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Interaction between Zucchini Yellow Mosaic Potyvirus RNA-Dependent RNA Polymerase and Host Poly-(A) Binding Protein

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Viral replication depends on compatible interactions between a virus and its host. For RNA viruses, the viral replicases (RNA-dependent RNA polymerases; RdRps) often associate with components of the host translational apparatus. To date, host factors interacting with potyvirus replicases have not been identified. The Potyviridae, which form the largest and most economically important plant virus family, have numerous similarities with the animal virus family, the Picornaviridae. Potyviruses have a single-stranded, plus sense genome; replication initiates at the viral-encoded, 3′ poly-(A) terminus. The yeast two-hybrid system was used to identify host plant proteins associating with the RdRp of zucchini yellow mosaic potyvirus (ZYMV). Several cDNA clones representing a single copy of a poly-(A) binding protein (PABP) gene were isolated from a cucumber (*Cucumis sativus* L.) leaf cDNA library. Deletion analysis indicated that the C-terminus of the PABP is necessary and sufficient for interaction with the RdRp. Full-length cucumber *PABP* cDNA was obtained using 5′ RACE; *in vitro* and *Escherichia coli*-expressed PABP bound to poly-(A)–Sepharose and ZYMY RdRp with or without the presence of poly-(A). This is the first report of an interaction between a viral replicase and PABP and may implicate a role for host PABP in the potyviral infection process.

INTRODUCTION

Successful systemic infection by a pathogen depends on compatible interactions between the pathogen and its host. This is particularly true for viral pathogens which have extremely small genomes and limited protein coding capacity. Several host proteins are involved in replication of RNA viruses, either as components of the viral replication complex or by binding directly to the viral genome (reviewed in Lai, 1998; Strauss and Strauss, 1999). For RNA viruses, a majority of the factors found in association with the viral replicase, the RNA-dependent RNA polymerase (RdRp), are subverted from the host RNA-processing and translational machinery. For example, elongation factors EF-1α and different subunits of eIF3 are associated with the replicase complexes of an array of bacterial, plant, and mammalian RNA viruses such as Qβ phage, brome mosaic virus, tobacco mosaic virus, vesicular stomatis virus, measles virus, and poliovirus (Lai, 1998; Strauss and Strauss, 1999). The exact roles of the translational machinery proteins in viral replicase complexes are not fully understood; virus replication and translation might be coupled or the host proteins may play different roles in virus replication than they do in host translation.

The Potyviridae, which resemble the animal virus family, the Picornaviridae, form the largest and one of the economically most important families of plant viruses; approximately 200 members cause serious diseases in a wide range of crop plants (Shukla *et al.,* 1994). The members of this family have a plus sense, single-stranded RNA genome of approximately 10 Kb, a VPg (viral protein genome linked) covalently linked to the 5′ end, and a poly-(A) tail at the 3′ end. The RNA encodes a single polyprotein, which is subsequently cleaved into nine proteins by viral-encoded proteases (Dougherty and Semler, 1993); function of the various potyviral proteins has been an active area of investigation. Among the potyviral proteins, NIb (nuclear inclusion b; originally named for its tendency to accumulate in the nucleus) functions as an RdRp (Hong and Hunt, 1996; Li and Carrington, 1995). Potyviral RdRps of tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV) interact with other potyviral proteins including P1, P3, and the NIa (nuclear inclusion a) protein, which is composed of the VPg at the amino terminus and the main viral proteinase at the carboxy terminus (Hong *et al.,* 1995; Li *et al.,* 1997; Fellers *et al.,* 1998; Merits *et al.,* 1999; Daros *et al.,* 1999).

Replication, which proceeds by copying of the plus strand to the complementary minus strand intermediate, followed by plus strand synthesis, occurs in membrane-associated, cytoplasmic fraction (Martin and Garcia, 1991; Schaad *et al.,* 1997). Cytoplasmic localization of the replication machinery is thought to be achieved by direction of a subset of the potyviral NIa protein proteolytic precursor (including the amino-terminal adjacent 6-kDa hydrophobic protein) toward the endoplasmic reticulum

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rather than the nucleus (Schaad *et al.,* 1997). The RdRp is, in turn, postulated to be recruited to the membrane fraction via interaction with the 6-kDa VPg–proteinase (NIa) complex.

Replication of the minus strand is initiated at the 3′ poly-(A) tail of the plus sense, genomic RNA. The presence of a poly-(A) tail is essential for replication of picornaviruses such as poliovirus, encephalomyocarditis virus, and rhinovirus (Cui *et al.,* 1993; Todd and Semler, 1996; Todd *et al.,* 1997; Agol *et al.,* 1999) and recent evidence suggests that it is also necessary for potyvirus replication (Tacahashi and Uyeda, 1999). Initiation of poliovirus replication requires uridylylation of the VPg which serves as a primer for minus strand synthesis (Agol *et al.,* 1999). The uridylylation is performed by the poliovirus RdRp ($3D^{pol}$ protein) and requires the presence of poly-(A) template (Paul *et al.,* 1998). It is not known whether viral poly-(A) tails exist as free poly-(A)s within the host or whether like eukaryotic mRNAs, they are found in association with host poly-(A) binding protein (PABP). Given the reported high abundance of PABPs, studies have indicated an approximately 75- to 95%-fold excess of free PABP over binding sites on cytoplasmic poly-(A) (Drawbridge *et al.,* 1990; Gorlach *et al.,* 1994), association of PABP with viral RNAs seems likely. PABP-dependent translation of viral genomes, analgous to the PABP-dependent translation of eukaryotic mRNAs, has been proposed (Gallie, 1998).

Poliovirus and other picornavirus RdRps do not bind to poly-(A) directly (Cui *et al.,* 1993; Paul *et al.,* 1994). The poliovirus $3D^{pol}$ is recruited to a complex secondary structure in the 3′ nontranslated region (NTR) upstream of the poly-(A) tail via interaction with the RNA-binding 3AB proteins (analagous to the potyviral 6-kDa VPg; 3A is involved membrane association; 3B is the VPg) (reviews: Xiang *et al.,* 1997; Agol *et al.,* 1999). These interactions are thought to provide template specificity. Potyviruses appear to have important secondary structure near the 3′ end of the genome which may include sequences within both the 3′ NTR and the adjacent coat protein coding region (Mahajan *et al.,* 1996; Haldeman-Cahill *et al.,* 1998). Although mutations in these regions interfere with replication, specific binding of viral proteins to these sequences has not been demonstrated for potyviruses.

