNP biogenesis, processing, and export (see Table 1 for the full listing). The respective mutant strains (*rna15-2*, *nup100Δ*, *gle2-1*, and the wild-type control are shown) with their chromosomal *PAB1* gene disrupted and the functional copy of *PAB1* provided on *CEN/URA3* plasmid, were transformed with either centromeric or high-copy (2 μ) plasmid-bearing p*GAL-PAB3* cassette, as indicated at *left*. Transformants were grown in synthetic complete medium with uracil and dilution series plated onto galactose or glucose-based synthetic medium with 5-FOA to select for the cells that have lost the *CEN/URA3/PAB1* plasmid. Only cells that grew on Gal/5-FOA, but not on Glu/5-FOA, resulted from complementation of the *pab1* deletion by the p*GAL-PAB3* expression cassette. Plating on the rich YPGal medium (to provide the total viable cell number) is also shown. All plates were grown for 6 d.

nylation in vitro (Brown and Sachs 1998), its possible requirement for the PAB3-mediated effect was also examined. Extracts from the YDB236-8 (*pab1Δ spb2Δ pan3Δ* + PAB3) were also prepared and assayed side-by-side with the extracts from the YDB220 (*pab1Δ spb2Δ* + Pab1p), YRP881 (*pab1Δ spb2Δ*), and YDB221 (*pab1Δ spb2Δ* + PAB3) cells in the 30-min endpoint assay, as well as in the kinetic assay, to follow the dynamic pattern of changes in the poly(A) tail length. Two main observations could be made. First, partial restoration of the poly(A) tail length control by PAB3 was completely dependent on the PAN function, as it was abolished in the *pan3Δ* genetic background. Thus, the PABP-stimulated, PAN-dependent control of the extent of the poly(A) tail addition may be an evolutionarily conserved phenomenon. Second, the kinetic behavior of the polyadenylation reaction was different in the Pab1p containing (strain YDB220, *pab1Δ spb2Δ* + Pab1p), PABP-deficient (strain YRP881, *pab1Δ spb2Δ*), and PAB3 containing (strain YDB221, *pab1Δ spb2Δ* + PAB3) extracts. In the YDB220 extracts, the maximal extent of polyadenylation was reached in 10 min, and no further changes in the poly(A) tail length occurred thereafter. In contrast, although the initial rate of polyadenylation in the *pab1Δ spb2Δ* (strain YRP881) extracts was similar, the poly(A) addition continued throughout the time course of the experiment. In the extracts from the PAB3-expressing cells (YDB221), although polyadenylation also continued past the 10-min time point, it proceeded with the much slower apparent rate. Importantly, in the extracts of YDB236-8 (*pab1Δ spb2Δ pan3Δ* + PAB3), the dynamic pattern of changes of the poly(A) tail length distribution was similar to those observed in the *pab1Δ spb2Δ* (strain YRP881) extracts.

Yeast Pab1p interacts directly with Pan3p (D. Mangus and A. Jacobson, pers. comm.). The PAN requirement for the partial restoration of the control the poly(A) tail length by PAB3 in vitro, together with the synthetic lethality between the PAB3 substitution for the yeast Pab1p and the loss of Pan3p (Chekanova et al. 2001), prompted us to test whether PAB3 and Pan3p might also interact physically. To this end, immunoprecipitation with anti Pan3p antibody was conducted from the total extract of the strain expressing PAB3 in the *pab1Δ spb2Δ* background (strain yDB221), as well as from an isogenic control strain YDB236-8 that lacked the *PAN3* gene (Fig. 4C). In addition, a YDB203 strain, which was complemented by PAB3, and therefore, required its expression for growth, and *pab1Δ spb2Δ PAN3* strain YRP881 were also tested. PAB3 was detected in the immunoprecipitates from the yDB221 and YDB203 cells, but not from the YDB236-8 (*pab1Δ spb2Δ pan3Δ* + PAB3) cells (Fig. 4C). Moreover, this interaction was not bridged by RNA, because it was insensitive to RNase A treatment (Fig. 4D). Thus, we conclude that PAB3 is able to partially control the extent of the poly(A) tail addition in yeast extracts in the PAN-dependent manner, and is able to interact with the PAN subunit in yeast.

PAB3 accelerates the entry of the newly synthesized mRNA into the translated pool

In considering possible mechanisms of complementation of the *pab1Δ* phenotype by PAB3, it seemed counterintuitive that just the acceleration of the mRNA decay, which results from the lag correction by PAB3, would be sufficient for the cell viability in the absence of the endogenous Pab1p. Rather, we reasoned that the expression of the plant PAB3 protein in the PABP-deficient yeast cells might have additional consequences on gene expression, other than just the correction of the lag prior to the entry of the mRNA into the decay pathway. We hypothesized that an acceleration of the rate of mRNA biogenesis and/or export might be a part of such a mechanism, because PAB3 functions in the 3′ end processing in yeast (above), and because the endogenous yeast Pab1p also has been implicated in mRNA biogenesis, and particularly in the 3′ end processing (Caponigro and Parker 1995; Amrani et al. 1997; Kessler et al. 1997; Minvielle-Sebastia et al. 1997; Brown and Sachs 1998; Mangus et al. 1998; Morrissey et al. 1999), which in turn is important for mRNA export in yeast (Brodsky and Silver 2000; Hilleren et al. 2001; Dower and Rosbash 2002; Hammell et al.

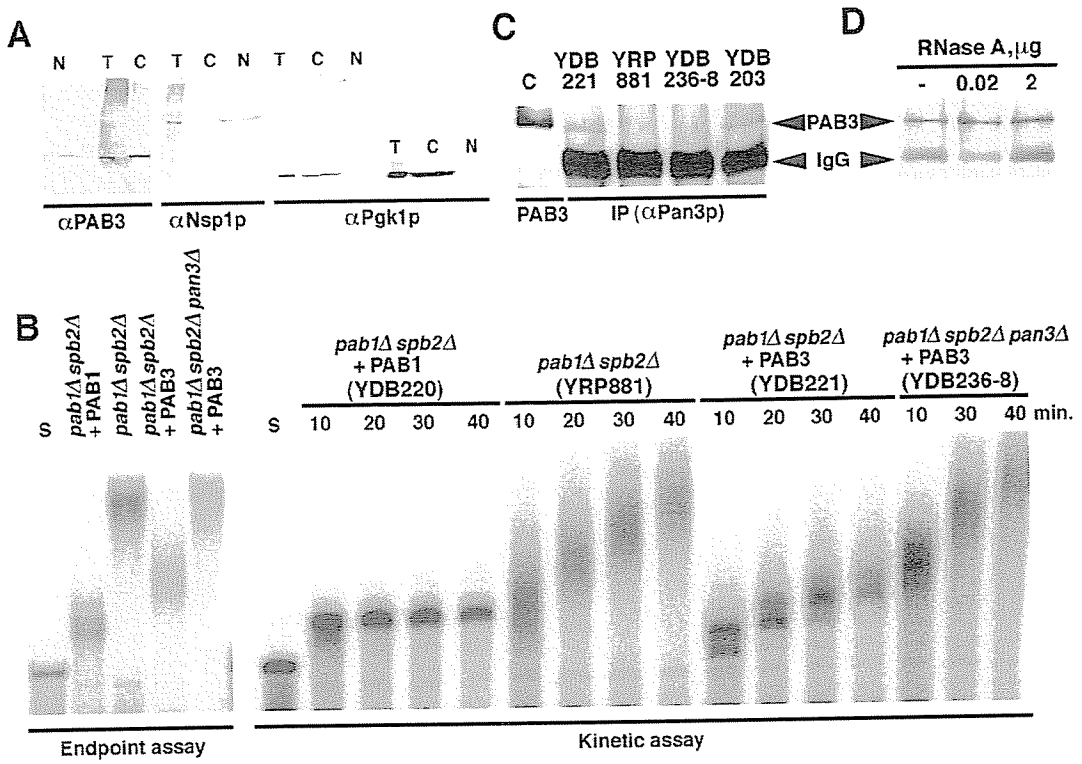

FIGURE 4. Evidence that PAB3 functions in the nucleus in yeast. (*A*) Results of the subcellular fractionation of the extract of the PAB3-complemented yeast strain YDB203. Equivalent amounts of the total unfractionated extract (T), as well as the nuclear (N) and cytoplasmic (C) fractions were immunoblotted and probed for PAB3, Nsp1p (nuclear marker) and Pgk1p (cytoplasmic marker). Overexposure of the immunoblotting for Pgk1p is shown at *right* to demonstrate the absence of the cytoplasmic contamination in the nuclear fraction. (*B*) Results of the endpoint (*left*) and kinetic (*right*) polyadenylation assays in vitro using the precleaved substrate. Relevant genotypes of the strains from which the extracts were prepared are shown at *top*. Lanes marked S contain the precleaved substrate RNA only. (*C*) PAB3 copurifies with the yeast Pan3p in a coimmunoprecipitation assay. Extracts from the yDB221 (*pab1Δ spb2Δ* + PAB3), yDB236-8 (*pab1Δ spb2Δ pan3Δ* + PAB3), and YDB203 (*PAN3* strain complemented by PAB3) cells were incubated with the antiPan3p antiserum immunoprecipitates captured on protein A agarose, extensively washed, and the bound material immunoblotted for PAB3. Recombinant PAB3 was loaded in the *leftmost* lane (marked C) as a positive control. Positions of the signals corresponding to PAB3 and IgG heavy chain are indicated by arrowheads. (*D*) Communoprecipitation of PAB3 and Pan3p from YDB203 extracts is resistant to treatment with 0.02–2 μg of RNase A for 40 min at room temperature.

2002). One expected consequence of such an activity of PABP in facilitating mRNP biogenesis would be an acceleration of the rate of entry of the mRNA into the translated pool.

To test this possibility, we have compared the kinetics of accumulation of the protein encoded by the reporter mRNA after its transcriptional induction in the PABP-deficient and PAB3-expressing cells, as an indirect measure of the kinetics of the entry of the reporter mRNA into the translated pool. An advantage of using PAB3-expressing strain in this analysis is that the *Arabidopsis* PAB3 supports neither the poly(A)-dependent enhancement of translation nor the poly(A)/cap synergy in yeast (Chekanova et al. 2001). Thus, any effect of PAB3 on the kinetics of the accumulation of the protein that is encoded by the reporter mRNA toward its steady state would be largely due to an effect of PAB3 on the kinetics of the entry of the mRNA into the translation cycle, rather than due to effects on the mRNA translation efficiency.

Because the very small size and an extreme hydrophobicity of the *MFA2*-encoded peptide made quantitative Western analyses difficult, a different reporter was used in this assay. Yeast *SSA4* mRNA, which encodes a Hsp70-type heat-shock protein Ssa4p, is virtually undetectable in cells grown at 28°C, but is rapidly induced after the shift to 42°C. In the YDB221 (*pab1Δ spb2Δ* + PAB3) cells, *SSA4* mRNA level peaked at a ~30-min time point after its induction by heat shock and then declined, which reflected its decay (Fig. 5A). In contrast, in the YRP881 (*pab1Δ spb2Δ*) cells, *SSA4* message was stable throughout the time course of the experiment. Thus, *SSA4* mRNA is also subject to the lag prior to the onset of mRNA decay, and this lag was corrected by the *Arabidopsis* PAB3 in yeast at 42°C. The critical observation was that PAB3 also reproducibly accelerated the rate of accumulation of the Ssa4p protein toward its steady-state level (Fig. 5B), so that the Ssa4p steady state was reached faster in the YDB221 (*pab1Δ spb2Δ* + PAB3) cells than in the YRP881 cells lacking PABP (15 min versus 30 min).

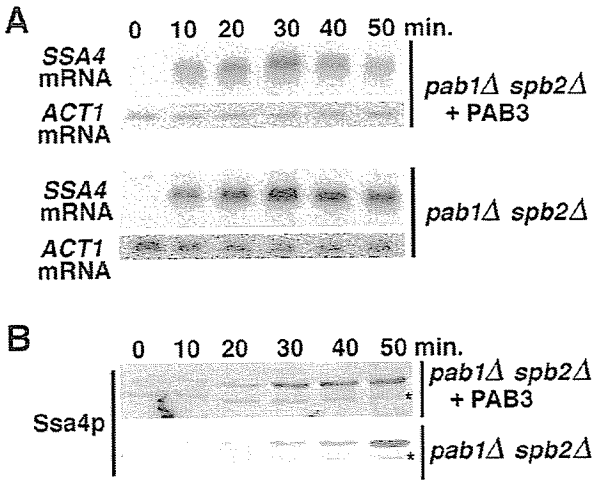

FIGURE 5. Kinetics of the accumulation of the *SSA4* mRNA (*A*, Northern blot) and Ssa4p heat-shock protein encoded by it (*B*, Western blot) in the YRP881 (*pab1Δ spb2Δ*) and YDB221 (*pab1Δ spb2Δ* + PAB3) strains after the shift to 37°C. *ACT1* mRNA (*A*) and nonspecific crossreacting asterisk (*B*) were used as loading controls.

Because PAB3 supports neither the poly(A)-dependent stimulation of the initiation of the protein synthesis in yeast, nor the 5′ cap/poly(A) translational synergy (Chekanova et al. 2001), we conclude that the PAB3-dependent acceleration of the rate of approach of the Ssa4p protein level toward its steady state reflects the acceleration of the entry of *SSA4* mRNA into the translated mRNA pool by PAB3.

PAB3 overexpression rescues the cold-sensitive phenotype and mRNA export defect of the yeast *nab2-1* mutant

The above observation that PAB3 was able to accelerate the rate of entry of the *SSA4* mRNA into the translated pool in yeast, as well as the known role of the yeast Pab1p in mRNA biogenesis, led us to test the possibility that the *Arabidopsis* PAB3 could affect the mRNA export in yeast. It has been demonstrated recently that overexpression of the yeast Pab1p, or targeting of the Pab1p into the nucleus, lead to the suppression of the cold-sensitive lethal phenotype and the mRNA export block of the *nab2-21* mutant (Hector et al. 2002). The Nab2p protein is one of the shuttling hnRNP proteins in yeast that associates with the poly(A)$^+$ mRNA in the nucleus, and plays an important role in facilitating the assembly of the export-competent mRNP (Green et al. 2002; Hector et al. 2002). In addition, Nab2p has a second function in the poly(A) tail length control during nuclear polyadenylation, and the *nab2* mutant cells accumulate hyperadenylated mRNA (Hector et al. 2002). Overexpression or nuclear targeting of Pab1p suppressed the cold-sensitive lethality and mRNA-export defect, although it had no effect on the poly(A) tail length control defect in the *nab2-21* cells (Hector et al. 2002). This suggests that Pab1p may have a role in the biogenesis of the export-competent mRNP that is distinct from the poly(A) tail length control per se.

We have asked whether the heterologous PAB3 would be able to restore the mRNA export in the *nab2* mutant cells. We chose a different *nab2* allele, *nab2-1*, as it showed much lower frequency of spontaneous suppressors and/or revertants than *nab2-21*. Also, *nab2-1* had a considerably less-pronounced poly(A) tail length-control defect, compared with *nab2-21* (Fig. 6A). However, much like with *nab2-21* allele, this poly(A) tail length defect was equally evident at both permissive and nonpermissive temperatures (Fig. 6A). The *nab2-1* mutant is also cold sensitive, and shows an accumulation of the poly(A)$^+$ mRNA in the nucleus at the nonpermissive temperature (Fig. 6C; Green et al. 2002).

We overexpressed the plant PAB3 from the *ADH1* promoter construct borne on a high-copy plasmid (this resulted in ~2.5–3-fold higher levels of PAB3 compared with the amount of Pab1p in the wild-type strain; data not shown), and also added one extra copy of the *PAB1* gene on a single-copy plasmid [approximately twofold overexpression of PAB1 was sufficient to rescue the mRNA export defect and cold sensitivity of *nab2-21* mutant (Hector et al. 2002)], in the *nab2-1* strain. In either case, the poly(A) tail length distribution was not changed noticeably (Fig. 6A). However, PAB3 reproducibly rescued the cold sensitivity of the *nab2-1* cells at 14°C, even though the rate of growth was still slower than in the cells containing one extra copy of *PAB1* (Fig. 6B). The effect of PAB3 on the mRNA export defect at 18°C was then tested by FISH with the oligo(dT) probe. Importantly, PAB3 partially reversed the nuclear accumulation of the poly(A)$^+$ mRNA observed in the *nab2-1* cells at 18°C (Fig. 6C), suggesting that PAB3 can stimulate mRNA biogenesis and/or export in yeast.

DISCUSSION

Evidence presented in this work strongly suggests that eukaryotic PABP has an evolutionarily conserved function in facilitating mRNA biogenesis and export. First, the *Arabidopsis* PABP, PAB3, accelerated the entry of the mRNA into the degradation pathway, as well as its entry into the translated pool when expressed in yeast. Second, plant PABP was present physically in the yeast cell nucleus in the complemented yeast strain, and it partially restored the poly(A) tail length control during polyadenylation reaction, in the PAN-dependent manner. Third, the substitution of the plant PAB3 for the endogenous yeast Pab1p resulted in synthetic lethality with *rna15-2*, an allele of the gene encoding the Rna15p subunit of the nuclear factor CFI, which is required for the pre-mRNA cleavage, as well as polyadenylation, and with *gle2-1*, a mutant allele of the gene encoding the NPC-associated protein. Fourth, overexpression of the plant PABP in yeast rescued the cold sensitivity and

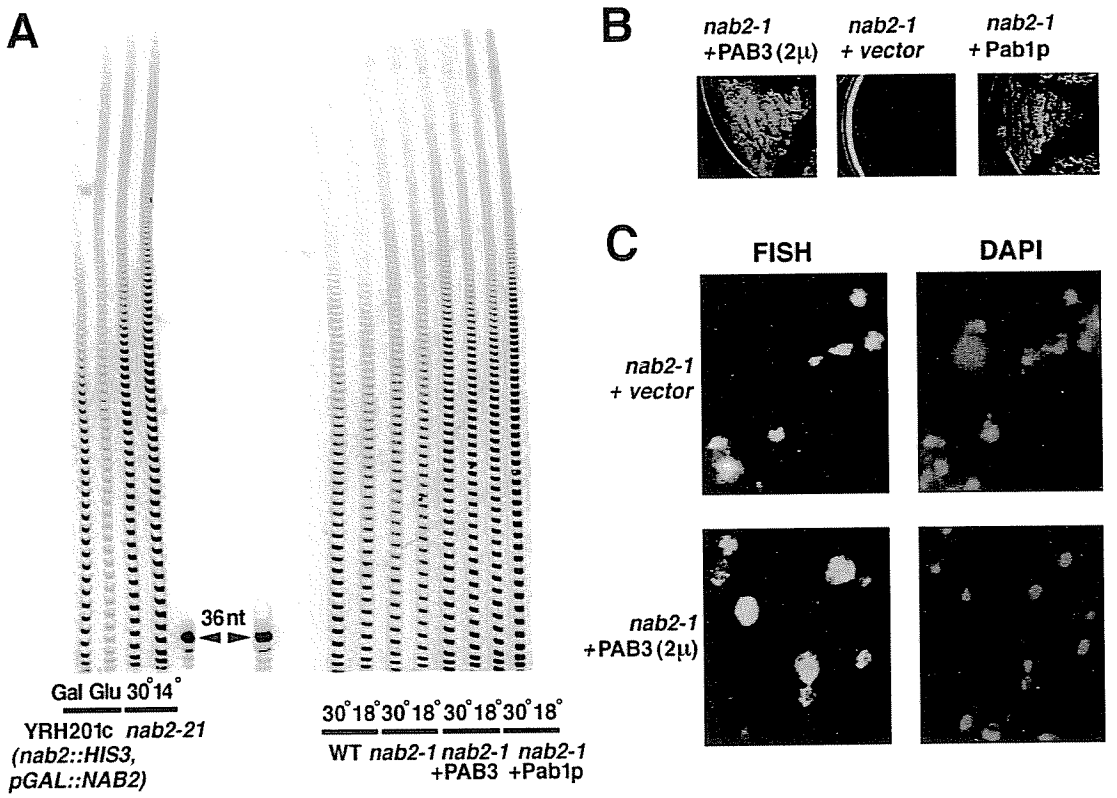

FIGURE 6. Consequences of the overexpression of PAB3 in the *nab2-1* mutant background. (*A*) A comparison of the poly(A) tail phenotypes of the *nab2-21* (*left*) and *nab2-1*, as well as *nab2-1* strain overexpressing PAB3 or Pab1p (*right*) at normal (30°C) and low (14 or 18°C, as indicated) temperatures. Total poly(A) tail length distributions in the YRH201c cells, in which Nab2p is expressed from *GAL* promoter, on galactose, and after the shift into glucose medium are also shown (*left*). A 36-nucleotide long DNA oligonucleotide was used as a size marker. (*B*) Phenotypic rescue of the cold sensitivity of the *nab2-1* mutant by overexpression of Pab1p or PAB3. The strains shown *above* the respective panels were streaked onto the selective synthetic medium and incubated at 18°C for 12 d. (*C*) FISH with the $(dT)_{70}$ probe of the *nab2-1* mutant cells transformed with the 2μ/PAB3 construct or an empty 2μ vector, as indicated, after the shift to the nonpermissive temperature (18°C) for 16 h. Staining for nuclei with DAPI is shown in the two *right* panels.

alleviated the mRNA export block of the *nab2-1* mutant strain, which is defective in one of the shuttling hnRNP proteins required for mRNA export.

