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Potato virus X-related single- and double-stranded RNAs

Characterization and identification of terminal structures

Valerian V. Dolja, Dmitrii P. Grama*, Sergei Yu. Morozov and Joseph G. Atabekov

Department of Virology, Moscow State University, Moscow 119899 and **D.K. Zabolotnyi Institute of Microbiology and Virology, Kiev 252142, USSR*

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Six species of 3′-coterminal poly(A)-containing RNAs of subgenomic (sg) size have been found in plants infected with potato virus X (PVX): two major (0.9 kb – the coat protein mRNA, and 2.1 kb) and four minor (1.4, 1.8, 3.0 and 3.6 kb). The 5′-end of the shortest sgRNA is located 26 nucleotides upstream of the initiating codon of the coat protein gene (812 nucleotides from the 3′-terminal poly(A) tract of the PVX genomic RNA). Double-stranded analogues have been found for most sgRNAs. The genomic-size double-stranded RNA (the replicative form) is shown to carry a poly(A)-poly(U) hybrid of a predominant length of 150–250 bp on one end, and an unpaired G residue on the other (the 3′-end of the negative chain). In contrast to this, the (−) chains of double-stranded 0.9 and 2.1 kbp sgRNAs lack the unpaired G and both end in C.

RNA; Potato virus X; subgenomic RNA; double-stranded RNA; (Plant virus)

1. INTRODUCTION

The filamentous potato virus X (PVX) is a type member of the potexvirus group, and possesses a positive RNA genome of about 6.4 kb. The 5′-end of PVX RNA is capped [1] and the 3′-end polyadenylated [2].

In vitro translation of PVX genomic RNA, as with other potexviruses, yields a set of large polypeptides of up to 150–180 kDa [3,4]. The gene for the PVX coat protein is localized in the genome 3′-terminal region [5] and is expressed in vivo through formation of subgenomic (sg)RNA of about 0.9 kb in length [6]. A similar sgRNA has been found for daphne virus X [7]. Only in one of the six potexviruses tested so far, namely the narcissus mosaic virus, can the coat protein sgRNA be encapsidated and form short virus particles [8].

Correspondence address: V.V. Dolja, Department of Virology, Moscow State University, Moscow 119899, USSR

This paper presents an attempt to characterize the set and structure of virus-specific single- and double-stranded RNAs formed in PVX-infected plants.

2. EXPERIMENTAL

PVX, wild strain, was propagated on *Datura stramonium* L. plants. The virus preparations, virion RNA, and total RNA from infected leaves were obtained as described [6]. Double-stranded RNA was isolated according to [9]. Oligo(dT)-cellulose chromatography, sucrose gradient centrifugation, agarose and PAG electrophoresis, and Northern hybridization were performed as in [10].

The clone pX-13B [5] containing an insert complementary to a region of PVX RNA that comprises a portion of the coat protein gene plus the extracistronic region (280 nucleotides in sum) was treated with *Hin*dIII and resolved in 6% PAG. The fragment thus generated was ligated into M13 mp8 [11] and the resulting clones were screened [12].

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The cDNA insert containing the 5′-terminal region of the coat protein gene was treated with *TaqI* and *PstI* to produce a fragment corresponding to region 721–792 from the poly(A) tail, which was used as a primer for locating the 5′-terminus of the coat protein sgRNA by the primer extension technique [13].

Single- or double-stranded PVX RNAs were labeled at their 3′-termini with [5′-^{32}P]pCp and RNA ligase; the 5′-termini after phosphatase treatment were labeled with [γ-^{32}P]ATP and phage T_4 polynucleotide kinase. Following phenol extraction, labeled RNA was electrophoresed in slab gels of 1.2% low-gelling-temperature agarose. RNA zones were excised and diluted 5–10-fold. Gel-purified RNA was denatured by boiling and rapid cooling and digested with RNase T_1 for 1 h at 37°C. After removing agarose by freeze-thawing and centrifugation, the samples were concentrated and electrophoresed in 15% PAG. The gels were autoradiographed, and labeled oligonucleotides were eluted when necessary.

