Nucleic acid-binding properties of a bacterially expressed potato virus Y helper component-proteinase

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The potyvirus helper component–proteinase (HC-Pro) is a multifunctional protein previously reported to have affinity for polyribonucleotides. To investigate further the ability of HC-Pro to bind nucleic acids, the potato virus Y (PVY) LYE84 isolate HC-Pro gene was amplified, cloned in an Escherichia coli expression vector and sequenced. HC-Pro was expressed as a fusion with the maltose-binding protein and purified by affinity chromatography. Electrophoretic mobility-shift assays demonstrated that HC-Pro acts as a sequence non-specific RNA-binding protein and suggest that more than one molecule of protein was bound per molecule of RNA. The HC-Pro RNA-binding activity was stable in 400 mM-NaCl and temperature sensitive. The recombinant protein preferentially bound ssRNA over DNA or dsRNA and showed little, if any, affinity for poly(A). The possible implications of the RNA-binding activity of HC-Pro in potyvirus replication and movement are discussed.

Introduction

Potyviruses possess a ssRNA genome of positive polarity that encodes a single polyprotein (for review see Riechmann et al., 1992). The polyprotein is processed into functional virus proteins by three virus proteinases (Carrington et al., 1990): the P1 proteinase, the helper component-proteinase (HC-Pro) and the nuclear inclusion-a proteinase.

The potyvirus HC-Pro is a multifunctional protein. It has a well-characterized C-terminal papain-like proteolytic domain responsible for the release of its own C terminus from the polyprotein (Carrington et al., 1989a). The N-terminal portion of HC-Pro is required for aphid-mediated plant-to-plant transmission (Atreya et al., 1992; Atreya & Pirone, 1993). However, in a recent report a point mutation within the conserved C-terminal proline-threonine-lysine (PTK) box of HC-Pro was shown to be associated with loss of aphid transmissibility of the virus but were also associated with attenuated virulence and symptom expression in infected plants (Atreya et al., 1992; Atreya & Pirone, 1993; Dolja et al., 1993). These data strongly suggest a role for the N-terminal domain of HC-Pro in potyvirus genome amplification.

A common feature of the N terminus of all potyvirus HC-Pro is the presence of a highly conserved arrangement of cysteines and a histidine that has been implicated in metal complex formation (Robaglia et al., 1989). Natural or site-directed mutations within this region not only abolished aphid transmissibility of the virus but were also associated with attenuated virulence and symptom expression in infected plants (Atreya et al., 1992; Atreya & Pirone, 1993; Dolja et al., 1993). These data strongly suggest a role for the N-terminal domain of HC-Pro in potyvirus genome amplification.

Recently, a mutational analysis of the tobacco vein mottling potyvirus genome revealed the presence of a defective virus that was able to replicate in protoplasts but unable to spread systemically within the host plant (Klein et al., 1994). Interestingly, the mutation mapped in the HC-Pro gene, suggesting an additional putative function for this protein. The functional importance of HC-Pro in systemic movement was further supported by Cronin et al. (1995) using a movement-defective tobacco etch potyvirus (TEV) containing a mutation in the central region of HC-Pro, complemented by transgenic plants expressing the functional HC-Pro.

Although interaction between HC-Pro and the virus coat protein (CP) has been proposed as an overall molecular model to explain some of the biological functions described (Pirone, 1991; Cronin et al., 1995), two lines of evidence suggest that HC-Pro also possesses a nucleic acid-binding activity that is related to its multifunctionality. HC-Pro purified from infected...
tobacco plants has affinity for homoribopolymers such as poly(U), poly(G) and poly(I) but not for poly(A) or poly(C) (Thornbury et al., 1985). In addition, virus cysteine-rich motifs similar to the one present in the N terminus of HC-Pro appear to be related to different classes of nucleic acid-binding proteins (Koonin et al., 1991). However, to date, there is no experimental confirmation for the presumed nucleic acid-binding activity of HC-Pro.

In this report we show that the potato virus Y (PVY) HC-Pro expressed in Escherichia coli as a fusion with the maltose-binding protein (MBP), as well as genuine HC-Pro generated by incubation of the fusion protein with factor Xa, possessed nucleic acid-binding activity and we discuss the biological significance of this activity in the potyvirus life cycle.

