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The Genome-linked Protein and 5′ End RNA Sequence of Plum Pox Potyvirus

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SUMMARY

The infectivity of plum pox potyvirus (PPV) RNA was decreased by treatment with proteases. Ribonuclease digestion of iodinated PPV RNA yielded material which had an electrophoretic mobility corresponding to M_r 22000. This protein presumably corresponds to the protease-sensitive structure needed for infectivity. A protein-linked RNase T1-resistant oligonucleotide, 38 nucleotides long, was sequenced and shown to correspond to the 5′ terminus of the RNA by sequence comparison to the RNAs of two other potyviruses, tobacco etch virus and tobacco vein mottling virus. A 12 nucleotide block was found to be completely conserved in the RNAs of the three viruses.

The RNA genomes of several animal and plant viruses have a protein covalently linked to their 5′ termini (Daubert & Bruening, 1984) and genome-linked viral proteins (VPgs) have been found attached to the RNAs of tobacco etch potyvirus (TEV) (Hari, 1981) and tobacco vein mottling potyvirus (TVMV) (Siaw *et al.*, 1985). In this paper we show that plum pox potyvirus (PPV) also has a VPg and show that proteolytic enzyme treatment decreases the infectivity of the viral RNA. Also, the existence of a terminal protein in the PPV genome has allowed us to sequence the 5′ end of the RNA directly.

PPV, Rankovic strain, was propagated in *Nicotiana clevelandii* and purified as described by Laín *et al.* (1988). RNA was extracted from purified virions using SDS and phenol (Zimmern, 1975) and was recovered from the aqueous phase by ethanol precipitation. In the ^{125}I-RNA labelling experiments the RNA was not precipitated; instead, to remove the contaminating capsid protein molecules, it was subjected to SDS–sucrose gradient centrifugation (Hellmann *et al.*, 1980) in a Beckman SW40 rotor for 9 h at 20 °C at 16000 r.p.m. The RNA band was then collected and ethanol-precipitated.

Results presented in Table 1 show that digestion with proteolytic enzymes always diminished the infectivity of PPV RNA, although some variability was observed between different experiments. This suggested the presence of a protein structure, presumably a VPg, associated with the genomic RNA. This result is in contrast with that obtained in similar experiments with TEV (Hari, 1981), in which the infectivity of TEV RNA was not decreased by treatment with proteinase K but, for undetermined reasons, considerably increased. When the influence of proteolytic treatments on the infectivity of the RNAs of several nepoviruses was studied (Mayo *et al.*, 1982), the effect observed was different and characteristic for each of them. Treatment with different proteases can cause different extents of decrease in the infectivity of the same RNA (Mayo *et al.*, 1982), which suggests that the peptides that remain attached to the RNA after the proteolytic treatments may be different, and thus may contribute differently to an early step of the viral life cycle. In the case of PPV both pronase and proteinase K digestions decreased the infectivity of the RNA although, as in the case of raspberry ringspot virus (Mayo *et al.*, 1982), pronase seemed to be more efficient. No data are available on the effect of pronase digestion on the infectivity of TEV RNA. In any case, these results might show not that intact

Table 1. *Effect of proteolytic treatments on the infectivity of PPV RNA**

Expt.	RNA concentration (μg/ml)	Infectivity after treatment with: Buffer only	Pronase	Proteinase K
1	3·5 (20)	4/7	0/7	
2	45 (60)	32/4	0/4	
3	70 (79)	76/10	3/10	
4	80 (50)	192/4	77/4	
5	100 (40)	276/6	2/6	
6	80 (59)	192/4		74/4 (a) 37/4 (b)

* PPV RNA, at the concentration indicated in parentheses, was incubated for 4 h at 37 °C in the absence or presence of pronase (Calbiochem) (0·8 mg/ml, or 0·4 mg/ml in expt. 3) or proteinase K (Merck) (0·2 mg/ml) in 10 mM-Tris–HCl pH 7·5, 5 mM-EDTA, 150 mM-NaCl, 0·5% SDS. Digestion with proteinase K was for 5 h (a) or 8 h (b). Both proteases were self-digested before use, and the integrity of the viral RNA after the protease treatments was confirmed by agarose gel electrophoresis. Treatments were stopped by phenol extraction and RNA was recovered by ethanol precipitation, resuspended at the indicated concentrations in 50 mM-phosphate pH 7·0 and used to inoculate *Chenopodium foetidum* plants. Infectivity is given as the number of lesions/the number of leaves inoculated.