Specific host factors interacting with potyviral replicases have not been identified yet. In this study we examined interactions between zucchini yellow mosaic potyvirus (ZYMV) and its cucumber (*Cucumis sativus* L.) host by identifying cucumber proteins that interact with ZYMV RdRp. Yeast two-hybrid analysis demonstrated reproducible and specific interactions between ZYMV RdRp and cucumber poly-(A) binding protein.

RESULTS

Screening of the cucumber yeast two-hybrid library with ZYMV RdRp

The yeast two-hybrid cDNA library was constructed from mRNA from leaf tissue of ZYMV- susceptible cucumber (cv. Straight 8). The library contained 5×10^6 pfu; greater than 90% of the excised plasmids encoded cDNA inserts ranging in size from 0.5 to 2.5 kb. The quality of the library was assessed by successful PCR amplification of the low-copy cucumber gene, *CS-ACS1* (ACC synthase; Trebitsh *et al.,* 1997). Following two separate screenings of the library using ZYMV RdRp expressed in the GAL4 binding domain vector as bait, 11 cucumber clones were found to have specific, reproducible interaction with the RdRp. Each clone induced the two reporter genes allowing for growth in the absence of histidine and expression of β-galactosidase activity. In each case, plasmids encoding putative interactors were isolated and retransformed back to yeast either alone or in combination with ZYMV RdRp, human p53, Lamin C, or binding domain vector alone. Each interacting clone failed to induce the reporter genes alone or in any combination except with RdRp.

Of the 11 verified clones showing interaction with RdRp, 3 encoded distinct, unknown proteins, 1 had significant sequence homology to DNA J and 1 to protein phosphatase, and 6 encoded a protein having high homology (ca. 70% nucleotide identity) to poly-(A) binding protein of *Arabidopsis thaliana* and wheat. The PABP-homologous cDNAs ranged in size from 0.5 to 1.5 kb, the longer the cDNA, the stronger the observed interaction with RdRp. The 6 RdRp-interacting cDNAs were reverse transcribed from at least three different mRNAs: cDNA NI 8, 60, and NI 351 have the same polyadenylation sites 149 nucleotides downstream of the stop codon; NI 7 has a poly-(A) addition site 201 nucleotides from the stop codon; and NI 439 and 447 have poly-(A) addition sites 250 nucleotides from the stop codon. Although PABPs are encoded by a multigene family in *Arabidopsis* and multiple isoforms have been observed in wheat (Belostotsky and Meagher, 1993; Hilson *et al.,* 1993; Le *et al.,* 1997), the overlapping sequence of all 6 cDNAs showed complete sequence identity, indicating that all of the clones were transcribed from the same cucumber *PAPB* gene.

The putative *PABP* clones were tested for interaction with two other ZYMV proteins, coat protein (CP) and helper component protease (HC-Pro) (Table 1). Although both the CP and the HC-Pro clones were capable of self-interaction, as would be predicted based on the ability of CP to polymerize to form capsids, HC-Pro to dimerize, and previous yeast two-hybrid studies (Hong *et al.,* 1995; Thornbury *et al.,* 1985; Urcuqui-Inchima *et al.,* 1999; Wang and Pirone, 1999), neither the ZYMV CP nor the HC-Pro interacted with

TABLE 1

Interactions among ZYMV Coat Protein (CP), Helper Component-Proteinase (HC-Pro), RNA-Dependent RNA Polymerase (RdRp), and Cucumber Poly-(A) Binding Protein (PABP) in the Yeast Two-Hybrid System

	BD-CP	BD-HC-Pro	BD-PABP	BD-RdRp
AD-CP	+++[a]	−	ND	−
AD-HC-Pro	−	++	ND	−
AD-PABP	−	−	−	+++
AD-RdRp	−	−	−	−

Note. ND, not determined.

[a] The strength of the interactions was based on the X-Gal filter assay. +++, colonies turned dark blue within 3 h; ++, colonies turned dark blue within 6 h to overnight; +, colonies turned light blue overnight; −, did not turn blue. All controls, including the clones by themselves or in combination with human p53 or Lamin C, remained white.

cucumber PABP. These results further suggest a specific interaction between cucumber PABP and the potyviral RdRp. The interaction between PABP and ZYMV RdRp did not occur when tested with RdRp fused to the GAL4 activation domain and PABP to the DNA binding domain. One-way interactions have been observed in numerous other two-hybrid combinations and may be due to conformational changes arising from the fusion protein (Fields and Sternglanz, 1994). PABP also did not interact with itself; this is consistent with previous studies indicating that multimerization of PABP requires the presence of poly-(A) (Kuhn and Pieler, 1996). The ZYMV RdRp did not interact with itself, CP, or HC-Pro.

Characterization of the cucumber PABP gene and interacting domains of the PABP and RdRp proteins

To further examine the interaction between the ZYMV RdRp and the putative cucumber PABP, the full-length cDNA was obtained by 5′ RACE (5′ rapid amplification of cDNA ends). In total, a 2597-bp cDNA sequence was obtained for the cucumber *PABP* gene (*CS-PAPB1;* GenBank Accession No. AF240679). In the 397-bp 5′ NTR of the cDNA, there are nine oligo-(A) clusters, ranging from 4 A to 9 A. These A-rich sequences were also found in PABPs from *Arabidopsis,* wheat, and other organisms (Belostotsky and Meagher, 1993; Hilson *et al.,* 1993; Le *et al.,* 1997). The 3′ NTR is 250 bp and three polyadenylation signal sequences are located at positions 2099, 2151, and 2201 bp.

