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The RNA helicase CI from plum pox potyvirus has two regions involved in binding to RNA

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Abstract The plum pox virus (PPV) protein CI is an RNA helicase, whose function in the virus replication is still unknown. Recently, an RNA binding domain was mapped to a region of the CI protein that includes the arginine-rich motif VI typical of RNA helicases of the superfamily SF2. In the present study, a second region involved in RNA binding activity of the CI protein has been identified. Northwestern assays with a series of maltose-binding protein fusions that contain different CI fragments showed that the RNA binding domain is located between residues 75 and 143. This segment contains the two most amino-terminal conserved domains of RNA helicases: I, involved in NTP binding, and Ia, of unknown function. The results can be explained in the context of a close interdependence between the protein regions involved in the NTPase and RNA binding activities that is expected for an RNA helicase.

Key words: RNA binding; RNA helicase; Cylindrical inclusion protein; Potyvirus; Plum pox virus

1. Introduction

A large number of proteins need to interact with RNA to carry out their functions. Different kinds of RNA binding domains have been identified both in proteins that recognize specific nucleotide sequences and in proteins that bind RNA without specificity [1]. RNA helicases are enzymes that use the energy derived from the hydrolysis of nucleoside triphosphates to melt regions of duplex RNA. Thus, binding to RNA is a property associated with RNA helicase activity. Experimental evidence and sequence analysis data have highlighted the role of RNA helicases in a variety of cellular processes such as transcription, RNA maturing, translation, and RNA replication [2–6]. Virus-encoded proteins with demonstrated or suggested RNA helicase activity have been identified in different groups of DNA viruses [7–9]. On the other hand, most RNA viruses from animals and plants encode at least one protein with a nucleoside triphosphate binding motif (NTPB motif). Functions in RNA replication and in virus or RNA traffic have been suggested for NTPB motif-containing proteins from RNA viruses. They are usually referred to as RNA helicase-like proteins, although such enzymatic activity has been demonstrated for the NTPB motif-containing proteins of only a few viruses [10–14].

The Potyvirus genus is the largest known group of plant viruses. Potyviruses belong to the picorna-like cluster of positive-strand RNA viruses [15] and express their 10-kb genome via a single polyprotein that is processed by three virus-encoded proteases [16,17]. It has been shown that the cylindrical inclusion (CI) protein of plum pox potyvirus (PPV) has RNA helicase activity [13]. The N-terminal half of the PPV CI protein has all the domains typical of helicases of the superfamily SF2 [3], including the NTPB motif. The C-terminal half of the CI protein shows no homology with RNA helicases, but small deletions in this region of a PPV CI protein synthesized in *E. coli* fused to the maltose-binding protein (MBP) drastically disturbed the RNA helicase activity [18]. Deletion analysis on the MBP-CI fusion product led to the identification of an RNA binding domain in a region that included the arginine-rich motif VI, the most carboxy-terminal conserved domain of RNA helicases [18]. The results presented in this paper indicate that, as has been reported for other proteins that interact with RNA, the PPV CI protein has a second RNA binding domain. This domain embraces a rather large sequence and is placed at the N-terminus of the protein region conserved in RNA helicases.

