Mapping of the RNA-binding domain of the alfalfa mosaic virus movement protein

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In-frame contiguous deletions were created in the movement protein gene of alfalfa mosaic virus by site-directed mutagenesis. The mutated movement proteins were expressed in Escherichia coli, extracted and then purified by denaturing gel electrophoresis and then renatured. Their binding ability with RNA was assayed by electrophoretic retardation and u.v.-crosslinking. Results indicated that a domain included within amino acids 36 to 81 was necessary for RNA binding.

The alfalfa mosaic virus (AMV) movement protein encoded by RNA3 (P3, 300 amino acids) has been shown to bind single-stranded nucleic acids in vitro (Schoumacher et al., 1992a and b), as do several other viral movement proteins. These are the 30K protein of tobacco mosaic virus (TMV) (Citovsky et al., 1990), the gene I product of cauliflower mosaic virus (CaMV; Citovsky et al., 1991), the 35K protein of red clover necrotic mosaic virus (RCNMV) (Osman et al., 1992), the ORF2 (open reading frame 2) product of foxtail mosaic potexvirus (Rouleau et al., 1993) and the putative 17K movement protein of potato leafroll luteovirus (Tacke et al., 1993). The nucleic acid binding domain(s) have been localized in the movement proteins of TMV (Citovsky et al., 1990, 1992) and RCNMV (Osman et al., 1992, 1993; Giesman-Cookmeyer & Lommel, 1993). Deletion mapping identified two binding domains in the TMV movement protein; one of them was active independently of the rest of the molecule and is qualified as 'independent' but the other was not (Citovsky et al., 1992). By the same approach, an independent RNA binding domain was detected in the RCNMV movement protein (Osman et al., 1993). In addition, alanine-scanning mutations affecting several other regions of the molecule decreased the affinity of the movement protein for RNA and the cooperativity of binding; mutations in one of these regions also affected the biological function of the protein (Giesman-Cookmeyer & Lommel, 1993).

The nucleic acid binding activities of the TMV and CaMV movement proteins are presumed to be relevant to the movement function because, upon binding, the nucleic acid molecule becomes elongated and 'shaped into a form suitable for transport through plasmodesmatal channels' (Citovsky et al., 1992). However, binding of the RCNMV movement protein was shown recently not to affect the overall length of RNA molecules (Fujiwara et al., 1993).

There is no evidence that the mechanisms of transport are identical for all plant viruses; indeed some evidence to the contrary is emerging (Atabekov & Talianksy, 1990; Harrison et al., 1990). In particular, the movement mechanisms of AMV and TMV may differ significantly because AMV, unlike TMV, seems to require its coat protein for cell-to-cell movement (van der Kuyl et al., 1991) and also the AMV movement protein does not have the same nucleic acid binding characteristics as the TMV 30K protein possesses (Schoumacher et al., 1992a and b). We undertook to locate the RNA binding domain(s) of the P3 protein by deletion mapping in order to compare its (their) structure with those of the TMV and RCNMV movement proteins. For this purpose, we created a set of eight contiguous in-frame deletions in the P3 gene ORF and expressed the corresponding proteins in Escherichia coli. The proteins were purified and their RNA binding activity was tested. The set of deletions of the P3 ORF shown in Fig. 1 was obtained by site-directed mutagenesis (creation of unique HindIII sites) and segment reassortment. Mutagenesis was carried out on the plasmid designated pSel1.P3N (Schoumacher et al., 1992b). The latter had been constructed by placing the complete P3 ORF between the SalI and BamHI sites of a pAlter-1 phagemid.

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Fig. 1. Mutagenesis of the P3 ORF. The wild-type P3 ORF is designated P3 wt and the deleted ORFs are designated P3A[x–y], where x and y indicate respectively the first and the last amino acid of the deleted portion, as numbered in the wild-type sequence (Ravelonandro et al., 1984). The positions of the HindIII restriction sites that were either originally present (▲) or created by mutagenesis are represented by vertical bars in the upper diagram (P3 wt), with the original dipeptides indicated underneath and amino acid numbering given above. In the deleted ORFs, the remaining portions of the wild-type sequence are represented by rectangles above which the inserted amino acids are indicated. The amino acids that border the deletions are shown within the rectangles, with changes indicated in bold type.

Facilitate subsequent insertion of the ORF in the expression vector pET-3d (Studier et al., 1990). After eliminating conservatively the natural HindIII restriction site of the P3 gene (encoding a KL dipeptide at positions 112 to 113 of the amino acid sequence), several other hexanucleotides, encoding identical or similar dipeptides (KL, KI or KM at nucleotide positions 35 to 36, 81 to 82, 156 to 157, 213 to 214 and 242 to 243) were substituted to create unique HindIII sites. In addition, a mutant (designated ‘A’) was created by inserting a HindIII site immediately upstream of the twenty-first codon. The original pSel.1.P3N plasmid and the mutagenized plasmids derived from it were digested by HindIII (in the P3 gene ORF) and by EcoRV (at a unique site located about 580 nucleotides upstream of the Ncol site). The internal deletions were created by reassembling the small EcoRV–HindIII fragment and the large HindIII–EcoRV fragment from two mutants carrying the HindIII site at successive locations. To obtain the N-terminally deleted gene, a linker carrying an Ncol site with the ATG
in phase with the rest of the sequence was inserted upstream of the HindIII site of mutant A. To obtain the C-terminally-deleted gene, a TGA triplet followed by a BamHI site was inserted after the 243rd codon. The sequence of 150 to 200 nucleotides bordering the mutations was confirmed with the dideoxynucleotide 'T7' chain termination system (Pharmacia), primed by the next mutagenic oligonucleotide downstream of the deletion created, or by a universal primer hybridizing to the phage T7 promoter adjacent to the polylinker of pAlter-1. Cloning techniques used in these and subsequent operations were as described by Sambrook et al. (1989).