The coding sequence is 1950 bp and predicts a 649-amino-acid protein with a molecular weight of 70.5 kDa, which is similar to other plant and animal PABPs (Belostostsky and Meagher, 1993; Le *et al.,* 1997). This gene (*CS-PABP1*) is most similar to the shoot-expressed *Arabidopsis PABP2* gene (Belostotsky and Meagher, 1993; Hilson *et al.,* 1993). A comparison of percentage amino acid identity shows greater homology between the putative cucumber *CS-PABP1* gene and *Arabidopsis PABP2* (69.7%) than between *Arabidopsis PABP2* and *Arabidopsis PABP5* (49.4%). Consistent with apparent transcription from a single gene, Southern blot analysis of cucumber genomic DNA indicated that the cucumber *PABP* clone hybridized with a single distinct genomic band with most restriction enzymes (Fig. 1); additional bands were not observed in low-stringency washes (data not shown). Although there is no *Eco*RI site within the coding region of the *CS-PABP1* gene, the two bands observed in the *Eco*RI digest are likely due an *Eco*RI site within an intron. Northern blot analysis of leaves, roots, and immature and mature flowers from susceptible "Straight 8" plants and leaves from the resistant lines "TMG-1" and "Dina-1" showed expression of *CS-PABP1* in all tissues tested; in each case hybridization was with a single band (data not shown). Higher expression was observed in leaves and immature flowers; there was no difference in expression between the susceptible and resistant genotypes.

The amino terminal two-thirds of the predicted PABP contains four RRMs (RNA recognition motifs), which are found in PABPs from all sources, including yeast and animals (Le *et al.,* 1997) (Fig. 2). Each RRM in turn has two conserved sequences, RNP-1 and RNP-2, which come in direct contact with RNA (Kuhn and Pieler, 1996). RRM-1 is responsible for the interaction with eIF4G in yeast (Kessler and Sachs, 1998). The two smaller cucumber cDNAs (NI439 and NI359) lacked these RRMs, suggesting that the RRMs are not involved in binding with ZYMV RdRp.

The carboxy-terminal third of the PABP protein is not as highly conserved as the amino terminus, and functions of the C-terminus are less well defined. At the C-terminus (amino acids 553–624), there is a 71-amino-acid region that is conserved among *Arabidopsis,* wheat, and cucumber. This CTC (C-terminal conserved) domain was also found within other characterized PABPs (e.g., yeast, vertebrate) and has been implicated in homodimerization and efficient poly-(A) binding (Kuhn and Pieler, 1996). The smallest PABP cDNA obtained from the two-hybrid screen (NI 351) encodes the last 130 amino acids and interacts with the ZYMV RdRp. This suggests that the CTC domain might be involved in the interaction with RdRp. Consistent with these results, deletion of the C-terminal 50 amino acids of PABP abolished the interaction in yeast, indicating that the C-terminus of PABP is essential for the binding with RdRp (Fig. 3A); we cannot rule out, however, the possibility that the failure to detect interaction with the C-terminal deleted protein is due to lack of stability of the deleted protein.

Deletion analysis was used to examine regions of the RdRp responsible for interaction with PABP (Fig. 3B). Deletions from both the amino- and the carboxy-termini

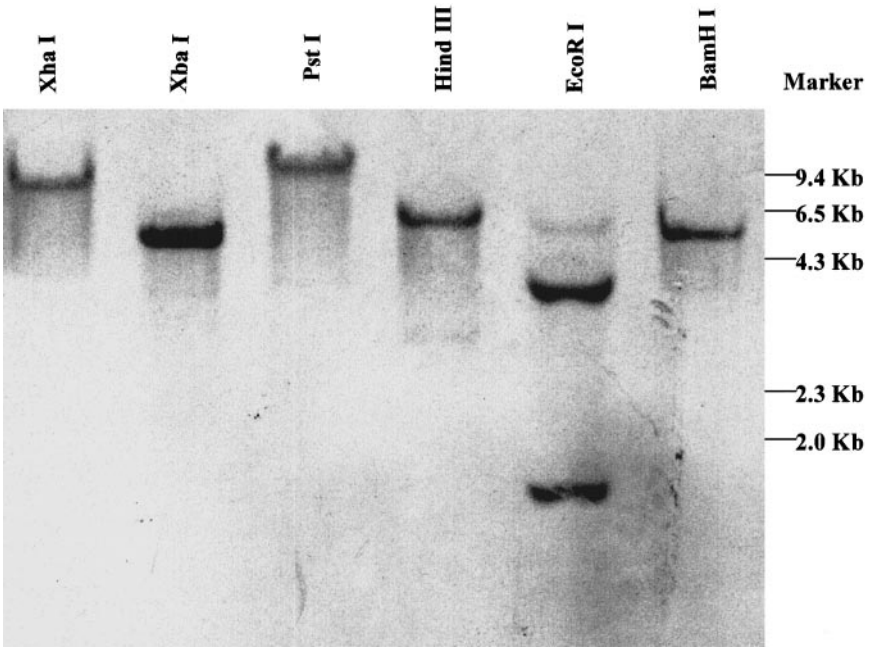

FIG. 1. PABP is a single or low-copy-number gene. Cucumber DNA (ca. 10 μg) was digested with the indicated restriction endonucleases, transferred to a nylon membrane, and hybridized with Dig-labeled PABP coding sequence. PABP cDNA sequence does not have internal recognition sites for these restriction enzymes.

of the RdRp abolished the interaction in yeast. Although we cannot eliminate the possibility that certain deletion products were unstable, it may be that a large portion of the RdRp is necessary for the interaction with PABP.

In vitro verification of the RdRp–PABP interaction

The *in vitro* expression product of the full-length PABP cDNA migrated in SDS–PAGE gels as predicted for an ca.