The possible role of the mRNA poly(A) tails, and by implication, PABP, in the nucleocytoplasmic mRNA transport has been a subject of a number of studies in a variety of eukaryotic systems. Early experiments by Krowczynska et al. (1985) showed that all mRNA that is exported to the cytoplasm is polyadenylated, whereas the cytoplasmic poly(A)$^-$ species are derived from those that are poly(A)$^+$ during transport. On the other hand, a study that used the polyadenylation inhibitor cordycepine, suggested that poly(A) is not strictly required for transport, although it might have a stimulatory role (Zeevi et al. 1982). The polyadenylated SV40 transcripts synthesized in vivo from the transfected templates were found to preferentially partition into the cytoplasm in *Xenopus* oocytes (Wickens and Gurdon 1983) and human cells (Connelly and Manley 1988), relative to the poly(A)$^-$ ones. Similarly, appending a RNA pol II 3′ end processing signal onto a reporter gene of bacterial origin resulted in an increase in its cytoplasmic-to-nuclear steady-state ratio in COS cells (Eckner et al. 1991). RNA microinjection studies in *Xenopus* oocytes also suggested that poly(A) is stimulatory, although it may not be absolutely required for export (Jarmolowski et al. 1994). The NS1 protein of influenza virus inhibits polyadenylation of cellular transcripts, and thereby prevents their export (Nemeroff et al. 1998). Evidence obtained in the COS cells argues that poly(A) tails promote export, but it appears that it is the dynamic process of the 3′ end formation, rather than just the mere presence of a monotonous run of Å residues near the 3′ end, is what is required for the efficient mRNA export (Huang and Carnichael 1996), because a poly(A) tract encoded in the body of the transcript was not sufficient.

In yeast, the RNA pol II transcripts that lack a polyadenylation signal were shown to be retained in the nucleus, as visualized by in situ hybridization (Long et al. 1995). Using another technology, on the basis of the tethering of GFP to the reporter transcripts, Brodsky and Silver (2000) have observed mRNA export defects in the *rna14-1*, *rna15-2*, *hrp1-3*, and *pap1-1* strains, which are conditionally defec-

tive in the components of CFI and PAP, respectively. Furthermore, in a screen for a failure to export the *SSA4* mRNA at an elevated temperature, Hammell et al. (2002) have found several alleles of *RNA15*, *RNA14*, *FIP1*, and *PAP1*. Dower and Rosbash (2002) have found that the T7 RNA polymerase transcripts accumulate in the nucleus, unless they are cleaved and polyadenylated by the machinery that normally processes the RNA pol II transcripts. All of these findings indicate the link between the mRNA 3′ end processing, polyadenylation, and export. Interestingly, the *rna15-1* mutant was found to have a very slow mRNA decay rate (Gonzalez et al. 2000). This could be due to a lag prior to the mRNA export, similar to the one that is observed in the *pab1* mutant strains, occurring because of improper maturation of the 3′ terminal domain of the mRNP.

Yeast Pab1p has been functionally linked previously to Pbp1p, a predominantly nuclear, Pab1p-interacting protein that facilitates proper polyadenylation (Mangus et al. 1998). However, the role of Pab1p in the nuclear steps of mRNA biogenesis may be broader than just the control of the length of the newly made poly(A) tails. This view is consistent with the results of Hector et al. (2002) that nuclear Pab1p can suppress the mRNA export defect of the *nab2-21* cells in a way that can be genetically uncoupled from the control of the poly(A) tail length by Pab1p. Rather, these findings, together with the ones presented here, suggest that the dynamic process of formation of the proper architecture of the 3′ domain of mRNP may be essential.

Our results may seem at odds with the results of Kadowaki et al. (1992), who found that inactivation of the temperature-sensitive *pab1-F364L* allele did not visibly change the nucleocytoplasmic distribution of the poly(A)$^+$ RNA, as visualized by in situ hybridization. However, the *pab1-F364L* allele that was used in that study has an extremely slow turnover rate (Sachs and Davis 1989). Thus, in the absence of definitive information as to whether it is temperature sensitive for function of the existing protein, or for the de novo synthesis of the functional polypeptide, these findings should be interpreted with caution. Second, this experiment, as well as many other studies examining the possible role of poly(A) and PABP in mRNA export, relied exclusively on the assays that are static in nature (such as FISH), and as such, did not directly address the possibility of more subtle changes in the rate of mRNA export. On the other hand, our data presented in this work suggest that PABP facilitates mRNA export, rather than being strictly required for it.

Interestingly, in *Schizosaccharomyces pombe*, PABP shuttles between the nucleus and the cytoplasm, and importantly, its overexpression could suppress conditional lethality, as well as an associated mRNA export defect of a *rae1-167 nup184-1* synthetic lethal strain (Thakurta et al. 2002). It should be pointed out that RAE1 of *S. pombe* is a homolog of the Gle2p of *S. cerevisiae*, which is functionally implicated in the evolutionarily conserved PABP function by our finding of synthetic lethality between the *gle2-1* allele and a substitution of the plant PAB3 for the yeast Pab1p. The ability of the *S. pombe* PABP to shuttle is directly related to its ability to rescue this mRNA export defect (Thakurta et al. 2002). However, this function of PABP in mRNA export is apparently redundant with other factors in *S. pombe*, as the loss of the *S. pombe* PABP does not lead to mRNA export defects, and, in fact, has no discernible phenotype at all. It is also significant in this context that the *S. cerevisiae* Pab1p interacts with Xpo1p, a nucleocytoplasmic transport receptor, and shuttles between the nucleus and the cytoplasm in a Xpo1p-dependent manner (Hammell et al. 2002). The nucleocytoplasmic shuttling ability seems to be conserved in PABP from other eukaryotic species, including human (Afonina et al. 1998) and *Leishmania* (Bates et al. 2000). Moreover, human PABP is also subject to arginine methylation (Lee and Bedford 2002), which is a modification that is characteristic of many shuttling proteins that are involved in nucleocytoplasmic transport (McBride and Silver 2001).

The findings presented here and elsewhere (Thakurta et al. 2002) of the genetic links between the nuclear function of PABP and Gle2p, a NPC associated factor, suggest the inner face of the NPC as another possible site of PABP action in the nucleus. On the other hand, a series of recent studies (Burkard and Butler 2000; Hilleren et al. 2001; Jensen et al. 2001a,b; Andrulis et al. 2002; Libri et al. 2002; Zenklusen et al. 2002) have led to a view of a release of the pre-mRNA from the transcription site as a distinct, regulated step in the biogenesis of functional mRNP that involves the nuclear exosome (for reviews, see Neugebauer 2002; Jensen et al. 2003). These results suggest an additional possibility (which is not mutually exclusive with the possible role in the NPC-associated step), that the role of PABP as an evolutionarily conserved facilitator of mRNA biogenesis may be linked to a release of the mRNP from the site of transcription. Future experiments will attempt to resolve, or reconcile these possibilities.

MATERIALS AND METHODS

Plasmid constructs

The *MFA2-MS2-RZ* construct used for the experiments shown in Figure 1 was pMM2-6 (Coller et al. 1998), kindly provided by Jeff Coller (University of Arizona). The constructs used for expressing Pab1p and PAB3, pDB464 (*TRP1/CEN/PAB1*), pDB419 (*TRP1/CEN/pGAL1-PAB3*), pDB463 (*TRP1/2μ/pADHI-PAB3*), and pDB488 (*G418^r/TRP1/2μ/pADHI-PAB3*) were described previously (Chekanova et al. 2001). The construct pDB493 is equivalent to pDB488, except that its *TRP1* marker was disrupted via linearization at the *Eco*RV site and limited digestion with *Exo*III and mung bean nucleases, followed by religation. The construct pDB490 (*G418^r/CEN/PAB1*) was similarly derived from the pDB489 (*G418r/TRP1/CEN/PAB1*; Chekanova et al. 2001) via disabling the *TRP1* marker.

Yeast strains and techniques

To construct the yeast strains for the synthetic lethal tests, strains bearing mutations in the various factors involved in mRNA processing or export (shaded gray in the Table 1) were first transformed with the pAS77, a *CEN/URA3/PAB1* plasmid (Sachs and Davis 1989). The chromosomal copy of the *PAB1* gene was then disrupted by transformation with linear DNA fragments containing *HIS3*, *LEU2*, or *TRP1* marker (depending on the strain used), flanked by the 300 bp from the native 5′ and 3′ flanking regions of *PAB1*. Chromosomal integrants were selected on the basis of the PCR assay using the pair of primers corresponding to the *PAB1* sequence, one within and one outside of the sequence present in the integrated fragment, and subsequently verified by Southern analysis. Then, the PAB3-expressing construct was introduced, which consisted of a p*GAL1-PAB3* cassette identical to that of pDB419, on both *CEN* and 2μ-based plasmids with either *LEU2*, *HIS3*, or Geneticin resistance marker, as dictated by the auxotrophies of the respective strains. Transformants were grown in synthetic medium containing uracil, and dilution series spotted onto YPD, SC galactose + 5 FOA, and SC glucose + 5-FOA plates. The carbon-source dependence of growth was used as a criterion to exclude the spontaneously arising extragenic suppressors of *pab1Δ* mutation. Other yeast strains used in this work are listed in Table 1. General yeast genetic methods were according to Guthrie and Fink (1991).

Purification of Xrn1p and cap status assays

The His-tagged Xrn1p was expressed in the yeast strain BJ5464 from the construct pAJ95 (*2μ/LEU2/pGAL10-XRN1-HA-His$_6$*, a kind gift from Arlen Johnson, University of Texas, Austin) as described previously (Johnson and Kolodner 1991), and protein purified on a Ni^{++} column as suggested by the manufacturer (QIAGEN). Treatment of total RNA with the purified Xrn1p was as per Boeck et al. (1998).

Subcellular fractionation

The YDB203 cells grown in YPGal to OD_{600} = 0.5 were converted to spheroplasts, lysed, and washed as described by Azad et al. (2001) and fractionated on Ficoll gradient according to Aris chapter (Dove et al. 1998). Equivalent proportions of total, nuclear, and cytoplasmic material were analyzed by immunoblotting as shown in Figure 4.

RNA analyses

RNA decay analyses, transcriptional pulse-chase experiments, and total poly(A) tail length analysis were done as described previously (Chekanova et al. 2001). The 7S rRNA precursor probe (oligo 020) and hybridization conditions were according to Mitchell et al. (1997).

Immuprecipitation and immunonoblotting

Antibodies were used in immunoblotting experiments in the following dilutions: against PAB3 (rabbit polyclonal), at 1:1000; against Pgk1p (mouse monoclonal, Molecular Probes), at 0.5 μg/mL; against Nsp1p (mouse monoclonal [Tolerico et al. 1999], a kind gift from John Aris, University of Florida, Gainesville), at 1:10000; and against Ssa4p (rabbit polyclonal, a kind gift from Elizabeth Craig, University of Wisconsin), at 1:3000. Immunoblots were quantitated using GEL Quant v. 1.0 (Multiplexed Biotechnologies, Inc.). Immunoprecipitation was conducted in 100 μL of buffer using a 1:1000 dilution of the rabbit antiserum raised against the Pan3p (Brown and Sachs 1998). The immunoprecipitates were captured on 20 μL of the protein A agarose beads (Santa Cruz), washed seven times with 500 μL of PBS, and bound material eluted into SDS-PAGE loading buffer, and probed with PAB3 antiserum. Immunoprecipitates shown in Figure 4D were treated with indicated amounts of RNase A for 40 min at room temperature prior to washes.

In vitro polyadenylation assays

Extracts were prepared by the liquid nitrogen homogenization method, and assays performed as described previously by Brown and Sachs (1998).

Fluorescent in situ hybridization

FISH with a Cy3-labeled oligo$(dT)_{70}$ probe was carried out as described in Vainberg et al. (2001).

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mRNA deadenylation by PARN is essential for embryogenesis in higher plants

SERGEI V. REVERDATTO,[1] JAMES A. DUTKO,[1] JULIA A. CHEKANOVA,[1] DOUGLAS A. HAMILTON,[2] and DMITRY A. BELOSTOTSKY[1]

[1]Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222, USA
[2]Department of Biology, Hartwick College, Oneonta, New York 13820, USA

ABSTRACT

Deadenylation of mRNA is often the first and rate-limiting step in mRNA decay. PARN, a poly(A)-specific 3′ → 5′ ribonuclease which is conserved in many eukaryotes, has been proposed to be primarily responsible for such a reaction, yet the importance of the PARN function at the whole-organism level has not been demonstrated in any species. Here, we show that mRNA deadenylation by PARN is essential for viability in higher plants (*Arabidopsis thaliana*). Yet, this essential requirement for the PARN function is not universal across the phylogenetic spectrum, because PARN is dispensable in Fungi (*Schizosaccharomyces pombe*), and can be at least severely downregulated without any obvious consequences in Metazoa (*Caenorhabditis elegans*). Development of the *Arabidopsis* embryos lacking PARN (AtPARN), as well as of those expressing an enzymatically inactive protein, was markedly retarded, and ultimately culminated in an arrest at the bent-cotyledon stage. Importantly, only some, rather than all, embryo-specific transcripts were hyperadenylated in the mutant embryos, suggesting that preferential deadenylation of a specific select subset of mRNAs, rather than a general deadenylation of the whole mRNA population, by AtPARN is indispensable for embryogenesis in *Arabidopsis*. These findings indicate a unique, nonredundant role of AtPARN among the multiple plant deadenylases.

Keywords: mRNA turnover, deadenylation

INTRODUCTION

The process of messenger RNA degradation is a principal component of the network of gene expression events that determine the ultimate steady-state concentration of every transcript in the cell under a myriad of conditions. Degradation of mRNA occurs through a defined sequence of steps, that is, along specific pathways. Studies in Fungi and Metazoa have led to a definition of the four major mRNA degradation pathways: (1) deadenylation-dependent decapping followed by 5′ → 3′ decay (for review, see Mitchell and Tollervey 2000; Tucker and Parker 2000; Butler 2002); (2) deadenylation-dependent 3′ → 5′ decay (Mitchell and Tollervey 2000; Tucker and Parker 2000; Butler 2002); (3) nonsense-mediated decay (NMD), that specifically targets mRNAs with premature termination codons (Chen and Shyu 2003; Lejeune et al. 2003; Mitchell and Tollervey 2003), and (4) nonstop decay, that specifically targets mRNAs lacking termination codon (Frischmeyer et al. 2002; van Hoof et al. 2002). Pathways (1), (2), and (4) are initiated by poly(A) tail shortening and/or its complete removal, collectively termed deadenylation, which often serves as a rate-determining step of the whole process. Furthermore, at least some cases of NMD also proceed via deadenylation as well (Chen and Shyu 2003; Lejeune et al. 2003; Mitchell and Tollervey 2003). Moreover, computational modeling of mRNA decay in yeast suggests that modulation of the rate of deadenylation is a very powerful way to manipulate the overall mRNA steady-state level (Cao and Parker 2001).

Deadenylation is also subject to regulation. The rate of mRNA deadenylation can be modulated by specific, portable sequence elements that are found in the mRNA 3′-UTR. For instance, AU-rich elements (AREs), such as the ones found in the 3′-UTRs of mammalian *c-fos* and GM-CSF, can confer rapid deadenylation kinetics upon the reporter mRNAs (Chen et al. 1995). Such elements appear to act as binding sites for the specific *trans*-acting factors. Re-

Reprint requests to: Dmitry A. Belostotsky, Department of Biological Sciences, State University of New York at Albany, Albany, NY 12222, USA; e-mail: dab@albany.edu; fax (518) 442-4368.

Abbreviations: PARN, poly(A)-specific 3′ → 5′ ribonuclease; PABP, poly(A) binding protein; NMD, nonsense-mediated decay; LM-PAT assay, ligation mediated poly(A) tail length assay; ARE, AU-rich element.

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cruitment of such factors could influence deadenylation via direct recruitment of deadenylases and/or stimulation of their activity, by altering the mRNA secondary structure, by resulting displacement from mRNA, or remodeling on mRNA, of the protecting RNA binding protein(s), for example, PABP, or by targeting PABP for degradation. Moreover, such *trans*-acting factors are the likely mediators of the regulation of the mRNA deadenylation rates by extracellular stimuli, acting through signal transduction pathways (Winzen et al. 1999; Vasudevan and Peltz 2001).

Poly(A) ribonuclease (PARN) is an evolutionarily conserved factor that has been implicated in mRNA deadenylation in metazoans (Wilusz et al. 2001). PARN belongs to the RNase D family of nucleases, which is characterized by the presence of the four conserved acidic amino acid residues in the active site that coordinate divalent metal ions for catalysis. Overall, the active site of PARN functionally and structurally resembles the active site of the 3′-exonuclease domain of *Escherichia coli* DNA polymerase I (Beese and Steitz 1991; Ren et al. 2002). Metazoan PARN specifically degrades only the poly(A) tail, but not the body, of the model polyadenylated mRNA substrates in vitro, and its activity and processivity are stimulated by the mRNA 5′ m^7GpppG cap, likely via an allosteric mechanism (Dehlin et al. 2000; Gao et al. 2000; Martinez et al. 2001). *Xenopus* PARN has been shown to deadenylate those maternal transcripts that lack cytoplasmic polyadenylation element (CPE), during oocyte meiotic maturation (Copeland and Wormington 2001), and mammalian PARN has been functionally implicated in accelerated decay conferred by AREs (Lai et al. 2003), as well as in NMD (Lejeune et al. 2003).

The occurrence of mRNA deadenylation during mRNA decay in higher plants has been mostly inferred, rather than directly demonstrated. It has been proposed, based on the examination of mRNA decay products, that ~25% of the oat phytochrome A mRNA pool degrades via a deadenylation-dependent 3′ → 5′ pathway (Higgs and Colbert 1994). Poly(A) tail length of the rice α-amylase mRNA, αAmy3, is regulated during development (Lue and Lee 1994). In a related study, αAmy3 3′-UTR has been shown to be responsible for destabilizing the αAmy3 transcript in high-sucrose media, possibly by promoting rapid deadenylation (Chan and Yu 1998). In the one of the best studied instances of the regulated mRNA deadenylation during plant development, some mRNAs in pollinated tobacco pistils were found to undergo a poly(A) tail shortening dependent on the plant hormone ethylene acting through a signal transduction cascade that involves phosphorylation and dephosphorylation events (Wang et al. 1996). Interestingly, poly(A) shortening in this system subsequently may or may not lead to the degradation of the body of the mRNA, depending on the transcript (Wang et al. 1996).

Here, we used reverse genetics to address the functional significance of the one of the potential *Arabidopsis* deadenylases, AtPARN, and to show that its function as deadenylase is essential for plant embryogenesis. Importantly, we found that only some, but not all, embryo-specific transcripts become hyperadenylated in the mutant embryos, suggesting that deadenylation of a select subset of mRNAs by AtPARN, rather than nonspecific deadenylation of the whole mRNA population, is essential for embryonic development. Furthermore, we found that PARN function is completely dispensable in *Schizosaccharomyces pombe*, and can be at least severely knocked down in *Caenorhabditis elegans* without any obvious phenotype. Thus, the essential requirement for the PARN function is not universal in eukaryotes, and could be unique to plants.

RESULTS

AtPARN is a predominantly cytoplasmic deadenylase

Two *Arabidopsis thaliana* genes, At1g55870 (hereafter called *AtPARN*) and At3g25430, encode proteins with high sequence similarity to metazoan PARN (BLAST E values of $6.5 \times e^{-39}$ and $9.8 \times e^{-21}$, respectively, upon using human PARN as a query, with sequence homology extending through at least N-terminal two-thirds of the amino acid sequence, Fig. 1). Both genes are expressed. However, whereas the *AtPARN* open reading frame (ORF) contains all of the four highly conserved acidic residues shown earlier to be essential for catalysis in the RNase D family nucleases, including human PARN (Ren et al. 2002), the At3g25430 ORF has nonconservative replacements in three of the four positions (Fig. 1). Thus, only the former is likely to encode a catalytically active PARN enzyme. This view is corroborated by the results of site-directed mutagenesis experiments on AtPARN described below, as well as the fact that the knockout of the At3g25430 gene causes no apparent phenotype (see Materials and Methods), in contrast to the embryonic lethal phenotype that is caused by the knockout of *AtPARN* (below). Purified recombinant AtPARN degraded only the poly(A) tail, not the body, of the model polyadenylated RNA substrate in vitro, and it did not degrade an otherwise identical polyuridylated substrate (Fig. 2A). Thus, AtPARN encodes a functional deadenylase enzyme.