VPg is required for the infectivity of the viral RNA, but that the peptide structure remaining attached to RNA after the proteolytic treatment interferes with RNA infectivity.

To demonstrate the presence of the putative VPg in the PPV RNA, gradient-purified viral RNA was iodinated using the Bolton and Hunter reagent, which is specific for proteins, as described by Siaw *et al.* (1985). Electrophoresis of ^{125}I-labelled RNA in 0·8% agarose gels indicated that the radioactivity was associated with RNA of genomic size (data not shown). Electrophoresis in an SDS–polyacrylamide gel of ^{125}I-labelled RNA digested with ribonuclease A revealed a band with an apparent M_r of approximately 22000 (Fig. 1, lane 2) that was absent from the undigested RNA (Fig. 1, lane 1). The faint band with higher mobility that also appeared in lane 2 was absent in other experiments and comigrated with the RNase A employed in the digestion and stained with Coomassie Brillant Blue. When the iodinated RNA was digested with RNase T1 (Fig. 1, lane 3) the new band had a mobility lower than the one produced after digestion with RNase A. This was expected because the 5′ regions of potyvirus RNA have few G residues. These bands disappeared upon treatment of the ^{125}I-labelled RNA with pronase (Fig. 1, lane 4). The putative ^{125}I-labelled VPg–5′ terminal oligonucleotides remained in the organic phase after phenol extraction, in agreement with the proteinaceous nature of the labelled material and a covalent linkage between it and the RNA.

Although the possession of genome-linked proteins seems to be a general characteristic of potyvirus RNA, there are great differences in the M_r of the VPgs as estimated by SDS–polyacrylamide gel electrophoresis. Values of 6000 and 24000 have been reported for the VPgs of TEV (Hari, 1981) and TVMV (Siaw *et al.*, 1985) respectively, and we have found that of PPV VPg to be 22000. These differences among three viruses that are quite similar in genomic structure and sequence (Allison *et al.*, 1986; Domier *et al.*, 1986; Maiss *et al.*, 1989; Laín *et al.*, 1989) are surprising. The potyvirus genome is expressed as a polyprotein that is autocatalytically cleaved into the functional polypeptides (Dougherty & Carrington, 1988). The cleavage sites are characterized by conserved series of amino acids, different for each potyvirus. The N terminus of TVMV VPg has been located at one of these sites (Shahabuddin *et al.*, 1988), but no such recognition sequence is available at its C terminus. Indeed, aberrant mobilities in polyacrylamide gel electrophoresis of the VPgs of several viruses are well known (Daubert & Bruening, 1984). This renders any discussion about the M_r of the potyvirus VPgs purely speculative, and points to the need for additional sequence data on the N and C termini of the VPgs.

The presence of the T1 oligonucleotide linked to the labelled protein in the organic phase after phenol extraction (see above) allowed its identification, purification and sequencing. If the labelled protein is an authentic VPg, this oligonucleotide should correspond to the 5′ terminus of