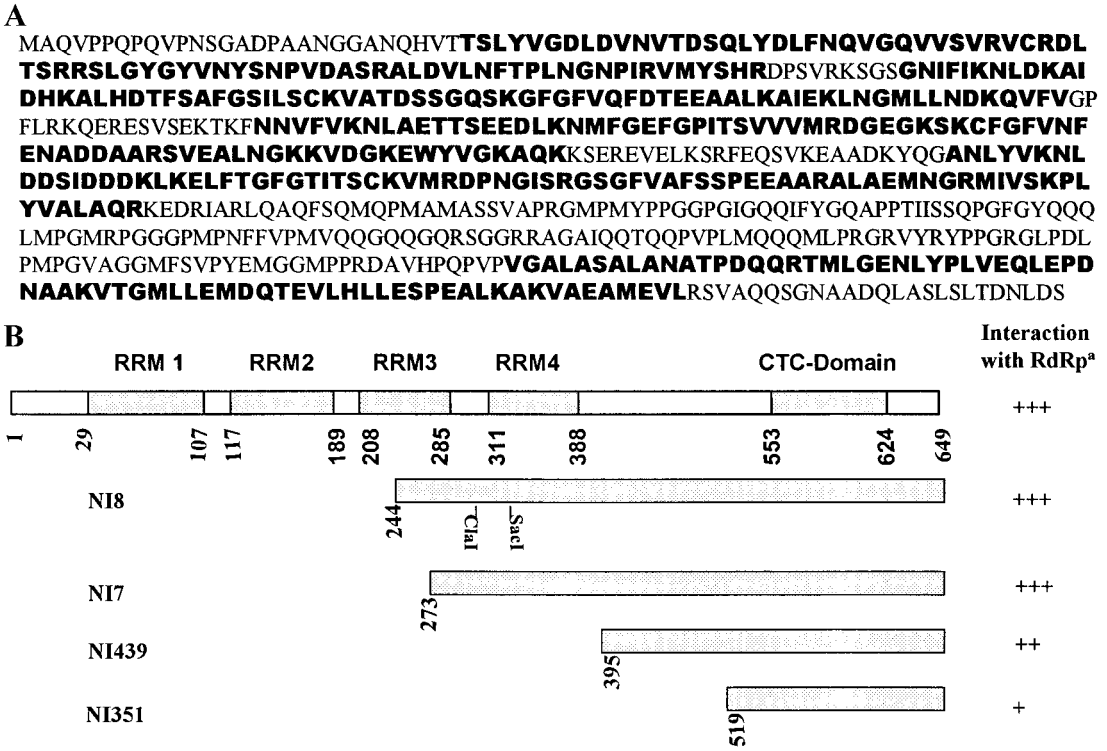

FIG. 2. Predicted amino acid sequence of cucumber poly-(A) binding protein. (A) Amino acid sequence predicted from full-length cDNA. The first four regions in bold are RNA recognition motifs (RRMs); the last region in bold is the C-terminal conserved (CTC) domain. (B) Schematic presentation of full-length PABP protein and products of four two-hybrid interacting PABP cDNAs (numbering of the clones is as in Table 1).

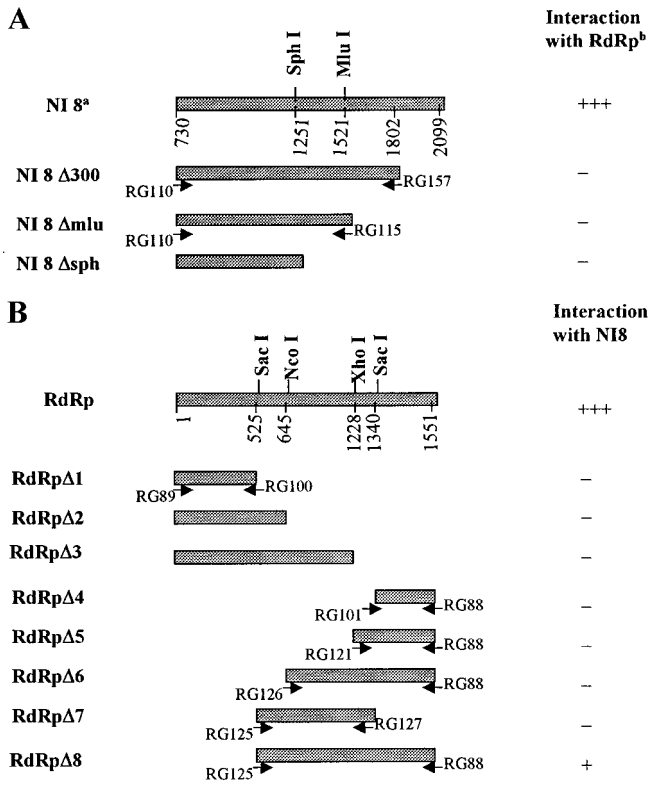

FIG. 3. Deletion analysis to determine regions in PABP and RdRp responsible for interaction. (A) Different sizes of cDNA NI8 were amplified by PCR or generated by restriction enzyme digestion. All of the deletions were tested against full-length RdRp in the yeast two-hybrid system. (B) Different sizes of RdRp were amplified by PCR or generated by restriction enzyme digestion. All of the deletions were tested against NI 8 in the yeast two-hybrid system. Numbering in each case corresponds to the full-length cDNA sequence.

70-kDa protein. The labeled cucumber PABP was able to bind to poly-(A)–Sepharose *in vitro* (Fig. 4A, lane 7), confirming that the cloned cDNA encodes a true PABP. Like other picornaviral RdRps (Cui *et al.,* 1993; Paul *et al.,* 1994), ZYMV RdRp alone did not bind directly to poly-(A) (Fig. 4A, lane 6). To demonstrate that the cucumber PABP can bind to ZYMV RdRp *in vitro,* full-length PABP was expressed in *Escherichia coli* as either a His–PABP fusion protein or a GST–PABP fusion protein and was immobilized on poly-(A)–Sepharose or glutathione–Sepharose. Approximately 10–20% of the input RdRp bound to these fusion proteins (Figs. 4B and 4C), but none bound to poly-(A)–Sepharose (Fig. 4B) or GST–Sepharose alone (Fig. 4C). Luciferase did not bind to the PABP bound to poly-(A)– or glutathione–Sepharose. These results suggested that the RdRp and PABP bind to one another specifically *in vitro.* In analogous experiments where RdRp was expressed in *E. coli* as a GST–RdRp fusion protein and immobilized on glutathione–Sepharose, approximately 10–20% of the *in vitro* labeled PABP was retained (data not shown). Since a comparable percentage of binding was observed whether the RdRp or PABP was immobilized and whether RdRp was expressed *in vitro* or in *E. coli* suggest a modest affinity between the two molecules. Whether this modest affinity reflects affinity *in planta* where other interacting factors may be involved is not known.

