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# Binding of a 50-kD Protein to a U-Rich Sequence in an mRNA Encoding a Proline-Rich Protein That Is Destabilized by Fungal Elicitor

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**The mRNA encoding the bean proline-rich protein PvPRP1 has been shown previously to be destabilized in elicitor-treated cells. In this study, we identified a 50-kD protein in cellular extracts that binds specifically to the *PvPRP1* mRNA by UV cross-linking assays. Using $^{32}$P-labeled RNAs transcribed in vitro from a series of 5′ deleted *PvPRP1* cDNA clones, we demonstrated that the *PvPRP1* mRNA binding protein (PRP-BP) binds to a 27-nucleotide U-rich (~60%) domain in the 3′ untranslated region. Poly(U) and, to a lesser extent, poly(A-U) competed for the PRP-BP binding activity. PRP-BP activity is redox regulated in vitro, as shown by the effects of sulfhydryl-modifying reagents on the RNA binding activity. Treatment of cellular extracts with the reducing agents DTT and β-mercaptoethanol increased binding activity, whereas treatment with the oxidizing agent diamide and the alkylating agent *N*-ethylmaleimide inhibited binding. In extracts from elicitor-treated cells, PRP-BP activity increased approximately fivefold prior to rapid *PvPRP1* mRNA degradation. The increase in PRP-BP activity was apparently due to post-translational regulation because control and elicitor-treated cell extracts supplemented with DTT showed high comparable levels of RNA binding activity. The kinetics of PRP-BP activation after elicitor treatment and its capacity for redox regulation in vitro suggested that PRP-BP may function in the elicitor-induced destabilization of *PvPRP1* mRNA.**

## INTRODUCTION

Regulation of mRNA stability has been demonstrated to be an important determinant controlling gene expression in diverse organisms (Peltz et al., 1991; Gallie, 1993; Green, 1993; Morris et al., 1993; Sachs, 1993). Individual mRNAs within eukaryotic cells can display a wide range of stabilities, with half-lives ranging from minutes to days. Transiently expressed mRNAs coding for lymphokines and protooncogene proteins are typically unstable with half-lives of 10 to 30 min (Laird-Offringa, 1992; Greenberg and Belasco, 1993). The rapid turnover of these mRNAs appears to be important in limiting the synthesis of the encoded proteins to brief periods during cellular replication and differentiation. Stability of individual mRNA species has also been shown to change in response to a wide variety of endogenous or environmental stimuli. Examples include destabilization of transferrin receptor mRNA in mammalian cells exposed to excess iron (Müller and Kühn, 1988) and heat shock destabilization of α-amylase transcripts in gibberellin-treated barley aleurone (Brodl and Ho, 1991).

The diversity in mRNA decay rates and regulation of mRNA stability implies the existence of specific *cis* elements on the mRNAs and *trans*-acting protein factors that can interact with them. The *trans*-acting factors affecting mRNA stability appear to be post-translationally regulated in several cases. An adenosine-uridine binding protein that interacts with AU-rich sequences within cytokine and lymphokine mRNAs is subject to regulation by redox alterations and phosphorylation (Malter and Hong, 1991). A cytosolic protein, IRE-BP, recognizes the iron responsive element (IRE) on the transferrin receptor mRNA (Klausner et al., 1993). Considerable evidence indicates that IRE-BP activity is regulated by the structural state of its Fe-S cluster (Haile et al., 1992) and protein sulfhydryls (Hentze et al., 1989). In plants, specific RNA binding proteins that stabilize chloroplast-encoded mRNAs have been identified (Stern et al., 1989; Gruissem and Schuster, 1993). Although poly(A) binding proteins have been characterized (Yang and Hunt, 1992), cytoplasmic mRNA binding proteins that interact with specific nuclear-encoded mRNAs have not been characterized in plants.

The response of plants to pathogens is characterized by increased expression of many genes concomitant with decreased expression of a number of other genes (Dixon and Harrison, 1990; Sauer et al., 1990). Many studies have investigated the mechanisms of positive regulation of plant defense-related gene expression, primarily at the transcriptional level. However, the mechanisms involved in repression of gene expression are virtually unknown. We have shown previously that elicitor-induced down-regulation of a bean

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transcript encoding a putative cell wall proline-rich protein, PvPRP1, is due to the destabilization of the mRNA (Zhang et al., 1993). The destabilization of the *PvPRP1* mRNA was found to be transcription and translation dependent, suggesting the involvement of a de novo–synthesized or labile protein factor(s). We have postulated that the synthesis of the PvPRP1 protein is reduced during the defense response because of its low tyrosine content, making it a poor candidate for wall strengthening by isodityrosine cross-linking compared to other tyrosine-rich proline-rich proteins (Sheng et al., 1991).

In this study, we identified a 50-kD protein, designated PRP-BP, in bean cellular extracts that specifically binds to a U-rich sequence in the 3′ untranslated region of *PvPRP1* mRNA. The RNA binding activity of PRP-BP is redox regulated in vitro, as shown by high-affinity binding that requires one or more sulfhydryl groups. PRP-BP activity increased rapidly in protein extracts prepared from *Colletotrichum lindemuthianum* elicitor-treated cells prior to the onset of *PvPRP1* mRNA degradation. Based on the kinetics of PRP-BP activation in cells treated with the fungal elicitor, we postulated that the interaction of PRP-BP with *PvPRP1* mRNA may be one component in the process of elicitor-induced *PvPRP1* mRNA degradation. In addition, the defense response in bean and many other species is accompanied by redox perturbations (Chai and Doke, 1987; Apostol et al., 1989; Edwards et al., 1991). The redox regulation of PRP-BP activity in vitro suggests a possible mode of regulation of this protein during the defense response.

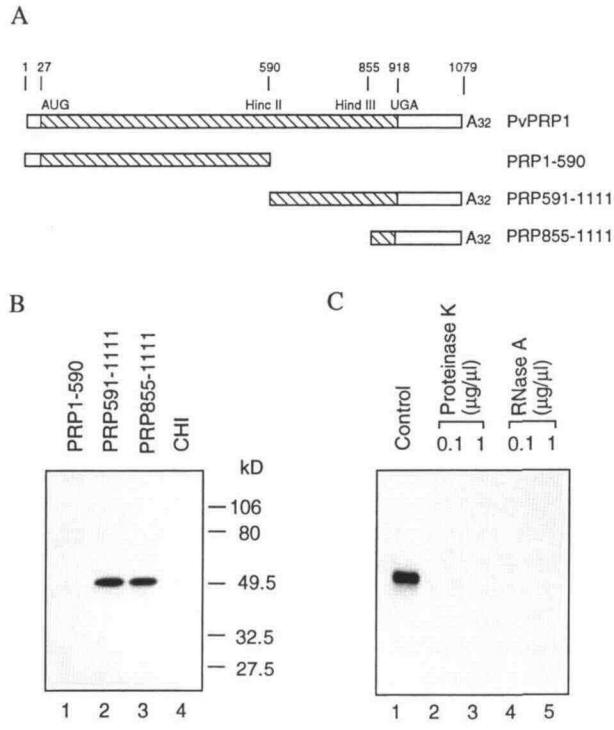


**Figure 1.** Interaction of the 50-kD PRP-BP with $^{32}$P-RNAs Analyzed by the UV Cross-Linking Assay.

**(A)** Diagrams of *PvPRP1* transcripts used in the UV cross-linking analysis. Shaded areas indicate coding regions. $A_{32}$ indicates the 32-nucleotide poly(A) tail.