RT-PCR based survey revealed that the *AtPARN* mRNA is broadly expressed in roots, stems, leaves, and flowers (data not shown). To address the subcellular distribution of the AtPARN polypeptide, we generated transgenic *Arabidopsis* plants bearing a genomic *AtPARN* construct containing the green fluorescent protein (GFP) ORF fused to the last exon of the *AtPARN* gene. The resulting AtPARN-GFP C-terminal fusion protein was functional, as it fully rescued the mutant phenotype of the *atparn-1* null allele (below). In the second approach, the GFP-AtPARN fusion construct driven by a strong, constitutive 35S promoter was introduced into onion epidermal cells using biolistic protocol. In both experiments, a predominantly cytoplasmic signal was

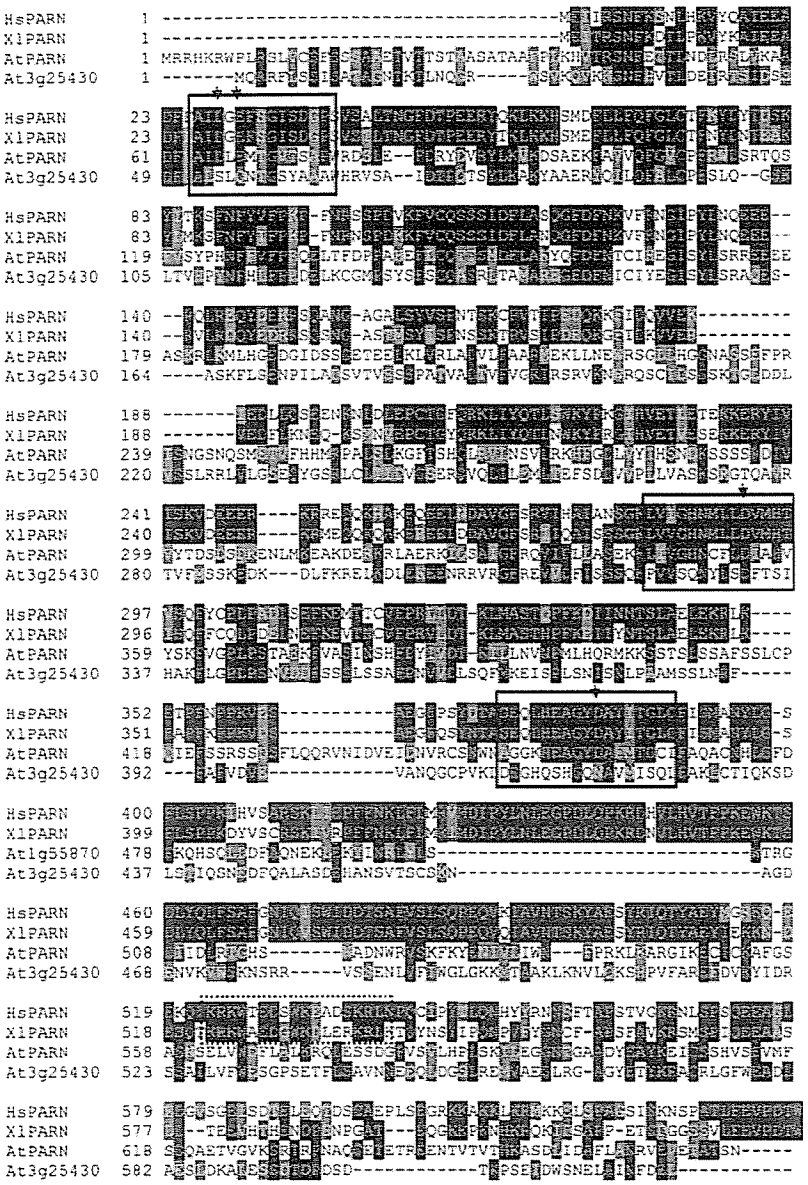

FIGURE 1. Sequence alignment of the amino acid sequences of human PARN, *Xenopus* PARN, AtPARN, and the *Arabidopsis* protein encoded by ORF At3g25430. Alignment was generated by ClustalW. The conserved ExoI, ExoII, and ExoIII domains are shown boxed. The bipartite NLS motif in human and *Xenopus* proteins is shown by dotted box. Black and gray shading designates residues that are identical or similar, respectively, in at least half of the sequences. The four acidic residues that coordinate the Mg^{++} ion for catalysis are marked by arrowheads. To produce D66A E68A mutant of AtPARN, D and E residues in the ExoI domain were replaced by alanines via site-directed mutagenesis.

observed (Fig. 2C,D). In contrast, human PARN exhibits both nuclear and cytoplasmic localization (Korner et al. 1998). In *Xenopus* oocytes, PARN is present in two isoforms, the nuclear 74-kDa and the cytoplasmic 62-kDa species, the latter probably derived by proteolytic cleavage from the 74-kDa polypeptide (Copeland and Wormington 2001). Both human and *Xenopus* PARN feature bipartite NLS in the C-terminal portion of the protein (Fig. 1). On the other hand, AtPARN lacks any obvious NLS motifs, which is consistent with its predominantly cytoplasmic localization.

Exoribonuclease activity of AtPARN is essential for plant embryogenesis

To investigate the significance of the AtPARN function for plant growth and development, several T-DNA insertional alleles of the *AtPARN* gene were isolated, and two of them (*atparn-1* and *atparn-2*, Fig. 3A) were characterized in detail. No homozygous mutant plants were recovered among the selfed progeny of plants heterozygous for either allele. Moreover, no viable transheterozygotes could be obtained by crossing the *atparn-1/+* and *atparn-2/+* plants, suggesting that the disruption of the *AtPARN* gene causes either zygotic or gametophytic lethality. However, reciprocal crosses have shown that the transmission of the mutant alleles through both male and female gametes was normal (data not shown), thus ruling out gametophytic lethality. On the other hand, one-quarter of the seeds obtained by selfing of the heterozygous *AtPARN/atparn* plants failed to accumulate chlorophyll and appeared white, in contrast to the normal seeds that underwent greening starting from the heart stage of embryo development (Fig. 3B). This is a classical hallmark of the embryonic lethal phenotype in plants (Meinke 1994). Dissection of the embryos from the green and white seeds and PCR-based genotyping showed that the white seeds contained smaller, underdeveloped embryos, all of which were *atparn/atparn* homozygous mutant (Fig. 3C). We noted that a previously described embryonic lethal mutation *emb25* maps very closely to the *AtPARN* gene (Meinke 1994); however, formal allelism tests showed that *AtPARN* and *EMB25* are distinct genes (see Materials and Methods). Thus, *Arabidopsis* PARN is a novel factor that is essential for embryogenesis.

The embryo-lethal *atparn-1* phenotype was fully rescued by the transformation with the *AtPARN* genomic fragment containing 0.9 kb of upstream flanking sequence. To dis-

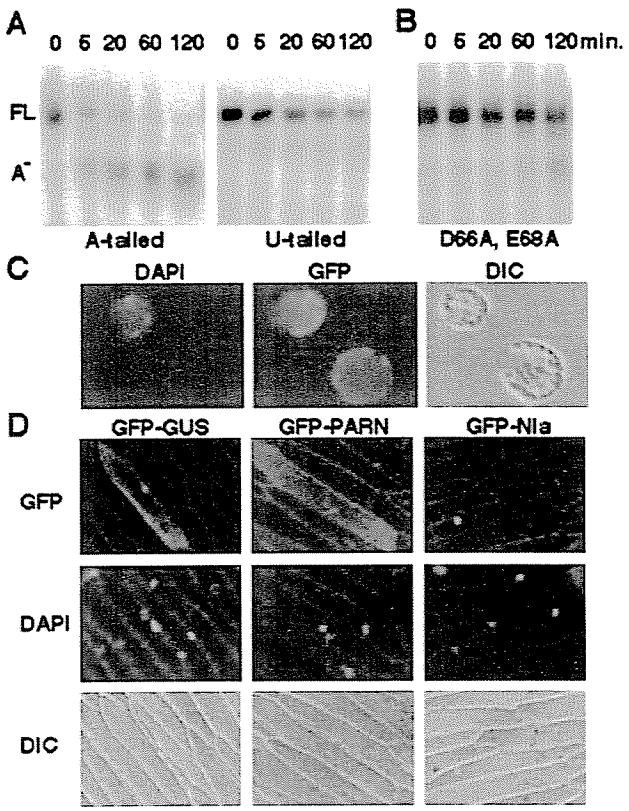

FIGURE 2. AtPARN is a predominantly cytoplasmic poly(A) exoribonuclease of an RNase D type. (*A*) Results of an in vitro assay using polyadenylated and polyuridilated synthetic RNAs of otherwise identical sequence, with wild-type recombinant AtPARN. (*B*) Results of an in vitro assay using polyadenylated RNA with the recombinant D66A E68A mutant AtPARN protein. (*C,D*) Localization of the AtPARN-GFP in transgenic *Arabidopsis* (*C*: protoplasts were prepared from the *atparn Arabidopsis* plants rescued by the AtPARN-GFP fusion; blue is DAPI staining for nuclei, red is chlorophyll autofluorescence of the chloroplasts) and of the GFP-AtPARN in onion epidermal cells using a biolistic assay (*D*: GFP-GUS and GFP-Nia are cytoplasmic and nuclear localized controls, respectively).

tinguish whether the lethality in the *Arabidopsis atparn* mutants occurred because of the loss of the AtPARN exoribonucleolytic activity, or of some other function associated with the AtPARN polypeptide, we substituted two of the four universally conserved acidic residues located in the ExoI domain that are essential for the activity of the RNase D type exonucleases (Ren et al. 2002) by alanines (D66A E68A mutant). Enzymatic assays of the D66A E68A mutant polypeptide that was expressed and purified from *E. coli* confirmed that these substitutions resulted in a loss of exoribonucleolytic activity (Fig. 2B). When expressed in planta, the AtPARN protein thus inactivated was unable to rescue the embryonic lethal phenotype of the *atparn* mutations, demonstrating that deadenylating activity of AtPARN is essential, at least during embryogenesis.

Microscopic examination of the selfed heterozygous plants revealed that the mutant embryos exhibited a pronounced retardation of development (Fig. 3D). *Arabidopsis* embryogenesis is characterized by a highly stereotypical progression through a series of morphologically distinguishable stages that are named according to the shape of the embryo: globular, heart, torpedo, walking-stick, and mature embryo (Bowman and Koornneef 1994). We found that by the time the normal embryos reached the heart stage of development, mutant seeds within the same siliques (seed pods) contained embryos that were still at the early globular stage; when normal embryos reached the walking-stick stage, the mutant sibling embryos were still at the late globular stage; by the time near-mature normal embryos had filled out most of the seed, mutant embryos within the same silique were still at the heart stage. We further verified that the morphology of the mutant embryos truly reflected a delayed pace of development by introducing, via crossing, the reporter gene fusions expressing β-galacturonidase (GUS) at the specific stages of embryogenesis, including FAE1::GUS (expressed from the early torpedo stage onward; Rossak et al. 2001), cotton α-globulin::GUS (expressed from the mid-torpedo stage onward; Sunilkumar et al. 2002), and 7S::GUS (expressed from the early bent cotyledon stage onward; Hirai et al. 1994; Apuya et al. 2001). For each of the three reporters, the mutant embryos were GUS-negative at the respective developmental stages, when the wild-type and heterozygous sibling embryos already exhibited robust GUS activity (Fig. 3E). The mutant embryos eventually did turn on the expression of these reporter genes (Fig. 3E, data for cotton α-globulin::GUS), but after a considerable delay. Furthermore, examination of the GFP gene trap reporter KS117, which normally expresses the green fluorescent protein throughout the endosperm during early embryogenesis but becomes restricted toward the posterior pole of the endosperm by the early globular stage (Sorensen et al. 2001), revealed that the *atparn* mutant seeds are impaired in the endosperm patterning: The posterior polarization of the KS117 expression failed to occur as late as the walking-stick stage of embryogenesis (Fig. 3F).

Mutant embryos beyond the early walking-stick stage were not observed, because at this point siliques entered desiccation. The onset of desiccation tolerance in the wild-type plants normally takes place toward the end of embryogenesis (Koornneef et al. 1989). Because of the retarded pace of development of the *atparn/atparn* mutant embryos, the seed desiccation in the *AtPARN/atparn* heterozygous plants commenced before the mutant embryos reached maturity. Thus, we reasoned that this might have explained the lethality of the *atparn* alleles. However, attempts to rescue the mutant embryos in culture did not produce any fully regenerated plants. Nevertheless, the *atparn* mutant embryos underwent multiple cell divisions, and showed cell differentiation and morphogenesis typical of normal seedling development. Many of the mutant embryos extended primary roots and produced root hairs that were correctly positioned in alternating epidermal cell files, and some of them also initiated the development of true leaves (Fig. 3G).

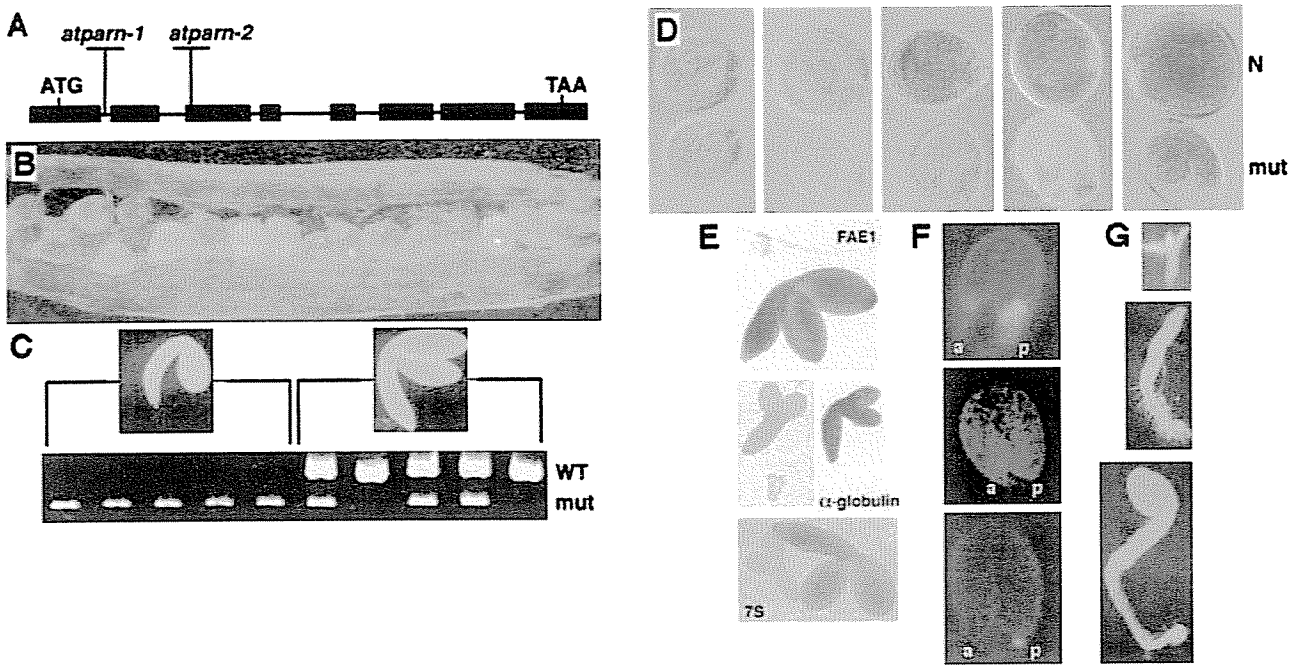

FIGURE 3. Null alleles of AtPARN lead to a slowed pace of embryogenesis and eventual arrest. (*A*) Schematic maps of the T-DNA insertion alleles *atparn-1* and *atparn-2*. (*B*) Dissected developing silique from the *atparn/AtPARN* heterozygote contains 1/4 white seeds, a classical hallmark of the embryo lethal phenotype. (*C*) PCR-based genotyping of the embryos dissected from the white and green seeds derived from the same silique. (*D*) DIC images of the developing seeds at progressively more advanced (from *left* to *right*) developmental stages; *top* row, phenotypically normal (wild-type and heterozygous) seeds, *bottom* row, mutant seeds. (*E*) Expression of the embryogenesis stage-specific marker genes in the mutant and phenotypically normal embryo pairs obtained from the selfed progeny seeds of the *atparn/AtPARN* heterozygous plants: FAE1::GUS (*top* panel), cotton α-globulin::GUS (*middle* two panels, *right* panel shows that the mutant embryos at near-terminal stages of development turn on the α-globulin::GUS reporter), and 7S::GUS (*bottom* panel). (*F*) Expression of the endosperm-specific GFP reporter KS117 becomes polarized toward the posterior pole (a, anterior; p, posterior) in the WT endosperm at the globular stage (*top*), but polarization fails to occur in the *atparn* seeds at a similar stage of development (*middle*, representative of 81 *atparn* mutant seeds examined). Normal siblings of the mutant seed (shown in the *middle* panel) contained mature embryos and showed distinct GFP expression in the posterior chalazal zone (*bottom*). (*G*) Incubation of the mutant embryos in an in vitro culture can lead to initiation of true leaves (*top left*), formation of the root hairs (*top right*), or both (*bottom* panel).

Therefore, quite extensive gene expression programs can occur in the complete absence of the AtPARN function.

Essential requirement for the PARN function is not universal across the phylogenetic spectrum

To assess the generality of the essential requirement for the function of the PARN deadenylase, representatives of Fungi and Metazoa were also examined. Remarkably, disruption of the gene encoding the PARN homolog in *S. pombe* had no apparent consequences on viability, growth rate, or sporulation (Fig. 4A–C). Moreover, no gross abnormalities in the total mRNA poly(A) tail length distribution were observed (Fig. 4D). This is likely due to a redundancy in mRNA deadenylation systems in *S. pombe* (see Discussion). In addition, the requirement for the PARN function in *C. elegans* was assessed by RNAi knockdown. *C. elegans* has two genes encoding PARN-like proteins, designated K10C8.1 and Y57A10A.25. Prior knockdown experiments targeting these two genes individually did not result in any apparent phenotype (Kamath et al. 2003). Therefore, we targeted these two genes simultaneously using a hybrid dsRNA, by both microinjection and feeding. In both cases, no visible phenotype in embryos, larvae, or adults was observed, whereas semiquantitative RT-PCR experiments showed that the expression level of both genes in feeding experiments was reduced by up to ~90% (data not shown). Although it cannot be ruled out that the residual amounts of these mRNAs were sufficient for full function in *C. elegans*, these data, taken together with the above finding in *S. pombe* and a complete absence of PARN homologs in *S. cerevisiae* and *D. melanogaster*, are consistent with the view that the essential requirement for the PARN function is not universal across the phylogenetic spectrum.

Loss of AtPARN affects poly(A) tail length distribution of only a select subset of embryonic transcripts

The effect of the loss of the AtPARN on mRNA deadenylation in embryos was assessed using a modified LM-PAT assay (Fig. 5A; Salles et al. 1999; Xie et al. 2003). In this assay, the total RNA pool extracted from the developing

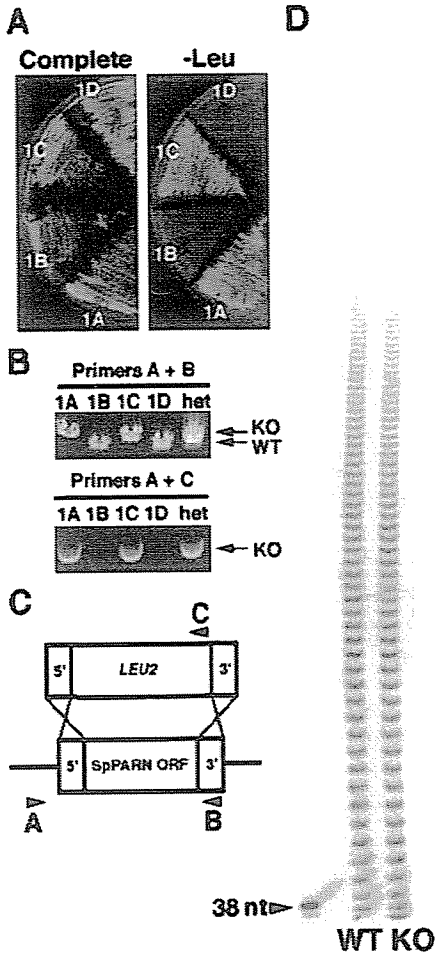

FIGURE 4. PARN function is dispensable in *S. pombe*. (*A–D*) PARN gene disruption in *S. pombe*. The *S. pombe* PARN gene was disrupted in a diploid strain as shown in *C*, and haploid meiotic progeny derived from resulting heterozygous strain were genotyped (*B*) and plated onto nonselective and selective media (*A*), as well as tested for alterations in the poly(A) tail length distribution (*D*).

wild-type or mutant seeds harvested at the torpedo stage of embryogenesis was tagged at the 3′ end via ligation of the RNA oligonucleotide whose own 3′ end was chemically blocked to prevent the formation of concatemers. The resulting tag was then used for priming the reverse transcription and for subsequent PCR using the tag-specific and the gene of interest-specific primer pair. Because the PCR amplification occurred across the poly(A) tail, the resulting PCR product length distribution was a function of the poly(A) tail length distribution of the embryonic transcript of interest. Furthermore, because the mutant seeds also contained, in addition to the zygotically derived homozygous mutant embryos, sporophytic diploid tissues that are heterozygous for the respective alleles, we examined the poly(A) tails of several transcripts selected based on the previously published information that they are expressed exclusively in embryos.

We detected no apparent differences in the poly(A) tail length distribution between mutant and wild-type embryos for cruciferin, oleosin, *LEC1*, and *LEC1*-like mRNAs. However, a significant elongation of the poly(A) tails of the *PROLIFERA* (*PRL1*) mRNA (Springer et al. 1995), which encodes the *Arabidopsis* homolog of MCM7 protein, a part of the MCM2–7 complex that functions in the initiation of DNA replication (Tye and Sawyer 2000), was observed in the mutant embryos (Fig. 5B). Importantly, no changes in the *PRL1* poly(A) tail length distribution were detected in the biotin biosynthesis-deficient *bio1-1* mutant, whose embryos arrest at a similar stage of embryogenesis. This demonstrates that the elongation of the poly(A) tails of *PRL1* mRNA is not a nonspecific consequence of an arrest of embryogenesis. It remains to be determined whether the apparent defect in the poly(A) tail shortening of the *PRL1* mRNA, which may lead to overexpression of the PRL1 polypeptide, is responsible for the defects in endosperm polarization, embryogenesis, and the eventual lethal phenotype of *atparn* mutants, but it is notable that the loss of function alleles of *PRL1* also display abnormal endosperm development and embryo lethal phenotype (Springer et al. 1995, 2000). Situations in which both overexpression and the loss-of-function alleles of a subunit of a multiprotein complex such as MCM cause similar phenotypes are not uncommon, probably because of the resulting imbalance in the concentrations of its components (Papp et al. 2003).

