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EVIDENCE FOR SUBGENOMIC RNAs IN LEAVES INFECTED WITH AN ANDEAN STRAIN OF POTATO VIRUS S

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Summary. - The genomic RNA (7.5 kb) and two subgenomic RNA species (2.5 kb and 1.1 kb) were detected by Northern hybridization in leaves infected with an Andean strain of potato virus S (PVS^A). The single-stranded RNAs contain a substantial poly-(A) tract as shown by oligo (dT)-cellulose chromatography and by an increased mobility on agarose gels following digestion with RNase H in the presence of oligo $(dT)_{12-18}$. In rabbit reticulocyte lysates, the 1.1 kb RNA directed the synthesis of the 34 kD virus coat protein which was not translated efficiently from full length genomic RNA isolated by sucrose gradient fractionation.

Key words: PVS (Andean); subgenomic RNAs; in vitro translation; carlavirus

Introduction

In vitro translation of American hop latent virus (AHLV). Helenium virus S (HelVS), and potato virus S (PVS), all members of the carlavirus group, have recently been described (Mackenzie *et al.*, 1989; Foster and Mills, 1991; 1990*a*). RNA extracted from these purified viruses, acts as a template for the synthesis of viral coat protein when translated *in vitro*. HelVS RNA was shown to contain two subgenomic RNAs of 3.3 kb and 1.5 in addition to genomic RNA (7.4 kb). The smaller subgenomic RNA was an efficient template for the synthesis of capsid protein (Foster and Mills, unpublished). A 1.3 kb subgenomic RNA was detected in RNA from purified PVS particles, encoding a 34 kD coat protein (Foster and Mills, 1990*a*). To date, no evidence has been reported to support a role for subgenomic RNAs in infected tissue.

In this paper we report on the viral RNA components found in tissue infected with an Andean strain of PVS. Evidence is presented that viral coat protein is translated from a polyadenylated subgenomic RNA present in virus-infected tissue.

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Materials and Methods

Virus purification and RNA extraction. Potato virus S (Andean strain) (PVS^A), isolated from breeders' line CE7260/12 from the Netherlands (Rose, 1983), was purified from systemically infected leaves of *C. quinoa* as described (Foster and Mills, 1990 *b*). The RNA was extracted from purified virus essentially as reported by Shields and Wilson (1987).

Total cellular nucleic acid extraction. Systemically infected leaves of *C. quinoa* were harvested, ground to a powder in liquid nitrogen and suspended in 10 volumes (w/v) of homogenization buffer (0.1 mmol/l NaCl, 50 mmol/l Tris-HCl pH 9, 50 mmol/l EDTA, 2 % SDS (w/v), 0.1 mg/ml protease K). The suspension was allowed to stand at room temperature for 15 min before addition of phenol- chloroform- isoamylalcohol (24:24:1). The slurry was agitated for 15 min prior to centrifugation (5000 g, 15 min). The aqueous phase was re-extracted with chloroform: isoamylalcohol (25:1) before precipitation with 0.1 vol NaCl (5 mol/l) and 2.5 vol ethanol at -20^{0}C. Total nucleic acid (NA) was recovered by centrifugation, resuspended in sterile distilled water and stored at -20 ^{0}C.

Isolation of poly (A)$^+$ RNA. Total cellular nucleic acid was extracted as above. Prior to ethanol precipitation the salt concentration was increased to 0.5 mol/l by addition of 5 mol/l NaCl. The solution was then shaken with 1 gram of oligo (dT)- cellulose (Collaborative Research, Type 2) before fractionation by column chromatography as described by Maniatis *et al.* (1982). Column fractions were assessed for RNA content either by ethidium bromide staining or by A_{260} reading. Fractions containing RNA were pooled, ethanol precipitated and resuspended in sterile distilled water.

RNA gel electrophoresis. RNA samples were initially electrophoresed under non-denaturing conditions in 1 % agarose gels in a TBE system (Maniatis *et al.*, 1982) containing 2.5 μg/ml ethidium bromide in both gel and running buffer. Accurate molecular weight measurement and RNA samples for transfer to nitrocellulose were run on denaturing formaldehyde agarose gels as described by Meinkoth and Wahl (1984).

RNA transfer and Northern hybridization. RNA was transferred to nitrocellulose and hybridized with cDNA as described by Perbal (1984).

cDNA synthesis. Viral RNA was used as template for cDNA production incorporating (^{32}P) TTP (29.6 TBq/mmol; Amersham) (Maniatis *et al.*, 1982) using either random fragments of calf thymus DNA or synthetic oligo $(dT)_{12-18}$ (Nicgene, Belfast) as primers (Taylor *et al.*, 1976).

