Beet necrotic yellow vein virus 42 kDa triple gene block protein binds nucleic acid in vitro

Claudine Bleykasten,* D. Gilmer, H. Guilley, K. E. Richards and G. Jonard

Institut de Biologie Moléculaire des Plantes du CNRS et de l’Université Louis Pasteur, 67084 Strasbourg Cedex, France

The triple gene block (TGB) of beet necrotic yellow vein virus RNA 2 is required for cell-to-cell movement of the virus RNA. The protein P42 encoded by the 5’-proximal gene of the TGB has consensus sequence motifs characteristic of an ATP/GTP-dependent helicase. P42 was over-expressed in *Escherichia coli* and shown to bind both single- and double-stranded RNA and DNA by Northwestern blotting. Site-directed mutagenesis located the nucleic acid-binding domain to the N-terminal 24 amino acids of the protein and a point mutation or deletions in the region of P42 containing the helicase consensus sequences did not affect nucleic acid-binding activity of the immobilized protein. Electrophoretic mobility-shift assays revealed that P42 also binds nucleic acids in solution and that deletion of the N-terminal region inhibits this binding. Mutations in both the N-terminal nucleic acid-binding domain and the helicase domain blocked infection of leaves, indicating that both regions of P42 are important for its activity in vivo.

Introduction

Beet necrotic yellow vein virus (BNYVV) is responsible for the rhizomania disease of sugarbeet. The genome of the virus consists of four plus-sense ssRNA molecules ranging from 6746 (RNA 1) to 1467 (RNA 4) nucleotides in length (for review see Richards & Tamada, 1992). RNA 1 encodes the virus replicase and is necessary and sufficient for infection of protoplasts. Moreover, RNA 1 combined with RNA 2 are necessary and sufficient for infection of leaves. RNAs 3 and 4 encode accessory functions involved in infection of roots and virus transmission by the fungal vector, *Polymyxa betae*. The genetic organization of BNYVV RNA 2 is shown in Fig. 1. The 5′-proximal pair of genes encodes the major and minor virus capsid proteins (Schmitt et al., 1992; Haeberle et al., 1994) and the 3′-proximal gene encodes a protein which regulates RNA 2 replication and coat protein expression (Hehn et al., 1995).

The central portion of RNA 2 contains a cluster of three slightly overlapping genes known as the triple gene block (TGB), encoding, in order, the proteins P42, P13 and a putative protein P15. Mutations to knock out each of these proteins separately did not interfere with infection of protoplasts but abolished lesion formation on leaves, demonstrating that the TGB plays a role in cell-to-cell movement of the virus RNA (Gilmer et al., 1992). TGB homologues (Petty & Jackson, 1990; Beck et al., 1991; Koonin & Dolja, 1993; Herzog et al., 1994) are present in the genomes of a number of other plant viruses, including the potex- and carlaviruses, barley stripe mosaic hordeivirus (BSMV) and peanut clump furovirus (PCV).

Little information is available concerning the mechanism by which the TGB proteins might facilitate cell-to-cell movement. It is known, however, that defective coat protein mutants of BNYVV and BSMV are infectious to leaves, indicating that virion formation is not required for passage from one cell to another (Petty & Jackson, 1990; Schmitt et al., 1992). This observation suggests that TGB-mediated cell-to-cell movement involves a virus protein capable of interacting with virus RNA. The first TGB protein of BNYVV, P42, has sequence motifs characteristic of a superfamily I DNA or RNA helicase (Koonin & Dolja, 1993), including a ‘P-Loop’ ATP/GTP-binding domain (Fig. 1). These motifs are also present in the corresponding TGB proteins of the other viruses referred to above. In this paper we have expressed BNYVV P42 in bacteria and showed that it possesses nucleic acid-binding activity. Site-directed mutagenesis revealed that the binding site is situated near the N terminus of the protein. The P-Loop motif is not required for nucleic acid binding. Deletions within both the N-terminal nucleic acid-binding domain and the

* Author for correspondence. Fax +33 88 61 44 42.
e-mail bleykasten@medoc.u-strasbg.fr

0001-3735 © 1996 SGM
helicase domain of P42 blocked local lesion formation on leaves of Chenopodium quinoa.

Methods

Bacterial strains. Escherichia coli strain DH5α (Sambrook et al., 1989) was used for all cloning steps and plasmid amplification and E. coli BW313 (Kunkel et al., 1987) was used to obtain uridylated ssDNA for site-directed mutagenesis. Protein expression was performed in E. coli BL21(DE3)pLysS (Studier et al., 1990).