The coding sequences to be expressed, obtained as NcoI–BamHI fragments from the pSeI1. P3N-derived plasmids, were inserted between the NcoI and the BamHI sites of the expression vector pET3d. The recombinant plasmids were cloned into E. coli strain BL21(DE3) and the proteins were extracted from inclusion bodies of the IPTG-induced bacteria with a urea-containing buffer (Schoumacher et al., 1992b). They were purified by SDS–PAGE in a 10% gel (Laemmli, 1970), eluted from the gel according to Hager & Burgess (1980), renatured by dialysis against 50 mm-Tris–HCl, pH 7.5, containing 10 mm-2-mercaptoethanol and 1% Tween-20 and stored at -20 °C after addition of glycerol (50% v/v final concentration). The purified mutant proteins were analysed in parallel with the full-length protein (Schoumacher et al., 1992b) by denaturing electrophoresis (Fig. 2a) and immunoblotting with an anti-P3 serum (Fig. 2b). A_gga scanning of the gel shown in Fig. 2(a) indicated that all these proteins were at least 90% pure. All the mutants reacted with the anti-P3 serum in the presence of 1% Tween-20, although the efficiency of detection was variable.

The renatured deleted proteins were tested for their ability to bind radioactively labelled RNA, using u.v.-crosslinking and electrophoretic retardation assays adapted from Citovsky et al. (1990). The incubations were done for 1 h at 27 °C in 40 mm-HEPES–NaOH, pH 7.0 (containing 12.5 mm-Tris–HCl, 2.5 mm-2-mercaptoethanol, 0-25% Tween-20 and 25% glycerol originating from the protein sample), in polypropylene microtubes which had been treated with dimethyldichlorosilane (2% solution in trichlorethane, obtained from LKB) and rinsed with deionized water. The RNA (141 nucleotides, collinear to the 5' end of AMV RNA3) was synthesized by the phage T7 RNA polymerase from an HaeIII fragment of the transcription plasmid 3BS(12.0) (Dore et al., 1990), in the presence of [α-32P]UTP (0.05 Ci/pmol).

The u.v.-crosslinking assay showed that all the deleted proteins (Fig. 3 lanes 2 to 9), except P3Δ[36–81] (Fig. 3 lane 4), could bind RNA as did full-length P3 (Fig. 3 lane 1). This was confirmed by the electrophoretic retardation assay (Fig. 4). Indeed all the mutant proteins except P3Δ[36–81] (Fig. 4 lane 4) decreased the mobility of RNA to some extent. Those which did bind RNA could be placed in two groups according to the degree of retardation observed. The first group comprising P3Δ[1–20] (Fig. 4 lane 2), P3Δ[21–34] (lane 3), P3Δ[113–156] (lane 6), P3Δ[215–243] (lane 8) and P3Δ[244–300] (lane 9) seemed to bind RNA as cooperatively as did full-length P3 (lane 1) because the only detectable species were the free and the fully retarded RNA. However, complete retardation of the bound RNA may have been due in some cases to aggregation of the protein. Aggregation, or incorrect conformation of the protein, could also cause the low efficiency of binding observed in lane 9 of Fig. 4. Indeed, P3Δ[244–300] had the characteristics of thermodynamically unstable proteins (Schein, 1990): poor solubility (of the order of 5 to 10 μg/ml after centrifugation for 20 min at 15000 g) and tendency to adsorb to polypropylene in spite of the presence of detergent. The lack of RNA binding activity of P3Δ[36–81] could also have been caused by incorrect renaturation. This seems unlikely however, because the latter protein did not aggregate or adsorb to plastic. An additional argument against improper renaturation is that this mutant had the same antibody reactivity as full-length P3 (Fig. 2). The second group of mutants, P3Δ[82–112] (Fig. 4, lane 5) and P3Δ[157–213] (Fig. 4 lane 7) bound the RNA with sufficient affinity to decrease its mobility but a significant proportion of the complexes (66% in the case of P3Δ[82–112] and 34% in the case of P3Δ[157–213]), evaluated by A_400 measurement) were incompletely retarded, indicating a low degree of cooperativity or a low binding constant. A similar
behaviour has been observed with some alanine-scanning mutants of the RCNMV movement protein (Giesman-Cookmeyer & Lommel, 1993).

In the summary the region spanning amino acids 36 to 81 of P3 appears necessary for RNA binding. For reasons explained above, we think it is unlikely that this region enables binding by an indirect effect on the conformation of the polypeptides as does the 65 to 86 amino acid region of TMV 30K (Citovsky et al., 1992). Thus the amino acids 36 to 81 region of P3 should interact directly with the RNA. Indeed, it includes the major portion of a positively charged domain: \((69)\) K\(^{+}\)E K\(^{+}\)K\(^{+}\)SILNR\(^{+}\)MLPK\(^{+}\)IGQR\(^{+}\)MYVH\(^{+}\)H\(^{+}\)H\(^{+}\) (90). Moreover, amino acids 69 to 72 have a higher surface probability than any other region of P3 according to Emini et al. (1985). The positive charge and high probability of surface exposure are shared by the independent RNA binding domains of the TMV and RCNMV movement proteins (Citovsky et al., 1992; Osman et al., 1993), but this is the only similarity. Other experiments now in progress will determine the relevance of the RNA binding domain of P3 to its biological activity.

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**References**


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