2. Materials and methods

2.1. Construction of expression vectors

All recombinant DNA procedures were carried out by standard methods [19]. *E. coli* strains JM109 and DH5α were used for the cloning of the plasmids. pcNCI, pcNCI196, pcNCI261 and pcNCI262-402 have been described previously [18]. Progressive deletions at the 3′ end of the PPV sequence cloned in pcNCI196 were carried out by removing the following restriction fragments from this plasmid: *Eco*RI (nt 3829)-*Hin*dIII (vector) after filling in with Klenow (for pcNCI60), *Nsi*I (nt 3875)-*Pst*I (vector) (for pcNCI74), *Bfr*I (nt 3945)-*Hin*dIII (vector) after filling in with Klenow at the *Hin*dIII end (for pcNCI97), *Nhe*I (nt 3990)-*Xba*I (vector) (for pcNCI114), *Sph*I (nt 4056)-*Hin*dIII after removal of the protruding ends with mung bean nuclease (for pcNCI134) and *Dpn*I (nt 4077)-*Hin*dIII (vector) after filling in with Klenow at the *Hin*dIII end (for pcNCI143). pcNCI61-196, pcNCI75-196, and pcNCI114-196 were the result of substituting, respectively, *Bgl*II-*Eco*RI, *Bgl*II-*Pst*I and *Bgl*II-*Stu*I restriction fragments of the vector plasmid pMal-c (New England Biolabs) for sequences of pcNCI196 spanning from the *Bgl*II site of the vector to the following restriction sites of the CI sequence: *Eco*RI (nt 3829) (for pcNCI61-196), *Nsi*I (nt 3875) (for pcNCI75-196) and *Nhe*I (nt 3990, after filling in with Klenow) (for pcNCI114-196). pcNCI144-196 was obtained by inserting into pMal-c digested with *Bam*HI and *Hin*dIII, the *Sau*IIIA (nt 4075)-*Hin*dIII (vector) fragment of pcNCI196. The numbering of nucleotides corresponds to the full-length sequence of PPV RNA [20]. The numbers in the names of the plasmids correspond to the last CI amino acid encoded by them, when the CI cistron is cloned from the first nucleotide, or to the first and the last CI amino acids, when the plasmid encodes an internal fragment of the CI protein. The accuracy of the constructions was verified by restriction analysis and by sequencing through the junctions of the ligated fragments.

2.2. Expression and isolation of the recombinant proteins

The expression of the recombinant plasmids and the partial purification of the corresponding MBP-CI fusion products were carried out essentially as previously described [18]. After growing at 30°C in LB medium containing ampicillin (100 μg/ml) and induction with 0.3 mM IPTG, transformed JM109 cells were collected by centrifugation and

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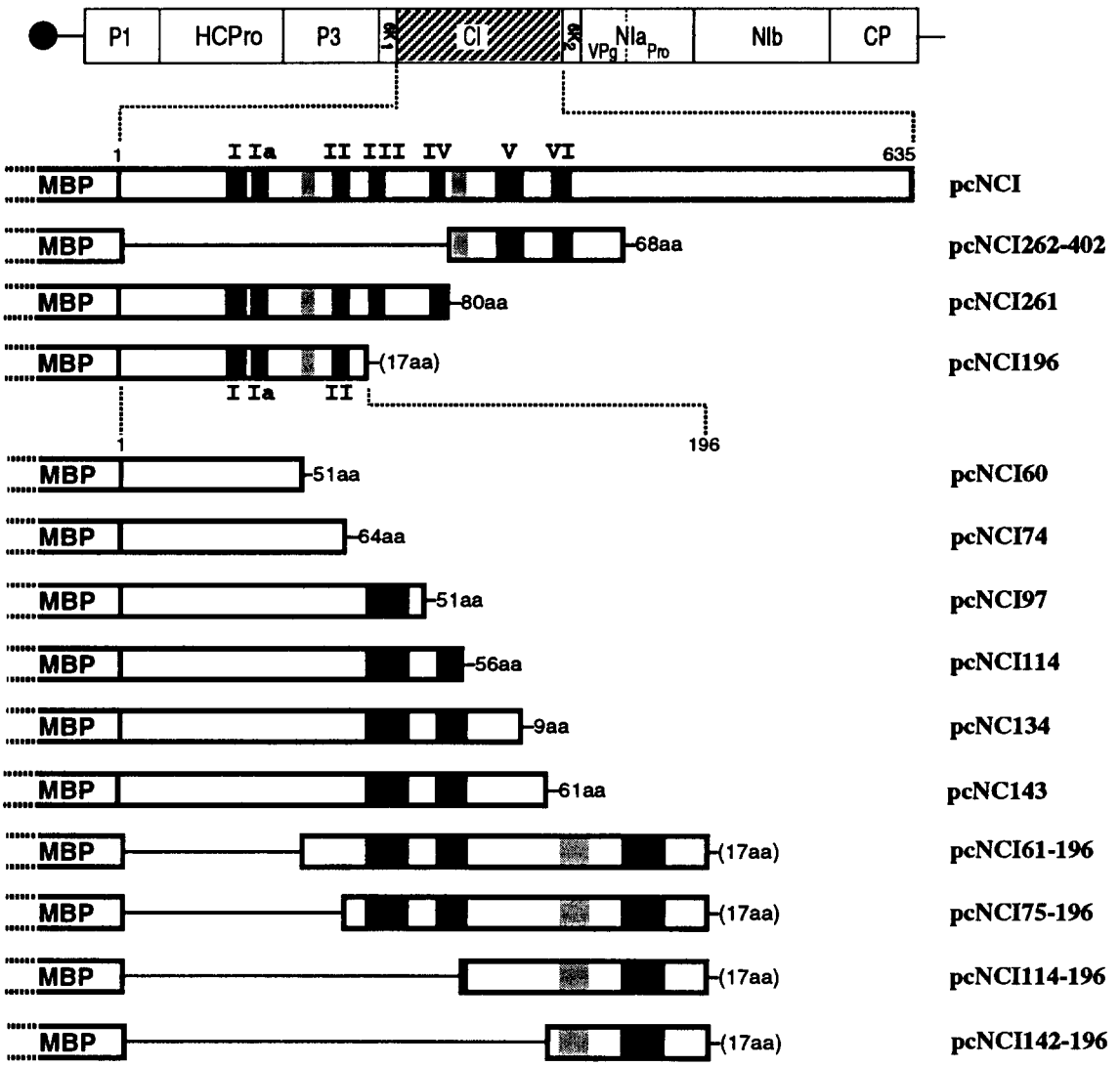