Construction of expression plasmids for wild-type and mutant P42. A BglII–PvuII restriction fragment (nucleotides 2077–3467; sequence coordinates refer to the complete RNA 2 sequence; Bouzoubaa et al., 1986) encompassing the P42 ORF was inserted between the BamHI and SmaI restriction sites of pBluescript BS(−) (Stratagene) to produce pBS-42. This plasmid was then modified by site-directed mutagenesis (Kunkel et al., 1987) to create an NcoI restriction site at the ATG initiation codon of the P42 ORF (nucleotides 2133–3286) and a BglII site immediately downstream of its termination codon. The P42 ORF of the resulting plasmid, pBS-42NB, was completely sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). The NcoI–BglII restriction fragment from pBS-42NB was cloned between the NcoI and BamHI sites of pET-3d (Studier et al., 1990) to give pET42. Another construct was obtained by PCR using primers designed to create an NcoI site at the ORF initiation codon and a six histidine ‘tag’ sequence (His6; Hochuli, 1990) plus a termination codon and a BamHI site at the 3' terminus. The PCR fragment was cut with NcoI and BamHI and ligated into NcoI-BamHI-cleaved pET-3d to produce pET42H. The insert in pET42H was shown to be free of errors by sequence analysis.

Mutant pET42HASN was obtained by removing an NcoI–SpeI fragment from pET42H, filling in the extremities and using the Klenow fragment of DNA polmerase I and nification. Mutant pET42HAS37 was produced by removing the SpeI–HpaI fragment, filling in the SpeI extremity with the Klenow fragment in the presence of dCTP and dTTP, removing the two 5'-protruding nucleotides with mung bean nuclease following the supplier's instructions (New England Biolabs) and ligation. pET42HAS12 and AH7 were obtained by exonuclease III (Pharmacia) deletion at the insert SpeI or HpaI restriction site following the supplier's instructions. For mutant pET42HAS37, pET42H was linearized with SpeI, subjected to partial digestion with exonuclease III followed by treatment with the Klenow fragment to produce blunt extremities. After digestion by SacI, an approximately 500 bp fragment was purified and inserted into pET42H which had been cut with SpeI (followed by treatment with the Klenow fragment) and then SacI. Mutants pET42AB1, AB2, AN and AHP were obtained by subcloning PCR fragments amplified with primers that introduced various in-frame deletions. All fragments generated by PCR were sequenced. The point mutant pET42HGAA, in which the P-Loop NTP-binding motif was modified to GAA, was obtained by site-directed mutagenesis. Mutagenesis was performed on pBS-42NB and the NcoI–BstXI fragment containing the mutation was cloned between the NcoI and BstXI sites of pET42H.

Plasmids containing full-length RNA 2 cDNA are not stable in most strains of E. coli but it was recently observed that a pBluescript construct containing full-length RNA 2 cDNA (pB2-14) could be maintained in E. coli strain MC1022 and that derived RNA transcripts were infectious (A. Hehn & D. Prüfer, personal communication). Using PCR-based mutagenesis, an NcoI site was introduced at the 5' terminus of the P42 ORF of pB2-14 to produce pB2-14Nco. This plasmid was linearized by partial digestion with NcoI, then cut with SacI and the NcoI–SacI restriction fragment of the full-length clone was replaced by

---

Fig. 1. Cloning and mutagenesis of the P42 ORF. The position of the P42 ORF in BNYVV RNA 2 is shown at the top. The P-Loop GXXXXGKS (NTP-binding motif) is represented by a filled circle (or an empty circle for p42HGAA) and helicase motifs I to VI by hatching. The C-terminal histidine tag in the translated protein is symbolized by (His)6. In the deleted proteins, the remaining portions of the wild-type sequence are represented by rectangles and the deletion by a dashed line. When cloning steps created a new codon at the border of a deletion, the corresponding extra amino acid is shown within the appropriate rectangle. Restriction sites used to create certain mutants are shown at the bottom.
the corresponding fragments from the pET42H mutants ASN, AS12, AS37, GAA, AB1, AB2 and AN to produce the RNA 2 mutants pB2-14ASN, pB2-14AS12 and so on. For simplicity, these full-length clones will sometimes be referred to as 2Nco, 2ASN, etc.