**(B)** Binding of a 50-kD protein to PRP591-1111, PRP855-1111, and CHI RNAs. Twenty femtomoles of $^{32}$P-labeled RNAs (PRP1-590, PRP591-1111, PRP855-1111, and CHI) were incubated with 2 μg of cellular extracts. After UV irradiation and RNase A treatment, polypeptides were separated on a 10% SDS–polyacrylamide gel. Labeled polypeptide was detected by autoradiography. Prestained protein size markers (Bio-Rad) were used as standards.

**(C)** Binding of the 50-kD PRP-BP to RNA was sensitive to both proteinase K and RNase A, indicating an RNA–protein interaction.

## RESULTS

### Identification of a 50-kD Protein, PRP-BP, That Specifically Binds to *PvPRP1* mRNA Sequences

Our previous study demonstrated that *C. lindemuthianum* elicitor-induced down-regulation of *PvPRP1* mRNA abundance is due to mRNA destabilization (Zhang et al., 1993). As part of our investigations of the molecular mechanisms responsible for this selective mRNA degradation, we employed the RNA–protein UV cross-linking assay to identify protein(s) that specifically interact with the *PvPRP1* mRNA. Cellular extracts were prepared from unelicited cells and incubated with $^{32}$P-labeled in vitro–synthesized transcripts corresponding to different regions of the *PvPRP1* mRNA, as shown in Figure 1A. The reaction mixtures were then cross-linked by UV irradiation, digested with RNase A, and separated by electrophoresis on 10% SDS–polyacryamide gels. RNA binding activity in the extracts was indicated by the radiolabeling of proteins (Wilusz and Shenk, 1988; Bohjanen et al., 1991). Figure 1B shows that protein was not radiolabeled under our assay conditions when extracts were incubated with PRP1-590, which includes 27 nucleotides of the 5′ untranslated region and 563 nucleotides of the coding region. In contrast, a 50-kD polypeptide was cross-linked to both PRP591-1111 and PRP855-1111 RNAs, which correspond to the HincII-EcoRI and HindIII-EcoRI fragments of the *PvPRP1* cDNA, respectively. This 50-kD protein was designated PRP-BP. Based on the fact that both $^{32}$P-labeled transcripts transferred radioactivity equally to the protein, the binding site for this protein must reside in the smaller PRP855-1111 transcript. Therefore, in all of the following experiments, the $^{32}$P-labeled PRP855-1111 transcript was used, unless otherwise specified.

To evaluate the RNA binding specificity of this protein, a nearly full-size transcript corresponding to a bean chalcone isomerase (CHI) cDNA (missing ~30 nucleotides at the 5′ end of the ~850 nucleotides total length) was tested for PRP-BP binding. CHI catalyzes a step in phytoalexin biosynthesis in bean, and its mRNA transiently accumulates in response to elicitor (Mehdy and Lamb, 1987). Figure 1B shows that the 50-kD protein was also labeled by the *CHI* transcript but at a barely

detectable level relative to the labeling by the *PvPRP1* 3′ region RNAs. The differences in protein labeling by different RNAs were not due to differences in the rates of degradation of these RNAs in the reaction mixtures (data not shown). Figure 1C shows that pretreatment of the cellular extracts with proteinase K or the $^{32}$P-labeled PRP855-1111 transcript with RNase A eliminated the radiolabeled band, demonstrating that the band is due to the interaction of a protein with high molecular mass RNA.

To further characterize the specificity of the interaction, the abilities of increasing concentrations of unlabeled RNAs to compete for PRP-BP were analyzed. Figure 2 (lanes 6 to 9) shows that unlabeled homologous PRP855-1111 resulted in a concentration-dependent reduction in the amount of radioactivity transferred to the PRP-BP protein. In contrast, unlabeled PRP1-590 or *CHI* RNAs had no detectable effect at up to a 250-fold weight excess (Figure 2, lanes 2 to 5 and lanes 10 to 13, respectively). These data indicate that the interaction between PRP-BP and the PRP855-1111 transcript was specific.

### PRP-BP Recognizes a 27-Nucleotide U-Rich Sequence in the 3′ Untranslated Region of *PvPRP1* mRNA

To further localize the PRP-BP binding site within the 3′ region of the *PvPRP1* mRNA, a series of 5′ deletion clones were prepared from pPRP591-1111, and $^{32}$P-labeled transcripts were synthesized in vitro. Figure 3A shows the diagrams of nine truncated *PvPRP1* RNAs that were tested for interaction with PRP-BP. Figures 3A and 3B show that comparable levels of PRP-BP binding were observed for the PRP940-1111 and all larger transcripts. In contrast, the level of radiolabeled

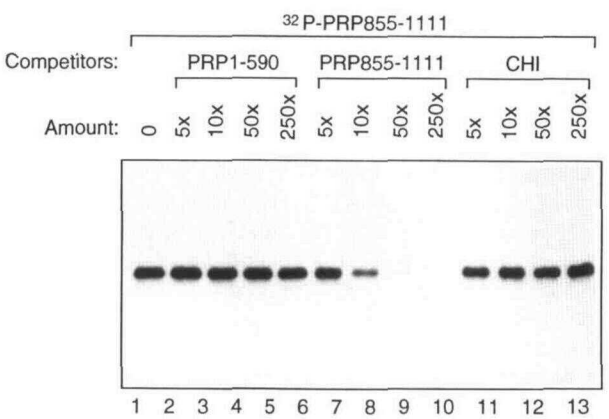


**Figure 2.** Specific Binding of PRP-BP to the 3′ Portion of *PvPRP1* mRNA as Shown by Competition Analysis.

Unlabeled PRP1-590 (lanes 2 to 5), PRP855-1111 (lanes 6 to 9), and *CHI* (lanes 10 to 13) were tested for their abilities to compete for the binding of PRP-BP using the UV cross-linking assay. $^{32}$P-labeled PRP855-1111 (20 fmol) was mixed with excess amounts of unlabeled RNAs (5, 10, 50, and 250 times by weight) before the addition of cellular extracts.