**Methods**

**Virus.** The LYE84 isolate of PVY was a kind gift from K. Gebré-Selassié (INRA, Avignon, France) and has been partially characterized (Tordo et al., 1995). It was propagated in Nicotiana tabacum var. Xanthi.

**RT-PCR amplification and cDNA cloning.** cDNA of the HC-Pro gene was obtained by RT-PCR performed on total mRNA extracted from tobacco plants infected with PVY LYE84. Polyadenylated mRNAs were isolated using oligo(dT) magnetic beads (Dynabeads) according to the manufacturer’s instructions. The sequences of the primers used in RT-PCR were based on the proposed potyvirus consensus cleavage sites between P1-HC-Pro and HC-Pro-P3 (Vance et al., 1992) and the known sequence of PVY F (previously named PVYn; Robaglia et al., 1989). First strand cDNA was synthesized using the primer 5' GCTCTAGATTAACCAACTCTATAATG Y, complimentary to nucleotides 2390-2405 of the virus genome (the stop codon introduced is in bold; numbering is according to Robaglia et al., 1989). The cDNA was then amplified using the primer described above and the primer 5' GGATATCCTGGATGCGCAATTTTTGG 3', homologous to nucleotides 1037-1057 of the virus genome. The primers contained an XbaI and an EcoRI restriction site (underlined) respectively, for direct cloning of the PCR product into the XbaI- and EcoRI-digested plasmid pMal-c (New England Biolabs) to yield pMal:HC-Pro (Fig. 1a). RT (Superscript; Gibco BRL) and Taq polymerase (Bioprobe) were used according to the suppliers’ instructions.

The amplified cDNA of PVY HC-Pro was inserted into the EcoRI- and XbaI-digested plasmid p204-linker, a derivative of p204 (Valle et al., 1992) in which an inserted polylinker replaced the sequence located between the Ncol and EcoRI sites. The resulting pT7:HC-Pro plasmid contained the HC-Pro coding sequence under the control of the T7 RNA polymerase promoter (Fig. 1b). Plasmid pT7:HCΔ699 was generated by digestion of pT7:HC-Pro with Ncol and Ndel (to remove the first 699 nucleotides from the 5' end of the HC-Pro coding sequence), filling with the Klenow fragment of DNA polymerase I and subsequent self-ligation (Fig. 1b).

Plasmid pTA216 contains the cDNA corresponding to the 5' region of turnip yellow mosaic tymovirus (TYMV) sRNA, under the control of the T7 RNA polymerase promoter (Boyer, 1993).

**cDNA sequencing.** The total nucleotide sequence of the HC-Pro coding region was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Sequenase Version 2 (USB). The reactions were carried out on denatured double-stranded plasmid templates derived from a single cDNA clone according to standard protocols. Internal oligonucleotide primers were synthesized and used to complete the sequence on both strands.

**Preparation of nucleic acids.** The 255 nt of the PVY-derived RNA probe (Fig. 1b) used for electrophoretic mobility-shift, UV crosslinking and competition assays were prepared by in vitro transcription (Kujawa et al., 1993) of BstEII-digested pT7:HCΔ699 with T7 RNA polymerase (Gibco BRL) in the presence of 50 μCi of [α-32P]CTP. For Northwestern blot assays, in addition to the above mentioned probe, a 32P-labelled non-specific RNA probe corresponding to the first 200 nt of the 5' region of TYMV was obtained by in vitro transcription with T7 RNA polymerase of SnaBI-digested pTA216 as described above (generating probe A'200). The sp. act. of the RNA probes varied from 1 to 3 x 106 c.p.m./μg. HindIII-linearized pMal-c (plasmid served as unlabelled dsDNA in competition assays. Single-stranded M13mp18, bovine tRNA, poly(A) and poly(U) were commercial preparations. Purified TYMV RNA was obtained from K. Séró (this laboratory) and yeast killer virus dsRNA from M. Wesolowsky (CGM, Gif-sur-Yvette, France).

**Expression, purification and analysis of MBP:HC-Pro and MBP:β-gal.** Expression and purification of the fusion proteins were essentially as described by Rodriguez & Carrasco (1993) with minor modifications.