**Protein expression and analysis.** Recombinant E. coli BL21(DE3)pLysS transformed by each construction in pET-3d were selected on ZB solid medium (10 g/l bacitracin, 5 g/l NaCl, 12 g/l agar) containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. Bacteria were grown at 37°C in L-broth (Sambrook et al., 1989) containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. After 5 h, lacUV5 promoter was induced by adding 0.4 mM IPTG and the cells were harvested 3 h after induction at 28 °C. Bacteria were lysed by boiling in 1 x Laemmli sample buffer (Laemmli, 1970) and the protein pattern was analysed by SDS-PAGE.

**Protein purification by one-step affinity chromatography.** For preparative-scale purification of P42H and mutants, a 300 ml culture of E. coli carrying the appropriate expression plasmid was grown at 37 °C to an OD600 of 0.5, cooled in ice and induced for 3 h with 0.4 mM IPTG at 28 °C. After centrifugation at 4000 g for 12 min, the pellet was resuspended in 5 ml 50 mM-Tris–HCl pH 8.0 supplemented with 1 mM- PMSF, followed by three cycles of freeze-thaw (liquid nitrogen –37 °C) and a few seconds of sonication. Heavy material was collected by 15 min centrifugation at 4000 g and the pellet was resuspended in 5 ml of B buffer (15 mM-Tris–HCl pH 8.0, 8 M-urea) plus 10 mM-imidazole. Recombinant protein was immobilized in batches on 2 ml Ni2+-NTA resin (Qiagen) equilibrated in B buffer. After 30 min, the supernatant was removed and the resin was washed extensively with B buffer plus 10 mM-imidazole and then with B buffer plus 50 mM-imidazole. Bound protein was eluted by adding one volume of E buffer (15 mM-Tris–HCl pH 7.0, 8 M-urea, 400 mM-imidazole) and this operation was repeated once. The combined eluates were supplemented with 0.5% Tween 20 and the concentration of recombinant protein was adjusted to 100 ng/μl by comparison to a standard of known concentration after SDS-PAGE.

The purified recombinant protein was renatured by dialysis at 4 °C against 21 of buffer SU (15 mM-Tris–HCl pH 8.0, 2 M-urea, 150 mM-NaCl, 0.5% Tween 20). After 1 h the original buffer SU was gradually replaced at the rate of 3 ml/min by buffer S (as buffer SU but without urea). After 24 h, the protein solution was further dialysed overnight against 21 of buffer S. The solution of renatured protein was then centrifuged at 18000 g for 30 min to remove aggregates that formed during the above procedure and the supernatant was stored at 4 °C in silanized Eppendorf microtubes.

**Nucleic acid probes.** 32P-labelled ssRNA probes were transcribed (Lemaire et al., 1988) with T3 or T7 RNA polymerase from linearized pBluescript containing a 12 kb cDNA fragment of the BNYVV genome (nucleotides 2324–3789 of RNA 2) or a 1.4 kb segment of the genomic cDNA of tomato black ring virus (TBRV) RNA 1, kindly provided by C. Fritsch (this laboratory).

For electrophoretic mobility-shift assays (EMSAs), an approximately 100 residue 32P-labelled RNA probe corresponding to the polylinker was obtained by T7 RNA polymerase transcription of XhoI-linearized pBluescript. After transcription, the DNA template was eliminated by treatment with DNase I and free NTPs were removed by passage through a Sephadex G50 minicolumn. Proteins were extracted by phenol–chloroform and the RNA was precipitated with two volumes ethanol plus 2 M-ammonium acetate. After centrifugation, the pellet was washed with 70% ethanol and suspended in 100 μl water. Immediately before use, the RNA was diluted 10-fold in 10 mM-Tris–HCl pH 8.0, 50 mM-NaCl, 1 mM-EDTA and 5 mM-magnesium acetate, heated at 65 °C for 2 min and slowly cooled to room temperature.