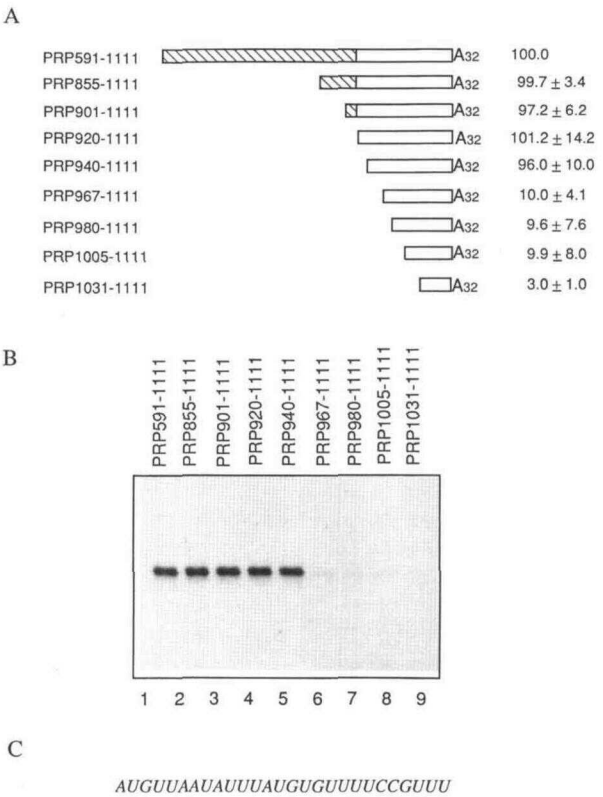


**Figure 3.** Identification of the 27-Nucleotide U-Rich Domain Containing the PRP-BP Binding Site in the 3′ Untranslated Region of *PvPRP1* mRNA.

**(A)** Transcripts synthesized in vitro from 5′ nested deletion clones used to localize the PRP-BP binding site. Shaded areas indicate coding regions. $A_{32}$ indicates the 32-nucleotide poly(A) tail. The relative binding of PRP-BP to each RNA shown on the right is the average value from three independent experiments.

**(B)** A representative autoradiogram showing the binding activities of PRP-BP to different RNAs, as measured by the UV cross-linking assay.

**(C)** The 27-nucleotide region containing the PRP-BP binding site.

complexes was reduced by ~90% in reaction mixtures containing PRP967-1111 or smaller transcripts. These data indicated that the PRP-BP binding site resides in the 27-nucleotide sequence between positions 940 and 967 within the 3′ untranslated region of the *PvPRP1* mRNA. It is unlikely that the loss of complex formation was due to a change in RNA secondary structure that accompanied the deletion of this 27-nucleotide sequence, because computer analysis revealed no significant secondary structure in this region. Figure 3C shows the sequence of the 27-nucleotide region containing the PRP-BP binding site. The region is U-rich, containing 60% uridylate residues. Interestingly, an AUUUA sequence is located at position 948 to 952. In mammals, this motif has been demonstrated to be a *cis* determinant affecting the degradation of numerous short-lived mRNAs (Vakalopoulou et al., 1991; Greenberg and Belasco, 1993).

The U-rich nature of the region containing the PRP-BP binding site prompted us to test the ability of various ribohomopolymers to compete for PRP-BP binding in competition titrations. Figure 4 shows that unlabeled poly(U) was most effective in competing for PRP-BP. Poly(A-U) was less effective compared to poly(U), whereas poly(A), poly(G), and poly(C) showed no evidence of competition, even at 1000-fold weight excess. In addition, PRP855-1111 transcripts labeled with $\alpha$-$^{32}$P-GTP instead of $\alpha$-$^{32}$P-UTP did not transfer radioactivity to the 50-kD PRP-BP protein, suggesting that G residues are not in close contact with the protein (data not shown).

To confirm the binding of PRP-BP to the 27-nucleotide U-rich sequence by a second assay, we performed an RNA band shift analysis. Radiolabeled PRP855-1111, which contains the 27-nucleotide sequence, or PRP980-1111, which does not contain these sequences, was incubated with cellular extracts. Heparin was added to reduce nonspecific binding and RNA–protein complexes were resolved by nondenaturing gel electrophoresis. Figure 5A shows that one shifted complex was formed in mixtures containing PRP855-1111 but not PRP980-1111. To determine whether the complex formed on PRP855-1111 contained the 50-kD PRP-BP, the reaction mixture for the RNA band shift assay was UV irradiated prior to electrophoresis on a nondenaturing gel. The labeled complex was excised from the gel and analyzed on an SDS–polyacryamide gel after treatment with RNase A. As shown in Figure 5B, the 50-kD PRP-BP was a component in the RNA–protein complex as revealed by the RNA band shift assay. Additional proteins that were not radiolabeled may be present in the complex as well.

## Interaction between PRP-BP and *PvPRP1* mRNA Sequences Is Redox Regulated in Vitro by Sulfhydryl Modification

To optimize the binding of PRP-BP to PRP855-1111, a number of reaction parameters were tested for their effects. The optimum pH for the PRP-BP activity was 7.0. The binding activity decreased slowly as the pH increased, with 70% of the maximal activity at pH 8.7, the highest pH tested, but activity dropped sharply when the pH was reduced, with only 15% of the activity at pH 6.7. The metal ions $K^+$, $Mg^{2+}$, and $Ca^{2+}$ inhibited PRP-BP activity with $I_{50}$ (concentration for 50% inhibition) values of 150 mM, 2 mM, and 100 $\mu$M, respectively (data not shown). Treatment of reaction mixtures with RNase A before UV cross-linking destroyed the RNA–protein complex (data not shown). In this regard, PRP-BP differs from several other RNA binding proteins that specifically bind to purine-rich sequences in the protooncogene mRNAs (Chen et al., 1992).

Reducing agents such as DTT and $\beta$-mercaptoethanol strongly stimulated the PRP-BP activity. Figure 6A shows that the pretreatment of cellular extracts with increasing concentrations of DTT resulted in increased RNA–protein complex

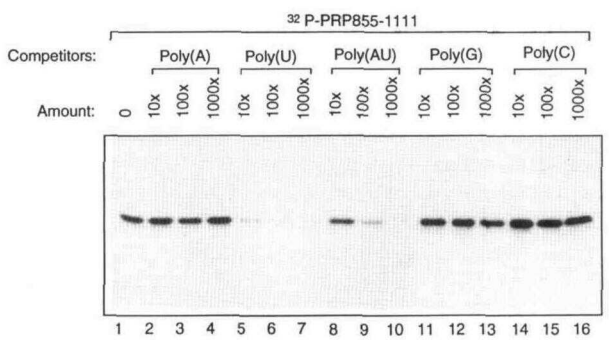


**Figure 4.** Poly(U) and Poly(A-U) Competition for the Binding of PRP-BP in UV Cross-Linking Assays.

Excess amounts (10, 100, and 1000 times by weight) of poly(A) (lanes 2 to 4), poly(U) (lanes 5 to 7), poly(A-U) (lanes 8 to 10), poly(G) (lanes 11 to 13), and poly(C) (lanes 14 to 16) were mixed with 20 fmol of $^{32}$P-labeled PRP855-1111 before the addition of the cellular extracts.