Most importantly, the transcript-specific differences in the effect of *atparn* null mutations on poly(A) tail length strongly indicate that AtPARN does not indiscriminately deadenylate all mRNAs in the embryos, but rather is specifically required for the poly(A) tail shortening of a select subset of embryonic transcripts. It is notable in this context that mammalian PARN was shown to be actively recruited to specific targets, such as those possessing AREs in their 3′-UTRs (Lai et al. 2003).

DISCUSSION

Mechanisms of mRNA decay in higher plants remain poorly understood. Most of the progress in this area has been made in defining and characterizing the *cis*-acting sequences that govern plant mRNA decay, such as DST (portable destabilizing element that confers constitutive instability; Gil et al. 1994; Gil and Green 1996), iLRE (light responsive element of pea *Fed-1*; Petracek et al. 1997), and possibly ARE (Ohme-Takagi et al. 1993), although no natural ARE-mediated decay substrates are known in plants, and premature termination codons (van Hoof and Green 1996; Isshiki et al. 2001). Furthermore, some plant genes have been shown and many more are suspected to be regulated at the level of mRNA of decay in response to various stimuli (e.g., ferredoxin Fed-1 by light [Petracek et al. 1997], α-amylase

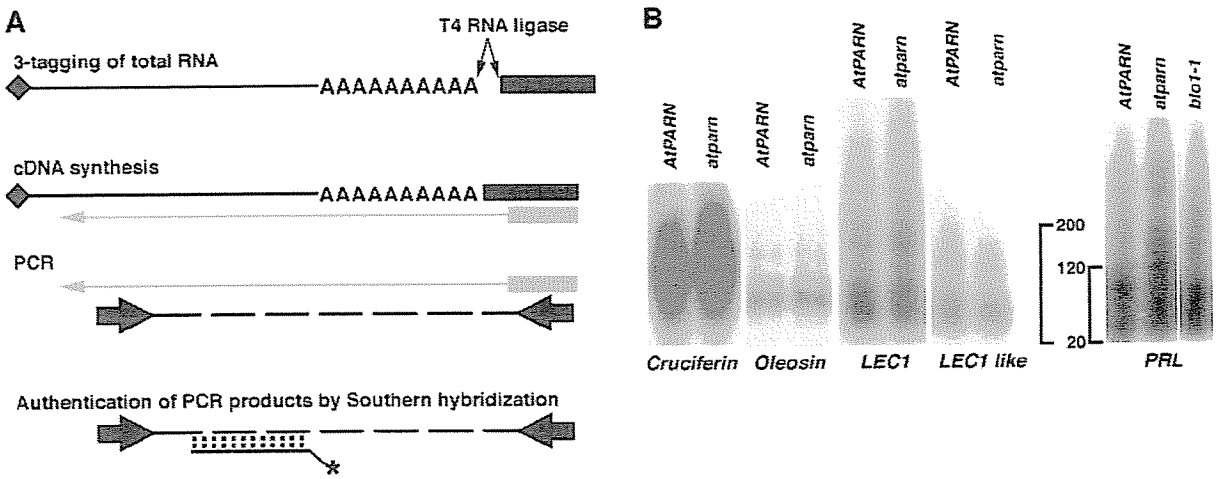

FIGURE 5. Poly(A) tail metabolism of some, but not all, embryo-specific mRNAs is impaired in the *atparn* mutant embryos. (*A*) Scheme of the LM-PAT assay used to visualize the poly(A) tail length distributions on selected embryo-specific transcripts. (*B*) LM-PAT assay results for cruciferin, oleosin, LEC1, *LEC1*-like, and PROLIFERA (*PRL*) mRNAs from *AtPARN*, *atparn*, and *bio1-1* seeds. Authenticity of the LM-PAT products in each panel was verified by hybridization with a gene-specific oligonucleotide probe. In the case of *PRL*, the validity of the length distribution of the hybridization products as a measure of the PRL mRNA poly(A) tail length distribution was additionally verified by carrying out an RNaseH/oligo(dT) cleavage prior to LM-PAT. In this case, the signal collapsed into a single band corresponding to a major polyadenylation site in PRL transcript (data not shown).

αAmy3 by carbon source [Sheu et al. 1996], and cystathionine γ-synthase by AdoMet [Chiba et al. 1999, 2003]). However, the pathways and governing principles of mRNA degradation in plants remain largely obscure, with the partial exception of the degradation pathway of oat phytochrome (*PHYA*) mRNA, that seems to undergo decay in both 5′ → 3′ and 3′ → 5′ directions (Higgs and Colbert 1994), and of soybean *SRS4* mRNA encoding the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit, whose degradation is initiated by endonucleolytic cleavages (Tanzer and Meagher 1995). The latter is an extremely abundant message, and thus its degradation pathway may be unusual. The AdoMet-regulated, translation-dependent decay of the cystathionine γ-synthase (CGS) mRNA may also be associated with endonucleolytic transcript cleavage. Yet, it is unclear how common this is, because even in case of CGS, this mechanism may not apply to other plants (Kreft et al. 2003). Yet another instance of the endonucleolytic cleavage occurs during microRNA-guided mRNA cleavage events (Llave et al. 2002; Palatnik et al. 2003; Xie et al. 2003). The latter pathway, which is related to the RNAi phenomenon in animals and posttranscriptional gene silencing (PTGS) in plants, apparently governs some key developmental decisions in plants (e.g., Chen et al. 2002; Boutet et al. 2003; Palatnik et al. 2003; Kidner and Martienssen 2004), but is unlikely to play a significant role in the general plant mRNA decay. Finally, nonsense-mediated decay (NMD) has been studied in *Arabidopsis* (van Hoof and Green 1996) and rice (Isshiki et al. 2001); however the sequence of steps, the factors involved, and the directionality of decay in both species remain unknown.

Identification of the plant mRNA decay enzymes using forward genetics in *Arabidopsis* has been attempted but has proven extremely difficult because such mutants are very rare (Johnson et al. 2000). On the other hand, in silico approaches allow investigators to tentatively identify potential homologs of many mRNA decay enzymes, including decapping enzymes (DCP1 and DCP2), 5′–3′ exonucleases (e.g., XRN1-like; Kastenmayer and Green 2000), exosome components (Chekanova et al. 2000, 2002), and multiple deadenylases that belong to several distinct classes (e.g., Dupressoir et al. 2001). Among the latter group, PARN is an evolutionarily conserved factor that has been implicated in mRNA deadenylation in metazoans (Wilusz et al. 2001). *Xenopus* PARN was shown to deadenylate those maternal mRNAs that lack cytoplasmic polyadenylation element (CPE) during oocyte meiotic maturation (Dehlin et al. 2000; Copeland and Wormington 2001), and mammalian PARN has been shown to act during NMD (Lejeune et al. 2003) and in ARE-mediated mRNA decay (Lai et al. 2003) in cultured mammalian cells. However, the significance of the PARN function at the whole-organism level has not been elucidated, and the division of labor, if any, between PARN and other deadenylation enzymes has not been addressed.

We have characterized the *Arabidopsis* PARN (AtPARN) and studied the consequences of the loss of the AtPARN function for plant viability. Results of the activity assays in vitro, coupled with the site-directed mutagenesis experiments, demonstrate that AtPARN is a poly(A)-specific exoribonuclease that belongs to the RNase D family. AtPARN appears to be localized predominantly in the cytoplasm, consistent with its role in mRNA turnover. We found that

deadenylation by PARN is essential for plant viability. The development of the *Arabidopsis* embryos lacking AtPARN, or expressing an enzymatically inactive protein, was markedly retarded, culminating in an arrest at the bent-cotyledon stage, although the *atparn* mutant embryos were capable of limited cell division, development, and differentiation in culture, indicative of the existence of AtPARN-independent gene expression programs. While the present report was in review, Chiba et al. (2004) also reported that the T-DNA insertional mutations in the *AtPARN* gene cause a lethal phenotype; however, those authors concluded that this could be due to either embryonic or gametophytic (i.e., haploid-phase) lethality. Importantly, however, neither formal analyses of male and female transmission by reciprocal crosses nor complementation experiments were conducted by those authors. In contrast, our phenotypic and genetic analyses, and in particular reciprocal crosses, rule out gametophytic lethality. We also extend our analyses to show that although AtPARN enzymatic activity is essential in a higher plant, it is dispensable in the representative of Fungi (*S. pombe*), and can be at least severely downregulated in a metazoan (*C. elegans*) without any obvious phenotype. Importantly, only some, but not all, embryo-specific transcripts examined were hyperadenylated in the mutant embryos, suggesting that deadenylation of a select subset of mRNAs, rather than of the whole mRNA population, is indispensable for embryogenesis in *Arabidopsis*. It is important to note in this regard that deadenylation of the ARE-containing mRNA substrates by mammalian PARN can be specifically stimulated by ARE-binding factors such as tristetraprolin (TTP), as was demonstrated by experiments in vitro and by cotransfection studies in human HEK293 cells (Lai et al. 2003). This stimulatory effect was dependent both on the *cis*-acting element (the ARE), and on the ability of the *trans*-acting factor (TTP) to recognize it, even though the direct TTP-PARN interactions could not be demonstrated. This could be because the interaction is transient and/or requires bridging factors, or because the TTP binding to the ARE causes remodeling of the mRNP structure, that is, it makes the polyadenylated mRNA substrate more accessible to PARN, rather than activating PARN enzyme directly. Regardless of the underlying mechanism, these and our findings suggest that the substrate specificity in PARN action is an evolutionarily conserved principle.

An important aspect of our findings is that the essential requirement of the PARN function is not universal across the phylogenetic spectrum. One potential explanation as to why Fungi and Metazoa may be more tolerant to the lack of PARN than plants is redundancy. In addition to PARN, two other deadenylation systems have been characterized in yeast and humans: poly(A) nuclease (PAN) and CCR4/CAF1 complex (Tucker et al. 2001; Uchida et al. 2004). However, the genome of *A. thaliana* encodes at least one of the PAN subunits, as well as six putative homologs of *CCR4*, at least five of which are expressed, and as many as 11 *CAF1* homologs, at least seven of which are expressed. Thus, redundancy alone cannot readily explain differences in the dependence on the PARN function between species. Rather, our findings suggest that AtPARN may be essential for regulating the poly(A) tail metabolism, and therefore translation efficiency and/or stability, of a specific subset of embryonic transcripts whose encoded products are critical for normal development.

MATERIALS AND METHODS

Plant material and genetic techniques

The *atparn* mutants were outcrossed to the wild-type WS plants twice prior to analysis. The *atparn-2* allele was subsequently moved into Ler background, in order to facilitate dissection, and used for the DIC microscopy of the embryos shown in Figure 3. Plants were grown at 23°C under 16 h light and 8 h dark. To establish that *AtPARN* is nonallelic with *EMB25*, the following criteria were used. When *EMB25/emb25* and *AtPARN/atparn2* plants were crossed, no white (i.e., mutant) seeds were observed among the F1 progeny seeds. Moreover, one-quarter of the selfed F2 progeny plants produced 50% white (mutant) seeds, rather than 25% white seeds. This could only be only possible if these plants contained two distinct embryo lethal mutations closely linked in *trans*. Furthermore, selfed F2 plants did not segregate any wild-type F3 progeny. Instead, all of the F3 progeny plants examined contained 50% white seeds, reinforcing the conclusion that their F2 parent plants contained *emb25* and *atparn* mutations closely linked in *trans*. Plant transformation was done by the floral dip method as described (Clough and Bent 1998). For the embryo rescue experiments, the early heart- to early torpedo-stage embryos were liberated from the developing seeds using insulin needles, placed on Murashige-Skoog medium with 1% sucrose and 0.7% Phytagar (Gibco), and incubated in a Percival growth chamber (23°C, 16 h light and 8 h dark) for 10–14 d, and surviving plantlets were transplanted to soil. Under these conditions, none of the mutant embryos (n = 46) and 70% of the wild-type and heterozygous (17 of 24) embryos were fully regenerated and gave raise to full-grown plants.

Plasmid constructs

Expression constructs

For initial expression trials, an ~2-kb cDNA fragment of AtPARN was amplified with primers oDB605/oDB606 and cloned into pET24a (Novagene) using NdeI and XhoI sites that are in frame with the C-terminal His tag (construct pDB574). The resulting His-tagged protein product was mostly insoluble. GST-tagged PARN (pDB586) expressed in insect Sf9 cells using a baculovirus Bac-to-Bac system (Invitrogen) was more soluble, but poorly bound to glutathione resin. For the expression in *E.coli* as a fusion with N-terminal maltose-binding protein (MBP), the cDNA fragment amplified with primers oDB632/oDB633 was cloned into both cytoplasmic (pMAL-c2X) and periplasmic (pMAL-p2X) ex-

pression vectors (New England Biolabs), using XhoI and HindIII sites, resulting in pDB578 and pDB579, respectively. Soluble recombinant AtPARN was obtained after the expression in pMAL-p2X or after refolding from the inclusion bodies as described below, obtained from the pMAL-c2X based construct. The latter preparation was free of contamination with nonspecific nucleases and was used for the experiment shown in Figure 2.

Plant constructs

All plant constructs were based on the binary vector pCAMBIA1300 (CAMBIA; Fig. 6). First, a 300-bp-long fragment containing the pea Rubisco small subunit 3'UTR/polyadenylation signal (GB #AF30985) was cloned into SalI/PstI sites, resulting in the construct pDB588. Next, the AtPARN fragment amplified with oDB613 and oDB614, that included 240 bp of the 5′ flanking sequence, was cloned into pDB588 via an intermediate construct, resulting in pDB596. Subsequently, a construct containing a larger fragment (895 bp) of the 5′-flanking region was created, by replacing the BamHI-BsrGI fragment of pDB596 with a oDB673/oDB614 PCR product, resulting in pDB604. To create the AtPARN-GFP fusion constructs, the GFP coding region from mGFP4 was amplified with primers oDB676/oDB677 and cloned in-frame as a C-terminal translational fusion with AtPARN ORF, at NotI and SalI sites. The AtPARN-GFP fusion with 240 bp of upstream sequence was named pDB600. Using a procedure similar to the one described above, BamHI-BsrGI region of pDB600 was replaced by the PCR products obtained using the *Arabidopsis* genomic DNA as a template and primers oDB674/oDB614 and oDB673/oDB614, resulting in the AtPARN-GFP fusion constructs containing 3.2 kb (pDB603) and 895 bp (pDB602) of the 5′-flanking sequence, respectively. For transient expression in onion epidermal cells, the AtPARN cDNA was cloned into the NcoI site of pAVA393 (von Arnim et al. 1998) under the control of the 35S promoter, in frame with the downstream mGFP5 gene (construct pDB614).

Catalytically inactive AtPARN constructs

To introduce the catalytically inactive form of AtPARN into *Arabidopsis*, the BamHI-BsrGI fragment of pDB604 was replaced by the corresponding DNA fragment with two amino acid substitutions, D66A and E68A. Self-complementary oligonucleotides oDB769 and oDB770 containing desired mutations were paired with the downstream (oDB730) and upstream (oDB673) primers, respectively, to amplify the two fragments of PARN gene, which were then fused by overlap extension PCR (Ho et al. 1989). The resulting mutant fragment was cut with BamHI and BsrGI, gel-purified, and ligated into the pDB604 cut with the same enzymes, producing plasmid pDB612. For production of the catalytically inactive AtPARN in *E.coli*, the BamHI-BsrGI fragment of pDB578 was exchanged for the equivalent piece obtained as described above, except that Z2590/oDB730 and oDB769/oDB730 primer combinations were used on the pDB578 plasmid as a template. The resulting construct was named pDB581.

T-DNA collection screening, plant genotyping, and expression and localization assays

T-DNA alleles of AtPARN

T-DNA populations (in WS ecotype) maintained at the University of Wisconsin Biotechnology Center were screened by PCR for T-DNA insertions in *AtPARN* using a T-DNA-specific primer, JL202 (all oligonucleotides used in this work are listed in Table 1) in combination with either gene-specific primer oDB556 or oDB557, according to the instructions provided on the UWBC Web site (www.biotech.wisc.edu/Arabidopsis). PCR products were verified by Southern analysis using the DNA fragment amplified by oDB556 and oDB557 as a probe. The T-DNA/genomic-DNA junctions were further authenticated by sequencing using a T-DNA left border-specific primer, JL270. Three T-DNA insertion

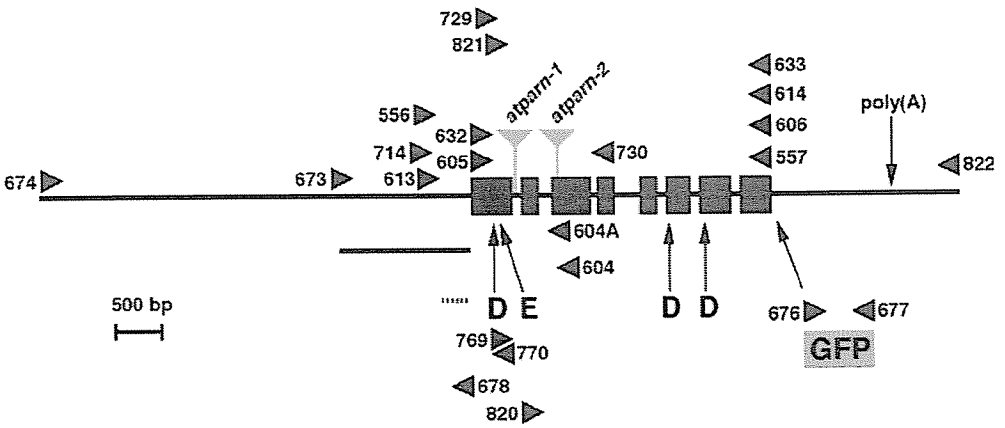

FIGURE 6. AtPARN constructs used in this work and positions of the oligonucleotides. The locations of the oligonucleotide primers mentioned in the Materials and Methods are indicated. Exons are shown as black bars. Approximate positions of the four conserved catalytically important residues, as well as the position of the polyadenylation signal are indicated. The green fluorescent protein (GFP) open reading frame (ORF) was fused onto the last exon of AtPARN for localization studies as shown. Transgene constructs containing either 900 bp or 3000 bp of the 5′ flanking sequence (indicated by solid black lines) were able to complement the *atparn* alleles, but the 240-bp 5′ flanking fragment construct (indicated by the dotted line) was unable to complement the mutant alleles.