**Northwestern blotting and filter-binding assay.** Total protein extracts from bacteria expressing wild-type P42H, the various P42H mutants (except for mutant P42HASH3), or control bacteria transformed with the empty vector pET-3d, were separated by SDS-PAGE on 10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was washed twice for 30 min with an aqueous solution of 6 M-urea and 0.1% NP-40 (Ossipow et al., 1993) and then with 10 mM-Tris–HCl pH 8.0, 1 mM-EDTA, 50 mM-NaCl, 2 mg/ml BSA, 2 mg/ml Ficoll and 2 mg/ml polyvinyl pyrrolidone (R buffer) for 1 h with two changes of buffer (Gramstat et al., 1990). The membrane was then incubated with 100 000 d.p.m./ml radiolabelled nucleic acid in R buffer for 1 h at room temperature. After incubation, the membrane was washed with 10 mM-Tris–HCl pH 8.0, 1 mM-EDTA and 200 mM-NaCl for 1 h with two changes of wash solution. Radioactive bands were identified by autoradiography. In some cases, the radioactivity in the bands was quantified using a Fuji XMAS1000 BioAnalyzer.

Extracts of bacteria expressing P42HASH3 were subjected to electrophoresis in a Tricine–SDS-PAGE system (Schägger & von Jagow, 1987) using a 14.5% polyacrylamide resolving gel (acylamide:bisacylamide 46:5:3). Electroblotting to nitrocellulose was performed in 10 mM-NaHCO3, 3 mM-Na2CO3 and 20% ethanol pH 9.9 (Rosenbaum et al., 1989) and, after transfer, the wet nitrocellulose was subjected to UV-crosslinking (0.05 J) in a UV Stratalinker 2400 (Stratagene). Otherwise, treatment of nitrocellulose and probe binding were carried out as described above.

For filter-binding assays, approximately 1 μg purified recombinant protein was fixed to nitrocellulose by filtration on a slot blot apparatus (Gibco BRL). Subsequent steps of nitrocellulose filter treatment and probe binding were as described for Northwestern blot assays.

**EMSAs.** RNA–protein binding reactions were performed at room temperature in a final volume of 15 μl using 80 000 d.p.m. of the 100 residue RNA radiolprobe and 500 ng of purified wild-type or mutant P42H. The reaction buffer contained 10 mM-Tris–HCl pH 9.0, 100 mM-NaCl, 0.5% Tween 20, 1 μg/ml RNasin, 1 mM-DTT and 0.5 mM-EDTA.

For 1 h, one-third volume of 50% glycerol and 0.1% bromophenol blue was added and the mixture was immediately loaded on a 6% polyacrylamide (acylamide:bisacylamide 37:5:1) non-denaturing gel which had been pre-run for 1 h. The gel was run in the gel and for electrophoresis was 0.5 × TBE (50 mM-Tris–borate, 1 mM-EDTA pH 8.3). Electrophoresis was performed at 7 V/cm for 3 h at room temperature. The gel was fixed for 15 min in 10% acetic acid and 20% ethanol and autoradiography was carried out directly on the wet gel.

**Protoplast and plant infection.** Wild-type RNA 1 transcript plus wild-type or mutant RNA 2 transcript were inoculated to C. quinoa protoplasts or leaves as described (Hehn et al., 1995) and accumulation of progeny virus RNA was assessed by Northern blot hybridization 48 h post-inoculation for protoplasts and 12 days post-inoculation for leaves.

**Results and Discussion**

*Expression of P42 in bacteria and its purification*

To obtain P42 in amounts suitable for biochemical characterization, the P42 ORF was inserted into a pET expression vector (Studier et al., 1990) and over-
expressed in *E. coli*. For cloning purposes, the sequence in the vicinity of the P42 initiation codon (AGGATGG; initiation codon underlined) was modified by site-directed mutagenesis to create an *NcoI* site (ACCATGG). To facilitate purification of the over-expressed protein, a sequence coding for His$_6$ was introduced in-frame at the 3' end of the coding region, followed by a termination codon and a *BamHI* site. The resulting P42H ORF was then cloned as an *NcoI–BamHI* fragment between the *NcoI* and *BamHI* sites of pET-3d to produce pET42H (Fig. 1). A second plasmid, pET42, which contained the 3' terminal *BamHI* site but did not code for the His$_6$ sequence, was constructed in a similar fashion.