Consistent with the ability of the C-terminal portion of the PABP to interact with the RdRp in yeast, the binding *in vitro* did not require the presence of poly-(A) (Fig. 4C). The NI 8 and NI 439 clones also weakly bound RdRp *in vitro,* but the shortest clone, NI 351, did not show detectable binding (data not shown). The difference between these results with the short clones *in vitro* and the yeast two-hybrid assay may reflect differences in sensitivity of the two methods. The shorter clones also showed reduced interactions in the yeast two-hybrid system as assayed by the speed and intensity of blue color development.

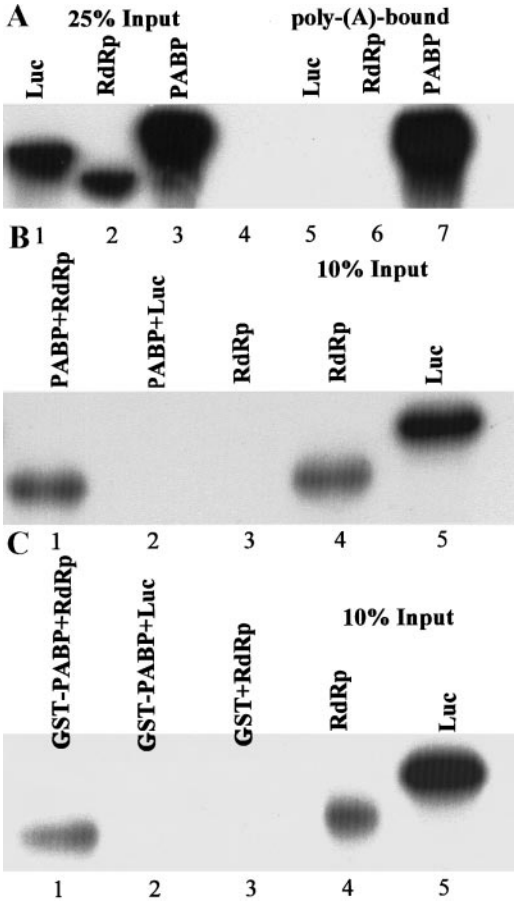

FIG. 4. Cucumber PABP can bind to poly-(A) and RdRp *in vitro.* (A) PABP can bind to poly-(A)–Sepharose *in vitro.* Lanes 1–3: 25% input of *in vitro* labeled luciferase (Luc), RdRp, and PABP. Lanes 5–7: proteins retained on the poly-(A)–Sepharose after six washes. (B) PAPB can bind to RdRp *in vitro* with the presence of poly-(A). Lanes 1 and 2 : His–PABP purified from *E. coli* was immobilized on poly-(A)–Sepharose and incubated with [^{35}S]RdRp (lane 1) or [^{35}S]Luc (lane 2), followed by six washes. Lane 3: RdRp was directly incubated with poly-(A)–Sepharose without His–PABP. Lanes 4 and 5: 10% input of [^{35}S]RdRp and [^{35}S]Luc. (C) PABP can bind to RdRp *in vitro* without the presence of poly-(A). GST–PABP (Lanes 1 and 2) or GST (lane 3) was immobilized on glutathione–Sepharose, and then incubated with [^{35}S]RdRp (lanes 1 and 3) or [^{35}S]Luc (lane 2), followed by six washes. Lanes 4 and 5 are 10% input of [^{35}S]RdRp and [^{35}S]Luc.

DISCUSSION

The above-described results indicate that the ZYMV potyviral RdRp is capable of specifically interacting with host PABP in the yeast two-hybrid system and in *in vitro* binding assays. To our knowledge, PABP has not been implicated as a component of any viral replicase complex nor has it been identified as playing a role in virus replication (Lai, 1998; Strauss and Strauss, 1999). The repeated and high-frequency isolation of PABP (6 of 11 clones) indicated that the interaction is reproducible. The interaction of the RdRp with PABP is particularly intriguing since potyviruses are polyadenylated at the 3′ end of the positive strand which is the site of initiation of minus strand synthesis.

Interaction was abolished by either amino- or carboxy-terminal deletions of the RdRp. This may reflect a complex secondary or tertiary structure of the RdRp. RdRps, like other types of DNA and RNA polymerases, consist of finger–palm–thumb domains resulting in intramolecular interactions between the amino- and carboxy-terminal portions of the molecule (O'Reilly and Kao, 1998; Lesburg *et al.,* 1999); such interactions also may be important for association with the PABP. Similar problems in assigning functions to specific RdRp domains were observed with the tobacco etch potyvirus RdRp. Loss of interaction between the TEV RdRp and the NIa protein were observed with both amino- and carboxy-terminal deletions of the TEV RdRp, and nuclear localization capacity was eliminated by deletions from either terminus and by small insertions at several positions in the protein (Li and Carrington, 1993; Li *et al.,* 1997).

In this study ZYMV RdRp did not interact with itself. Previous two-hybrid analysis with TEV and tobacco vein mottling virus potyviral RdRps have provided different results (Hong *et al.,* 1995; Li *et al.,* 1997). Self-interaction was observed for TVMV RdRp but not TEV RdRp. The reasons for these differences among the potyviral RdRps are not known; they may reflect real differences or may be an artifact due to an interfering effect of the GAL4 domains in one or both fusion proteins. We also did not observe the interaction between RdRp and CP as was reported for TVMV (Hong *et al.,* 1995). In that study, interaction was observed only when the RdRp was fused to the binding domain. Despite known interactions between HC-Pro and CP *in vivo* (e.g., for aphid transmission) and demonstrations of interaction using *in vitro* binding assays (Blanc *et al.,* 1997; Peng *et al.,* 1998), we did not observe an interaction between HC-Pro and CP in our yeast two-hybrid assay. The failure to bind in the yeast two-hybrid assay may reflect burying of the amino-terminus of the CP in the fusion protein construct. The CP amino-terminus is normally externally located on the virion and is critical for the CP/HC-Pro interaction.