TABLE 1. Oligonucleotides used (see Fig. 6 for primer locations)

Name	Sequence
JL202	5′-CATTTTATAATAACGCTGCGGACATCTAC
JL270	5′-TTTCTCCATATTGACCATCATACTCATTG
oDB556	5′-TCACAAAACGGTACCGTTTCCTCGGAATC
oDB557	5′-ACTCGTAGCAGTTTCGACTTCAACTCTAT
oDB604	5′-GCTGGTGGATCAAATGTGAGTTCTTGACGA
oDB604A	5′-TGTGAGTTCTTGACGAGGAA
0DB605	5′-CCGTTCACATATGCGCCGGCACAA
oDB606	5′-ATTACTCGAGGCAGTTTCGACTTCAACTCTATT
oDB613	5′-TTGGATCCTGTTGTTGTTCATCGGGAAAA
oDB614	5′-TCCATCTTCTCTTTTCCATGGCATTACTCGTAGCAGTTTCGACTTCAACTC
oDB632	5′-TCTCGAGATGGGTCATCATCATCATCATCATGAAAATTTGTATTTTCAAGGTATGCGCCGGCACAAGCGA
oDB633	5′-GGAAGCTTAATTACTCGTAGCAGTTTCGACT
oDB635	5′-CAGCTGTTGCCCCGTCTCACTGGTGAA
oDB663	5′-GCGTAATACGACTCACTATAGGCTATCTCAGCAGAGAAGTC
oDB664	5′-TGTGAGCAAGCTTCAAGAA
oDB665	5′-CTATCTCAGCAGAGAAGTC
oDB666	5′-GCGTAATACGACTCACTATAGGTGTGAGCAAGCTTCAAGAA
oDB667	5′-AAGCATTCTATCCGTTGGA
oDB668	5′-GCGTAATACGACTCACTATAGGAAGCATTCTATCCGTTGGA
oDB669	5′-GTAGGTTGGAATCGACGTTCTCCGGAACACATATT
oDB670	5′-GTCGATTCCAACCTACGCAAGGAGACAACGAGATC
oDB671	5′-TCAGCCATCAGGGTTCTTC
oDB672	5′-GGGTAATACGACTCACTATAGGTCAGCCATCAGGGTTCTTC
oDB673	5′-GCATTTTGCATGGATCCATGCTCTG
oDB674	5′-GGTTAACACAGCCAATACATATACAACATC
oDB676	5′-ATAATCATGAGTAAAGGAGAAGAAC
oDB677	5′-TATGTCGACTTATTTGTATAGTTCATCCA
oDB678	5′-CATTTTTGAACGGAGAGAGAGA
oDB714	5′-CCGCCATGACGATTCGAGTTCCCAAGC
oDB729	5′-CAGGCGCGCCGTGGCGAGACTCCTTAG
oDB730	5′-GGGCCCATGTACACAAGATCTCCAAAATGCTT
oDB743	5′-TTTCTAGAACATGCACTGATAATATGCTA
oDB744	5′-TTGAGCTCTTCTTGCGTTAGGAGTTCGATGA
oDB745	5′-TTTCTGCAGCAATAGTACAGGTGGCTGATTAC
oDB746	5′-TTCTCGAGTCAACAATGCCTCGAATGCTCCC
oDB769	5′-GTGGCGATTGCCCTTGCGATGACTGGCGTG
oDB770	5′-CACGCCAGTCATCGCAAGGGCAATCGCCAC
oDB777	5′-CTTAACGTAGAGACCTTAGAA
oDB778	5′-GTAGAAGTGATCTCTTGATTG
oDB786	5′-GTACAAAGTCACATCTAAATTTCC
oDB802	5′-ACAAGGTACTCTACAACGTAT
oDB803	5′-GGTACTCTACAACGTATGATG
oDB820	5′-CCACCAGCTCATGAATTTCTCTGTCA
oDB821	5′-ACTCCGCCGAGAAATTCGCC
oDB822	5′-CGTTCTCCACACGATTGCCG
oDB824	5′-ATATAGACCACCGTTACCAAC
oDB825	5′-TGTGTTTCTATATGTTGTCAA
oDB826	5′-GCAAGTGTTGTGTTGTACTTT
oDB827	5′-AAGGAGTTGCATTTTACAGGT
oDB828	5′-AATGAACCTCGAGTACTGTAA
oDB829	5′-ATGTGACTAGTTTTGTTGAGG
oDB833	5′-ATTGACATACTTTACCACAAG
oDB834	5′-GTGTTTTTCATCTGATCTGT
oDB835	5′-TAAGCGAAAACAATGCTGCT
0DB853	5′-UACUCAUCAUACGUUGUAGAGUACCUUGUAidT
oDB938	5′-AAAGGGCAGAGAGCGAACTGGAGAA
oDB950	5′-GGTGCAGGACATACCGTTCCAGAAT
Z2590	5′-CGACGCATATGAAAATCGAAGAAGGTAAACTG
Z2658	5′-ATCGCTGCAGTTGGACGATCGATGATAAG
Z2858	5′-GCGTCTAGAACACACAGGGGCGCTATCGCA

alleles were found in the BASTA population: 557B14 (*atparn-3*), 556B14 (*atparn-1*), and 557B40 (*atparn-2*), located 364 bp, 408 bp, and 728 bp downstream of the ATG codon, respectively. Actual sequences of the T-DNA/genomic-DNA junctions are as follows (T-DNA left border sequences are italicized, short scrambled sequences between the T-DNA and genomic DNA are shown in lowercase):

557B14 (*atparn-3*)—364 bp downstream of ATG codon: *TTTC CCGGACATGAAGCCATTTACA*-tgaagccatcg-TACCCGTTAG TTTCTCTTTCTCTTTTCTTTTAAATACCCGTTA;
556B14 (*atparn-1*)—408 bp downstream of ATG codon: *TTTCCCG GACATGAAGCCATTTACAATTGAATATATCCTG*-ttagttaa-ACAC AATTGAAGCTAAAGAGAAAGAGAAAGA;
557B40 (*atparn-2*)—728 bp downstream of ATG codon: *TTTCC CGGACATGAAGCCATTTACAATTGAATATATCC*-TGAAAA TGCTACACGGCGAGGACGGAATTGATTCATCGGGTG.

T-DNA allele of At3g25430

The null allele of At3g25430 (line SALK_078011) was generated by Joseph Ecker's group at the Salk Institute (Salk Institute Genome Analysis Laboratory) as a part of the published collection of T-DNA insertion lines (Alonso et al. 2003), and obtained via the Arabidopsis Biological Resource Center at Ohio State University. The T-DNA in SALK_078011 is inserted in exon 1 of At3g25430. Screening of this line's progeny was conducted using PCR primer pairs oDB950 (sense) and oDB938 (antisense), which produce the 744-bp-long product from the wild-type allele only, and oDB635 (T-DNA-specific primer) and oDB938. The latter pair produce a 400-bp product from the mutant allele only. Numerous homozygous mutant plants were identified, and none had any obvious phenotype.

Expression survey of AtPARN

RNA was prepared using an adaptation of the guanidinium thiocyanate/acid phenol extraction method (Chomczynski and Sacchi 1987). *Arabidopsis* tissue (50–100 mg) was harvested into 1.5-mL Eppendorf tubes, and homogenized in 400 µL of homogenization buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 1.5% sodium sarcosyl, 1% β-mercaptoethanol) with a plastic pestle. Extract was supplemented with 40 µL of 3 M sodium acetate (pH 5.2) and 500 µL of phenol-chloroform mix, vortexed, incubated on ice for 10 min and centrifuged. The supernatant was removed, and nucleic acids precipitated by adding an equal volume of isopropanol. The RNA pellet was redissolved in 150 µL of homogenization buffer, reprecipitated with isopropanol, and reprecipitated again with lithium chloride. After the DNAse digestion and extraction with phenol-chloroform, RNA was precipitated with sodium acetate/ethanol. The final pellet was washed with 70% ethanol, dried, redissolved in 27 µL RNAse-free water, and stored at −80°C. The cDNA synthesis was performed using Superscript II (Invitrogen) on 10 µL of the above total RNA preparation with 0.5 µg dT_{17} primer in a standard 20 µL reaction. After 1 h at 37°C, the reaction was heated at 75°C for 15 min, diluted to 100 µL with TE, and stored at −80°C. For the 50-µL PCR reaction, 1 µL of this preparation was used as template with AtPARN gene-specific primers oDB605 and oDB606.

Biolistic assays

Onion epidermal peels were prepared for transformation as described (Varagona et al. 1992). The protocol for DNA preparation and biolistic delivery was essentially as described by Sanford et al. (1993). Briefly, 30 mg of 1.6 µM gold particles were suspended in 120 µL of sterile 50% ethanol and kept suspended by constant vortexing. Then, 25 µL of solution (enough for 3 bombardments/construct) was added to a microfuge tube containing 2.5 µL of DNA (1 µg/µL) of the appropriate plasmid construct. To this tube was added 25 µL of 2.5 M $CaCl_2$ and then 10 µL of 1 M spermidine (free base) with continued vortexing for about 3 min. The DNA-coated particles were spun down for 15 sec, washed with 70% ethanol, and resuspended in anhydrous 100% ethanol.

For transformation, the gold particles were propelled using a "flying-disc" helium-driven biolistic device that uses a fast-opening valve to generate the pressure shock wave. A total of 8 µL of DNA-coated particles were spread on each disc and placed under a gentle stream of air which rapidly evaporated the 100% ethanol, leaving evenly distributed particles. These discs were then mounted into holders and used to transform onion epidermal peels.

Genotyping assays for the complementation of the null allele of AtPARN

PCR on leaf clips (Klimyuk et al. 1993) was used for plant genotyping, as well as for the verification of transformants. The presence of AtPARN gene T-DNA knockouts was established by means of PCR analysis employing combination of left border primers JL202 or JL270 with one of the downstream (oDB604 or oDB730) or upstream (oDB556 or oDB729) primers. Amplification with primer pairs oDB604/oDB556 or oDB729/oDB730 was used to establish the presence of the wild-type AtPARN allele in the segregating progeny of the heterozygous knockout plants. Multiplex PCR for simultaneous detection of the AtPARN T-DNA insertions and the wild-type AtPARN was carried out using primer combinations oDB729/oDB730/JL270 or oDB729/oDB730/JL202.

Following the selection of the transformants on hygromycin plates, the presence of the AtPARN transgenes was confirmed by PCR with primers M13/oDB678 for the wild-type AtPARN transgene constructs, or with primers oDB676/oDB677 for the PARN-GFP fusions. Complementation of the null allele by all of the introduced AtPARN transgenes except the construct pDB596 (below) was then assayed using multiplex PCR with primers oDB820/oDB821/oDB822. In this case, the PARN fragment introduced on a transgene cannot be amplified, because the primer oDB822 lies outside of the region included in the construct. Amplification from the endogenous *AtPARN* gene produced two bands, that is, products of the oDB821/oDB822 and oDB820/oDB822 primer pairs, with a size difference of 200 bp. The absence of the larger of the two products due to the inserted T-DNA would be indicative of the complementation.

For genotyping of the plants transformed with the construct pDB596, the primer pair oDB714/oDB604A was used, which amplify the fragment from the endogenous wild-type, but not from the introduced AtPARN transgene, across the position of the T-DNA insertion. Absence of such a band would mean the absence of noninterrupted wild-type PARN gene. The band was present in all plants studied. The numbers of plants that were tested for the

complementation with the different AtPARN constructs and the numbers of complemented positives found are listed in Table 2.

Microscopy

For observation of embryos (Fig. 3), developing seeds were fixed in HistoChoice (Amresco), cleared using modified Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 mL glycerol, and 30 mL water), mounted, and viewed on an Olympus BX60 using Nomarski optics. GFP fluorescence (Figs. 2, 3) was viewed using the 41001 filter cube. Protoplasting of the leaves from transgenic plants to facilitate the visualization of GFP was conducted as described by Abel and Theologis (1994).

AtPARN enzymatic assays

Soluble AtPARN, expressed in *E.coli* as MBP fusion in pDB579 (based on pMAL-p2X), exhibited specific poly(A) degrading activity. However, such preparations were often contaminated with copurifying nonspecific nuclease activity. Therefore, the recombinant protein was refolded from the inclusion bodies as described below, which resulted in the active enzyme preparation essentially free from nonspecific nucleases. For the AtPARN assays, a 102-bp DNA fragment containing T7 promoter and 20 nucleotide poly(A) or poly(T) tail (Chekanova et al. 2000) was used as a template to generate radiolabeled RNA substrates. RNA was synthesized with an Ampliscribe T7 kit (Epicentre) in the presence of [^{32}P]-UTP (3000Ci/mMol, Amersham). Resulting RNAs were treated with DNAse, extracted with phenol/chloroform, and precipitated. Purified RNA substrates were incubated with protein samples under conditions similar to those described for human PARN (Ren et al. 2002), except that the reaction buffer used did not contain polyvinyl alcohol. Aliquots of 30 µL (1–3×10^{4}cpm and 1–5 µg of recombinant MBP-AtPARN per time point) were taken at specified time intervals and immediately mixed with 60 µL of 2× protease buffer (200 mM Tris-HCl pH7.9, 300 mM NaCl, 25 mM EDTA, 2%SDS) and 30 µL of Proteinase K solution (800 µg/mL Proteinase K, 200 µg/mL Glycogen, 80 µg/mL total yeast RNA), incubated at 37°C for 1 h, and precipitated with NaOAc/EtOH overnight. Resulting RNA samples were separated on 8% PAAG containing 7 M urea, and exposed to a PhosphorImager screen (Molecular Dynamics).

Expression and purification of AtPARN

Manufacturer (New England Biolabs) recommended procedures were employed for expression and purification of the MBP-AtPARN fusions. Generally, 0.5 L of rich media, or LB supplemented with 0.2% glucose and 0.1–0.15 mg/mL ampicillin was inoculated with 1/100 volume of overnight culture of appropriate construct in BL21 or Origami B (Novagen) host. Cultures were grown at 37°C to OD_{600} of 0.6–0.8, cooled, and induced with 0.3 mM IPTG overnight at room temperature. After harvesting, the cell pellet was resuspended in 20 mL of column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10% glycerol, 0.2% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM DTT) and subjected to three freeze-thaw cycles. Cells were lysed by sonication, and the soluble fraction was separated by centrifugation at 40,000*g* for 45 min. The pellet was resuspended in fresh 20 mL of column buffer and stored at −80°C. Two mL of this suspension was mixed with 14 mL of 7M urea and left rocking overnight at 4°C. The solution was clarified by centrifugation (40,000*g*, 30 min), and diluted 1.5-fold by addition of 8 mL of AtPARN storage buffer SB (20 mM HEPES pH 8.2, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT). After another 6 h of rocking at 4°C, solution was again clarified, and two more twofold SB dilution cycles were performed with mixing at 4°C for 6 h to overnight. The final clarified dilution containing ~1.15 M urea was concentrated 10-fold on a Vivaspin 10-kDa ultraconcentrator (Vivascience), and dialyzed against two 2-L changes of SB supplemented with 1 mM oxidized/0.2 mM reduced glutathione. No visible precipitate formed at this stage. Dialyzed protein was concentrated to a final volume of 0.5 mL and stored at −80°C.

An aliquot of 250 µL of refolded AtPARN was diluted with 5 mL of column buffer and mixed overnight with 1 mL of washed amylose resin at 4°C. The column was drained, washed with column buffer supplemented with 1 M KCl, and then with SB and eluted with 10 × 0.5 mL aliquots of 10 mM maltose in SB. Fractions containing MBP-AtPARN were pooled and concentrated.

TABLE 2. Results of the complementation tests with the various AtPARN constructs

Construct	5′-upstream region, bp	# Plants tested	# Complemented
pDB596	240	63	0
pDB603	3220	15	1
pDB602	895	81	12
pDB600	240	26	0
pDB604	895	26	13
pDB612	895	101	0

S. pombe constructs and techniques

To prepare the *S. pombe* PARN gene disruption cassette, the following three PCR-generated DNA fragments were amplified and joined: (1) the 994-bp fragment from the *S. pombe* PARN gene 5′ flanking region, generated with primers oDB744 and oDB743 with genomic *S. pombe* DNA as template; (2) the *S. cerevisiae LEU2* gene (which complements the *S. pombe leu1* mutation), generated with primers Z2658 and Z2858 using Ycp111 (Gietz and Sugino 1988) as template; and (3) the 1084-bp fragment from the *S. pombe* PARN gene 3′ flanking region, generated with primers oDB745 and oDB746 with genomic *S. pombe* DNA as template. The resulting fragments were digested with SacI + XbaI, XbaI + PstI, and PstI + XhoI, respectively, joined via three-way ligation and cloned into Bluescript SK(+). The resulting disruption cassette was excised with SacI + XhoI, and integrated into the diploid *S. pombe* strain produced by mating of FY527 (*h⁻ his3-D1 ura4-D18 leu1-32 ade6-M216*) and FY528 (*h⁺ his3-D1 ura4-D18 leu1-32 ade6-M216*; Liang and Forsburg 2001). Integrants were verified by PCR analysis and Southern blotting, sporulated, and patched onto Leu⁺ and Leu⁻ media to isolate *parn⁻* and *parn* mutant progeny. The general *S.pombe* techniques and media were according to Moreno et al. (1991).

C. elegans techniques

Double-stranded RNA targeting the two *C. elegans* PARN homologs, K10C8.1 and Y57A10A.25, were produced as follows. For

microinjection, the 740-nt-long K10C8.1 dsRNA was produced by annealing of the T7 transcripts made from the templates that were amplified by PCR using oDB663 and oDB664 (top strand, T7 polymerase promoter included in oDB663) and oDB665 and oDB666 (bottom strand, T7 polymerase promoter included in oDB666). This dsRNA targeted exon 3 (the longest exon) of K10C8.1. For Y57A10A.25, templates were produced by overlap extension PCR (Ho et al. 1989) -mediated fusion of two PCR fragments, 480 bp and 536 bp, corresponding to the exons 2 and 3 of Y57A10A.25. Exon 2 fragment was amplified with oDB667 and oDB669, and exon 3 with oDB670 and oDB670. Overlap introduced into the 5′ halves of the primers oDB669 and oDB670 was used to anneal the products, and the fused 1016-bp product was amplified by the external primers oDB667 and oDB671. The T7 promoter for transcribing either top or bottom strands of the resulting fusion fragment was then introduced through reamplification with oDB668 (which contains the T7 promoter sequence, for top strand) and oDB671, or with oDB667 and oDB672 (T7 promoter for transcribing the bottom strand). The in vitro transcripts were quantitated and annealed, and the extent of annealing was verified by agarose gel electrophoresis prior to microinjection. *C. elegans* culture and dsRNA microinjection to induce RNAi were done as described by Grishok et al. (2000).

For RNAi by feeding, the PCR-derived templates were first cloned into the dual T7 promoter vector L4440, as follows. First, the Y57A10A.25 exon 2/3 PCR chimera was cleaved at the internal SacI sites, which resulted in removal of ~60 bp from the 5′ end and ~110 bp from the 3′ end, and cloned into the SacI site of L4440. Into the resulting plasmid, the K10C8.1 exon 3 fragment was then cloned as NcoI/blunt fragment after cleaving off some 120 bp from its 3′ end using an internal NcoI site. RNAi by feeding was induced according to the protocol described by Timmons et al. (2001). Neither the feeding random-sequence RNA nor the dual RNAi against the two *C. elegans* PARN homologs has any effect on brood size, embryo hatching, or adult worms. As a control, *POS-1* RNAi knockdown performed in parallel resulted in near 100% embryonic lethality

RNA techniques and LM-PAT

Polyadenylated and polyuridilated RNA substrates for AtPARN assays were synthesized from templates and according to procedures described by Chekanova et al. (2000). Capped RNA was produced according to Stripecke and Hentze (1992). Total RNA isolation from *S. pombe* and measurements of the poly(A) tails lengths were carried out as described by Chekanova et al. (2001) and Sachs and Davis (1989).

LM PAT assay for poly(A) tail length was adapted from the siRNA 3′ end identification procedure described by Xie et al. (2003). Briefly, 50–100 seeds containing embryos from torpedo to bent cotyledon stages of development were collected, and total RNA was purified using an Arcturus PicoPure kit according to the instructions provided by the manufacturer (including the DNAse treatment step). Total RNA was 3′ end-tagged with the 5′ phosphorylated RNA adaptor oligonucleotide oDB853 and RNA ligase, and reverse transcribed using SuperScript II enzyme (Life Technologies) and the oDB802 primer that is complementary to the adaptor oligonucleotide. Diluted cDNA was used as a template for PCR with oDB802 as antisense primer and oDB777 (for PRL1), oDB828 (for cruciferin), oDB825 (for oleosin), or oDB835 (for LEC1-like) as sense primer, using a touchdown profile (initial annealing at 59°C, then decreasing by 1°C per cycle until 48°C was reached, and the 33 additional cycles at 48°C annealing). Diluted first-round products were subjected to the nested PCR under the same conditions, using oDB803 as antisense primer and oDB778 (for PRL1), oDB829 (for cruciferin), oDB826 (for oleosin), or oDB834 (for LEC1-like) as sense primer. Products were authenticated by Southern hybridization with the following gene-specific oligonucleotides as probes: for PRL1, oDB786; for cruciferin, oDB827; for oleosin, oDB824; and for LEC1-like, oDB833. RNaseH treatments prior to LM PAT were carried out as described previously (Chekanova et al. 2001).

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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'/2μ/pADHI-PAB3*)	this work
YDB239	*trp1, ura3, leu2, his4, cup1::LEU2pm, spb2::URA3, pab1::URA3, pan3::TRP*	pDB489 (*G418'/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.