pET42H and pET42 were transferred into *E. coli* BL21(DE3)pLysS and expression of the inserted protein was induced with IPTG. The total protein content of the bacteria was assessed 3 h post-induction by SDS-PAGE. Coomassie Brilliant Blue staining revealed that the bacteria transformed by pET42 and pET42H (Fig. 2a, lanes 2 and 3) produced abundant amounts of an approximately 42 kDa species which was absent in the protein profile from bacteria transformed with the empty vector pET-3d (Fig. 2a, lanes 1 and 13). In Western blot experiments, the 42 kDa band was recognized by a P42-specific antiserum (Niesbach-K16sgen *et al.*, 1990; Fig 2b, lane 2), confirming its identity as P42. About 100 μg of P42 was produced per ml of bacterial culture, corresponding to 20 μg P42 protein per mg of fresh bacterial pellet. Fractionation experiments following bacterial lysis revealed that the bacterially expressed P42 (with or without a His$_6$ tag) was almost exclusively associated with cellular debris, presumably as inclusion bodies (data not shown). Attempts to produce P42 and P42H in a soluble form by modification of the conditions of bacterial culture or IPTG induction were not successful.

Fig. 2(a), lanes 4–12 and 14 show the total protein content of bacteria transformed with plasmids expressing the mutated forms of P42H described in Fig. 1. All mutant proteins were found to accumulate in the induced bacteria in amounts roughly comparable to wild-type P42H and were detected by the P42-specific antiserum (data not shown).

**Nucleic acid-binding activity of immobilized P42H**

The affinity of P42H for nucleic acids was tested by Northwestern blot assay. Following induction, total protein from bacteria expressing the recombinant protein was subjected to electrophoresis in a 10% polyacrylamide gel under denaturing conditions, electroblotted onto nitrocellulose and renatured *in situ* (see Methods). The blot was then incubated with a $^32$P-labelled probe consisting of an RNA transcript complementary to nucleotides 2324–3789 of BNYVV RNA 2. After washing to eliminate excess probe, radioactive RNA fixed to the immobilized protein was visualized by autoradiography. A band corresponding to the position of P42H was visible on the autoradiogram (Fig. 3a, lane 3) but was absent when a protein extract derived from bacteria transformed with the empty vector, pET-3d, was subjected to the same procedure (Fig. 3a, lane 1). Note that, in addition to the recombinant P42H, several bacterial proteins in the extract also displayed nucleic acid-binding activity in some experiments (Fig. 3a).

A ssRNA probe also bound to immobilized P42 (Fig. 3a, lane 2), proving that the C-terminal His$_6$ tag of P42H was not responsible for the *in vitro* RNA-binding activity. No labelling was observed when the blot was incubated
Nucleic acid-binding of BNYVV P42 protein

Fig. 3. Binding of immobilized P42/P42H to nucleic acid probes. (a) Northwestern blot of proteins from recombinant E. coli BL21(DE3)pLysS expressing empty vector pET-3d (lane 1), P42 (lane 2) and P42H (lanes 3–7). Radioactive probes were 1.2 kb BNYVV RNA transcript (lanes 1–3), 1.4 kb TBRV RNA transcript (lane 4), ssDNA (lane 5), dsDNA (lane 6) and dsRNA (lane 7). The position of P42 and P42H is indicated by a triangle and bacterial nucleic acid-binding proteins by dots. (b) The effect of salt concentration on the binding of purified P42H to ssRNA. Binding is expressed relative to the value observed at 50 mM-NaCl (1.0). (c) Northwestern blot of proteins from E. coli BL21(DE3)pLysS expressing P42HGAA (lane 1), P42HASN (lane 2), P42HASN2 (lane 3), P42HASN37 (lane 4), P42HASN1 (lane 5), P42HASN2 (lane 6), P42HASN (lane 7), P42HASN17 (lane 8), P42HASN (lane 9), empty vector pET-3d (lane 10) and 4 μg, 2 μg, 1 μg or 0.5 μg of P42HASN (lanes 11–14). Electrophoresis was performed in a 10% SDS-polyacrylamide gel (lanes 1–9) or in a Tricine-SDS-14.5% polyacrylamide gel (lanes 10–14). The radioactive probe was single-stranded BNYVV transcript. (d) The sequence of the N-terminal 24 amino acids of P42. Basic residues are underlined.