RNA fractionation by sucrose density gradient centrifugation. Poly (A)$^+$ RNA from infected tissue was size fractionated on sucrose gradients as described by Harbison *et al.* (1984).

RNase H digestion. RNA samples (1 μg) were incubated at 45^{0}C for 15 min with 1 μg oligo $(dT)_{12-18}$, cooled to room temperature and digested with 1 unit RNase H (Amersham) in 20 mmol/l Tris-HCl pH 7.5, 10 mmol/l $MgCl_2$, 0.1 mmol/l dithiothreitol for 30 min at 37 °C. The reaction mixture was extracted with phenol, chloroform and RNA ethanol precipitated.

In vitro translation. Translation mixtures contained 66 % (v/v) reticulocyte lysate (P and S Biochemicals), 82 mmol/l KCl, 0.4 mmol/l $MgCl_2$, 8 mmol/ l creatine phosphate, 40 mol each of essential amino acids except methionine, 37 MBq/ml L-(^{35}S)-methionine (29.6 TBq/mmol; Amersham) 1 unit/μl Human placental ribonuclease inhibitor (HPRI) (Amersham) and 40 μg/ml RNA. All incubations were at 30^{0}C in a standard reaction volume of 50 μl. Translation products were separated on 12.5 % polyacrylamide gels (Laemmli, 1970) and visualized by autoradiography.

Immunoprecipitation. Antiserum to PVS (Bioreba AG) was used for immunoprecipitation essentially as described by Mayo and Reddy (1985).

Results

Northern transfer and hybridization of RNA from infected leaves

To determine whether subgenomic RNA may be present in infected plant material, total nucleic acid and poly (A)+ RNA were isolated from healthy and infected leaves and analysed by agarose gel electrophoresis and Northern hybridization using viral cDNA as the probe. Genomic RNA (7.5 kb) was evident in both total and poly (A)+ RNA samples when stained with ethidium bromide (Fig. 1A). Genomic RNA was almost exclusively selected for by oligo (dT)-cellulose chromatography, with only small quantities of host RNA species present.

When total NA and poly (A)+ samples were analysed using Northern hybridization with cDNA to viral RNA, three bands were detected in both samples

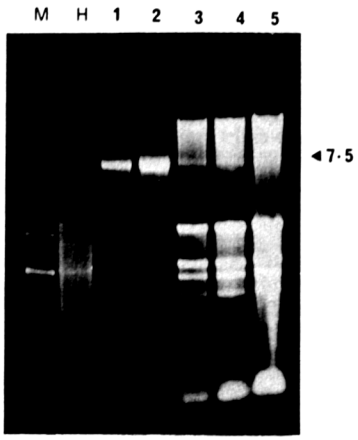


Fig. 1A

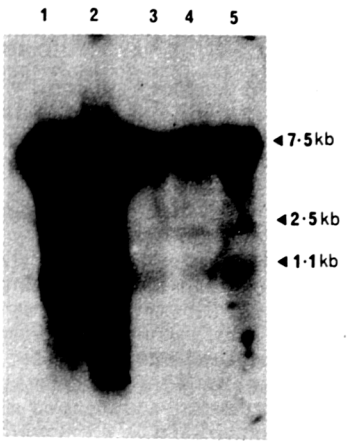


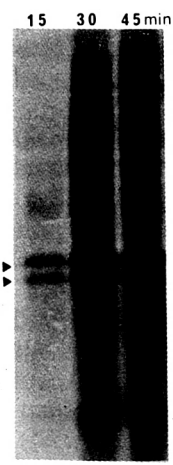
Fig. 1B

Fig. 1C

Fig. 1A. Agarose gel analysis of RNA from healthy and infected leaves. (M) *E. coli* ribosomal RNA markers (1.5 kb & 3.1 kb); (H) healthy *C. quinoa* poly (A)+ RNA (2μg); lanes 1 & 2 PVS^A infected *C. quinoa* poly (A)+ RNA (4 μg & 8 μg); lanes 3, 4 & 5 PVS^A infected *C. quinoa* total nucleic acid (10 μg, 15 μg, 20 μg). Position of PVS^A genomic RNA (7.5 kb) is indicated.

Fig. 1B. Northern blot hybridization of RNA from infected leaves. RNA in agarose gel shown in Fig. 1A was transferred to nitrocellulose and hybridized with viral cDNA probes. Lanes 1–5 as for Fig. 1A. Positions and sizes of RNA species are indicated.