Fig. 1. Diagrammatic representation of the MBP-CI fusion proteins encoded by the different recombinant plasmids. The numbers of non-specific vector amino acids present at the end of each fusion product (in parentheses, when they are the product of readthrough over a stop codon suppressed by the JM109 bacteria) are written next to the bars which represent the protein coding sequences. Domains conserved among RNA helicases (named according to [3]) are shown as black or gray (lower conservation) areas. The position of the CI cistron in the PPV genome is indicated at the top of the figure. The CI sequence present in pcNCI196 (first and last amino acids are indicated) is enlarged to facilitate the observation of further deletions.

lysed by grinding with alumina. The crude extract was loaded onto an amylose resin column (New England Biolabs) equilibrated in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 M NaCl. The non-retained proteins were successively washed with the same buffer containing 1, 0.5, 0.2 M NaCl and without NaCl, whereas the products specifically retained were eluted with buffer containing 10 mM maltose and no NaCl.

2.3. Northwestern assay for RNA binding

The partially purified proteins were subjected to SDS-PAGE and transferred to nitrocellulose using a Trans-Blot apparatus (Bio-Rad). The membrane was incubated at room temperature in a renaturation solution containing 1 mM EDTA, 60 mM NaCl, 0.1% Triton X-100, 1×Denhardt's reagent and 10 mM of MES (pH 5, 5.5 or 6) or HEPES (pH 7, 7.5 or 8). This step was repeated four times. The membrane was then incubated in the same buffer containing 6×10^4 cpm of RNA labelled with [α-^{32}P]UTP (400 Ci/mmol). This radioactive probe was prepared by in vitro transcription of plasmid pT4ps2 digested with *Eco*RI and *Pvu*II, followed by removal of unincorporated nucleotides by spun column centrifugation through Sephadex G-50 [13]. After several washes in the renaturation buffer, the nitrocellulose membrane was dried in air, stained with Ponceau red and exposed to X-ray film.

3. Results

3.1. pH dependence of RNA binding of the PPV CI protein

Binding of the potyvirus CI protein to RNA has been shown by several in vitro techniques, such as retention in nitrocellulose membranes [21], UV cross-linking [10,21], and Northwestern assays [18]. In a previous study we reported that, at pH 7.5, different PPV CI fragments that contained the amino acids from 350 to 402, fused to the maltose-binding protein (which does not bind RNA by itself), conferred RNA binding activity on the fusion products. By contrast, PPV CI fragments containing the first 261 residues of the protein were not able to bind RNA. The fact that several RNA binding proteins have two RNA binding sites at separate locations [22–25], together with results obtained by Eagles et al. [10]