Fig. 4. EMSA for nucleic acid-binding with purified P42-related proteins. (a) Purity of affinity chromatography-purified P42-related proteins assessed on a silver-stained 10% SDS-polyacrylamide gel (Sambrook et al., 1989); total bacterial proteins prior to purification (lane 1), purified P42H (lane 2), P42HGAA (lane 3), P42HASN (lane 4), P42HASN1 (lane 5) and an extract from bacteria carrying empty pET-3d after being subjected to affinity chromatography (lane 6). (b) EMSA for RNA probe-binding carried out with no added protein (lane 1), the ‘purified’ protein from bacteria transformed with pET-3d (lane 2), P42H (lanes 3–5 and 9–10), P42HASN (lane 6), P42HGAA (lane 7), P42HASN1 (lane 8) or 500 ng BSA (lane 11). In lane 4, the P42H protein was eliminated by adsorption to Ni²⁺-NTA-agarose before incubation with the probe and in lane 5, the Ni²⁺-NTA matrix was washed with EDTA prior to use. For the competition experiment (lane 9), 300 ng poly(U) was present during the probe-binding reaction. In lane 10, the binding reaction was performed at 450 mM-NaCl. The position of unbound probe is indicated by a black triangle, major bands displaying retarded mobility are indicated by empty triangles and the gel pockets by ‘P’.

with [α-32P]UTP alone or with [γ-32P]UTP in the presence of non-labelled RNA (data not shown), ruling out the possibility that the radioactivity associated with the immobilized P42/P42H was due to binding of [α-32P]UTP which had not been incorporated into the transcript. Finally, the ionic strength dependence of the binding of ssRNA was examined using a filter-binding assay. Binding of RNA probe at different salt concentrations displayed a broad maximum between 250–300 mM-NaCl (Fig. 3b) but, unless otherwise noted, probe fixation in the Northwestern blot experiments was carried out at 50 mM-NaCl.

Immobilized P42H was able to bind a ssRNA transcript derived from an unrelated virus (TBRV; Fig. 3a, lane 4) and to ssDNA (Fig. 3a, lane 5), dsDNA (Fig. 3a, lane 6) and, with somewhat lower apparent affinity, to dsRNA (Fig. 3a, lane 7). Thus, the in vitro binding of nucleic acid by P42/P42H is not sequence-specific and displays no discrimination between single-stranded and double-stranded RNA and DNA, a novel feature among known and putative plant virus movement proteins. The lack of sequence specificity in in vitro assays is a property common to other nucleic acid-binding, putative movement proteins (Citovsky et al., 1990; Osman et al., 1992; Schoumacher et al., 1992; Rouleau et al., 1994). However, it is possible that, in vivo, movement proteins
display greater binding specificity (Fujiwara et al., 1993) or that binding specificity is conferred on P42 by either of the other two TGB proteins.

**Location of a nucleic acid-binding domain on P42**

To localize the region of P42 possessing nucleic acid-binding activity, the collection of P42H mutants (Fig. 1) was tested by Northwestern blot assay (Fig. 3c, lanes 1–9 and 11–14). All mutants reproducibly displayed RNA-binding activity except for P42HASN (Fig. 3c, lane 2), which lacks amino acids 2–49. Similar results were obtained when the RNA probe was replaced by a ss- or dsDNA probe (data not shown). The fact that mutant P42HAS12 (amino acids 25–71 deleted) still possessed nucleic acid-binding activity (Fig. 3c, lane 3) suggested that the binding domain is located between amino acids 2 and 24. Note that mutant P42HASH3, which contains only the N-terminal residues 1–49 and C-terminal residues 342–384, could still bind the RNA probe (Fig. 3c, lanes 11–14), indicating that 75% of the P42 molecule is dispensable for RNA-binding activity. Because it is unlikely that an extensively deleted protein such as P42HASH3 could fold in a manner closely resembling the wild-type protein, the ability of P42H to bind nucleic acid, at least in the Northwestern blot assay, is probably conformation independent.

The N-terminal portion of P42 is rather hydrophilic (Fig. 3d) and is predicted by the computer program Peptidestructure (Devereux et al., 1984) to have a high probability of being localized on the surface of the folded protein. There are five basic amino acids in residues 2–24 of P42 and some or all of these may be important in RNA binding. Sequence comparisons revealed no obvious sequence homology with the RNA-binding domains of the tobacco mosaic virus 30 kDa movement protein (Citovsky et al., 1992) or with the RNA recognition motif of the 70 kDa U1 small nuclear ribonucleoprotein (Kenan et al., 1991).

**Nucleic acid-binding activity in solution: EMSA**

Wild-type P42H and the mutants P42HASN, P42HGAA and P42HΔB1 were purified by affinity chromatography with Ni²⁺-NTA under denaturing conditions. SDS-PAGE followed by silver staining revealed that this procedure eliminated the bulk of the bacterial proteins present in the total extract (Fig. 4a, lane 1) and produced a major band of recombinant protein of the expected size (Fig. 4a, lane 2).