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**Fig. 1.** Schematic representation of relevant portions of the HC-Pro cDNA-containing plasmids. (a) pMal:HC-Pro expression plasmid showing the position of the MBP (open box) and HC-Pro (shaded box) coding regions. The size (kDa) of the resulting protein is indicated below the corresponding box. Part of the nucleotide sequence present at the junction of the fused gene is indicated and the EcoRI site used for cloning is underlined. The corresponding amino acids are shown below the nucleotide sequence with the first amino acid of HC-Pro (Ser) represented in italic. The factor Xa cleavage site is indicated by an arrow. (b) pT7:HC-Pro and pT7:HCΔ699 transcription plasmids containing the T7 promoter (open box). Plasmid pT7:HC-Pro was restricted to generate pT7:HCΔ699, which was used for in vitro transcription. The horizontal black rectangle shown below the pT7:HCΔ699 map corresponds to the 255 nt RNA transcript used as probe in the different binding studies. Restriction enzyme sites in HC-Pro cDNA used for construction of the described plasmids are shown.
E. coli BL21(DE3) cells were transformed with pMal-c, which encodes an MBP-β-galactosidase-α-peptide fusion (MBP-β-galz) used as control, or with pMal:HC-Pro to express the MBP:HC-Pro fusion protein, and were grown in LB medium containing ampicillin to an OD₆₀₀ of 0.7, followed by addition of IPTG to 0.3 mM and induction for 2 h at 24 °C. All subsequent steps were performed at 4 °C. Cells were collected by low-speed centrifugation, resuspended in lysis buffer (10 mM-Tris-HCl pH 7.8, 200 mM-NaCl, 1 mM-DDT, 0.25% Tween 20 and 1 mM-PMSPF) and stored at -20 °C. After thawing, 2 mg/ml of lysozyme were added followed by 30 min of incubation. Further lysis of the cells was achieved by sonication. After centrifugation at 9000 × g for 30 min, the supernatant was diluted 1:5 with column buffer (10 mM-Tris–HCl pH 7.8, 5 mM-EDTA, 200 mM-NaCl, 1 mM-DTT, 0.25% Tween 20 and 10% glycerol) for 25 min at room temperature. The reaction mixtures were loaded onto a 5% non-denaturing polyacrylamide gel (acrylamide: bisacrylamide 30:0.8, pre-run for 50 rain at 60 V) and electrophoresed at 60 V in 1 × TAE (40 mM-Tris-acetate, 1 mM-EDTA pH 8.5) at room temperature.

For binding assays, increasing amounts of purified MBP:HC-Pro (from 50 ng to 1200 ng) were incubated with 10 ng of RNA probe in binding buffer. The radiolabelled complexes and free RNA probe were detected by autoradiography of the gels.

For competition assays, a standard binding reaction containing constant amounts of fusion protein (100 ng) and RNA probe (2 ng) and including 20 units of RNasin (Promega) was performed as described above, except that the RNA probe and increasing amounts of the unlabelled competitor nucleic acid [1- to 1000-fold (w/w) excess] were mixed where indicated prior to addition of the fusion protein.

To investigate the influence of increasing salt concentrations on the stability of the protein-RNA complex, a standard binding reaction was performed as described for the competition assays, except that 0 to 600 mM-NaCl was added to the binding buffer. For quantitative analysis, the radioactivity in the gels was measured using a PhosphoImager and ImageQuant software (Molecular Dynamics).

UV cross-linking and Northwestern blot assays. For UV cross-linking experiments, 25 ng of ³²P-labelled PVY-derived RNA probe were incubated with 1 µg of purified MBP:HC-Pro as described for the mobility-shift assays. The reaction mixtures were then irradiated for 15 min (1.8 J) on ice in a Stratallinker 1800 (Stratagene) at 8 cm from the light source (Rouleau et al., 1994). Three µg of RNase A (4-49 U/mg; Worthington Biochemical) were then added and samples were incubated for 20 min at 37 °C to degrade excess non-cross-linked RNA. Cross-linked proteins were analysed by SDS-PAGE, dried and exposed to an X-ray film. Northwestern blot assays using the PVY- or TYMV-derived RNA probes were carried out as described (Fernández et al., 1995). The transferred proteins were visualized by Ponceau-S staining. MBP-β-galz and MBP were visible after transfer to nitrocellulose, but were no longer detected after the Northwestern blot assay.