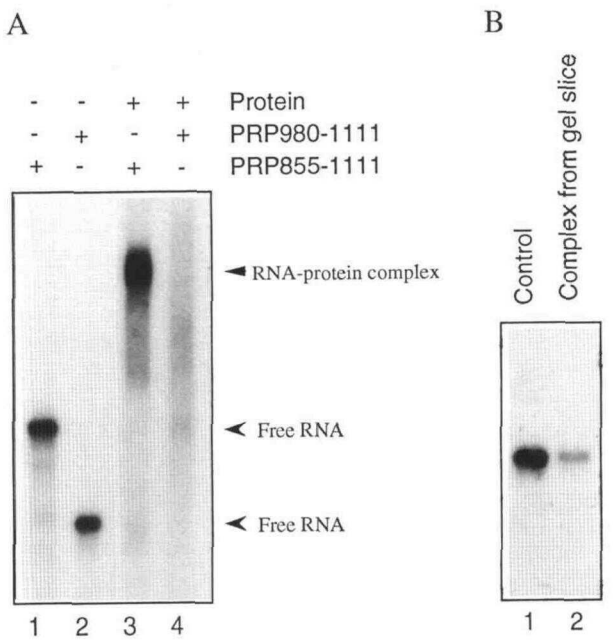


**Figure 5.** A Single RNA–Protein Complex Was Resolved by RNA Band Shift Assay and the Complex Shown to Contain PRP-BP.

**(A)** RNA band shift assay revealing a single shifted complex on PRP855-1111. Protein (5 $\mu$g) and $^{32}$P-labeled PRP855-1111 or PRP980-1111 (20 fmol) were incubated at room temperature for 10 min. After the addition of heparin to reduce the nonspecific binding, the mixtures were resolved on a 4% nondenaturing polyacryamide gel. +, presence; –, absence.

**(B)** The shifted RNA–protein complex containing the 50-kD PRP-BP. The reaction mixture was UV cross-linked just before loading onto the nondenaturing gel. After wet film exposure, the band containing the PRP855-1111 RNA–protein complex was excised, the gel slice was digested with RNase A (final concentration of 2 $\mu$g/$\mu$L) for 30 min at 37°C, and loaded on a 10% SDS–polyacrylamide gel. The control was processed according to the standard UV cross-linking assay as given in Methods.

formation. More than fivefold stimulation in activity was observed with 10 mM DTT (Figure 6B). Similar stimulatory effects were observed with β-mercaptoethanol.

Studies of UV irradiation–induced covalent linkages between nucleic acids and amino acids have shown that cysteine is one of the most reactive amino acids (Smith, 1976). To exclude the possibility that the DTT stimulation of PRP-BP activity in cellular extracts is an artifact that resulted from increased availability of cysteine residues for cross-linking due to reduction of protein disulfide bands, the effect of DTT on PRP-BP activity was also determined using the band shift assay. The shifted complex increased in abundance with DTT concentrations in the same range, confirming that DTT increased the amount of RNA–protein complexes formed. In addition, the DTT stimulation of the interaction was not attributable to the inhibition of ribonucleases by DTT because comparable amounts of intact RNA remained in the band shift assay reactions with different DTT concentrations (data not shown).

To further investigate whether the stimulation of PRP-BP activity was due to the reduction of a sulfhydryl group(s), the effects of diamide, a reversible –SH oxidizing agent, and *N*-ethylmaleimide (NEM), a –SH group alkylating agent, were tested. The minimal concentrations of diamide or NEM needed for complete inhibition of PRP-BP activity were determined by preincubating cellular extracts with various concentrations of these agents at room temperature for 10 min (data not shown). Figure 7 (lanes 4 and 5) shows that PRP-BP RNA binding activity was undetectable in cellular extracts pretreated with 10 mM diamide or 1 mM NEM. Incubation with 1% β-mercaptoethanol after the 10-mM diamide treatment restored the PRP-BP activity, whereas the effect of NEM was irreversible (Figure 7, lanes 6 and 7). Treatment with diamide or NEM after the formation of RNA–protein complexes but before UV cross-linking also abolished the transfer of radioactivity to the protein (Figure 7, lanes 8 and 9). Together, these data established that a sulfhydryl group(s) is critical for maximal RNA binding activity of PRP-BP.

Although the RNA binding activity of PRP-BP was inhibited by the oxidant diamide, it was not sensitive to molecular oxygen (data not shown). Therefore, cellular extracts can be handled under aerobic conditions without the loss of PRP-BP activity.

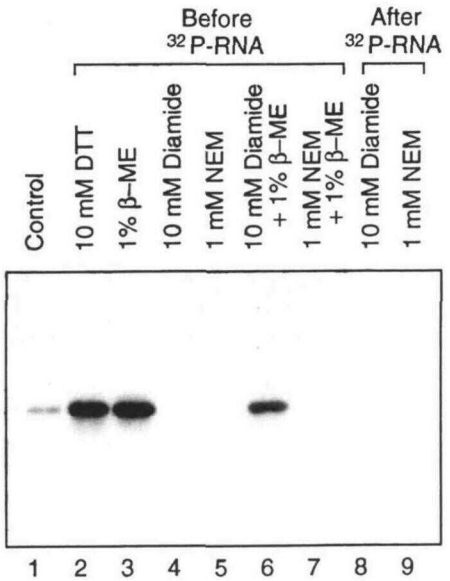


**Figure 7.** Redox Regulation of PRP-BP RNA Binding Activity in Vitro.

Cellular extracts were treated as labeled above each lane. In lanes 2 to 7, the cellular extracts were treated with DTT (lane 2); β-mercaptoethanol (β-ME; lane 3); diamide (lane 4); NEM (lane 5); diamide, and then β-mercaptoethanol (lane 6); or NEM, and then β-mercaptoethanol (lane 7) before the addition of $^{32}$P-PRP855-1111. In lanes 8 and 9, the $^{32}$P-PRP855-1111 (20 fmol) was added to cellular extracts first, and after the formation of RNA–protein complex, the reaction mixtures were treated with either diamide (lane 8) or NEM (lane 9). All treatments were before UV cross-linking and each treatment was for 10 min at room temperature.