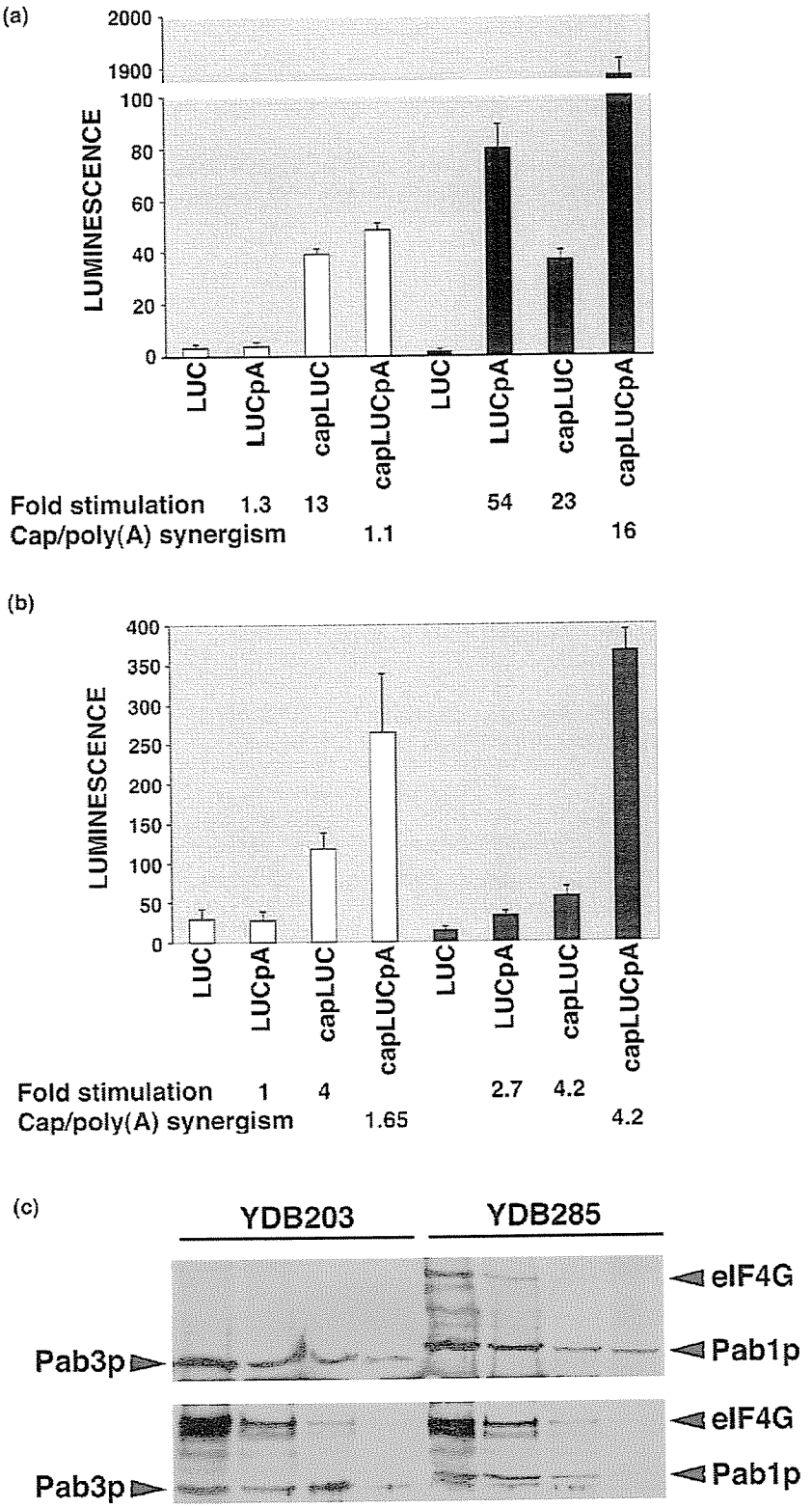
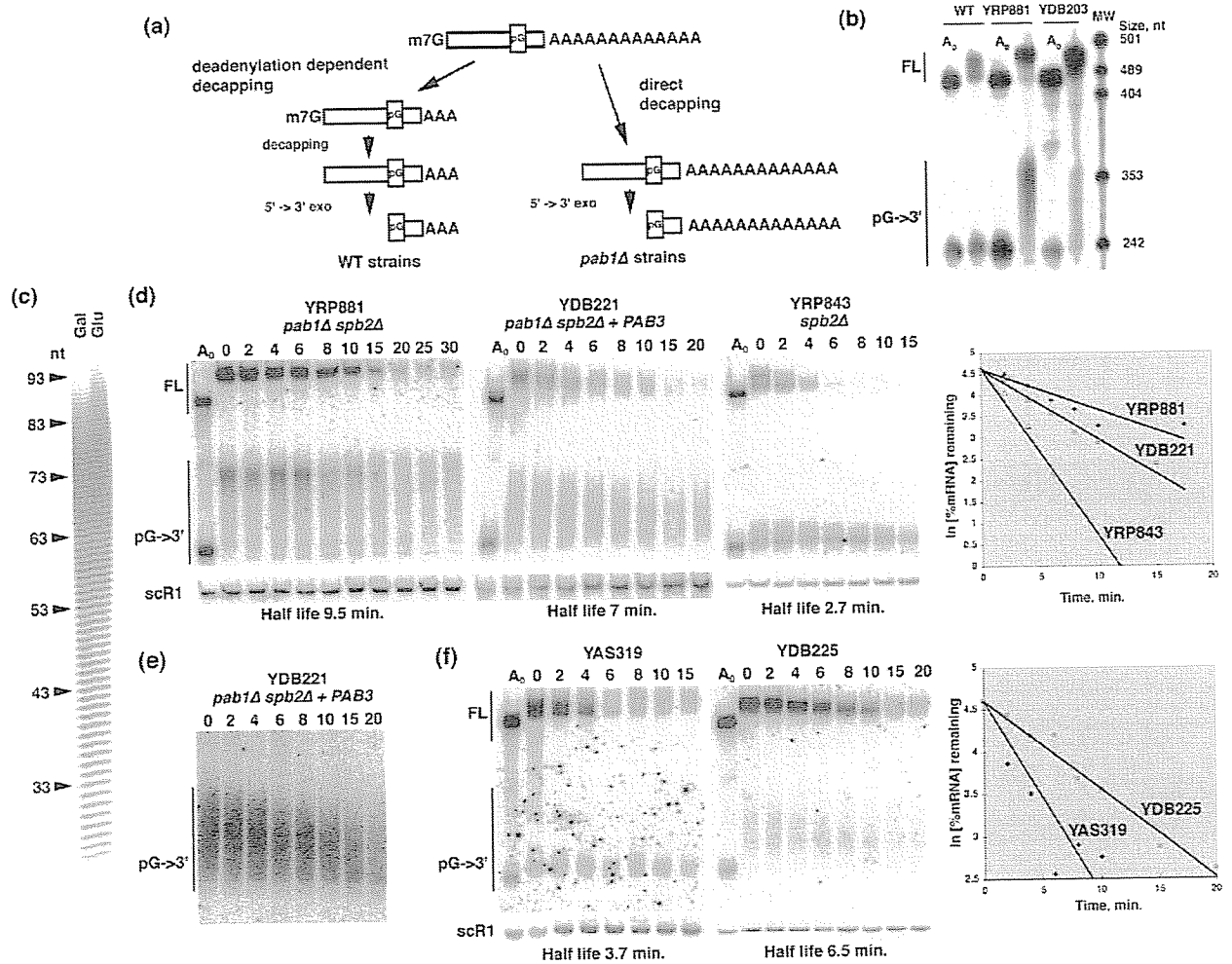


Figure 2

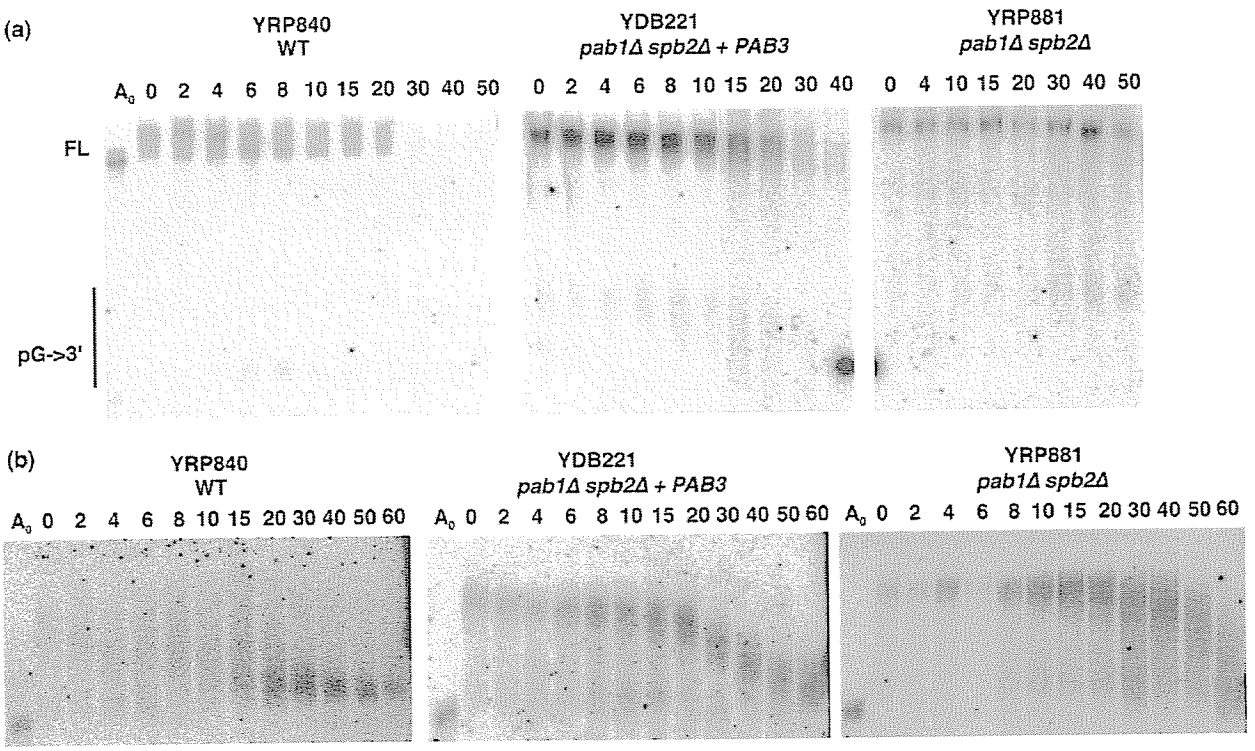


Pab3p promotes *MFA2pG* mRNA deadenylation and decay in yeast but does not prevent mRNA decapping prior to deadenylation. **(a)** A diagram of the deadenylation-dependent decapping pathway of mRNA decay in the wild-type and in *pab1Δ* yeast strains, as revealed by examining poly(G)-containing reporter transcripts. **(b)** Northern blot analysis of the *MFA2pG* mRNA in the wild-type strain YRP840 (WT), *pab1Δ*, *spb2Δ* 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_0 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 *pab1Δ*, *spb2Δ* strain (YRP881), the *PAB1 spb2Δ* strain (YRP843), and the *pab1Δ*, *spb2Δ* strain expressing *Arabidopsis* Pab3p (YDB221). Time points after the transcriptional repression are shown above the lanes. The lane labeled A_0 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 *pab1Δ*, *spb2Δ* strain were observed (Figure 2d). First,

Figure 3



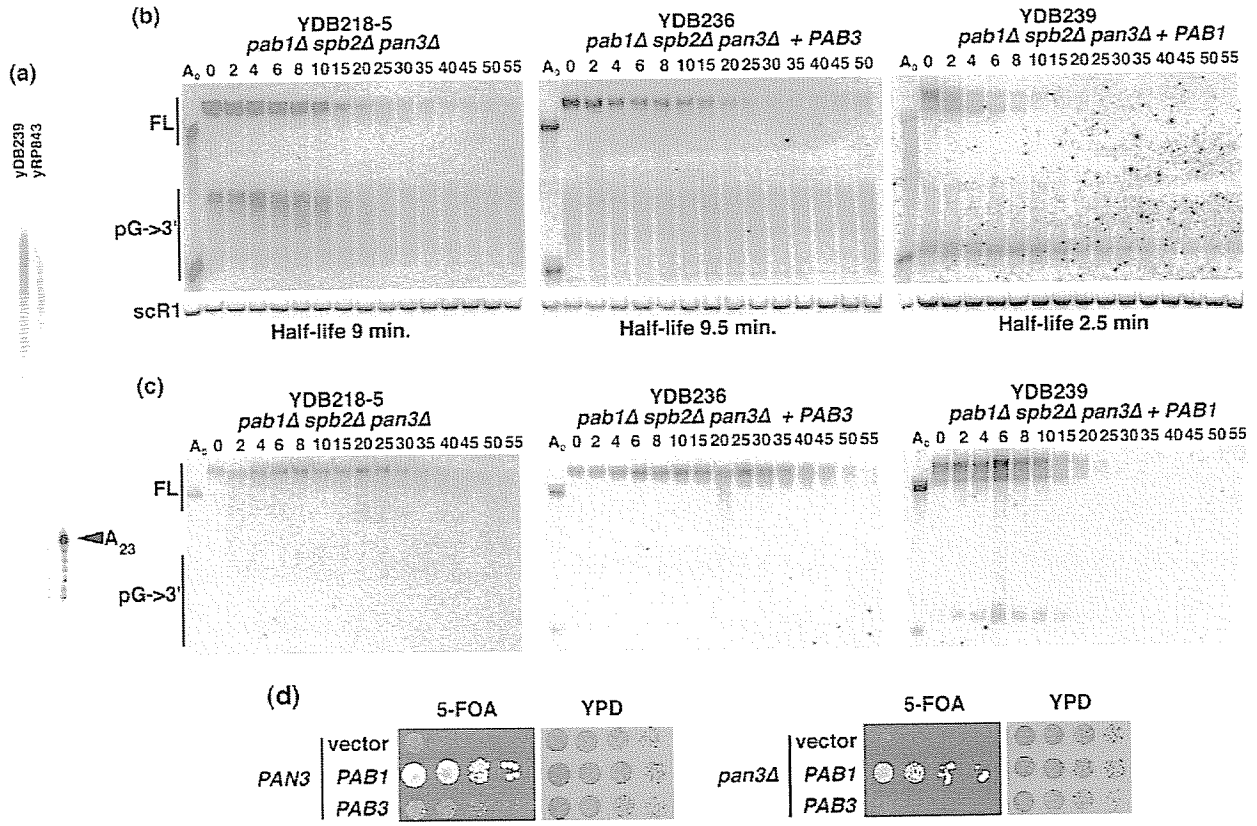
Pab3p corrects the lag prior to the onset of *MFA2pG* mRNA decay in yeast. Transcriptional pulse-chase analysis in the wild-type strain (YDB840), the *pab1Δ*, *spb2Δ* strain (YRP881), and the *pab1Δ*, *spb2Δ* strain expressing *Arabidopsis* Pab3p (YDB221) of the **(a)** *MFA2pG* mRNA and the **(b)** *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 *pab1Δ*, *spb2Δ* 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 *pab1Δ*, *spb2Δ* 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 *spb2Δ* 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 *pab1Δ*, *spb2Δ* 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 *pab1Δ* 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 *pan3Δ* strain yDB239 to near-isogenic *PAN3* strain yRP843). **(b)** Transcriptional chase from steady state and **(c)** transcriptional pulse-chase in the *pab1Δ*, *spb2Δ*, *pan3Δ* strain (YDB218-5) and *pab1Δ*, *spb2Δ*, *pan3Δ* strain expressing the yeast Pab1p (YDB239) or *Arabidopsis* Pab3p (YDB236). **(d)** Pab3p expression restores the viability of the *pab1Δ* mutant containing the wild-type *PAN3* gene, but not in the presence of the *pan3Δ* mutation. Yeast strain YAS319, which contains its only copy of the *PAB1* gene on a *URA3* plasmid, or its *pan3Δ* 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 *pan3Δ* 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 *pab1Δ*, *spb2Δ* + 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 *pab1Δ*, *spb2Δ* 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 (*pab1Δ*, *spb2Δ*, *pan3Δ*) 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.

Acknowledgements

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ELSEVIER

Plant gene expression in the age of systems biology: integrating transcriptional and post-transcriptional events

Dmitry A. Belostotsky[1] and Alan B. Rose[2]

[1]Department of Biological Sciences, State University of New York at Albany, 1400 Washington Ave, Albany, NY 12222, USA
[2]Molecular and Cellular Biology, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA

The extensive mechanistic and regulatory interconnections between the various events of mRNA biogenesis are now recognized as a fundamental principle of eukaryotic gene expression, yet the specific details of the coupling between the various steps of mRNA biogenesis do differ, and sometimes dramatically, between the different kingdoms. In this review, we emphasize examples where plants must differ in this respect from other eukaryotes, and highlight a recurring trend of recruiting the conserved, versatile functional modules, which have evolved to support the general mRNA biogenesis reactions, for plant-specific functions. We also argue that elucidating the inner workings of the plant 'mRNA factory' is essential for accomplishing the ambitious goal of building the 'virtual plant'.

Exploring the plant 'mRNA factory'

Progress in our understanding of eukaryotic mRNA biogenesis has been largely driven by mechanistic studies performed using *in vitro* systems in yeast and mammals. By contrast, the comparable *in vitro* tools in plants do not yet exist. Instead, most of the available knowledge is derived from three main sources: inferences based on bioinformatic analyses, examination of the behavior of DNA or RNA-based reporter constructs in cells or whole plants, and analysis of mutants. Curiously, many of the mutants that have allowed us a glimpse into the inner workings of the plant 'mRNA factory' have been identified serendipitously. A reluctance to seek such mutants directly might have been partly because of the relative paucity of the appropriate molecular assays and a view that the respective genes are essential for viability – an assumption that might seem reasonable, but nonetheless has not always proven valid.

The paradigm

The synthesis and maturation of mRNA, and the assembly of export-competent mRNP complexes (see Glossary), has been shown in recent years to be carried out by an integrated 'mRNA factory' that comprises RNA polymerase II (RNAP II) and numerous processing and export factors [1–8]. As in a real factory, a complex network of quality control checkpoints must be passed for the product (mRNA) to be successfully exported and enter cytoplasmic transactions (Figure 1).

The most important element that facilitates the interconnection of multiple steps of mRNA biogenesis is the C-terminal domain (CTD) of the largest subunit of RNAP II. Acquisition of the CTD was an important step in the evolution of complex patterns of regulated gene expression because it provided a scaffold for extensive interactions with factors involved in all steps of mRNA production [9]. The resulting close association of

Corresponding authors: Belostotsky, D.A. (dab@albany.edu); Rose, A.B. (abrose@ucdavis.edu).

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Glossary

AtCPL1–AtCPL4: *Arabidopsis* proteins related to FCP (C-terminal domain phosphatase-like).
CBC: nuclear cap-binding complex, composed of CBP80 and CBP20 subunits.
CF1, CF2: cleavage factors 1 and 2, parts of the mRNA 3′ end processing machinery.
CPSF: cleavage and polyadenylation specificity factor.
CStF: mammalian cleavage stimulation factor.
CTD: C-terminal domain of RNAP II.
eIF4F, eIFiso4F: cytoplasmic cap binding complexes (heterodimers of eIF4E/eIF4G and eIFiso4E/eIFiso4G, respectively).
EJC: exon junction complex, deposited as a consequence of splicing.
FCP: TFIIF-associating component of CTD phosphatase. Dephosphorylates Serine 2 of the CTD.
GT: guanylyltransferase component of the capping enzyme.
GUS: β-glucuronidase.
hnRNP: heterogeneous nuclear ribonucleoprotein.
mRNP: messenger ribonucleoprotein.
MT: N^7G methyltransferase component of the capping enzyme.
NMD: nonsense-mediated decay, a specialized mRNA degradation pathway. Targets mRNAs containing premature stop codons.
PABP: major poly(A) binding protein, predominantly cytoplasmic at steady state but has an important role in nuclear 3 end processing as well.
PABPN: nuclear-specific poly(A) binding protein, regulates PAP.
PAP: poly(A) polymerase.
P-TEFb: transcription elongation factor composed of cyclin T and CDK9 kinase.
RNAP II: RNA polymerase II.
RT: RNA triphosphatase component of the capping enzyme.
SCP: small CTD phosphatase, dephosphorylates Serine 5 of CTD.
snRNA: small nuclear RNAs, key constituents of the spliceosome.
SR proteins: Ser/Arg-rich proteins participating in many aspects of splicing.
TAR: *trans*-activating response region.
TBRP: *trans*-activation response RNA-binding protein.
TFIIH: transcription initiation factor that contains CDK7 kinase and cyclin H.

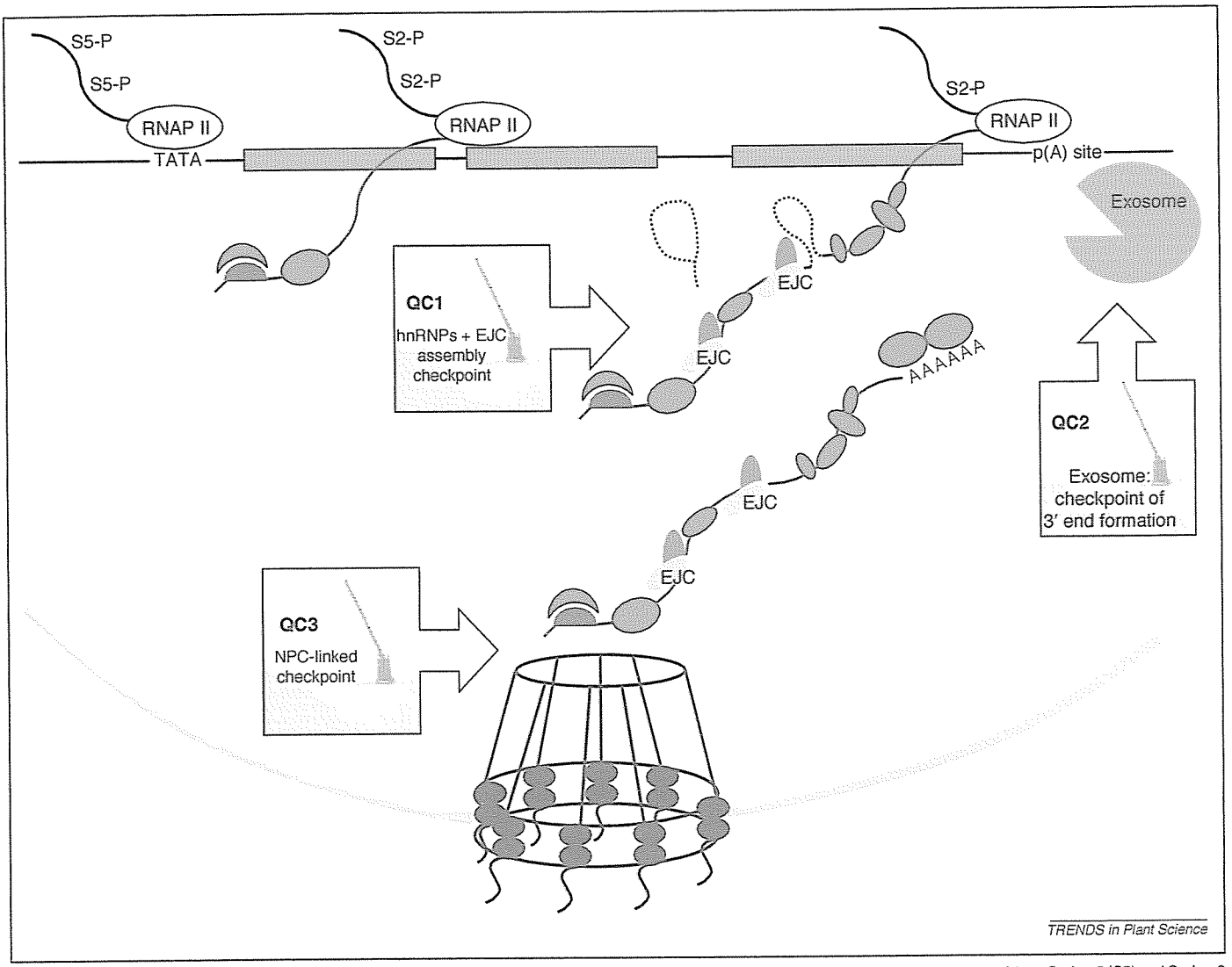

Figure 1. Key features and quality control checkpoints of the 'mRNA factory'. In the CTD of RNAP II, the changing phosphorylation of repeat residues Serine 5 (S5) and Serine 2 (S2) during transcription regulates the dynamic interactions of the CTD with enzymes of mRNA maturation (capping, splicing and polyadenylation factors, not shown). The 5′ cap is added early in transcription, whereas splicing of introns usually occurs cotranscriptionally but can happen later as well. Splicing leads to a deposition of the exon junction complex (EJC) upstream of the splice junctions. Numerous hnRNPs and other RNA binding proteins (represented as green symbols of different shapes and sizes), including cap binding protein eIF4E and poly(A) binding protein, associate with maturing transcripts; the complement of these factors is likely to be distinct for different mRNAs. The appropriate assembly of hnRNP proteins and completion of 3′ end formation is monitored at quality control checkpoints QC1 and QC2, respectively; messages improperly matured at these stages are subject to retention at the transcription site and degradation by the exosome complex. The nuclear pore complex-linked checkpoint QC3 ensures that only spliced transcripts are exported from the nucleus and causes mRNA to be kept at the site of transcription when the nuclear-pore complex-linked export step is blocked [69].