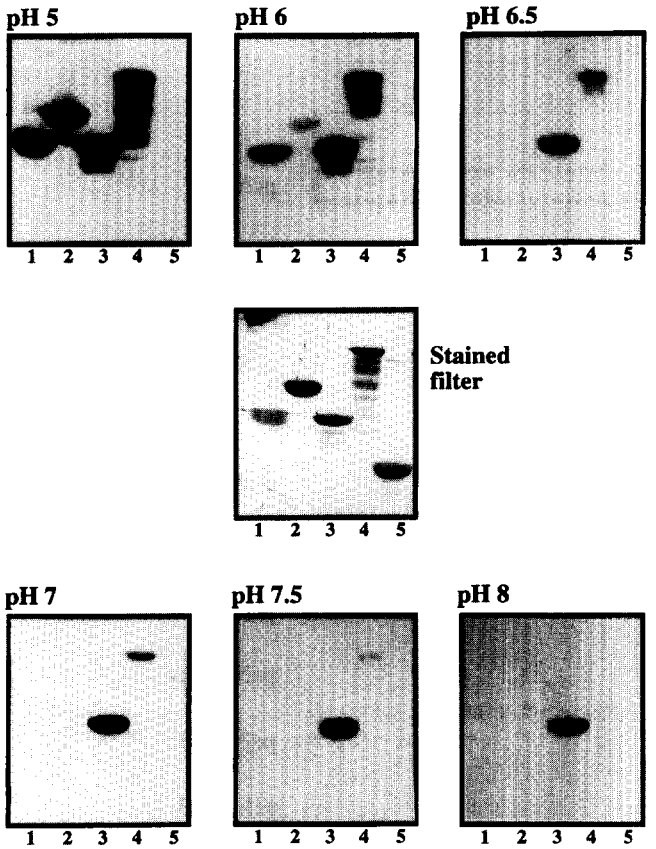

Fig. 2. RNA binding of CI deletion mutants (Northwestern assay) at the pH conditions indicated in each panel. The MBPCI fusion proteins (see Fig. 1) were subjected to SDS-10% PAGE, transferred to nitrocellulose and incubated with a [^{32}P]RNA probe. Different amounts of the partially purified fractions were loaded in the gel to have equivalent bands of the full-length products. Proteins were detected by Ponceau red staining (central panel), and RNA binding by autoradiography. Lanes 1, MBP-CI196 (10 μg); lanes 2, MBP-CI261 (10 μg); lanes 3, MBP-CI262-402 (5 μg); lanes 4, MBP-CI (20 μg); lanes 5, MBP-βgal (10 μg).

which showed that 7.5 is in the limit of the pH range in which the CI protein of tamarillo mosaic potyvirus could be cross-linked to RNA by UV irradiation, prompted us to further analyze the RNA binding capacity of the different regions of the PPV CI protein.

Not only RNA binding experiments (retention to nitrocellulose [21] and UV cross-linking [10]), but also enzymatic assays (stimulation by poly A of ATPase activity [21]), have indicated that the CI-RNA interaction is very sensitive to salt concentration. Our preliminary Northwestern experiments led to the same conclusion (data not shown). We therefore decided to carry out the RNA binding assays at 60 mM NaCl, a salt concentration that can be considered quite stringent according to our results and to previous reports [10,18]. In agreement with the UV cross-linking assays of Eagles et al. [10], the RNA binding activity of the PPV MBP-CI fusion product analyzed by Northwestern declined rapidly when the pH was increased, being quite low at pH 7.5 and undetectable at pH 8 (Fig. 2, lanes 4). The CI fragment spanning residues between 262 and 402 and, hence, including the previously characterized RNA binding domain conferred to MBP-CI262-402 RNA binding activity even at pH 8, although a clear reduction of binding was observed at the higher pHs (Fig. 2, lanes 3). Interestingly, although MBP-CI261 and, more markedly, MBP-CI196 did not bind RNA at pH 6.5 and over, they were able to do it at pH 5 and 6 (Fig. 2, lanes 1 and 2). Since RNA binding of MBP-βgal (MBP fusion product encoded by the pMal-c vector, which includes 94 aa from the polylinker and the β-galactosidase gene) could not be detected at any of the pH conditions (Fig. 2, lanes 5), we conclude that a second RNA binding domain was present in the N-terminal first 196 aa of the PPV CI protein.