Results

cDNA sequence

The entire ORF of the PVY LYE84 HC-Pro, including an artificial stop codon (UAA), was amplified by RT-PCR. The complete nucleotide sequence of the cloned HC-Pro gene (1368 bp) was determined and the amino acid sequence deduced (data not shown). The cDNA sequence displayed a high level of identity with previously established sequences of potyvirus HC-Pro. It shares 93% and 91% (at the nucleotide level) and 96% and 95% (at the amino acid level) identity with the corresponding sequence of potato virus C (Thornbury et al., 1990) and PVY Fr (Robaglia et al., 1989), respectively. The presence of a lysine at position 50, a residue shown to be important in HC-Pro-mediated aphid transmission of potyviruses (Atreya et al., 1992; Atreya & Pirone, 1993), is in agreement with the aphid-transmissible phenotype of this virus (K. Gebrê-Sélassié, personal communication). No amino acid alterations were observed in regions known to be highly conserved between potyviruses (Granier et al., 1993), except the central CCC box described by Cronin et al. (1995) which was CSC in PVY LYE84.

Expression and purification of MBP:HC-Pro

The entire reading frame of HC-Pro was cloned into the E. coli expression vector pMal-c, to generate a fusion protein between the E. coli MBP and HC-Pro (Fig. 1a) whose expression was under the control of an IPTG-inducible promoter (Maina et al., 1988). Attempts to recover the recombinant fusion protein after induction and incubation of the cells for 2 h at 37 °C were unsuccessful, since the fusion protein was totally insoluble (data not shown). Induction and expression at 24 °C rendered most of the fusion protein soluble. Large amounts of fusion protein accumulated after induction by IPTG (Fig. 2a; compare lanes 1 and 2) and over half of the MBP:HC-Pro remained in the soluble fraction after centrifugation at 9000 × g (compare lanes 3 and 4). Purified MBP:HC-Pro produced a major band with the expected mobility (92 kDa) by SDS-PAGE (lane 5) but minor amounts of lower molecular mass species were also observed. In contrast, no corresponding band was observed in preparations of purified MBP-β-galz (52 kDa; lane 7). The recombinant fusion protein and some of the breakdown products reacted with anti-PVY HC-Pro antibodies in immunoblot assays (Fig. 2b). MBP:HC-Pro could be cleaved by factor Xa (Fig. 2a; lane 6) to yield MBP (42 kDa) and HC-Pro (50 kDa).
However, the resulting HC-Pro became insoluble after this treatment. Consequently, with the exception of the Northwestern blot assays (for which genuine HC-Pro, in spite of its insolubility, was used after separation from MBP by SDS-PAGE) only the intact fusion protein was used to investigate the nucleic acid-binding properties of HC-Pro.

**Determination of the RNA-binding properties of MBP:HC-Pro**

To investigate the RNA-binding properties of MBP:HC-Pro, increasing amounts of fusion protein were mixed with a constant amount of the 32P-labelled PVY-derived RNA probe (Fig. 1b) and mobility-shifts were determined in non-denaturing polyacrylamide gels. The results presented in Fig. 3(a) clearly indicate that the fusion protein has affinity for the RNA. Protein concentrations above 200 ng (lanes 5 to 8) completely shifted the labelled RNA probe, leading to the formation of complexes that barely entered the gel matrix. No intermediate bands between the free RNA probe and the shifted complexes were observed. This 'all or none' profile suggests that more than one molecule of MBP:HC-Pro is bound per molecule of RNA.

To ascertain that binding was not the result of non-specific interaction caused by the experimental conditions, MBP:β-galα and BSA were used in place of MBP:HC-Pro. Neither of these control proteins elicited a shift of the RNA probe (Fig. 3b, lanes 4 to 6) as opposed to the fusion protein (lane 2). Similar results (data not shown) were obtained using other control proteins including the MBP-paramyosin fusion protein (New England Biolabs), confirming that binding activity was specific for PVY HC-Pro. Heating MBP:HC-Pro at 65 °C for 5 min completely abolished RNA-binding activity (lane 3), showing that the ability to bind RNA was temperature-sensitive. RNA binding could be competitively inhibited by a 1000-fold (w/w) excess of bovine tRNA (lane 8), indicating that binding activity of MBP:HC-Pro lacks nucleic acid sequence specificity. However, protein–RNA complex formation was apparently unaffected when a 1000-fold (w/w) excess of poly(A) was added to the binding reaction (lane 7), in agreement with a previous report (Thornbury et al., 1985) suggesting that HC-Pro has little, if any, affinity for poly(A).