3. RESULTS AND DISCUSSION

Blot hybridization was performed for RNA preparations from purified PVX virions (fig.1f) and from virus-infected plants (fig.1e); the poly(A)$^+$ fraction of the latter preparation was also analysed (fig.1d). The radioautograph shows that the PVX virions contain only genomic-size RNA. By contrast, infected cells, besides this, also display two major RNAs 0.9 and 2.1 kb in length and at least four minor ones of 1.4, 1.8, 3.0 and 3.6 kb. All these RNAs are polyadenylated (cf. fig.1d,e) and are 3′-coterminal since the DNA probe used was complementary to the 3′-end of the PVX genome. Specific hybridization with cDNA to the coat protein gene fragment confirms the identity of 0.9 kb sgRNA with the recently described mRNA for the PVX structural protein [6].

The 5′-end of the 0.9 kb sgRNA was located with the use of a DNA primer complementary to the coding region of the coat protein gene in the reverse transcription of poly(A)$^+$ RNAs from infected plants. The size of the major run-off product locates the 5′-end of the sgRNA 26 or 27 bases upstream of the initiating codon in the coat protein gene (see fig.3). This position corresponds

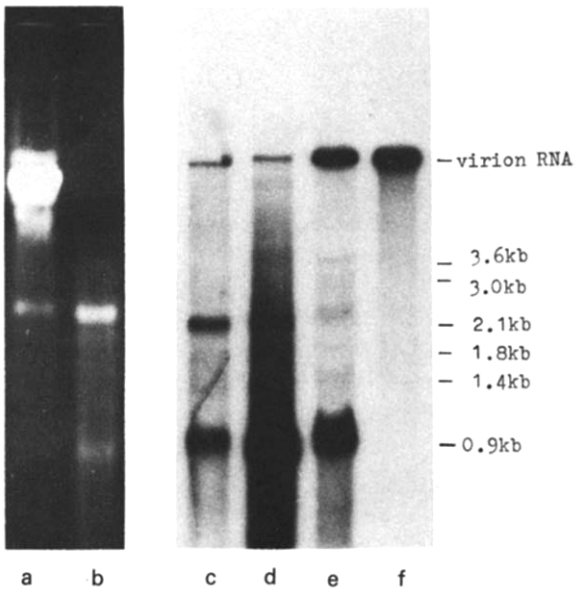

Fig.1. (a,b) Agarose gel electrophoresis of ds PVX-specific RNAs, stained with ethidium bromide; (a) heavy and (b) light portions of the sucrose gradient peak; (c–f) Northern hybridization of glyoxal-denatured RNA – (c) preparation of ds PVX RNAs, (d) ss poly(A)$^+$ RNA from PVX-infected plants, (e) total ss RNA from PVX-infected plants, (f) RNA extracted from purified virus particles. The positions of hybridizing species are indicated.

to the 812th or 813th G residue of the PVX genomic RNA, counting from the 3′-terminal poly(A) tract. The 5′-end of sgRNA carries the sequence GGUUAAGUUUCCAU which has also been found in the region adjacent to the coat protein gene of potato aucuba mosaic virus [14]. The preservation of this sequence in two different potexviruses indicates that it may be essential, for instance, to formation of the 5′-end of the coat protein sgRNA.

The second part of this work was devoted to the analysis of double-stranded (ds) PVX-specific RNAs. As can be seen in fig.1a,b, PVX-infected cells yield a set of ds RNAs. The most prominent zone in fig.1a corresponds to the replicative form (RF) of the genomic RNA (6.4 kbp). One can also see a number of lighter varieties (5.0, 2.1, 1.8, 1.4 and 0.9 kbp). Upon glyoxal denaturation of these ds RNAs and blot hybridization with the same DNA probe we obtained the radioautograph