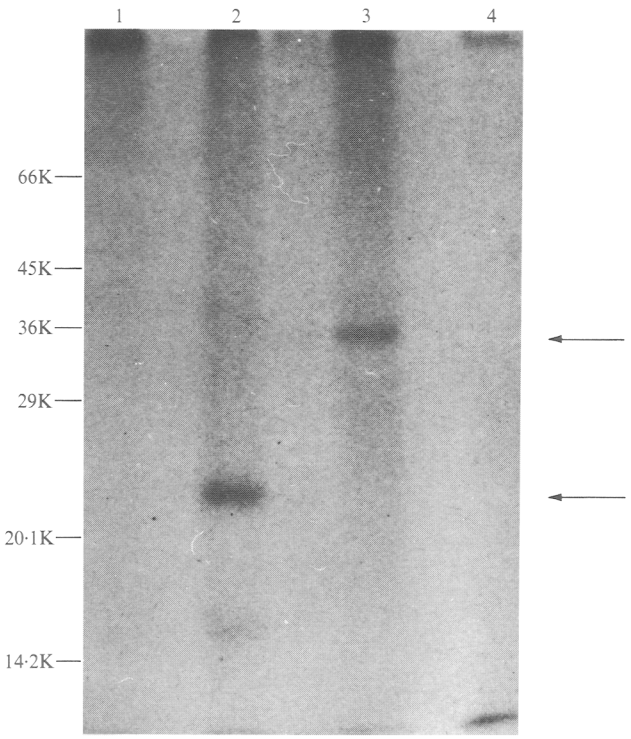

Fig. 1. Analysis by SDS–PAGE and autoradiography of the protein released by RNase digestion of ^{125}I-labelled PPV RNA. Electrophoresis was in a discontinuous SDS gel system (Laemmli, 1970). The separating gel was 15% acrylamide, 0·25% bisacrylamide, 0·1% SDS. Lane 1, no treatment; lane 2, after RNase A digestion; lane 3, after RNase T1 digestion; lane 4, after pronase digestion. The numbers at the left refer to the M_r values of marker proteins.

PPV RNA. A sample of 20 μg of PPV RNA was treated with RNase T1 (55 units; Boehringer) and alkaline phosphatase (120 units; Boehringer) in a reaction volume of 50 μl, essentially as described by Fon Lee & Fowlks (1982). The incubation mixture was extracted three times with phenol–chloroform and the resulting organic phases were combined, re-extracted with 20 mM-Tris–HCl pH 7·5, 2 mM-EDTA (TE 2 ×) and then mixed with 2·5 volumes of ethanol. The recovered material, presumably the VPg-linked 5′-terminal T1 oligonucleotide, was 3′ end-labelled with 40 μCi [5′-^{32}P]pCp (3000 Ci/mmol; Amersham) and T4 RNA ligase (New England Biolabs) (Fon Lee & Fowlks, 1982), ethanol-precipitated and resuspended in TE containing 0·125% SDS. The products of the labelling reaction, either intact or digested with pronase, were analysed in a 7 M-urea/20% acrylamide gel (Fig. 2*a*). Besides a large amount of contaminating oligonucleotides not removed by the phenol extractions, a band that was absent from the untreated material (Fig. 2*a*, lane 1) appeared when it was digested with pronase (Fig. 2*a*, lane 2). This band should correspond to the T1 oligonucleotide linked to the remaining amino acids after the proteolytic treatment. The intact protein–oligonucleotide band was not seen, presumably because it could not enter the gel.

This procedure to obtain the putative 5′-terminal T1 oligonucleotide was slightly modified to get an oligonucleotide preparation with a higher specific radioactivity to enable its sequencing. After digestion of 15 μg of PPV RNA with RNase T1 and alkaline phosphatase as above, the mixture was subjected to three successive cycles of phenol extraction and ethanol precipitation of the organic phases. After the last one, the precipitated material was resuspended in 0·125% SDS in TE and treated with pronase, phenol-extracted again, and labelled with 40 μCi [5′-^{32}P]pCp. The 3′ end-labelled putative 5′-terminal oligonucleotide was purified by polyacryl-