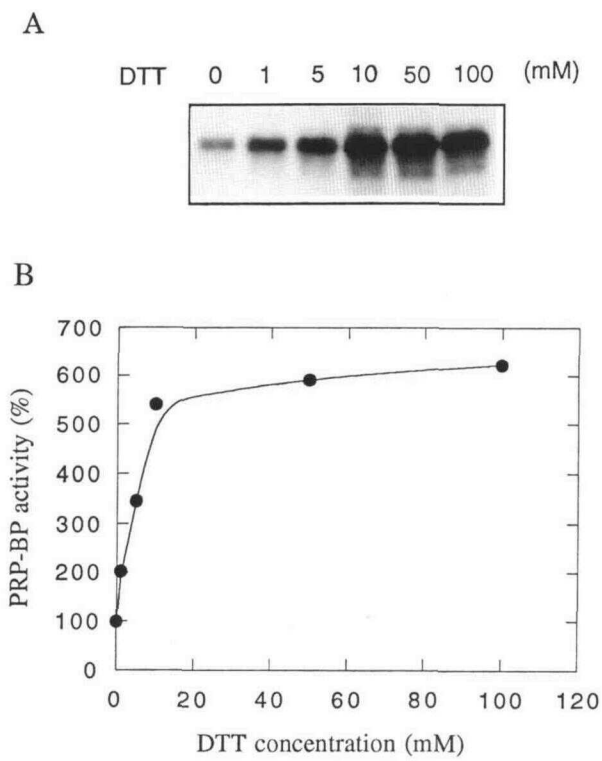


**Figure 6.** Stimulatory Effect of DTT on the RNA Binding Activity of PRP-BP.

**(A)** Cellular extracts were treated with different concentrations of DTT at 25°C for 10 min before the RNA–protein UV cross-linking assay.
**(B)** Quantitative analysis of the autoradiogram shown in **(A)**.

### Post-Translational Up-Regulation of PRP-BP Activity Precedes the Rapid Degradation of *PvPRP1* mRNA in Elicitor-Treated Cells

The *trans*-acting factors involved in the regulation of mRNA stability are generally believed to be cytoplasmic. To localize PRP-BP activity, total cellular and nuclear extracts were compared. The nuclear extracts contained little PRP-BP activity, suggesting that the active factor is predominantly cytoplasmic (data not shown).

If PRP-BP is involved in the regulation of *PvPRP1* mRNA destabilization upon elicitor treatment, then PRP-BP activity may be expected to change in parallel with the change in *PvPRP1* mRNA levels. Therefore, protein extracts were prepared from cells treated with elicitor for various times, and the PRP-BP binding activities were analyzed. Figure 8A (lanes 1 to 5) shows that the PRP-BP activity increased to a maximum in extracts prepared from 1-hr elicitor-treated cells and then decreased to lower but higher than basal levels. Figure 8B shows that approximately a fivefold increase in PRP-BP activity preceded the rapid degradation of *PvPRP1* mRNA in the same cells. Because the PRP-BP activity is redox regulated in vitro, we tested the PRP-BP activity in the same set of cellular extracts after preincubation with 10 mM DTT. Under these conditions, PRP-BP activities were high and comparable in all of the extracts (Figure 8A, lanes 6 to 10). These results suggested that the elicitor-induced increase in PRP-BP activity is due to post-translational regulation.

To address whether the lower PRP-BP activity in 0-hr extracts was due to the inactivation of the protein during extraction, we performed mixing experiments. Extracts were prepared using identical procedures from triplicate aliquots of cells corresponding to 0-hr, 1-hr, and a mixture of equal weights of 0- and 1-hr elicited cells. After dialysis, the cellular extracts were analyzed by the UV cross-linking assay. The relative levels of PRP-BP activity were 1.00 ± 0.10 (SD), 3.04 ± 0.32, and 4.77 ± 0.79 in 0-hr, mixed, and 1-hr cellular extracts, respectively. The PRP-BP activity in mixed cell extracts was aproximately the expected average of activities in the 0- and 1-hr cell extracts, indicating that lower activity in 0-hr cellular extracts was not due to changes in extractability. In addition, these data suggested that it is unlikely that an inhibitor was responsible for the lower PRP-BP activity in the 0-hr cell extracts.

To better distinguish the PRP-BP activities existing in cellular extracts with or without DTT pretreatment, a saturation titration study was performed. Cellular extracts from unelicited cells with or without 10 mM DTT pretreatment were incubated with increasing amounts of $^{32}$P-labeled PRP855-1111. As shown in Figure 9, a two-component titration curve was observed when the cellular extracts were not treated with DTT, indicating the presence of two populations of PRP-BP with different affinities. In contrast, a single component was detected after the extracts were pretreated with 10 mM DTT. It is not possible to assign accurate $K_D$ values for the different PRP-BP populations because of the presence of endogenous

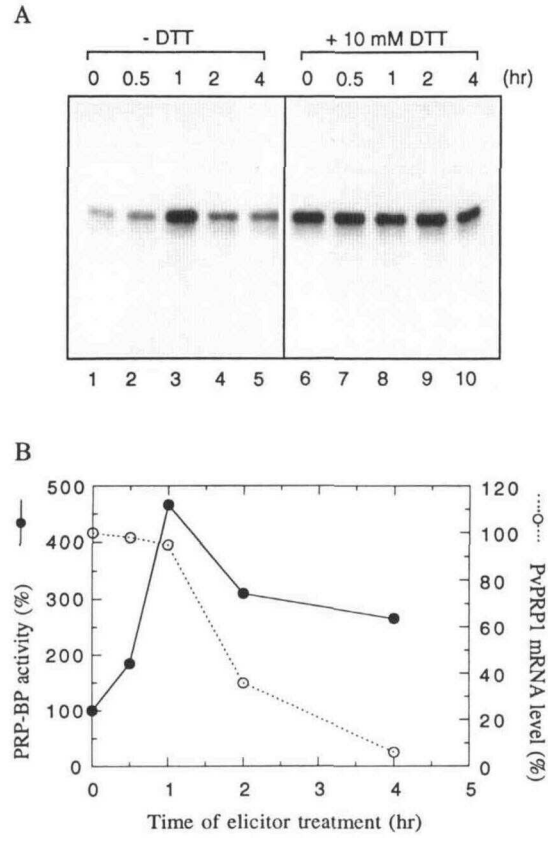


**Figure 8.** Rapid *PvPRP1* mRNA Degradation Was Preceded by Increased PRP-BP Activity in Cells Treated with Fungal Elicitor.

**(A)** Cellular extracts from cells treated with elicitor for various times were assayed for PRP-BP activity by UV cross-linking analysis without (lanes 1 to 5) or with (lanes 6 to 10) 10 mM DTT pretreatment. Lanes 1 to 5 were from an autoradiogram exposed for 20 hr, whereas lanes 6 to 10 were from an autoradiogram exposed for 4 hr.