PABP belongs to a large family of RNA binding proteins that contain highly conserved RNA recognition motifs. Although PABPs generally exist as multigene families, complete sequence identity in overlapping regions of the interacting cucumber clones indicated that a single cucumber PABP was interacting with the ZYMV RdRp. Southern blot analysis showed only a single hybridizing band, which may reflect the high sequence variability among different members of the PABP family (Belsotosky and Meagher, 1993; Hilson *et al.,* 1993). These results suggest specificity in the RdRp–PABP interaction, although the possibility of developmental or tissue-specific expression of the different PABPs such that only a single gene family member was expressed in leaves at the time they were harvested for cDNA synthesis cannot be eliminated; within *Arabidopsis* different members of the PABP gene family are expressed in different tissues (Belostosky and Meagher, 1993; Hilson *et al.,* 1993). Northern analysis indicated that the *CS-PABP1* gene was expressed in all tissues tested including leaves, roots, and immature and mature flowers.

PABP has been the subject of a good deal of recent research indicating that it plays a critical role in eukaryotic translation (Jacobson, 1996; Gallie, 1998; Sachs *et al.,* 1997). PABP is an essential component of eukaryotic cells; deletion of PABP in yeast can cause lethality (Sachs *et al.,* 1987) and reduction in PABP levels, either by cleavage or by sequestration by viral proteins, can result in shutdown of host translation (Chen *et al.,* 1999; Joachims *et al.,* 1999; Piron *et al.,* 1998). Recent evidence has shown that PABP also facilitates initiation and maintenance of efficient translation by promoting interactions between the 5′ and 3′ termini of messenger RNAs (reviews: Gallie, 1998; Sachs *et al.,* 1997). A typical mRNA molecule has a 5′ cap and 3′ poly-(A) tail; the two termini function synergistically to promote translation through protein–protein interactions: at the 5′ end, eIF4E, which is a subunit of eIF4F (containing subunits eIF4E, eIF4G, and eIF4A), binds to the cap structure; at the 3′end, PABP binds to the poly-(A) tail; PABP then binds to eIF4G. These interactions result in a circular mRNA molecule. By bringing the 5′ and 3′ ends close to each other, translation of full-length message is promoted and reinitiation of translation is facilitated. Although not capped, the potyviral 5′ NTR of TEV conferred synergistic enhancement of translation when in combination with a poly-(A) tail (Gallie *et al.,* 1995).

Viral RdRps frequently have been shown to interact with components of the host translational apparatus (Lai, 1998; Strauss and Struass, 1999). The results presented here showing interaction of PABP with the ZYMV RdRp suggest that an additional component of the host translational machinery associates with a viral replicase and raises some intriguing questions. Perhaps, similar to its role in eukaryotic translation, PABP facilitates intramolecular interactions relevant to potyviral replication. Cellular proteins binding viral RNA may serve to bring spatially separate regions, including 3′ and 5′ termini, of viral

RNA template together to form replication complexes. The joining of plus and minus strand leader sequences is a critical step in mouse hepatitis virus RNA synthesis (Lai, 1998). Perhaps the interaction between RdRp and host PABP serves to promote interaction between RdRp and the viral poly-(A) tail, either by helping to recruit RdRp to the poly-(A) tail or by facilitating removal of PABP from the poly-(A) tail and allowing access of the RdRp for initiation of replication. Deletion analyses and partial clones obtained in the yeast two-hybrid screen indicated that interaction with the RdRp occurred via the carboxy-terminus and not the RNA binding motifs in the amino-terminal half of the protein. Studies with *Xenopus* PABP showed that the conserved CTC domain was important for polymerization of PABP leading to enhanced PABP binding (Kuhn and Pieler, 1996). Perhaps association of the RdRp with PABP interferes with the PABP polymerization and facilitates removal from the poly-(A) tail.

Recent studies have shown that viral-induced shutdown of host protein synthesis, which is thought to facilitate viral infection by increasing accessibility of host factors for viral purposes, can be mediated, at least in part, by sequestration or cleavage of PABPs. PABPs were sequestered by NS1 protein during influenza A virus infection (Chen *et al.,* 1999), were removed from interaction with eIF4F during rotavirus infection (Piron *et al.,* 1998), and were cleaved by viral proteases during Picornavirus infection (Joachims *et al.,* 1999). In each case there was an associated reduction in host protein synthesis that could be related to an effect on PABP. Interestingly, the interaction between influenza A NS1 and human PABPII, which takes place in the nucleus and results in hnRNAs with poly-(A) tails that are too short to allow for export, occurs via the carboxy terminus of PAPBII.

Shutdown of host translation is less well studied for plant viruses. In the one system that has been examined, infection by pea seed borne mosaic potyvirus, inhibition of host gene expression and virus-mediated mRNA degradation occurred in a reversible manner during the course of infection (Wang and Maule, 1995; Aranda *et al.,* 1996; Aranda and Maule, 1998). There appears to be a widespread loss of many host mRNAs, indicating degradation. If RdRp serves to remove PABP from the poly-(A) tail, this could result in decreased mRNA stability. Such an observation would not be inconsistent with sequestration of PABP. During potyvirus infection, RdRp is expressed in large quantities and can accumulate in the nucleus as an inclusion body (NIb). Its function in the nucleus is unknown since the viral life cycle is completed within the cytoplasm. It is possible that the NIb/RdRp sequesters PABP and keeps it from binding to hnRNA in the nucleus, inhibiting RNA processing.

The possible involvement of the RdRp–PABP interaction in viral replication by recruitment to the poly-(A) tail or participation in translational inhibition is not necessarily mutually exclusive. RdRp may interact with PABP to facilitate viral replication, while at later stages increasing quantities of RdRp might inhibit host translation. Our future research will examine possible functions of the interaction between potyviral RdRp and cucumber PABP.

MATERIALS AND METHODS

Strains and plasmids

Plasmid pBluescript KS and *E. coli* strain XL1-Blue (Stratagene) were used for general DNA manipulation. Yeast (*Saccharomyces cerevisiae*) strain YRG2 and the GAL-4-based two-hybrid plasmids pBD-GAL4, p53, pLAMINC, pSV40, and pAD-GAL4 were purchased from Stratagene. pBD-GAL4 and pAD-GAL4 (binding domain and activation domain, respectively) were used to express coat protein, helper component-proteinase, RNA-dependent RNA polymerase, and poly-(A) binding protein in yeast as GAL4 binding domain or activation domain fusion proteins. pGEM-T EASY vector (Promega) was used for cloning 5′ RACE (rapid amplification of cDNA ends) products. pGEX-5x-1 (Phamacia) was used to produce fusion proteins GST–PABP or GST–RdRp in *E. coli* strain Xa 90, and pET-28a-1 (Novagen) was used to produce His–PABP in *E. coli* strain BL21(DE3).