RNA-processing enzymes increases the efficiency of mRNA biogenesis by concentrating the key players near the site of action. The unique structure of the CTD, which comprises numerous tandem heptapeptide repeats with a consensus sequence YSPTSPS [10], provides ample opportunities for regulation via phosphorylation and conformational isomerization by peptidyl-prolyl *cis/trans* isomerases. The rich repertoire of conformational and phosphorylation states allows the CTD to not only modulate the activity of the polymerase, but also to recruit different multiprotein complexes involved in mRNA processing at specific points in the transcription cycle.

The CTD is hypophosphorylated during recruitment into preinitiation complexes. Phosphorylation of the CTD, particularly on Serine 5 by the CDK7 kinase of the initiation factor TFIIH (Figure 2), coincides with transcription initiation and also promotes the binding of mRNA capping factors. Capping occurs when the nascent transcript is 20–30 nt long; at this step, some of the capping enzyme subunits dissociate from the polymerase. The transition into elongation is marked by the removal of the phosphate groups from Serine 5 by the small CTD phosphatase (SCP) while Serine 2 phosphorylation increases as a result of the complex interplay between the transcription elongation factor P-TEFb (composed of cyclin T and CDK9 kinase) and phosphatase FCP (Figure 2). Concomitantly, a subset of splicing and polyadenylation factors adheres to the CTD, either by direct binding or via transfer from the preinitiation complex [11]. The polyadenylation factors that bind to the CTD near the start of a gene are carried along with the transcription machinery to the 3′ end where they and other factors that join the complex at the later stages of the transcription cycle participate in processing the 3′ end of the mRNA. Splicing can occur

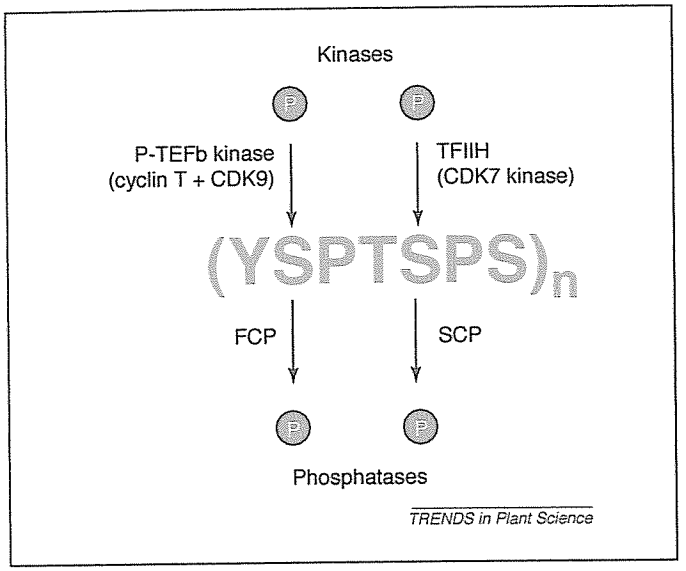

Figure 2. Factors regulating CTD phosphorylation. The consensus sequence of the heptapeptide repeats that constitute the CTD is shown and mammalian enzymes that phosphorylate and dephosphorylate the serine residues at positions 2 and 5 are highlighted. For simplicity, only those factors mentioned in the text are indicated. The complete details of all the enzymes that can modify the CTD and the residues on which they act remain unclear, and the substrate specificities of the enzymes shown are not absolute *in vitro*.

any time after an intron is transcribed until the mRNA is exported, but is usually completed while the nascent RNA is still tethered to the template DNA by the polymerase [12]. As a consequence of splicing, a collection of proteins known as the exon junction complex (EJC) is deposited 20–24 nt upstream of the former site of the intron [13]. The EJC has diverse roles in different species, such as facilitating mRNA export by direct interactions with proteins that associate with the nuclear pore complex, promoting association of the mRNA with polysomes, and helping to identify aberrant mRNAs containing premature stop codons for degradation via nonsense-mediated mRNA decay (NMD) [14].

To what extent does the scenario just described (based primarily on results from yeast and mammals) also occur in plants? The presence in plants of genes encoding presumptive homologs of most of the aforementioned factors that govern the processes of capping, splicing, polyadenylation, export and mRNA surveillance (listed on the accompanying website www.albany.edu/faculty/dab/IPGE_HUB.html) is consistent with the view that it should be fundamentally similar in plants. However, there are some remarkable and puzzling omissions, as discussed below. In addition, the overall similarity of the parts list contrasts with the many known differences of the gene expression reactions between plants and other organisms. Finally, the relatively mild consequences of mutations in several genes that are thought to be essential suggests that either many processes in plants can be carried out by functional homologs that lack sequence similarity or that plants are more different than we realize.

RNA polymerase II and its regulatory factors

The CTD in *Arabidopsis* has 40 repeats, 36 of which match the YSPTSPS consensus, and thus falls in the middle of the range defined by yeast and human CTDs (26 and 52 repeats, respectively). Consequently, the *Arabidopsis* CTD is capable of binding multiple factors, although the network of interactions might not be as extensive as it is in mammals. However, the machinery that governs CTD phosphorylation in plants might be more complex than it is in other kingdoms. For example, *Arabidopsis* differs from both fungi and vertebrates in having at least two CDK7-like kinases capable of phosphorylating Serine 5 [15]. Moreover, each of these can be found in two distinct, large complexes that further differ in their ability to phosphorylate the CTD. On top of this, a distinct kinase (At4g28980) acts upstream to activate both of these CDK7-like kinases. Therefore, a somewhat shorter CTD in *Arabidopsis* could be more than compensated for by the complex interplay of multiple activities that modify it.

In contrast to the TFIIH-associated CDK7-like kinases, the similarity of the presumptive *Arabidopsis* homologs of other kinases and phosphatases that govern the CTD phosphorylation (Figure 2) to their respective mammalian counterparts is low and/or limited in length. Nevertheless, At5g10270 and At5g64960 are likely to represent *bona fide* homologs of CDK9, a subunit of a kinase (P-TEFb) that can phosphorylate Serine 2 of the repeats, because they interact in two hybrid assays with the other P-TEFb subunit, cyclin T [16]. At least four genes (AtCPL1– AtCPL4) contain regions of similarity to the catalytic domain of FCP, and AtCPL1 and AtCPL2 specifically dephosphorylate Serine 5 but not Serine 2 *in vitro* [17]. Remarkably, mutations in two of these (AtCPL1/FRY2 and AtCPL3) result in increased expression of a subset of cold- and abscisic acid (ABA)-regulated genes [18,19].

How can a generic RNAP II regulatory factor such as FCP have such a specific function in plant stress response? In contrast to classical FCPs, the AtCPL proteins contain additional domains suggestive of function. AtCPL1 (as well as AtCPL2) are unique in that they also contain RNA binding domains resembling the human TRBP protein that binds the HIV TAR RNA. TAR RNA, in turn, is the interaction site for the HIV Tat protein that associates with the CTD kinase that hyperphosphorylates RNAP II. Extending the analogy, one could envision that AtCPL1 binds to a subset of messages in a sequence-specific fashion and regulates the mRNA biogenesis at specific loci via modulating the RNAP II phosphorylation state in *cis*. However, AtCPL3 is unique among all known CTD phosphatases in having a region homologous to CES1 (capping enzyme suppressor) in yeast. Thus, AtCPL3 could be directly regulating the 5′ cap status of specific messages.

Capping enzymes and cap binding factors

The mRNA 5′ cap is a focal point of many documented regulatory pathways [20]. Formation of the mature 5′ cap involves three distinct enzymatic activities: RNA triphosphatase (RT), guanylyltransferase (GT) and N7G methyltransferase (MT). The RT and GT activities are a property of a single, bifunctional enzyme in humans but reside in separate polypeptides in yeast. Moreover, the human and fungal enzymes are regulated differently by the RNAP II CTD, and the requirements for the interaction between the RT and GT subunits are also different, even within the

kingdom fungi [21]. Plants, like humans, have bifunctional RT/GT enzymes but, unlike humans, *Arabidopsis* has three distinct RT/GT genes with distinct expression patterns [22], which might indicate their functional specialization.

The cap binding complex (CBC), a heterodimer of CBP20 and CBP80, binds to the cap during transcription and is subsequently replaced by translation initiation factor eIF4E in the cytoplasm following a 'pioneer' round of translation that occurs while the mRNA is still associated with CBC [23]. Unexpectedly, the *Arabidopsis* mutant *abh1*, which is defective in CBP80, was isolated in a screen for ABA hypersensitivity: it shows ABA-hypersensitive regulation of seed germination, stomatal closure and cytosolic calcium increases in guard cells [24,25]. It is also remarkable that even though the *Arabidopsis* CBP80 is encoded by a single-copy, ubiquitously expressed gene, the *abh1* mutation has no major effect on plant growth and development beyond the ABA responses and alters the expression of only a small subset of genes. The relatively minor consequences of eliminating CBP80 are particularly surprising because CBC is thought to participate in multiple steps of mRNA metabolism, including the splicing of cap-proximal introns, 3′ end processing and the export of snRNAs.

Another unique aspect of the machinery that interfaces with the mRNA 5′ cap in plants is the presence of the two highly divergent cytoplasmic cap binding complexes, eIF4F and eIFiso4F (eIF4E/eIF4G and eIFiso4E/eIFiso4G heterodimers, respectively). The cap affinity and abundance of the two isoforms are different, and eIF4F is more efficient in supporting cap-independent translation and translation of structured RNA than is eIFiso4F *in vitro* [26], hinting at possible functional specialization *in vivo*. Indeed, the eIFiso4E subunit of eIFiso4F plays a nonredundant role in the replication cycle of plant potyviruses. Surprisingly, plants lacking eIFiso4E otherwise grow and develop completely normally [27,28].

Splicing

Homologs of most of the protein and RNA factors involved in splicing in other eukaryotic systems can be identified in the *Arabidopsis* genome, suggesting overall conservation of the basic splicing mechanisms [29–32]. The conserved sequences found at the 5′ and 3′ splice sites and branchpoints are also similar in plants, yeast and mammals; their importance is supported by parallel mutational analyses in all three systems. Given these similarities, it might seem puzzling that plant introns are poorly spliced in mammals and *vice versa*.

This incompatibility with the mammalian splicing machinery might be because splicing in plants is more complex. For example, *Arabidopsis* contains almost twice as many Ser/Arg (SR) proteins than the human genome does [33]. SR proteins have multiple roles in mediating constitutive and alternative splicing through numerous protein–protein interactions between themselves and involving the splicing apparatus [34]. Plant SR proteins help define exons and introns, recruit spliceosomal proteins onto pre-mRNA, influence splice site choice by binding to exonic and intronic splicing enhancer and silencer signals, and/or bridge the 5′ and 3′ splice sites. Thus, improper interpretation of the divergent instructions encoded in typical plant and mammalian introns might preclude splicing by the heterologous apparatus.

Additional considerations are that plant introns often lack recognizable polypyrimidine tracts (but are usually U-rich throughout, particularly in dicots), and the way in which introns are distinguished from exons might also be different. In vertebrates, the relatively short exons are usually separated by large introns and they are differentiated primarily by factors that bind to exon sequences (exon definition [35]). By contrast, plant introns are generally short and are identified either mostly by intron-binding factors (intron definition) or through a combination of exon and intron definition [29–32]. Thus, plant introns might have additional features that are not properly recognized in mammalian systems.

Introns are known to significantly stimulate gene expression in plants, mammals, yeast, nematodes and insects, suggesting widely conserved interactions between splicing and other steps of gene expression. A connection between splicing and transcription, particularly involving CTD phosphorylation, has been demonstrated in mammals and yeast [36]. Transcript initiation is stimulated by interactions between U1 snRNA, which anneals to the 5′ splice site in the first step of spliceosome assembly, and a kinase that phosphorylates Serine 5 of the CTD [37]. Furthermore, introns also stimulate RNAP II elongation via interaction of snRNPs with the elongation factor TAT-SF1; TAT-SF1 in turn binds to P-TEFb, which phosphorylates Serine 2 of the CTD [38].

Evidence for a similar connection between splicing and transcription in *Arabidopsis* comes from *in vivo* studies in which the position of a single intron was varied throughout a stably integrated transgene. The intron-mediated stimulation of mRNA accumulation declined with distance from the promoter until it was lost entirely about a kilobase from the start of the gene [39]. Although these findings are most consistent with an effect of introns on transcript elongation, direct evidence for intron-mediated stimulation of transcription in plants has not been reported [40,41].

The *Arabidopsis* protein HUA2 is unique to plants but contains homology to factors that interact with components of the general transcription machinery and has similarity to the domain in a rat SR protein that mediates its interaction with the CTD [42]. Furthermore, HUA2 interacts with a homolog of the yeast Prp40p splicing factor, which is known to bind to the phosphorylated CTD [43]. Thus, HUA2 might also link splicing and transcription. The *hua2* mutation affects the expression of only a few genes, including *AG* [42], *FLC* and *FLM* [44]. This illustrates a recurring trend of adaptation of the functional modules, which originally evolved to serve the general mRNA biogenesis reactions, for various plant-specific processes by combining conserved domains with sequence-specific RNA binding domains and/or protein-interacting domains.

Polyadenylation

Formation of the 3′ end involves cleavage of the nascent transcript and addition of a poly(A) tail whose length is

species-specific and to some extent message-specific [45]. The cleavage event is guided by *cis*-acting sequence determinants that act as binding sites for the two key multisubunit factors (called CPSF and CStF in mammals) that associate with cleavage factors. Poly(A) polymerase (PAP) then adds the tail whose length is limited by PABPN, a nuclear protein that controls the processivity of PAP, and/or by exonucleolytic trimming of the newly synthesized tails in a manner dependent on PABP – a conserved poly(A) binding protein. Importantly, an *Arabidopsis* PABP, PAB3, partially complements this function in yeast [46].

Although the *cis* requirements for polyadenylation in plants are reasonably well understood [47], the reaction mechanism and the composition of complexes that carry it out remain to be elucidated. The *Arabidopsis* genome contains many homologs of the factors found in yeast and mammals (www.albany.edu/faculty/dab/IPGE5.html). Among the few that have been studied, both copies of CPSF73-like genes are essential [48] (Q. Li, personal communication). However, so far, none of these factors have been experimentally proven to act in polyadenylation *in planta*.

In yeast and mammals, the functional coupling between polyadenylation and transcription is extensive and includes (but is not limited to) direct interactions of several polyadenylation factors with phosphorylated CTD [49]. Such interactions, if any, are yet to be demonstrated in plants. However, genetic dissection of flowering time control in *Arabidopsis* has led to an unexpected insight into the plant-specific factors interfacing with the polyadenylation apparatus. The *Arabidopsis* gene *FCA* encodes an RNA-binding protein that promotes flowering by counteracting the floral repressor FLC (FLOWERING LOCUS C) [50]. *FCA* expression is negatively autoregulated through alternative processing of its own pre-mRNA, brought about by the FCA protein promoting cleavage and polyadenylation within *FCA* intron 3 [51]. The resulting truncated transcript encodes an inactive polypeptide. Efficient selection of this promoter-proximal polyadenylation site depends on the interaction of FCA with FY, a homolog of the yeast Pfs2p subunit of the cleavage and polyadenylation factor (CPF). Moreover, the FCA–FY interaction is also required for the downregulation of the floral repressor *FLC* [52]. Thus, it is likely that FCA controls 3′ end formation of a select subset of transcripts in an FY-dependent manner.

The Pfs2p in yeast and the CstF subunit CstF50 in mammals are believed to be functionally equivalent in spite of having dissimilar sequences [53]. *Arabidopsis* has genes encoding homologs of both proteins, *FY* (At5g13480) and At5g60940, which raises the possibility that plants possess two different polyadenylation complexes, perhaps with distinct functions.

mRNA export

At all steps of mRNA biogenesis and export, messages are engaged in dynamic association with many proteins so that export occurs in the form of large messenger ribonucleoprotein (mRNP) complexes. Export is mediated by nuclear export receptors, the most important and conserved of which is TAP (Mex67 in yeast) [54]. Such receptors are recruited to the mRNA via adaptor proteins and help dock the mRNP substrate to specific sites at the nuclear pore complexes. The mRNP is then translocated through the central aqueous channel of the pore, followed by disengagement and remodeling of the mRNP on the cytoplasmic side. Homologs of many of the key players involved in these processes can be recognized in the *Arabidopsis* genome, with the striking exception of Mex67p/TAP. Thus, the factor responsible for the recruitment of the mRNA to the nuclear pore complex in plants remains mysterious.

mRNA export in fungi and metazoa is intimately connected to splicing. On the one hand, splicing acts to promote export through interactions between the EJC proteins and export factors such as TAP/Mex67p [14]. Conversely, unspliced transcripts are actively retained in the nucleus [55]. Whether either mechanism operates in plants remains unclear. On the other hand, there is tentative evidence that mRNA export in plants is linked to transcript elongation because the CTD kinase subunit of the *Arabidopsis* P-TEFb interacts with a RNA binding protein similar to the yeast and mammalian hnRNPs that engage in nucleocytoplasmic shuttling [16].

The yeast Dbp5p protein is a RNA helicase believed to be involved in remodeling or disassembly of the mRNP cargo on the cytoplasmic side of the nuclear pore. Two mutant alleles of the *Arabidopsis* homolog of yeast Dbp5p affect plant responses to cold, but in opposite ways [56]: *los4-2* is chilling resistant and heat sensitive, whereas *los4-1* is chilling sensitive. The *los4-2* allele also shows an ABA hypersensitive response, which led the authors to hypothesize that this specific allele influences the export of transcripts involved in ABA signal transduction [56]. Nevertheless, mRNA export is also globally perturbed in *los4-1* at all temperatures and in *los4-2* at elevated temperatures only.

mRNA export in animals and yeast is also coupled to proper mRNA 3′ end processing. In tobacco, *GUS* mRNA whose 3′ ends are generated by ribozyme-directed cleavage, rather than by normal processing, is preferentially detected in the nucleus [57]. This finding seems to indicate that proper mRNA 3′ end processing is also required for export in plants, although an alternative possibility (i.e. that mRNA is exported from the nucleus but rapidly degraded in the cytoplasm) cannot be ruled out.

Translation

Several of the EJC proteins remain bound to the mRNA in the cytoplasm where they enhance translation by promoting its association with ribosomes. They also facilitate the recognition of premature termination codons because they mark the exon–exon junctions, which typically occur no further than 50 nucleotides downstream of authentic stop codons in mammals. In this context, the peculiarities of the few known examples of NMD in plants are intriguing. First, NMD of the *waxy* mRNA in rice depends on splicing of an intron that is upstream of the premature stop codon [58], whereas in mammals downstream introns trigger NMD. Second, most of the genes shown to be subject to NMD in plants have no introns [59–62], demonstrating that plant NMD might be totally independent from intron

recognition. Perhaps *cis*-acting sequence determinants play an important role in NMD in plants, as they do in yeast [63].

Nevertheless, *Arabidopsis* has homologs of most of the components of the EJC in mammals, including several that are missing in yeast. In plants, as in mammals, the inclusion of an intron in a gene apparently provides a modest (two to fourfold) increase in translation [39,64–66]. The magnitude of the enhancement in *Arabidopsis* was similar even for introns that differed widely in their effect on mRNA accumulation and was relatively insensitive to changes in intron position [39]. These findings are consistent with a role for the EJC in either enhanced export or more efficient ribosome association in plants. Interestingly, the plant homolog of EJC protein Magoh has been identified as a pollen guidance mutant [67], providing yet another example of a seemingly generic mRNA biogenesis factor acting in a specific pathway.

Prospects

Understanding the mechanistic details of the fundamental reactions of gene expression as well as their interconnections will be essential for making the concept of the 'virtual plant' [68] a reality. The most striking feature of the evidence gained to date is the contrast between the plausible expectation, based on sequence homology, that gene expression operates by conserved mechanisms in all eukaryotes, and the frequent failure of plant mutants to display the phenotypes predicted by analogy with mammals and yeast. The mild consequences of mutating a factor thought to be essential, or lethality caused by disrupting a gene that is dispensable in other organisms, are instructive in demonstrating that bioinformatic analysis must be coupled with direct 'invasive' experimentation to generate an accurate understanding of gene expression in plants.