**(B)** Quantitation of PRP-BP activity in cellular extracts prepared from cells treated with elicitor for different times (●). The activity at 0 hr was normalized to 100%. The *PvPRP1* mRNA levels in the same cells were measured by RNA gel blotting and quantitated using densitometry (○). The mRNA abundance at 0 hr was normalized to 100%.

nuclease activities in the cellular extracts that partially degraded the RNA over the course of the incubation. However, these data suggested the existence of two forms of PRP-BP with different RNA binding affinities whose proportion appears to depend on the redox states of PRP-BP protein, or alternatively, a regulatory protein that then modifies PRP-BP activity.

## DISCUSSION

In this study, we identified a 50-kD protein, PRP-BP, that specifically binds to the 3′ untranslated region of *PvPRP1* mRNA.

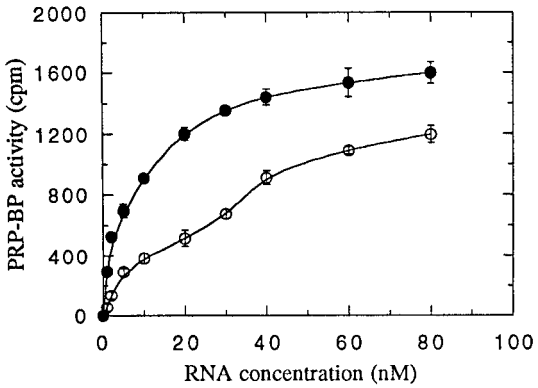


**Figure 9.** Saturation Titration of PRP-BP Activity in Cellular Extracts.

Cellular extracts from unelicited cells were preincubated with (●) or without (○) 10 mM DTT at room temperature for 10 min. The complexes formed in the reaction mixture of the protein (2 μg) and the increasing concentration of $^{32}$P-labeled RNA were measured according to the standard UV cross-linking assay, except that lower specific activity ($10^7$ cpm/μg) of $^{32}$P-labeled PRP855-1111 was used. Error bars represent the SD from three repetitions.

Based on the binding activity of PRP-BP to a series of 5′ deleted *PvPRP1* transcripts, the PRP-BP recognition site was localized to a 27-nucleotide U-rich domain within the 3′ untranslated region of *PvPRP1* mRNA. Several RNA binding proteins that interact with chloroplast mRNAs have been characterized (Stern et al., 1989; Schuster and Gruissem, 1991; Gruissem and Schuster, 1993). In this study, we presented direct evidence of a protein that binds specifically to a plant nuclear-encoded mRNA, although several cDNAs with an RNA binding motif have been cloned (Bar-Zvi et al., 1992; Ludevid et al., 1992; van Nocker and Vierstra, 1993). It is likely that additional specific RNA binding proteins will be identified for other mRNAs, including short-lived mRNAs, such as the oat phytochrome mRNA (Seeley et al., 1992) and small auxin-up RNAs (*SAUR*s; Newman et al., 1993), as well as mRNAs that have been shown to be post-transcriptionally regulated, such as pea ferredoxin I mRNA (Dickey et al., 1992) and the ribulose-1,5-bisphosphate carboxylase small subunit mRNA (Shirley and Meagher, 1990; Thompson and Meagher, 1990; Fritz et al., 1991).

The 27-nucleotide region in the *PvPRP1* mRNA that interacts with PRP-BP is U rich and contains an AUUUA motif. Considerable evidence suggests that the *cis* determinants conferring cytoplasmic instability for several short-lived mammalian mRNAs, such as c-*fos* and lymphokine mRNAs, include an AUUUA motif flanked by AU-rich sequences within the 3′ untranslated regions of these mRNAs (Bohjanen et al., 1991; Gillis and Malter, 1991; Greenberg and Belasco, 1993). A number of recent studies using either the RNA band shift or UV cross-linking assay have identified proteins that specifically recognize AU-rich mRNA destabilization elements in vitro (Malter, 1989; Bohjanen et al., 1991; Brewer, 1991; Gillis and Malter, 1991; Vakalopoulou et al., 1991; You et al., 1992). Based on the similarity between the sequences containing the PRP-BP recognition site and the AU-rich sequences in mammalian mRNAs, it is tempting to speculate that the PRP-BP recognition site also functions as a *cis* determinant affecting *PvPRP1* mRNA stability. It is interesting to note that at least two other transcripts that are down-regulated in plants also contain AUUUA motifs within their 3′ untranslated regions. These are the bean hydroxyproline-rich glycoprotein mRNA, *Hyp2.11*, whose level decreases in cell cultures treated with a fungal elicitor (Sauer et al., 1990) and the apple *pSD4* mRNA, whose level decreases in cell cultures after cytokinin treatment (Watillon et al., 1991).

A second AUUUA motif occurs at position 1042 to 1046 further downstream in the *PvPRP1* 3′ untranslated region. However, our studies showed that PRP-BP binding to this region is very low. Sequence alignment of the 27-nucleotide PRP-BP binding region and the second AUUUA-containing region revealed only 29% homology. These considerations indicate that the presence of the AUUUA motif alone is insufficient for PRP-BP binding. Studies of AUUUA-containing mammalian mRNAs have demonstrated the importance of flanking sequences in the protein–RNA interactions (Gillis and Malter, 1991).

Poly(U) and, to a lesser extent, poly(A-U) competed for the binding of PRP-BP to the $^{32}$P-labeled PRP855-1111 transcripts containing the 27-nucleotide U-rich domain. Several other RNA binding proteins have also been shown to bind poly(U), such as the 32-kD adenosine-uridine binding protein that binds to the AU-rich domain in the 3′ untranslated region of short-lived mRNAs (Vakalopoulou et al., 1991). In our competition experiments, poly(U) was a more efficient competitor than PRP855-1111 on a weight basis, as evidenced by quantitative comparison of their relative abilities to displace radiolabeled PRP855-1111 from PRP-BP (10-fold excess results shown in Figures 2 and 4, and 5- and 50-fold results, data not shown). Although, we do not know the length of poly(U) required for PRP-BP binding, it is likely that poly(U) contains many more PRP-BP binding sites per molecule than contained within one PRP855-1111 transcript. Thus, the 27-nucleotide U-rich region in *PvPRP1* mRNA sequences may be a more efficient competitor on a molar basis. Nevertheless, the fact that poly(U) can compete for binding of the factor suggested that PRP-BP will bind to mRNAs lacking the 27-nucleotide U-rich domain but containing a uridylate stretch of sufficient length.