The ZYMV RdRp gene was amplified by PCR with Vent DNA polymerase (New England Biolabs) using ZYMV cDNA [Connecticut (CT) isolate; Grumet and Fang, 1990] as template. The amplified product was inserted as an *Eco*RI–*Sal*I fragment into pBD-GAL4, pAD-GAL4, and pGEX-5x-1 vectors in frame to form pBDRdRp, pADRdRp, and pGEXRdRp, respectively, and sequenced. The primers were designed as follows (restriction sites are italicized): 5′ end primer, 5′ CCG*GAATTC*AGCAAGCGAGAAAGATG 3′, and 3′ end primer 5′ AGA*GTCGAC*TTGGAGCATCACAGTGT 3′.

The helper component-protease gene was amplified as above using the ZYMV NAA isolate cDNA (Gal-On *et al.,* 1992) as template, inserted as an *Eco*RI–*Sal*I fragment into pBD-GAL4 and pAD-GAL4 vectors in frame to form pBDHC and pADHC, and sequenced. The primers were 5′ end primer 5′ CCG*GAATTC*AGCGAAGTTGACCAC 3′ and 3′ end primer 5′ AGA*GTCGAC*ACCAACTCTGTAATG 3′.

The ZYMV-CT isolate coat protein gene in the pTL37-CP construct (Fang and Grumet, 1993), was digested with *Nco*I, filled in with Klenow fragment (Gibco BRL), and then digested with *Pst*I. The blunt-*Pst*1 CP fragment was then ligated to pUC119, which had been digested with *Eco*RI, filled in with Klenow, and then digested with *Pst*I. The *Eco*RI–*Pst*I CP fragment was then inserted into pBD-GAL4 and pAD-GAL4 vectors to form pBDCP and pADCP and sequenced.

The yeast two-hybrid full-length cucumber PABP clones were generated as follows. PCR (Vent polymerase) was used to amplify the 5′ end of the full-length

PABP clone (obtained by 5′ RACE as described below) and to add an *Eco*RI site to the start codon. The 5′ end primer was 5′ TA*GAATTC*ATGGCTCAGGTTCCACC 3′. The 3′ end primer was the GSP2 primer used for 5′ RACE (see below). The PCR product was digested with *Eco*RI and *Cla*I, ligated to the *Eco*RI–*Cla*I digested plasmid pADNI8, which had the partial PABP clone, to form pAD-PABP. The full-length PABP was also inserted as an *Eco*RI–*Sal*I fragment into pBD-GAL4, pGEX-5x-1, and pET-28a-1 to form pBDPABP, pGEXPABP, and pETPABP.

Construction of the cucumber leaf two-hybrid cDNA library in λ HybriZAP-2.1 vector

Total RNA was isolated from young leaves of cucumber cultivar Straight 8 as described by Chomczynski and Sacchi (1987); mRNA was further purified using the Promega PolyATract mRNA isolation system II. Approximately 5 μg of mRNA was used for cDNA synthesis following the protocol of the HybriZAP 2.1 two-hybrid cDNA synthesis kit, and cDNAs (ranging from 0.5 to 2.5 kb) were inserted into the λ HybriZAP-2.1 vector as *Eco*RI–*Xho*I fragments. The primary library contained $\sim 5 \times 10^6$ PFU (plaque forming units). The primary λ cDNA library was amplified once and then converted to a plasmid (pAD-GAL4) library by *in vivo* mass excision according to the Stratagene protocol.

Screening the library with RdRp and testing for interactions between viral proteins

Yeast transformation, growth media, and X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) filter assays were performed following the supplier's procedures (Stratagene). Yeast YRG2 cells were first transformed with pBDRdRp and then transformed with CsCl-purified cDNA library plasmid DNA. The transformants were plated onto SD (synthetic dropout) medium without leucine, tryptophane, or histidine (SD-L-T-H). Colonies that grew on selection medium were restreaked onto SD-L-T-H, transferred to nitrocellulose membrane (Schleicher & Schuell), and assayed for expression of β-galactosidase activity (Lac Z) by X-Gal filter assay. Colonies that survived in medium without histidine and turned blue in the X-Gal assay were considered putative positives. Plasmids were recovered from putative positive yeast colonies using Zymoprep yeast plasmid miniprep (Zymo Research, CA), transformed to *E. coli,* and amplified. The recovered plasmids were then transformed back to YRG2 yeast either alone or in combination with pBD-GAL4, p53, pLamin C, or pBD-RdRp. The transformants were plated onto SD-H or SD-L-T-H and assayed for Lac Z activity. Those that can turn on two reporter genes only in the presence of pBD-RdRp, but cannot turn on reporter genes, either alone or in combination with pBD-GAL4, p53, or pLamin C, were considered positives.

5′ RACE

A 5′ primer, GSP1, 5′-TCATTCTTCCATTCATCTCAG-CAA-3′), and 3′ primer, GSP2 (5′-TTGTCATCATCGATGC-TATCAT), complementing the 5′ end of the longest PABP cDNA (NI 8) near the *Sac*I and *Cla*I sites, respectively, were synthesized as gene-specific primers for 5′RACE. The fragments were then amplified according to the 5′ RACE protocol (Gibco BRL) and cloned to pGEM-T EASY vector using AT cloning (Promega protocol). The two longest cDNA fragments, 1.2 and 1.4 kb, were sequenced. The overlapping sequences of the two cDNAs were the same. One had a 397-bp 5′ NTR, and the other had a 216-bp 5′ NTR.