To verify that mobility-shift was not due to the presence of a contaminating *E. coli* protein in the MBP:HC-Pro preparation, two different approaches were used. First, a UV cross-linking assay was performed, with the proteins visualized as shown in Fig. 4(a). After UV irradiation, MBP:HC-Pro and a more slowly migrating band were covalently linked to the labelled RNA probe (Fig. 4b; lane 1). The latter band probably corresponds to large cross-linked RNA–protein complexes that are observed when proteins reported to possess co-operative binding activity are cross-linked (Citovsky et al., 1990). Omission of the UV irradiation step or treatment of the irradiated sample with proteinase K eliminated the radioactive bands (Fig. 4b; lanes ‘-’ and PK respectively). No detectable complex (lane 2) was formed with MBP:β-galα and possible minor contaminant *E. coli* protein(s). Furthermore, no apparent alteration in the electrophoretic mobility of the cross-linked complex versus the non-
Nucleic acid-binding properties of PVY HC-Pro

Fig. 3. Electrophoretic mobility-shift assays of MBP:HC-Pro binding to a 32P-labelled PVY-derived RNA probe (10 ng). Both panels are autoradiographs of native polyacrylamide gels. (a) Incubations without or with increasing amounts of fusion protein. Lane 1, RNA probe alone; lanes 2–8, 50, 100, 200, 400, 600, 800 and 1200 ng of MBP:HC-Pro, respectively. (b) Specificity of binding of MBP:HC-Pro to ssRNA. Lane 1, RNA probe alone; lane 2, 300 ng of MBP:HC-Pro; lane 3, same as lane 2 but protein was heated at 65 °C for 5 min before incubation with the probe; lanes 4 and 5, 1 μg and 2 μg of MBP:β-galz, respectively; lane 6, 1 μg of BSA; lane 7, same as lane 2 with 1000-fold excess (w/w) of poly(A); lane 8, same as lane 2 with 1000-fold excess (w/w) of bovine tRNA.

Fig. 4. RNA-binding activity of purified MBP:HC-Pro fusion protein analysed by SDS-PAGE after UV cross-linking. (a) Coomassie blue staining showing the position of purified MBP:HC-Pro (lane 1) and MBP:β-galz (lane 2); lane M, protein markers in kDa. (b) One μg of each protein shown in (a) (lanes 1 and 2) was incubated with 25 ng of 32P-labelled PVY-derived RNA probe, irradiated with UV light, treated with RNase A, resolved by SDS-PAGE and autoradiographed. Lane 1, purified MBP:HC-Pro; lane 2, purified MBP:β-galz; lane (−), same as lane 1 but the UV irradiation step was omitted; lane PK, same as lane 1 but the irradiated sample was treated with proteinase K (1 mg/ml) for 30 min at 37 °C. In (b), the values (kDa) to the right are from prestained protein markers (Bio-Rad) analysed on a parallel lane of the same gel.

cross-linked fusion protein was observed, as is the case for other virus RNA-binding proteins (Citovsky et al., 1990; Schoumacher et al., 1992).

Second, Northwestern blot assays were performed to determine whether RNA-binding activity could be associated with genuine HC-Pro (Fig. 5). MBP:HC-Pro intact, or cleaved by factor Xa to generate genuine HC-Pro, and also control MBP:β-galz, were subjected to SDS-PAGE, transferred to nitrocellulose and after a renaturation process, incubated with the 32P-labelled TYMV-derived RNA probe A’200. Labelling was specific for intact MBP:HC-Pro (Fig. 5b; lanes 1 and 2) and HC-Pro (lane 3). Two additional faster migrating protein species were weakly labelled in the sample containing mature HC-Pro (lane 3). The appearance of more than one band is probably due to non-canonical cleavage by factor Xa within the HC-Pro portion of the fusion protein. Although factor Xa recognizes a specific cleavage site (Maina et al., 1988) present at the junction of the fusion protein (Fig. 1a), non-canonical cleavages by this proteinase have been reported (Rodríguez & Carrasco, 1993). The same results (data not shown) were obtained using a second non-specific RNA probe (complementary to TYMV A’200) and the PVY-derived probe, thus confirming the lack of sequence specificity.