Fig. 1C. RNase H digestion. Poly (A)+ RNA digested with RNase H in the presence of oligo $(dT)_{12-18}$. Lane 1 digested (A)+ RNA; lane 2 control undigested poly (A)+ RNA; lane 3 *E. coli* ribosomal RNA markers.



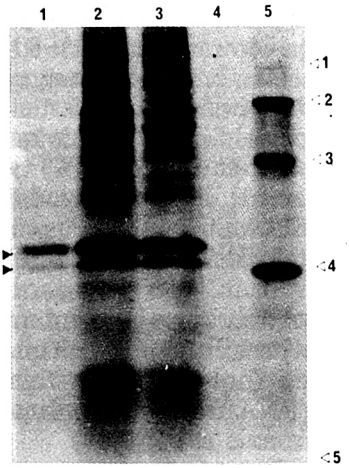


Fig. 2A **Fig. 2B** **Fig. 2C**

Fig. 2A. In vitro translation of poly (A)$^+$ RNA from healthy and infected plants. (H) healthy plant poly A$^+$ RNA; (M) ^{14}C-protein molecular weight markers (as for Fig. 2C); (I) infected plant poly (A)$^+$ RNA.

Fig. 2B. Timecourse translation of infected plant poly (A)$^+$ RNA. Equal volumes were removed at the times indicated (min) and separated on 12.5 % polyacrylamide gels.

Fig. 2C. Immunoprecipitation. Lane 1, products immunoprecipitated with PVS antiserum; lanes 2 & 3, control translations; lane 4 products immunoprecipitated with pre-immune antisera; lane 5, ^{14}C-markers (Amersham), (1) phosphorylase b (M.W. 92,500), (2) bovine serum albumin (69,000), (3) ovalbumin (46,000), (4) carbonic anhydrase (30,000), (5) lysozyme (14,300).
(V) corresponsds to the *in vitro* translation of RNA obtained from purified virus particles.
The position of the 34 K coat protein and 31 K related peptide is indicated on all figures.

(Fig. 1B). The sizes of these bands were determined as approximately 7.5 kb, 2.5 kb and 1.1 kb with all three binding well to oligo (dT)-cellulose, suggesting a terminal poly (A) tract. The increase in mobility of RNA digested with RNase H in the presence of oligo $(dT)_{12-18}$ was assumed to correspond to the loss of the terminal poly (A) tract and was estimated at approximately 100–300 bases in length (Fig. 1C). Mock inoculated *C. quinoa* did not contain RNA species detectable by cDNA hybridization.

In vitro translation of poly (A)$^+$ RNA from healthy and infected plants

To determine whether the translation products of poly (A)$^+$ RNA from infected plants differed from those of RNA extracted from purified virus particles, poly (A)$^+$ RNA was isolated from healthy and infected plants, translated *in vitro* and the products analysed by gel electrophoresis. Poly (A)$^+$ RNA

from healthy plants produced a range of products up to 51 kD, with one major product at 23 kD. In contrast, poly (A)+ RNA from infected plants, containing three viral RNA species, produced a range of products similar to those seen for RNA extracted from purified virus particles, (Fig. 2C, Lane V) with no plant products being produced in detectable quantities (Fig. 2A).

Timecourse experiments of poly (A)+ RNA from infected plants revealed peptides of 34 kD and 31 kD synthesized shortly after initiation (Fig. 2B). The 34 kD peptide was shown to be the virus coat protein by its co-migration in gels with PVS coat protein (data not presented) and by its specific immunoprecipitation with antiserum to PVS virus particles (Fig. 2C). The co-precipitated product of 31 kD possibly arose by an internal initiation codon or early termination within the coat protein gene. Small quantities of a larger 44 kD peptide also immunoprecipitated (not visible on this exposure), confirming the result obtained for viral RNA from both ordinary and Andean strains (Foster and Mills, 1990*a*; Mackenzie *et al.*, 1989).