RdRp and PABP deletions

All the PABP deletions were made from the longest cDNA obtained from the two-hybrid screen, NI8 (Fig. 3). NI8Δ300 was amplified by PCR, using RG110 and RG157 as 5′ and 3′ terminal primers. RG110 was located at the 5′ end of NI8 and included an *Eco*RI site, 5′-AAA*GAATTC*GGCTTTGTAAATTTTGAG-3′. RG 105 complemented the region from positions 1801 to 1819 (position denoted according to full-length *CS-PABP* cDNA) of NI8 and included a *Xho*I site, 5′-TCT*CTCGAG*CAAATGTAGAACCT-CAGT-3′. The PCR product was inserted into pADGAL4 as an *Eco*RI–*Xho*I fragment to form pADNI8Δ300. NIΔmlu was amplified with RG110 and RG115, which complemented the region from positions 1476 to 1495, with *Xho*I, *Sph*I, and *Sac*I sites at the 5′end, 5′-GTA*CTCGAGCAT-GCGAGCTC*AAAGGTACAGGCTGCTGG-3′. The PCR product was inserted as an *Eco*RI–*Xho*I fragment into pADGAL4 to form pADNI8Δmlu and into pBluescript to form pBSNI8Δmlu. pBSNI8Δmlu was then digested with *Sph*I and religated to form pBSNI8Δsph. The *Eco*RI–*Xho*I NI8Δsph fragment was then subcloned to pADGAL4 to form pADNI8Δsph.

All the RdRp deletions were made from full-length RdRp (Fig. 3). RdRpΔ1, -Δ4, -Δ5, -Δ6, -Δ7, and -Δ8 were amplified by PCR using primers as indicated in Fig. 3; all the PCR products were inserted into pBDGAL4 as *Eco*RI–*Sal*I fragments. The sequences of primers RG100, 101, 121, 125, 126, and 127 were 5′-AGA*GTCGAC*CCTGACTTTCTCAAGC-3′, 5′-CCG*GAATTC*TGCGCTGCGATGATT-3′; 5′-ACT*GAATTC*-CTCGAGAAAGAGAGAAT-3′; 5′-GTG*GAATTC*CCAATTCTT-GCTCCTGA-3′; 5′-TTA*GAATTC*GAGCTCAGGCCGCTT-3′, and 5′-TTC*GTCGAC*TCTCGAGTTTTGGAGTG-3′, respectively. RdRpΔ2 was generated as follows: pBDRdRp was digested with *Nco*I, filled in with Klenow fragment, and digested with *Eco*RI. This RdRp fragment was ligated to pBDGAL4, which had been digested with *Sal*I, blunted, and then cut by *Eco*RI to form pBDRdRpΔ2. pBSRdRp (RdRp in pBluescript) was cut with *Eco*RI and *Xho*I, and the *Eco*RI–*Xho*I RdRpΔ3 fragment was then subcloned to pBDGAL4 as pBDRdRpΔ3.

In vitro expression and poly-(A)–Sepharose binding of PABP and RdRp

Full-length PABP and RdRp were cloned to pET28a-1 to produce histidine-tagged PABP or RdRp. Each pET plasmid and the Promega TNT system control construct expressing luciferase without a histidine tag was expressed in a 50-μl volume using 2 μg of plasmid DNA in the Promega TNT quick coupled transcription/translation system with the addition of 0.4 mM magnesium acetate, 30 mM KCl, and [^{35}S]methionine. Five microliters of the 50-μl reaction product was incubated with poly-(A)–Sepharose (Sigma) for 1 h on ice in 100 μl of binding buffer [100 mM sodium acetate, 5 mM $MgCl_2$, 5 mM KCl, 40 mM HEPES, pH 7.6, 10% glycerol, protease inhibitor cocktail (Sigma), 0.2% Triton X-100, 5 mM β-mercaptoethanol]. The beads were washed six times with 500 μl of binding buffer plus 200 mM NaCl. The beads were then boiled in 24 μl of loading buffer and analyzed by SDS–PAGE followed by autoradiography overnight.

Expression of PABP and RdRp in *E. coli*

The *E. coli* stain Xa 90 harboring pGEXPABP was grown at 37°C in LB with 50 mg/L ampicillin to OD_{600} = 1.0. IPTG (β-D-thiogalactoside) was added to 0.1 mM and the cells were grown for another 3 h at 37°C and then harvested. The *E. coli* stain Xa 90 harboring pGEXRdRp was grown at 37°C in LB with 50 mg/L ampicillin to OD_{600} = 0.3–0.4. IPTG was added to the medium and the cells were then grown at 16°C overnight prior to harvesting. GST-tagged PABP and RdRp were purified as recommended by the supplier (Phamacia); yield of protein was estimated by the Bio-Rad assay. *E. coli* strain BL21 (DE3) harboring pET-PABP was grown at 37°C in LB with kanamycin to OD_{600} = 0.6. After the addition of IPTG to 1.0 mM, the cells were grown for 3 h at 37°C. His-tagged PABP was purified as recommended by the supplier (Novagen).

In vitro analysis of protein–protein interaction

Five micrograms GST or GST–PABP purified from *E. coli* was immobilized on 30 μl of glutathione–Sepharose (Pharmacia) and incubated with 5 μl of *in vitro* ^{35}S-labeled RdRp or luciferase (prepared using Promega TNT quick coupled transcription/translation system) in 90 μl of binding buffer plus 10 μl of bovine serum at room temperature for 1 h. The beads were then washed six times with 500 μl of binding buffer plus 200 mM NaCl, boiled in 24 μl of loading buffer, and analyzed by SDS–PAGE followed by autoradiography overnight. For binding to poly-(A), 5 μg of His–PABP was immobilized on poly-(A)–Sepharose, and 5 μl *in vitro* ^{35}S-labeled luciferase or RdRp was added. Binding, washing, SDS–PAGE, and autoradiography were as above.

DNA and RNA hybridization

Genomic DNA was extracted from young cucumber leaves according to the protocol of Dellaporta *et al.* (1985). Ten micrograms of restriction-enzyme-digested DNA was subjected to electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Micron Separations) by capillary blotting (Sambrook *et al.,* 1989). Total RNA was isolated from cucumber leaf, root, and immature and mature flowers as described by Chomczynski and Sacchi (1987); 10 μg of each was used for Northern blot (Sambrook *et al.,* 1989). Full-length *CS-PABP1* was labeled using the DIG DNA labeling and detection kit (Boeringer Mannheim). High-stringency hybridization and wash conditions were performed according to Sambrook *et al.* (1989). Low-stringency hybridization and wash conditions were performed following Hilson *et al.* (1993).

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