The combined results are convincing evidence that the RNA-binding activity is specific for HC-Pro and that MBP alone is devoid of such activity.

Characteristics of the RNA binding activity of MBP:HC-Pro

The stability of the fusion protein–RNA complex was assayed by electrophoretic mobility-shift assays and evaluated by measuring the amount of labelled, bound complex formed in the presence of increasing salt

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Fig. 5. Northwestern blot analysis of RNA binding to immobilized HC-Pro. Purified proteins were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with a 32P-labelled TYMV-derived RNA probe (A200). (a) Nitrocellulose membrane stained with Ponceau-S after Northwestern blot assay. Lanes 1 and 2, 300 and 600 ng of MBP:HC-Pro, respectively; lane 3, 2 μg of MBP:HC-Pro digested with factor Xa; lane 4, 500 ng of purified MBP:β-gal; lane M, prestained protein markers (Bio-Rad) in kDa. (b) Autoradiograph of the nitrocellulose membrane shown in (a) to detect RNA binding. The position of each protein is indicated in (a).

Fig. 6. Effect of increasing NaCl concentrations on the stability of the MBP:HC-Pro–RNA complex. A standard binding reaction containing 2 ng of 32P-labelled PVY-derived RNA probe and 100 ng of purified MBP:HC-Pro was performed in the absence or presence of increasing salt concentrations. The complexes were resolved in native polyacrylamide gels. The relative amount of specific complex formed at each NaCl concentration was measured using a PhosphorImager.

Table 1. Inhibition of MBP:HC-Pro–RNA complex formation by different nucleic acids

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Type*</th>
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<tr>
<td>TYMV RNA</td>
<td>ssRNA</td>
<td>5</td>
</tr>
<tr>
<td>Poly(U)</td>
<td>ssRNA</td>
<td>10</td>
</tr>
<tr>
<td>M13mp18</td>
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<tr>
<td>Yeast killer virus</td>
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* Type of competitor nucleic acid added to standard binding reaction.
† Ratio (w/w) of competitor to probe required to reduce complex formation by 50%. The amount of specific complex formed was measured using a PhosphorImager.

formation was reduced at salt concentrations ranging from 50 to 400 mM-NaCl, significant binding activity persisted in these conditions (77% to 54% of maximum binding). In contrast, complex formation was strongly reduced (to 21%) at 600 mM-NaCl. Thus the MBP:HC-Pro–RNA complex remained (> 50%) stable at ionic strengths up to 400 mM-NaCl.

The nucleic acid-binding specificity of HC-Pro was assessed by measuring the ability of different unlabelled nucleic acids to compete for binding with a fixed amount of labelled RNA probe in a mobility-shift assay. It was determined by comparing the ratio of competitor to probe required to reduce complex formation by 50%. Table 1 presents the results of a typical experiment.
TYMV genomic RNA (6318 nt; Morch et al., 1988) and poly(U) competed efficiently with the labelled RNA probe for binding to MBP:HC-Pro, since 50% inhibition was reached at competitor:probe (w/w) ratios of 5 and 10, respectively. Single-stranded DNA (M13mp18; 7250 nt; Yanisch-Perron et al., 1985), dsDNA (pMal-c2 linearized with HindIII; 6646 bp) and dsRNA (yeast killer virus; 5200 bp, 2900 bp and 2200 bp; Fried & Fink, 1978) competed similarly but less efficiently than ssRNA, with 50% inhibition observed at ratios ranging from 22 to 45. Taken together, these results show that HC-Pro has a preference for ssRNA [with the exception of poly(A)] rather than for DNA (ss or ds) or dsRNA.

Discussion

The study of the biochemical functions of the potyvirus HC-Pro has long been hampered by the difficulties encountered in obtaining large amounts of the protein. HC-Pro has been reported as highly insoluble when expressed in heterologous systems and attempts to solubilize these insoluble inclusions and renature the resulting products failed to yield active HC-Pro (Thornbury et al., 1993; I. G. Maia & F. Bernardi, unpublished results). To date, with the exception of the proteinase domain that is active in E. coli and insect cells (Carrington et al., 1989b; Thornbury et al., 1993), no in vitro function has been ascribed to bacterially expressed HC-Pro.