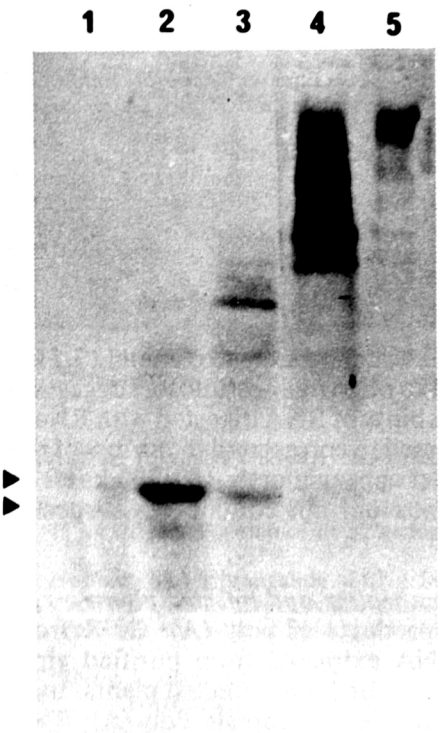


Fig. 3
In vitro translation of gradient fractionated poly (A)+ RNA. Poly (A)+ RNA from infected leaves was fractionated on sucrose density gradients and translated *in vitro*. Lanes 1, 2 & 3 fractions containing subgenomic RNAs; lanes 4 & 5 fractions containing genomic RNA.

In vitro translation of gradient fractionated poly (A)+ RNA

Poly (A)+ from infected leaves was fractionated on sucrose density gradients and translated *in vitro*. Gradient fractions containing low molecular weight RNA directed the synthesis of the 34 kD and 31 kD peptides (Fig. 3, Lanes 1 and 2). As RNA size increased in later fractions, 40 kD and 44 kD peptides appeared in addition to the 34 kd and 31 kD (Fig. 3, Lane 3). Fractions containing genomic RNA (7.5 kb) directed the synthesis of large molecular weight products from 50 kD up to 190 kD (Fig. 3, Lanes 4 and 5). Genomic RNA also directed the synthesis of trace amounts of the 44 kD, 40 kD, 34 kD and 31 kD peptides. This may be caused by translation of artificially produced subgenomic RNA molecules produced by ethanol precipitation and/or the reticulocyte lysate as described for other plant viruses (Dougherty and Hiebert, 1980; Bendena *et al.*, 1985).

Discussion

In this paper, we demonstrate that leaves infected with an Andean strain of PVS contain 2 subgenomic RNA species (2.5 kb and 1.1 kb) in addition to the 7.5 kb genomic RNA. Digestion with RNase H in the presence of oligo $(dT)_{12-18}$ suggests that genomic RNA possesses a substantial poly (A) tract. Subgenomic RNAs were also able to bind to oligo (dT)-cellulose and may have a similar length poly (A) tract at or near the 3'terminus. *In vitro* translation products of poly (A)+ RNA isolated from infected plants were similar to those obtained from viral RNA, with the 34 kD coat protein and 31 kD related peptide synthesized early in timecourse experiments. Sucrose gradient fractionation of poly (A)+ RNA from infected tissue revealed a 1.1 kb subgenomic RNA probably encoding the coat protein and 31 kD peptide. Fractions containing the 2.5 kb subgenomic RNA produced 44 kD and 40 kD products in addition to smaller quantities of the 34 kD and 31 kD products.

Sequence data reported for an Andean strain of PVS (Mackenzie *et al.*, 1989) predicted a 42 kD open reading frame (ORF) with the 33 kD coat protein (initiating from an internal AUG codon) immediately upstream of a 3' proximal 11 kD product. A subgenomic RNA of 1.2 kb would therefore be too small to include the first AUG for the 42 kD peptide, but would expose the AUG for the coat protein. This agrees well with our estimate of 1.1 kb for the subgenomic RNA which encodes the coat protein for an Andean strain *in vivo* and with the reported 1.3 kb encapsidated subgenomic RNA encoding the 34 kD coat protein of an ordinary strain of PVS (Foster and Mills, 1990*a*). From sequence data, a triple gene block, of 25 kD, 12 kD and 7 kD was identified 5' to the coat protein gene. Extensive homology was found between these proteins and the corresponding proteins of the triple gene block of the potexviruses (Guilford and Forster, 1986; Dolja *et al.*, 1987; Abouhaidar and Lai, 1989). If, by analogy

to potexviruses, PVS uses a subgenomic RNA to express these proteins, a 2.4 kb RNA would be required. The 2.5 kb subgenomic RNA, reported here may therefore be used for this purpose *in vivo*. There was no direct experimental evidence for the production of these proteins *in vitro*, but detection would have been unlikely partially due to peptide size, but also, if homologous to sequence predicted proteins (Mackenzie *et al.*, 1989) due to low methionine content.

The evidence presented here for the existence of polyadenylated subgenomic RNAs *in vivo*, reinforces the overall similarity in genome organisation between PVS and members of the potexvirus group already suggested from sequence information (Mackenzie *et al.*, 1989; Rupasov *et al.*, 1989).

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