The 6 M-urea in the solutions of purified proteins was eliminated by dialysis and the affinity of wild-type or mutant P42H proteins for nucleic acids in solution was tested by EMSA. In the absence of protein, the α²P-labelled RNA probe migrated in a 6% non-denaturing polyacrylamide gel as a major, rapidly migrating band which was accompanied in some experiments by a minor band of lower electrophoretic mobility and a small amount of material remaining in the pocket (Fig. 4b, lane 1). The major and minor bands are believed to represent conformational isomers because only a single band was observed when a sample from the same preparation of probe was subjected to electrophoresis in denaturing conditions (data not shown). When incubated with 500 ng of P42H prior to electrophoresis, the amount of rapidly migrating, unbound probe diminished and two or more bands of lower mobility appeared, presumably as a result of complex formation between the probe and P42H (Fig. 4b, lane 3). A considerable amount of radioactivity also remained in the gel pocket (Fig. 4b), which may attest to the formation of higher order aggregates of P42H and probe.

No retardation of probe mobility was observed when the probe was incubated with 500 ng BSA (Fig. 4b, lane 11) or with a protein extract obtained by the same affinity chromatography purification procedure from bacteria transformed with the empty vector pET-3d (Fig. 4b, lane 2). If P42H was preincubated with Ni²⁺-NTA resin to sequester the His⁶-tagged protein before incubation with the probe, no band-shift was observed (Fig. 4b, lane 4), but this effect could be reversed if the Ni²⁺ ligand before exposure to P42H (Fig. 4b, lane 5). These latter experiments tend to rule out the possibility that the band-shift apparently provoked by P42H was due to binding of the probe by small amounts of a co-purifying contaminant. The P42H-induced mobility-shift of the RNA probe still occurred at high salt concentrations (450 mM-NaCl; Fig. 4b, lane 10), but was inhibited by addition of excess unlabelled poly(U) (Fig. 4b, lane 9), indicating that P42H has affinity in solution for other RNA species.

Complex formation occurred when the RNA probe was incubated with P42HGAA (Fig. 4b, lane 7) and P42HΔB1 (Fig. 4b, lane 8). We conclude that the ATP/GTP-binding activity presumably associated with the P-Loop is not required for RNA fixation. In the case of P42HΔB1, most of the radioactive probe reproducibly remained in the pocket rather than entering the gel (compare Fig. 4b, lanes 3 and 8). This may indicate that the deletion in P42HΔB1 alters the mode of interaction of the mutant protein with the probe or promotes self-association of the protein. On the other hand, no band-shift was observed when P42HASN was incubated with the probe (Fig. 4b, lane 6). Taken together, these EMSA results confirm the findings from the Northwestern blotting experiments that the N-terminal portion of P42 is required for RNA-binding while other portions of the molecule (or at least the P-Loop) are not required.
Nucleic acid-binding of BNYVV P42 protein

Fig. 5. Test for virus accumulation in protoplasts (a) and leaves (b) inoculated with RNA 1 transcript plus RNA 2 transcripts carrying mutations in P42. The protoplasts or leaves were mock-inoculated (lane 1) or inoculated with RNA 1 transcript plus different RNA 2 transcripts corresponding to the wild-type (lane 2), pB2-14Nco (lane 3), 2ASN (lane 4), 2A12 (lane 5), 2Δ37 (lane 6), 2GAA (lane 7), 2ΔB1 (lane 8), 2ΔB2 (lane 9) and 2ΔN (lane 10). In (a), lane 11 shows RNA from protoplasts inoculated with virion RNA of isolate Stras 12 (Quillet et al., 1989). Virus RNAs were detected in the total RNA extracts using 32P-labelled antisense probes specific for BNYVV RNAs 1 and 2 (Hehn et al., 1995).

Effect of P42 mutations in vivo

A transcription vector (pB2-14Nco) was constructed carrying the full-length RNA 2 cDNA, but with the sequence around the P42 initiation codon (AGGATGGT) modified to contain an NcoI restriction site (ACCATGGT). When co-inoculated with RNA 1 transcript, both the pB2-14 and the pB2-14Nco transcripts replicated efficiently in C. quinoa protoplasts (Fig. 5a, lanes 2 and 3). These transcript mixtures were also infectious to C. quinoa leaves (Fig. 5b, lanes 2 and 3) and produced typical symptoms.