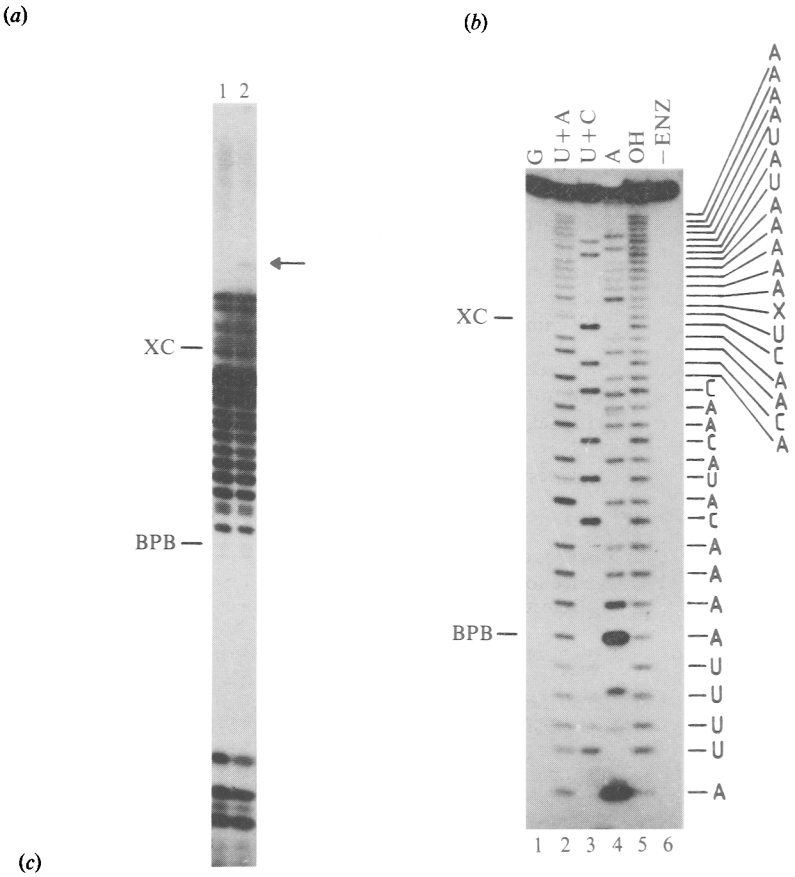

Fig. 2. Identification and sequencing of the 5′-terminal T1 oligonucleotide of PPV RNA. (*a*) PPV RNA digested with RNase T1 and 3′ end-labelled with [5′-^{32}P]pCp analysed in a 20% acrylamide/7 M-urea gel. Lane 1, no treatment; lane 2, after pronase digestion. (*b*) Autoradiograph of a sequencing gel (20% polyacrylamide/7 M-urea) showing the first 36 nucleotides of the PPV 5′-terminal RNase T1 oligonucleotide labelled at its 3′ end and sequenced by partial digestion with RNases. The bands corresponding to the two last nucleotides were absent because they migrated out of the gel in this experiment. Digestions were performed with RNase T1 (lane 1), RNase Phy M (lane 2), RNase BC (lane 3), RNase U2 (lane 4), 50 mM-$NaHCO_3$ for 5 min at 100 °C (lane 5) or no enzyme (lane 6). XC, xylene cyanol marker dye; BPB, bromophenol blue. (*c*) Comparison of the 5′ end RNA sequences of PPV, TEV and TVMV.

amide gel electrophoresis and sequenced by partial digestion with the site-specific ribonucleases T1, Phy M, BC and U2 (P-L Biochemicals RNA sequencing enzyme kit, employed according to the supplier's instructions) (Fig. 2*b*). A 38 nucleotide long sequence was obtained. There was one uncertainty, at position 13, where a band appeared in the alkali ladder but was not present in any RNase lane. The artefactual bands that appeared at positions 20, 33 and 34 were absent in gels from other experiments. The sequence obtained showed significant homology with the 5′ terminus of the TEV (Allison *et al.*, 1986) and TVMV (Domier *et al.*, 1986) RNAs, confirming that we had identified a VPg linked to the 5′ end of the PPV RNA. As the linkage between VPg and RNA was not expected to be cleaved by RNases and the largest bands appeared at the same

level in the alkali ladder and RNase Phy M lanes (Fig. 2*b*) it can be inferred that the sequence up to the first 5′ ribonucleotide had been obtained and that the bond between the latter and the VPg was not cleaved by the mild alkali conditions employed. There is a 12 nucleotide block completely conserved among the three potyvirus RNAs compared (Fig. 2*c*). This sequence conservation at the 5′ end of the RNAs is in contrast with the diversity of their 3′ non-coding regions (Laín *et al.*, 1988), suggesting that this nucleotide block could play an important role in a step of the virus life cycle in which either only the 5′ end is involved, such as encapsidation or translation, or where both ends participate but in different ways, such as replication.

Note. After submission of this paper the complete nucleotide sequence of the RNA of a non-aphid-transmissible strain of plum pox virus (PPV-NAT) was published (Maiss *et al.*, 1989). Its 5′-terminal sequence is identical to that reported here for the PPV Rankovic strain.

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