Sequence comparisons between the 27-base *PvPRP1* sequence and other sequences within DNA data bases showed no significant sequence homologies beyond U-rich character. Specifically, the PRP-BP binding site has no similarity to the DST (represents downstream element) mRNA destabilization elements within the 3′ untranslated regions of *SAUR*s transcripts (Newman et al., 1993). Computer modeling of the *PvPRP1* 3′ untranslated region failed to demonstrate a consistent secondary structure. Analysis of a number of AUUUA-containing mammalian RNAs also found no evidence of a defined secondary structure (Gillis and Malter, 1991).

The activity of PRP-BP was shown to be redox regulated in vitro, and one or more sulfhydryl groups are required for high affinity RNA binding. RNA saturation titrations revealed

the presence of at least two populations of PRP-BP binding activity whose proportion appears to depend on the redox states of sulfhydryl group(s) in the PRP-BP protein. Because our study was conducted with crude cellular extracts, we cannot determine whether PRP-BP or another regulatory protein that modifies PRP-BP activity is reduced. The nature and functional roles of the protein sulfhydryls remain to be established. We found that extracts prepared from elicitor-treated cells exhibited increased PRP-BP activity compared to unelicited cell extracts. The increased activity appears to be due to post-translational mechanisms rather than an increase in the amount of protein. The nature of the elicitor-induced activation of PRP-BP is unknown. One possibility suggested by the redox regulation of PRP-BP activity in vitro is that elicitor treatment results in an increase in the pool of reduced PRP-BP characterized by a higher RNA binding activity.

The capability for redox regulation of PRP-BP activity makes this protein a good candidate to serve as a sensor for redox changes in cells during the defense response. Redox perturbation including generation of superoxide anions and $H_2O_2$ is one of the earliest responses of plants to microbial infection. It has been proposed that the oxidative burst might function as a signal for the defense responses (Chai and Doke, 1987; Apostol et al., 1989). In addition, certain sulfhydryl agents and thiols stimulate the production of phytoalexins and other defense responses (Gustine, 1987; Wingate et al., 1988). Bean cells treated with fungal elicitor accumulate homoglutathione within 2 hr (Edwards et al., 1991). The increase in the concentration of homoglutathione and possibly other thiols may directly or indirectly contribute to the postulated reduction of PRP-BP in elicited cells. The kinetics of accumulation of homoglutathione are consistent with a regulatory role for PRP-BP in *PvPRP1* mRNA degradation because the onset of mRNA degradation occurred within 1 to 2 hr after elicitor treatment. The hypothesis that the redox state of PRP-BP is regulated in vivo raises the question of whether such a reversible modification could be physiologically relevant in the reducing environment of the cytosol. The oxidation equilibrium constants for particular sulfhydryls can vary over many orders of magnitude, depending on the protein environment and the ratio of reduced to oxidized glutathione or total thiols in the cytoplasm. There is evidence that sulfhydryl groups regulate the activity of IRE-BP in vivo (Hentze et al., 1989). A study with 3-hydroxy-3-methylglutaryl coenzyme A reductase showed that oxidized sulfhydryls can predominate in a cytosolic glutathione redox buffer system (Cappel and Gilbert, 1988).

Based on its up-regulation in elicitor-treated cells, we suggest that PRP-BP protein functions in destabilization of the *PvPRP1* mRNA in elicited cells rather than in the stabilization of the mRNA in unelicited cells. The apparent location of PRP-BP activity in the cytoplasm is consistent with this function because mRNA degradation is generally believed to occur in the cytoplasm. Possible roles of the PRP-BP include its function as sequence-specific endoribonuclease or as a recognition factor for a larger degradation complex. Our previous results show that ongoing transcription and translation are needed for the rapid degradation of *PvPRP1* mRNA in elicited cells and suggest the involvement of either a de novo–synthesized or labile protein factor(s) (Zhang et al., 1993). The present study showed that PRP-BP is clearly not synthesized de novo, and there is no indication that the protein is labile. Under our assay conditions, only PRP-BP was shown to interact directly with *PvPRP1* mRNA, although it is possible that other proteins may bind to the same RNA or to a complex of PRP-BP and the RNA but cannot be cross-linked efficiently by UV light and thus cannot be detected. We believe it is likely that protein factors in addition to PRP-BP are needed for maximal *PvPRP1* mRNA degradation in response to fungal elicitor. There is evidence that the instability of mRNAs containing AUUUA motifs is mediated through a greater than 20S degradation complex, which includes multiple protein components (Savant-Bhonsale and Cleveland, 1992). Purification of PRP-BP and analysis of the stability of RNAs containing the 27-nucleotide U-rich domain in transgenic plants will be required to further evaluate the role of PRP-BP in the *PvPRP1* mRNA degradation process.

## METHODS

### Plant Cell Culture and Treatment

Cell suspension cultures of French bean (*Phaseolus vulgaris* cv Immuna) were initiated and maintained in darkness at 23°C as described previously (Dixon and Bendall, 1978). Experiments were conducted with 8- to 10-day-old liquid cell cultures. Elicitor from *Colletotrichum lindemuthianum* was used at a final concentration of 60 μg of glucose equivalents per mL. Cells were harvested by filtration through two layers of Miracloth (Calbiochem), frozen in liquid nitrogen, and stored at −70°C.

### Preparation of Total Cellular and Nuclear Extracts

Cells (~1 gram) were ground in liquid nitrogen and extracted with buffer A (20 mM Hepes, pH 7.5, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/mL of aprotinin, 2 μg/mL of leupeptin, 5% glycerol [v/v]) (2 mL of extraction buffer per g of cells) at 4°C. The insoluble material was removed by centrifugation for 20 min at 15,000 rpm in a microcentrifuge or for 4 hr at 40,000 rpm in an ultracentrifuge (TL-100; Beckman) at 4°C. The supernatant was dialyzed in dialysis tubing (Spectra/Por 7; Spectrum Medical Industries, Inc., Los Angeles, CA, molecular weight cutoff of 10,000) against three changes of buffer B (10 mM Hepes, pH 7.5, 0.5 mM PMSF, 5% glycerol [v/v]) overnight. For small volumes of samples from cells treated with elicitor for different times, the cellular extracts were dialyzed in a microdialyzer (System 500; Pierce, Rockford, IL) with dialysis membrane (molecular weight cutoff of 10,000) for 6 hr with three changes.

Nuclear extracts were prepared as described by Armstrong et al. (1992) with some modifications. Bean nuclei were isolated as described previously (Zhang et al., 1993). The nuclear pellet was resuspended in high-salt buffer (buffer A plus 500 mM NaCl) and incubated on ice for 30 min with slow stirring. After centrifugation at 40,000 rpm for 30

min in an ultracentrifuge (TL-100; Beckman), the supernatant was dialyzed against buffer B in the microdialyzer as given above.

The cellular extracts could be stored for more then 1 year at −70°C without significant loss of RNA binding activity. Protein concentration was determined by using a protein assay kit (Bio-Rad) with BSA as the standard.