To determine their effect on the activity of RNA 2 in vivo, all but two of the P42 mutations analysed in the nucleic acid-binding experiments were introduced into pB2-14Nco. Constructs containing the two most 3′-proximal deletions of the P42 ORF, corresponding to mutants ΔH7 and ΔHP (see Fig. 1), were not used in this study because of concern that the deletions would interfere with production of the subgenomic RNA thought to direct synthesis of P13 (Gilmer et al., 1992).

When co-inoculated with RNA 1 transcript into protoplasts, all the mutant RNA 2 transcripts were efficiently replicated (Fig. 5a, lanes 4–10), demonstrating that the mutations did not disable cis-acting sequences on RNA 2 necessary for its replication. When inoculated along with RNA 1 to C. quinoa leaves, however, none of the mutant RNA 2 transcripts produced an infection. No local lesions were observed and no virus RNA was detectable in the inoculated leaves by Northern blot hybridization (Fig. 5b, lanes 4–10).

The fact that deletion of the N-terminal nucleic acid-binding site in pB2-14ASN is lethal for infection of whole plants supports (but does not prove) the idea that the binding activity detected in vitro is relevant to virus movement in vivo. The fact that mutations in the helicase domain of P42 are also lethal in whole plants indicates that this region of P42 is important for movement as well.

Proteins containing the P-Loop (domain 1) and the associated motifs II–VI of the helicase are present in the genomes of a large number of RNA viruses (Koonin & Dolja, 1993). These proteins are generally assumed to possess RNA helicase activity although this has been

![Fig. 6. Schematic representation of the structure of the TGB protein analogue of BNYVV P42 in different viruses. Hatching represents the helicase motifs and the filled circle the ATP/GTP-binding domain, as in Fig. 1. PVX, potato virus X; WCIMV, white clover mosaic virus; PVM, potato virus M; BSMV, barley stripe mosaic virus; PCV, peanut clump mosaic virus. Size group (1) and (2) TGBs are defined in the text.](image)
demonstrated experimentally in only a few cases (Lain et al., 1990; Shuman, 1992; Eagles et al., 1994). The putative helicase domains of members of the Sindbis-like superfamily of viruses (including the tobamoviruses) and the picornavirus-like virus superfamily (including the potyviruses) are believed to be involved in virus RNA replication, although participation in other functions cannot be ruled out.

Viruses with a TGB all belong to the Sindbis-like superfamily and thus possess two copies of the helicase consensus sequence: one presumably associated with RNA replication (present on the RNA 1 gene product of BNYVV; Bouzoubaa et al., 1987) and one mapping to the first protein of the TGB. For those viruses possessing a TGB, it is interesting to note that the 5'-proximal ORF of the TGB, which contains the helicase domain, falls into two size classes (Fig. 6): (1) approximately 25 kDa for the potex- and carlavirus and (2) about twice this size for BNYVV, PCV (51 kDa; Herzog et al., 1994) and BSMV (58 kDa; Gustafson & Armour, 1986). The helicase domain comprises the C-terminal 20 kDa or so of each protein. Thus, the TGB members of size class 2 possess a long N-terminal extension which is absent from the members of size class 1. The nucleic acid-binding domain of P42 maps within this extension, raising the possibility that the potex- and carlavirus helicases may bind to RNA by a different mechanism. It is not yet known if the N-terminal extensions of the PCV 51 kDa and BSMV 58 kDa proteins also possess nucleic acid-binding activity.

The RNA-binding subdomain of the plum pox potyvirus CI protein (helicase superfamily 2) has been mapped to the region containing helicase motif VI (Fernandez et al., 1995). Helicase motif VI of the eIF-4A protein has also been shown to possess RNA-binding activity (Pause et al., 1993). In the case of BNYVV P42, motif VI can be deleted (mutants P42HAVH7 and DSH3) without affecting RNA-binding activity in the North-western blot assay. Furthermore, no RNA binding was detected when motif VI was retained and the N-terminal region was deleted (mutant P42HAVSN), indicating that motif VI of P42 does not independently bind RNA to a significant extent, at least in our assay conditions. These results suggest that the mechanism of reaction between P42 and RNA may be different from that of the virus helicase domains thought to be involved in RNA replication.

References


---

*(Received 7 November 1995; Accepted 9 January 1996)*