It has been shown that, although the PPV CI protein is not able to unwind dsDNA, it binds ssDNA, and poly dA stimulates its ATPase activity [21]. In agreement with these results, both MBP-CI262-402 and MBP-CI196 bind ssRNA and a 37-mer oligodeoxynucleotide with similar pH requirements (data not shown).

3.2. Mapping of the RNA binding domain

To define the sequences of the N-terminal region of the CI protein involved in RNA binding more accurately, progressive deletions from both ends of the cloned CI sequence were performed in pcNCI196 (Fig. 1). MBP-CI143 bound RNA at pH 6 to the same degree as MBP-CI196, but further deletions to create MBP-CI134 and MBP-CI114 caused a drastic reduction in the binding activity, and MBP-CI100, MBP-CI74 and MBP-CI60 were inactive (Fig. 3A, lanes 7–13). Thus, the C-terminal limit of the RNA binding domain is located around aa 143. In agreement with this conclusion, no discernible RNA binding of MBP-CI142-196 was detected (Fig. 3A, lane 3). MBP-CI114-196 (CI fragment starting at aa 114) had RNA binding activity similar to that of MBP-CI196 (Fig. 3A, lanes 4 and 7). However, a much stronger signal was observed when aa 75–114 were also present in the fusion product (MBP-CI75-196 and MPB-CI61-196) (Fig. 3A, lanes 5 and 6), indicating that sequences in this region are involved in the RNA binding. The stronger RNA affinity conferred by the 61–196 and 75–196 segments when compared with the complete 1–196 fragment was confirmed by the fact that only MBP-CI61-196 and MBP-CI75-196, but not MBP-CI196, bound RNA at pH 7 (Fig. 3B, lanes 5–7). The binding of these proteins was similar to that of MBP-CI262-402 that bears the formerly identified RNA binding domain (Fig. 3B, lane 1). The extra amino acids encoded by the vector which are present at the C-ends of the recombinant proteins are not responsible for the RNA binding since they also form part of MBP-βgal or of other MBP-CI fusion proteins that did not bind RNA. All these results denote the existence of an RNA binding domain between residues 75 and 143 of the PPV CI protein.

4. Discussion

In general, RNA binding domains are broad and have a rather low degree of overall sequence conservation. It is usual to find more than one copy of an RNA recognition motif, or more than one type of motif, in a protein, that can be placed in very distant regions of the molecule [1,26]. It has been postulated that in some cases a domain can make specific contacts with defined RNA sequences, while a second one contributes specifically to the strength of the binding [24,26].

It has been shown by nitrocellulose filter retention [21], Northwestern assay [18] and UV cross-linking [10,21] that the potyvirus CI protein binds RNA in a non-specific fashion.

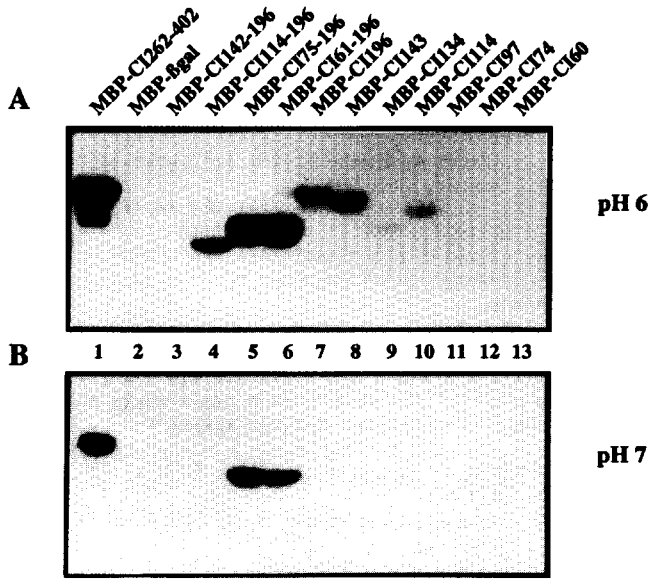

Fig. 3. Deletion analysis of the RNA binding domain present in the PPV CI fragment encoded by pcNCI196. Approximately 6 μg of MBP-CI262-402 and 10 μg of the rest of the MBP-CI fusion proteins indicated at the top of the panels were subjected to SDS-10% PAGE, transferred to nitrocellulose and incubated with a [^{32}P]RNA probe at pH 6 (panel A) or at pH 7 (panel B). RNA binding was detected by autoradiography.