Even though conventional *in vitro* assays for many mRNA processing reactions might never be an option, perhaps because essential but delicate interconnections between steps are much more difficult to preserve during preparation of extracts from plant cells than from mammalian or yeast cells, several alternatives are likely to continue providing useful information. Foremost among these is the analysis of complete and partial loss-of-function mutants in genes whose sequence suggests a role in gene expression. As indicated by the precedents cited above, such analyses can yield a few surprises. Cross-species complementation, both *in vivo* and *in vitro*, will be another useful tool to determine the extent that processes are conserved. Finally, chromatin immunoprecipitation has the potential to detect changes in the transcription machinery (and associated factors) as it traverses a gene. Establishing the biochemical details of the various steps of gene expression in plants, and demonstrating the functional interactions between them, is a major challenge that must be met to accomplish the goal of the 2010 Project – to determine the function of all the genes in *Arabidopsis*.

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Dmitry Belostotsky: Research Program

My research group is investigating the mechanisms and the significance of posttranscriptional events in gene expression, using two model systems, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. In *Arabidopsis*, we are specifically interested in the role of the posttranscriptional regulatory mechanisms in reproductive development, fate specification and gene imprinting. On the other hand, in *Saccharomyces* our focus is on the most fundamental aspects of posttranscriptional processes. These two lines of study have both common and distinct attributes, resulting in a synergy that benefits both plant side and yeast side of the lab.

The Plant Exosome Complex: Genetics, Genomics, Proteomics and Development

Exosome complex is a large, stable, and evolutionarily conserved macromolecular machine that comprises as many as 9 or 10 active 3'-5' exoribonucleases of two catalytic types: phosphorolytic and hydrolytic. Exosome carries out at least three very distinct types of RNA processing and/or degradative reactions: (i) processing of the precursors of stable RNAs (e.g. rRNA, snRNA and snoRNA) into mature products, (ii) degradation of mRNA in the cytoplasm, as a part of its normal turnover, and (iii) cotranscriptional surveillance of mRNA which ensures that the transcripts that are not properly processed or packaged become actively retained at the sites of transcription and eventually destroyed. In addition, undermodified or misfolded tRNAs also suffer similar fate as a result of surveillance by the exosome.

Compared to yeast, our knowledge of the exosome functions in multicellular organisms lags far behind, and whether or not exosome even exists in plants was not known prior to our studies. We have shown that *Arabidopsis* AtRRP41 protein is a *bona fide* exosome subunit via complementation of the respective yeast mutant, as it rescues not only its temperature sensitivity, but also a highly characteristic "stuttering" during the processing of 5.8S rRNA from the 7S precursor. We have further demonstrated that AtRRP41 is a part of a large complex *in planta*, and hypothesized that this complex represents plant exosome.

With the goal to carry out a detailed analysis of its composition and functions, we have isolated insertional mutations in all of the putative subunit genes of the *Arabidopsis* exosome, identified by various computational tools. In the next step, we have created TAP tag-complemented lines that completely rescue the phenotypes of the null alleles of *AtRRP41* and AtRRP4 genes, which encode prototypical phosphorolytic and hydrolytic exosome subunits, respectively. We have then successfully optimized the TAP protocol for *Arabidopsis,* and conducted a MS/MS analysis of the purified complex, which allowed us to identify all of the predicted subunits and an additional one that was missed by the genome annotators. To my knowledge, this is the first successful application of the TAP tag-assisted proteomics to a multiprotein complex in plants that was not previously identified by other means.

We have found that remarkably, while the composition of plant exosome is conserved, loss of its individual subunits causes very distinct phenotypes, which is in sharp contrast with the

situation in other eukaryotes. First, the *rrp41* null is female gametophytic lethal. It is not transmitted through female parent, due to an arrest of female gametophyte development after the first mitotic division (in contrast, transmission through the male parent is normal). This manifests itself genetically as semisterility. On the other hand, the *rrp4* null allele shows no female gametophyte problems, but is instead embryo lethal: 1/4 of the seeds obtained by selfing of the *rrp4* heterozygote arrest at, or prior to, globular-embryo stage. Thus, these distinct developmental arrest phenotypes reveal an unexpected functional specificity among the core exosome subunits in *Arabidopsis*. Our ongoing site-directed mutagenesis and transgenic rescue experiments will address whether this is due to distinct catalytic, structural, or substrate recognition functions of these subunits.

Detailed analysis of the *rrp4* mutant produced two additional observations that are novel and exciting. First, the *rrp4* null seeds ectopically express endosperm-specific markers in the embryo, thus indicating that *rrp4* null embryos assume partial endosperm identity – an unprecedented phenotype that has never been previously observed by plant biologists. This finding opens new avenues towards elucidating how the embryo and endosperm identities are established during seed development.

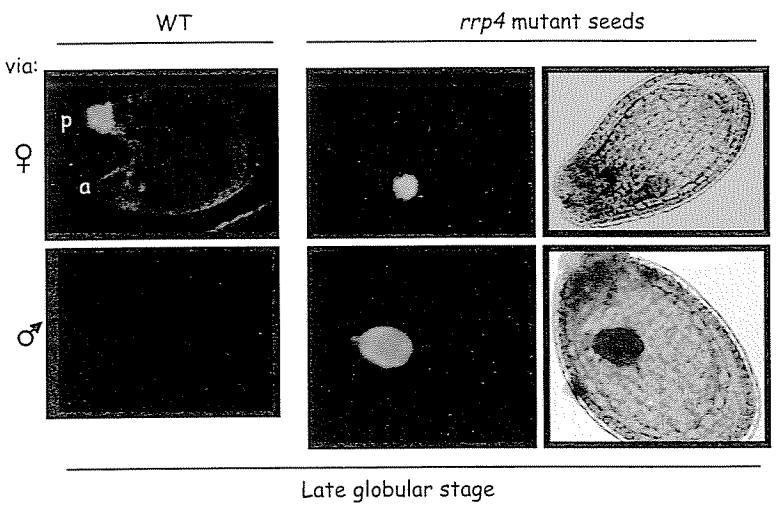

Fig.1. In WT seeds at the late globular stage, the GFP gene trap reporter KS117 is expressed only in the posterior (chalazal) endosperm, in a monoallelic (imprinted) fashion. In contrast, in the *rrp4* mutant seeds, KS117 is ectopically expressed in the embryo (also note the abnormally large embryo size, bottom right) and no longer follows the imprinted monoallelic pattern of expression.

Second, a detailed examination of the behavior of one of these reporters has revealed that its expression no longer follows the normal, maternally imprinted monoallelic pattern. This is the first precedent of the involvement of any posttranscriptional regulatory complex in regulating the expression of imprinted genes in plants. Moreover, it is highly intriguing that the *rrp4* seeds also show an excessive proliferation of the cells in the embryo - a phenotype also observed in *mea* mutants. MEA gene encodes a Polycomb group protein and a likely histone methyltransferase that controls the imprinting of several genes in developing seed, including that very marker whose expression is affected by the *rrp4* mutation!

These findings, taken together with current literature on imprinting in plants and on PcG proteins in general, have led us to propose the ***working hypothesis*** that the *Arabidopsis* exosome affects the biogenesis and/or metabolism of certain RNA species (possibly small RNAs) that act as guides to direct the chromatin modifying factors, such as PcG proteins, to their targets, such as imprinted genes. We are presently testing this hypothesis via generating the conditional exosome alleles and using them for transcriptome profiling experiments utilizing whole-genome tiling arrays.

To generate such alleles, we have successfully harnessed an inducible RNAi, by targeting AtRRP4 and AtRRP41 with intron-spliced hairpin RNAs expressed under the control of an estradiol-regulated chimeric transcription factor. As expected, the estradiol-regulated RNAi of AtRRP4 or AtRRP41 causes an arrest of seedling growth, accompanied by a highly specific defect in the processing of 5.8S rRNA, quite similar to what we have seen in yeast. However, in the course of characterizing these inducible RNAi lines, we have also discovered that depletion of AtRRP4 or AtRRP41 leads to an accumulation of 3' end-extended species of 7SL RNA - a RNAP III transcript encoding a RNA subunit of the signal recognition particle. This is remarkable, because 7SL is not acted upon by the exosome in other species. These 3' end-extended species of 7SL span the normal RNAP III terminator site and end at one of the several cryptic ones. This suggests a novel, exosome-dependent pathway of processing of 7SL species from 3' end-extended precursors in plants.

This unexpected finding illustrates the power of our approach to identifying novel substrates of plant exosome, and serves as just one example of many additional exosome substrates and functions that remain to be discovered by tiling array studies that are presently ongoing (a collaboration with Joe Ecker at Salk).

***Saccharomyces*: poly(A) tails, poly(A) binding protein, mRNP biogenesis and export**

The role of the mRNA poly(A) tails in gene expression (and by implication, of the machinery that produces, recognizes and removes them from the mRNA) has been the subject of debate for several decades. This debate has now been largely settled as far as the cytoplasmic mRNA transactions are concerned, however, the nuclear role of poly(A) in early mRNA biogenesis and export remains poorly understood.

Our recent contributions to this field began as a cross-species complementation of the lethal yeast mutation in the gene encoding poly(A) binding protein (PABP). PABP mediates several key posttranscriptional events, such as mRNA turnover and the initiation of protein synthesis. Therefore, it is not surprising that PABP is ubiquitous in *Eukarya*, and its function is usually essential for viability. The ability of PABP to stimulate translation is mechanistically well understood, and is largely due to its interaction with the translation initiation factor eIF4G. eIF4G interacts simultaneously with the cap binding protein eIF4E and with PABP, thus causing circularization of translated mRNA, which facilitates ribosome recycling. In contrast, the role of PABP in mRNA degradation appeared contradictory. On the one hand, PABP inhibits mRNA deadenylation, as well as mRNA decapping, and yet, on the other hand it is also ***required*** for the proper rate of deadenylation.

Our detailed cross-species complementation study of the yeast *pab1Δ* mutation with the *Arabidopsis* PABP gene, PAB3, has led to the resolution of this apparent paradox. In short, we have found that one previously overlooked, yet evolutionarily conserved function of PABP is to facilitate mRNA biogenesis and export, and thereby promote the entry of the mRNA into the decay pathway. Specifically, via a separation of the overall process of the mRNA decay

into two distinct phases - an initial period elapsing between the transcriptional induction and the onset of mRNA decay from the mRNA decay *per se* - we have shown that PABP acts to accelerate the initial ***rate of entry*** of mRNA into the decay process, rather than to speed up its actual decay. Subsequent experiments have led us to conclude that this accelerated commitment to decay by PABPs reflects its role in facilitating the export of mRNA from the nucleus (e.g. Fig. 2A, B). Moreover, not only our findings demonstrate that the function of PABP in mRNA biogenesis is conserved between yeast and plants, but they also further show that this function ***alone*** can be sufficient for viability in yeast.

Simultaneously with our efforts, Michael Rosbash's group at Brandeis have shown that the mRNAs whose 3' ends are generated by a cis-acting ribozyme (artificially engineered into the message) and therefore not properly processed and polyadenylated, become retained at the site of transcription. Moreover, the internally encoded, rather than posttranscriptionally added, poly(A) tails suffice, at least in some cases, for rescuing the transcription-site retention and export block of such mRNAs. PABP is an obvious candidate for the role of the trans-acting factor(s) that mediate this effect.

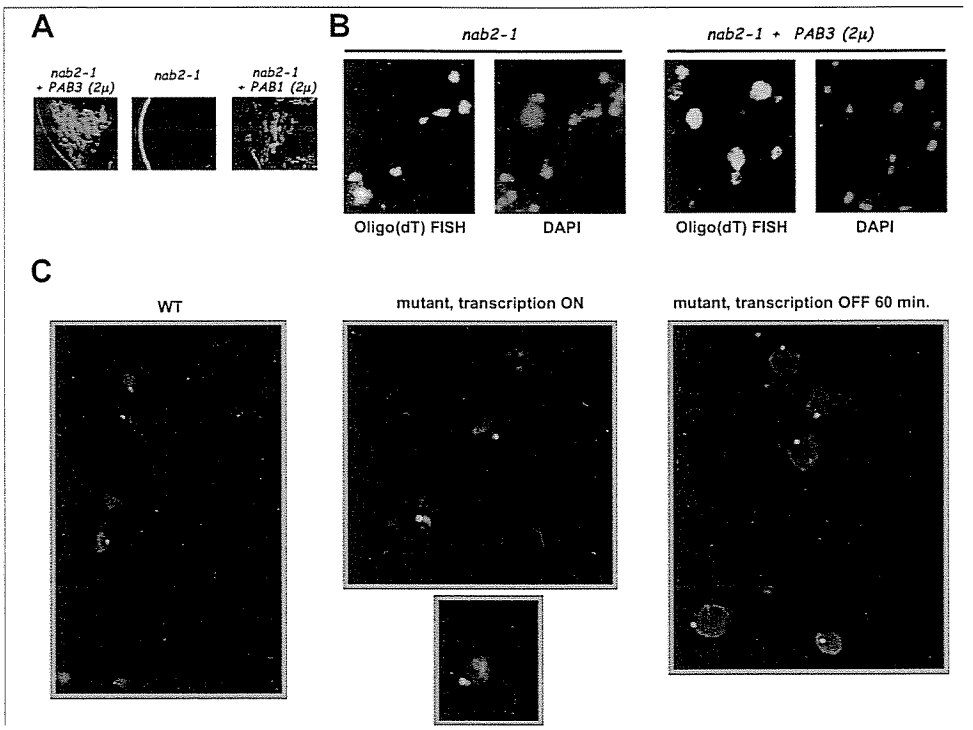

Fig.2. (A, B) Evolutionarily distant (plant PAB3) PABP protein rescues cold sensitivity (A) and mRNA export defect (B, in situ hybridization for total mRNA in red, DNA specific stain in blue) of the nab2-1 cells that are defective in the major hnRNP protein. (C) The quality of the mRNA 3' end maturation has profound downstream effects. Left panel: Ribozyme-terminated transcripts are retained near (red), yet not coincident with the gene of origin (green). Middle: example of mutants with altered "nuclear mRNA dot" morphology. Right: separation of the "nuclear dot" from the gene of origin in one of the mutants.

A natural affinity between these two lines of study has led to collaboration, culminating in a recent 6 months sabbatical in Rosbash lab that has been extremely fruitful. During this time,

we have adapted the high throughput method of systematic genetic analysis for the screening for altered transcription-site mRNA retention. Several factors whose loss causes such informative alterations and which thereby are implicated in transcription-site retention have been found (Fig. 1C). In a related effort, we have begun to investigate the relationship between the retained pool of the defective transcript (visualized by FISH as "nuclear mRNA dot") and its locus of origin ("locus dot", visualized using the TetR-GFP bound to appropriately integrated array of Tet operators). The key findings here so far include (1) realization that, quite unexpectedly, the "nuclear mRNA dot" represents mRNA population that not only is not nascent, but is also distinct from the locus of origin (although it is adjacent to it); (2) nevertheless, this non-nascent, retained mRNA pool remains closely associated with the locus from which it was produced long time after transcription ceases and RNA Pol II is cleared from the gene in the WT cells, and (3) in one of our mutants, this close association is lost after transcription ceases. These exciting findings uniquely position our teams (as this line of study has now become a long-term collaboration with Rosbash lab) close to elucidating the real nature of the "nuclear mRNA dots" as well as of the tethers that hold them in close association to the genes of origin.

Poly(A) Ribonuclease: mRNA-Specific and Plant-Specific Essential Functions

The three major mRNA degradation pathways in eukaryotes are initiated by the poly(A) tail shortening and/or its complete removal. i.e. deadenylation, which often serves as a rate-determining step of the whole process. Computational modeling studies suggest that modulation of the rate of deadenylation is a very powerful way to manipulate the overall steady state level of a given mRNA. mRNA deadenylation is also subject to regulation by the environmental signals, conveyed to gene expression machinery by various signal transduction pathways. A total of three mRNA deadenylation systems are known in eukaryotes: Ccr4p/Caf1p complex, PAN complex and the poly(A) ribonuclease PARN. We have discovered that the PARN function is uniquely essential in *Arabidopsis*, and therefore have chosen this factor for an in-depth study.

We have isolated several null alleles of AtPARN, and discovered that they share an embryonic lethal phenotype manifest at the early-cotyledon stage of development. Through the complementation studies with the transgene constructs encoding the WT or catalytically disabled AtPARN polypeptides, we have shown that the essential role of AtPARN in plant embryogenesis requires its catalytic function. We have also expressed the respective WT and site directed mutants as recombinant proteins and characterized them biochemically.

We further extended our studies of PARN to test whether it is likewise essential in other species. To this end, we have created a knockout of the sole *S. pombe* PARN homolog, as well as performed a double RNAi knockdown of the two homologs of *C. elegans* PARN. Remarkably, and in sharp contrast to essential requirement of PARN for plant growth and development, neither fungal nor metazoan loss-of-function studies resulted in any deleterious phenotype. Thus, the essential requirement for PARN function appears to be unique to plants.

Using a modification of the poly(A) tail assay that we have developed, we have discovered

that the requirement for the PARN deadenylation activity during plant embryogenesis is not global. Rather, we have uncovered an unexpected specificity in the AtPARN action towards only a subset of the embryonic transcripts. We hypothesize that AtPARN is actively recruited to its "preferred mRNA clients" by sequence-specific RNA binding proteins. AtPARN could interact with such sequence-specific RNA binding proteins directly, or alternatively, AtPARN and sequence-specific RNA binding proteins may participate in larger common complexes. To address both of these possibilities, we are undertaking two complementary approaches to identification of the interacting partners of AtPARN: affinity tagging of AtPARN *in vivo* in conjunction with MS/MS-assisted proteomic analysis (similar to what is described above for the exosome complex) and two hybrid system screen. In addition, an inducible RNAi strategy, as described above in the exosome section, is being used to comprehensively identify the targets of AtPARN. To this end, we will undertake a laser capture microdissection-assisted, microarray-based whole-transcriptome analysis of the *atparn* mutant embryos, as well as of the AtPARN-depleted seedlings. Functional studies of the rice AtPARN (as well as of plant exosome) are also being initiated, on collaboration with Gynheung (Gene) An's group at POSTECH (Pohang, Korea).

Poly(A) binding proteins in plants: complex biology and a potential biotech application

The remarkable feature of the plant kingdom is high complexity and antiquity of the PABP gene families. We have identified a total of 8 PABP genes in *Arabidopsis* that fall into the three evolutionarily ancient classes, based on the three distinct criteria (phylogenetic analyses of their encoded amino acid sequences, of their exon/intron structures, and of their expression patterns). Class I is comprised of the two reproductive tissues-specific *PAB* genes (*PAB3* and *5*), Class II of the three broadly and strongly expressed genes (*PAB2*, *4* and *8*), and Class III of the tissue specific, weakly expressed *PAB6* and *7* genes. This is the largest known PABP multigene family in any species so far. Moreover, the degree of sequence divergence among the *Arabidopsis* PABPs greatly exceeds the divergence observed within the PABP multigene families of other eukaryotes. The high degree of the amino acid sequence divergence and vastly different expression patterns of the Class I, II and III PABPs argue for the existence of functional differences between classes, and possibly even between members of the same class.

To address the possible functional specialization among the divergent plant PABP classes, we have constructed a comprehensive collection of insertional mutants (two or more null alleles per gene). This unique resource enabled us to initiate the rigorous experimental testing of their functions and of issues of redundancy and/or functional specialization. So far, we have uncovered nonredundant role of Class I in plant reproductive development, as well made a totally unexpected and exciting finding that the Class II PABPs have a specific role in attenuation of heat stress response. This finding may have commercially important implications, and is presently a subject of the patent filing. In addition, in collaboration with the group of Prof. Orna Elroy-Stein (Tel Aviv), we are investigating the role of plant PABPs in internal initiation of translation – an unconventional mode of initiating protein synthesis that also can be harnessed for the purposes of agricultural biotechnology.

Dmitry Belostotsky: Teaching Interests

As will be clear from the listing below, I have taught in a variety of courses, and also have developed several new courses, either by myself or as a part of a team. This hopefully demonstrates that I possess a considerable versatility in the ability to teach a range of subjects, both individually and as a part of the team.

At Bloomington, I would be particularly interested in teaching courses in molecular genetics / genomics / gene expression on both undergraduate and graduate levels. In the future, I would be interested to participate in, and possibly even help organizing, a course or courses in systems biology / chemical genetics. Furthermore, I would be an enthusiastic contributor to the success of the graduate training grants such as NSF-IGERT.

Courses currently taught

BIO365	Biochemistry (undergraduate course required of all Biology majors), 100%
BIO504	Cell Biology (graduate course required of all M.S. and Ph. D. students), 50%

Complete list of all courses taught:

Undergraduate:

BIO365	Biochemistry
BIO369	Computer Applications for Molecular Biologists (under HHMI grant)
BIO323	Plant Physiology
BIO102N	General Biology for non-majors

Graduate:

BIO524	Advanced Molecular Biology
BIO603	Topics in Genetics: Focus on Epigenetic Phenomena
BIO504	Cell Biology
BIO506	Molecular Biology of Plants
BIO629	Advanced Genetics
BMS606	Biology of Model Organisms

New courses developed:

Undergraduate:

BIO369	Computer applications for molecular biologists (100%)
BIO323	Plant Physiology (new course syllabus, jointly with Dr. J. Mascarenhas)

Graduate:

BIO603	Topics in Genetics: Focus on Epigenetic Phenomena (100%)
BIO629	Advanced Genetics (50%)
BMS606	Biology of Model Organisms: segment on Arabidopsis genomics (20%)