### Plasmid Constructs

Plasmids that served as templates for in vitro synthesis of sense strand RNA transcripts were prepared as follows. Plasmids pPRP1-590, pPRP591-1111, and pPRP855-1111 were obtained by subcloning the HincII-HincII, HincII-EcoRI, and HindIII-EcoRI fragments of the bean proline-rich protein gene, *PvPRP1*, cDNA into corresponding restriction sites of pGEM-3Zf(+) plasmid (Promega). The numbers in the name of plasmids and RNA transcripts define the starting and ending nucleotides corresponding to their positions in the cDNA sequence of *PvPRP1* (Sheng et al., 1991). Plasmids pPRP901-1111, pPRP920-1111, pPRP940-1111, pPRP967-1111, pPRP980-1111, pPRP1005-1111, and pPRP1031-1111 were obtained by a 5′ exonuclease III–nested deletion of pPRP591-1111 (Erase-a-Base; Promega). Exonuclease III digestion was performed at 15°C, and samples were taken at 30-sec intervals after 2 min. Polymerase chain reaction with T7 and SP6 primers followed by electrophoresis on 6% nondenaturing polyacryamide gel was used to select clones with the desired inserts. The sequence of each clone was confirmed using double-stranded DNA sequencing (Sequenase 2.0; U.S. Biochemical Corp.). Plasmid pSP65-CHI was constructed by inserting the chalcone isomerase (CHI) cDNA in the EcoRI site of pSP65 (Mehdy and Lamb, 1987).

### Preparation of RNA Transcripts

In vitro transcriptions were performed using the Maxiscript kit according to the manufacturer's instructions (Ambion, Austin, TX). Labeled RNA transcripts were produced using SP6 RNA polymerase in reactions containing $\alpha$-$^{32}$P-UTP or $\alpha$-$^{32}$P-GTP (800 Ci/mmol; Amersham). Electrophoretic analysis on a 5% sequencing gel showed that greater than 95% of the transcripts were full length. The specific activities of $^{32}$P-labeled RNAs were $\sim 10^8$ cpm/μg. For the saturation titration experiment, $^{32}$P-PRP855-1111 of lower specific activity ($10^7$ cpm/μg) was synthesized by including 10-fold more cold UTP in the in vitro transcription reaction. Unlabeled transcripts were synthesized using similar protocols.

The RNA probes were prepared from EcoRI-linearized plasmids described above as follows: PRP1-590, a 652-nucleotide sense transcript (62 nucleotides were from the plasmid polylinker) containing 27 nucleotides of the 5′ untranslated region and 563 nucleotides of the N-terminal coding region of *PvPRP1*; PRP591-1111, a 553-nucleotide sense transcript (32 nucleotides were from the plasmid polylinker) containing 230 nucleotides of the C-terminal coding region and 191 nucleotides of the 3′ untranslated region, including the poly(A) of *PvPRP1*; PRP855-1111, a 271-nucleotide sense transcript (14 nucleotides were from the plasmid polylinker) containing 66 nucleotides of the C-terminal coding region and 191 nucleotides of the 3′ untranslated region, including the poly(A) of *PvPRP1*. PRP901-1111, PRP920-1111, PRP940-1111, PRP967-1111, PRP980-1111, PRP1005-1111, and PRP1031-1111 are sense transcripts of 219 (8 nucleotides were from the polylinker), 207 (15 nucleotides were from the polylinker), 188 (16 nucleotides were from the polylinker), 159 (14 nucleotides were from the polylinker), 148 (16 nucleotides were from the polylinker), 121 (14 nucleotides were from the polylinker), and 96 (15 nucleotides were from the polylinker) nucleotides from EcoRI-linearized pPRP901-1111, pPRP920-1111, pPRP940-1111, pPRP967-1111, pPRP980-1111, pPRP1005-1111, and pPRP1031-1111, respectively. Sense *CHI* RNA was synthesized from BamHI-linearized pSP65-CHI.

### RNA–Protein UV Cross-Linking and RNA Band Shift Assay

Unless otherwise specified in the text, the RNA–protein UV cross-linking assay was performed as follows. Cellular extracts from unelicited cells (2 μg of protein) were incubated with 20 fmol of $^{32}$P-labeled RNA in 10 mM Hepes, pH 7.5, 0.5 mM PMSF, and 5% glycerol in a final volume of 10 μL at room temperature for 30 min. After UV cross-linking for 5 min in a UV Stratalinker 1800 (Stratagene), the mixture was digested with RNase A (final concentration: 1 μg/μL) at 37°C for 30 min. The proteins were then separated by electrophoresis on 10% SDS–polyacrylamide gels under reducing conditions. The dye front together with $^{32}$P-labeled oligonucleotides from RNase digestion were run out of the gel to avoid contamination during handling. Gels were fixed, dried, and then exposed to x-ray film at −70°C. The relative RNA binding activities of PRP-BP were quantitated by scanning densitometry of the autoradiograms using an absorbance/fluorescence detector (model UA-5; ISCO, Lincoln, NE).

For the RNA band shift assay, 4 units per μL of RNasin (Promega) was included, and the mixtures were incubated at room temperature for 10 min. Heparin was then added to a final concentration of 4 μg/μL to reduce nonspecific binding. After the addition of 1 μL of tracking dye (0.5% xylene cyanol [w/v] per 0.5% bromphenol blue [w/v] per 50% glycerol [v/v]), the samples were separated by electrophoresis on a 4% nondenaturing polyacryamide gel (acryamide-bis, 60:1) at 15 V/cm for ~2.5 hr. Gels were dried on paper (3MM; Whatman) and exposed at −70°C for 2 to 5 hr. Alternatively, the radiolabeled band was cut out of the nondenaturing gel, pulverized, and treated with RNase A (final concentration 2 μg/μL) at 37°C for 30 min. After the addition of SDS sample buffer and boiling for 3 min, the sample was run on a 10% SDS–polyacryamide gel.

### Computer Homology Search and RNA Secondary Structure Modeling

Homologous sequences to the PRP-BP binding site were obtained by comparing the 27-nucleotide U-rich domain with the entire GenBank and EMBL data bases with the IntelliGenetics Suite (IntelliGenetics, Inc., Mountain View, CA). Computer modeling of the secondary structure of *PvPRP1* mRNA 3′ untranslated region was performed using a DNA sequence analysis system (HIBIO DNasis; Hitachi America, Ltd., San Bruno, CA).

### RNA Isolation and Gel Blot Analysis

Total RNA was isolated from ~1 g of cells for each sample by the phenol–SDS method. The RNA gel blot analysis and the quantitation of *PvPRP1* mRNA abundance were performed as described previously (Zhang et al., 1993).

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