The RNA binding of the CI protein is very susceptible to high ionic strength and pH, indicating that ionic interactions are fundamental for the binding activity [10]. In agreement with these data, a 52-aa fragment that includes the arginine-rich motif VI of PPV CI protein was shown to confer RNA binding activity [18]. Motif VI is the most carboxy-terminal conserved domain of RNA helicases of the superfamily SF2, and has been shown to be also involved in the RNA binding activity of the prototype of the superfamily, the translation initiation factor eIF-4A [27]. In this paper we describe the localization of a second RNA binding domain at the N-terminal region of the PPV CI protein. A similar arrangement of two regions involved in RNA binding, an N-terminal segment and a C-terminal domain involving an Arg-rich motif, has been reported for the poliovirus NTPB protein, the 2C protein [23]. The activity of some RNA binding domains has been reported to depend on the protein environment in which they are embodied; thus, some deletions that leave the primary sequences responsible for RNA binding untouched abolish this activity, which can be regained by additional deletions [22,23,28]. In the same way, the RNA binding domain placed at the N-region of the CI protein, although active in all the tested constructions in which it is present, confers a stronger binding capacity that is less dependent on pH conditions, when it forms part of MBP-CI61-96 and MBP-CI75-196 than when it is held in fusion proteins that have larger CI fragments such as MBP-CI196 and MBP-CI261 (Figs. 2 and 3). Also, like other domains involved in binding to RNA [29–32], the N-terminal RNA binding domain of the CI protein consists of a rather broad sequence. Non-overlapping subregions, such as those present in MBP-CI114 and MBP-CI114-196, have some RNA binding activity although they are much less efficient than the complete domain (Fig. 3).

The deletion analysis mapped boundaries of the RNA binding domain within residues 75 and 143. There is no obvious similarity between the amino acid sequence of this region or its predicted secondary structure and those of other known RNA binding domains. However, the region presents a quite considerable level of homology among potyviruses (34 out of 69 residues are strictly conserved in 12 or 13 of 13 potyvirus CI proteins compared, data not shown). It includes two motifs, I and Ia, typical of the superfamily SF2 of RNA helicase-like proteins. It is well established that motif I (the A segment of the Walker box) is directly involved in NTP binding [33], and it seems unlikely that it plays a significant role in RNA binding. By contrast, a function for the Ia motif has still not been established. It is tempting to speculate that it could constitute the core of the N-terminal RNA binding domain of the CI protein. However, when the binding activities of MBP-CI143 and MBP-CI134 are compared, a drastic reduction was observed as result of the deletion of only 9 aa, which are outside the sequences of motif Ia conserved in the helicase-like superfamily SF2. Thus, residues downstream from motif Ia are presumably relevant for the interaction with RNA.

The results described in this report are congruent with the expected role of RNA binding during the unwinding process. Sliding over an RNA chain disrupting secondary structures or opening a double-stranded molecule seems to require weak and non-specific nucleic acid binding, such as that found for the CI protein. On the other hand, RNA binding and NTP hydrolysis should be perfectly coordinated to carry out the helicase activity. Direct interaction between domain II (involved in NTPase activity) and domain VI (involved in RNA binding) has been postulated on the basis of the strict correlation observed in the RNA helicase-like proteins of superfamily SF2 between the amino acids present at two conserved positions of domains II and VI. Proteins having the signature DEAD in segment II had HxxGRxxR in segment VI, and proteins with DExH in segment II had Gln in place of His in motif VI [3,34]. The identification in the PPV CI protein of an RNA binding domain adjacent to, or overlapping, sequences involved in NTP hydrolysis also supports the close coupling between these two biochemical activities. The collaboration of two distant sequences for an effective RNA binding that we have found in the PPV CI protein seems to be a strategy adopted by a large number of proteins that interact with RNA. Further genetic and biochemical analysis on the RNA helicase activity of the PPV CI protein should help to unravel how both RNA binding domains are engaged in the protein function.

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