Here we show that PVY-HC-Pro expressed in E. coli as a fusion with MBP is partly soluble when expressed at 24°C and can be purified easily. The purified fusion protein acts as a sequence non-specific RNA-binding protein, since it binds both PVY-derived and unrelated RNA molecules. HC-Pro RNA-binding activity is stable at ionic strengths up to 400 mM-NaCl and is inactivated by heat. The behaviour of RNA binding to HC-Pro is reminiscent of RNA–protein interactions believed to reflect co-operative processes (Citovsky et al., 1990; Osman et al., 1992; Schoumacher et al., 1992; Soumounou & Laliberté, 1994). However, in view of recent results obtained by Thomas & Maule (1995) with the gene I protein of cauliflower mosaic virus (CaMV), identification of a co-operative RNA-binding activity using mobility-shift assays should be interpreted with caution. Finally, several control experiments provide support for the observed HC-Pro RNA-binding activity. To our knowledge, this is the first direct demonstration of a function of HC-Pro using a purified bacterially expressed protein.

The specificity of RNA binding to HC-Pro is similar to that observed for other plant virus RNA-binding proteins, such as the potyvirus P1 proteinase (Brantley & Hunt, 1993) and the gene I protein of CaMV (Citovsky et al., 1991), which bind ssRNA preferentially to ssDNA or double-stranded nucleic acids. However, it differs from the binding activity of the movement proteins (MP) of red clover necrotic mosaic virus (Osman et al., 1992) and tobacco mosaic virus (TMV; Citovsky et al., 1990) which bind to ssRNA and ssDNA equally well. The very low affinity for poly(A) is consistent with previous results obtained during purification of HC-Pro from infected tobacco plants (Thornbury et al., 1985). The significance of this finding remains unexplained. Nevertheless, it demonstrates that a correlation exists between HC-Pro purified from infected plants and the corresponding bacterially expressed protein with respect to their affinity for poly(U) and their very low, if any, affinity for poly(A).

Searches in available databases failed to show any amino acid sequence similarity between HC-Pro and known RNA-binding motifs (Burd & Dreyfuss, 1994). The N-terminal cysteine-rich motif conserved in all potyvirus HC-Pro could be responsible for binding to RNA. The fact that cysteine-rich motifs have been involved in nucleic acid binding in some proteins of plant (Gramstat et al., 1990) and animal viruses (Gorelick et al., 1988; Eagle & Klessig, 1992) further supports this hypothesis. However, more work is required to identify the functional RNA-binding domain of HC-Pro.

The exact biological significance of RNA binding by HC-Pro is unknown. Plant virus proteins displaying a similar sequence non-specific and co-operative RNA-binding activity have usually been implicated in cell-to-cell movement of viruses (Deom et al., 1992), a well-studied example being the P30 MP of TMV (Citovsky et al., 1990, 1992). For potyviruses, mutational analyses have established that the virus CP is involved in cell-to-cell spread of TEV (Dolja et al., 1994, 1995), suggesting that potyviruses probably move as encapsidated RNA. The identification of an RNA-binding activity related to HC-Pro implies that potyviruses might alternatively use an HC-Pro-mediated RNP complex for movement. To date no potyvirus protein has been identified as being a specific MP. The presumed candidate, the RNA-binding P1 proteinase, was recently shown to play little, if any, role in cell-to-cell movement (Klein et al., 1994; Verchot & Carrington, 1995).

A second potential role of RNA binding by HC-Pro is related to potyvirus replication. Experimental evidence suggests a role for the N-terminal domain of HC-Pro in genome amplification (Atreya et al., 1992; Atreya & Pirone, 1993; Dolja et al., 1993) and the RNA-binding activity demonstrated here is consistent with this possibility. Although it is difficult to explain how a sequence non-specific protein such as HC-Pro could participate in regulation of virus replication, this could be modulated by association of HC-Pro with another (host or virus)
protein to specifically recognize the virus RNA, or by a special compartmentalization process.

Using the expression system described here, we have demonstrated an additional function for PVY HC-Pro. It will now be interesting to investigate whether the recombinant HC-Pro can mediate aphid transmission of PVY. A number of important functions are being assigned to HC-Pro, providing support for its multifunctionality during the potyvirus life cycle.

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