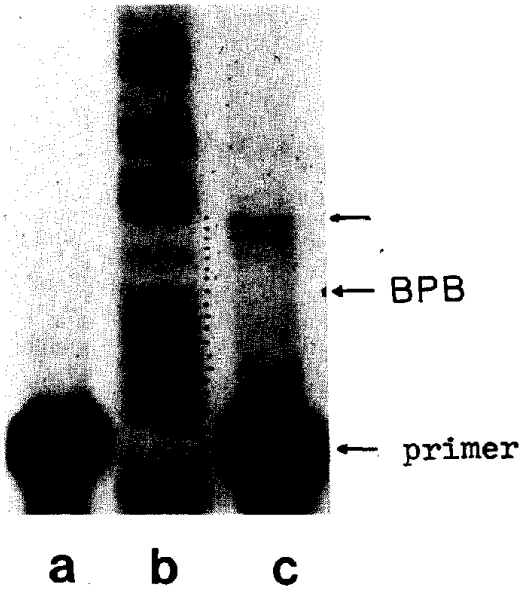

Fig.2. Primer-extension analysis of the 5′-terminus of the coat protein sgRNA. (a) The cDNA products of the reverse transcription of genome PVX RNA, (b) sequencing ladder, (c) the cDNA products of the reverse transcription of poly(A)$^+$ RNA from PVX-infected plants. The positions of the major primer-extension product, bromophenol blue, and labeled primer are shown.

presented in fig.1c. This result is clearly very similar to that obtained for single-stranded (ss) sgRNAs (cf. fig.1d,e). In other words, ds analogues of most part of PVX sgRNAs can be isolated from infected cells.

To study the structure of PVX ds RNA, it was labeled at the 3′-ends with [5′-^{32}P]pCp, and its individual fractions were purified by agarose electrophoresis. After denaturation and RNase T_1 treatment the labeled 3′-terminal oligonucleotides were analysed in PAGs. As can be seen in fig.4a, the PVX genomic-size ds RNA (RF) has two types of labeled termini. Comparison with PVX virion ss RNA (fig.4b) shows that one terminus belongs to the RF (+) chain and consists of a poly(A) tail of 10–250 nucleotides. The lengths predominant for both the RF (+) chain and virion RNA are 70 and especially 150–250 nucleotides. The 3′-end of the RF (−) chain upon T_1 hydrolysis yields a hexanucleotide, as evident from the comparison of fig.4a,b. This hexanucleotide was eluted from PAG and subjected to complete digestion with RNase T_2. Cellulose thin-layer two-dimensional chromatography demonstrated that the 3′-terminal nucleotide of the RF (−) chain is G (not shown).

Further, PVX RF was labeled at the 5′-termini with [γ-^{32}P]ATP in the presence of polynucleotide kinase, gel-purified, and subjected to T_1 hydrolysis. PAG electrophoresis revealed only a poly(U) tract of the RF (−) chain of exactly the same length as the poly(A) in the (+) chain (fig.4b,d). To identify the 5′-terminal residue of the (+) chain, labeled RF was subjected to complete hydrolysis with nuclease P_1. Two-dimensional thin-layer chromatography (not shown) demonstrated that the bulk of the label is associated with G, which thus appears to be 5′-terminal in the (+) chain.

The data obtained allow the conclusion that the 'right' end of PVX RF is represented by a poly(A)-poly(U) hybrid 150–250 bp in length. The presence in the (−) chain of the poly(U) tract testifies to the template copying of the poly(A) 3′-tail in the PVX genome. The 5′-terminus of the RF (+) chain has an ultimate G and appears to carry no cap since it is efficiently labeled by the kinase. The 3′-terminus of the (−) chain also ends in G. Therefore the 'left' end of the RF cannot be 'blunt'. In all probability it is the extra G in the

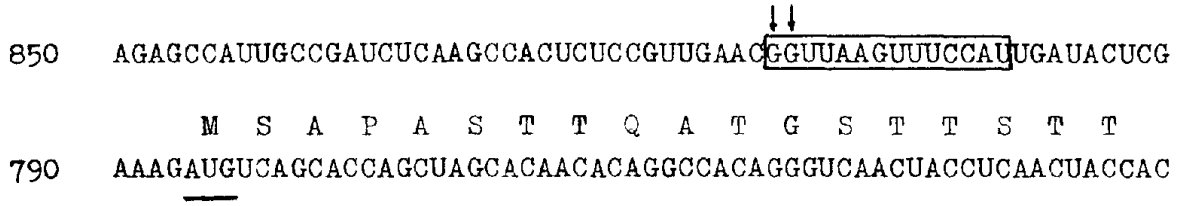

Fig.3. The sequence in the PVX genomic RNA [5] corresponding to the 5′-end (arrowed) of the coat protein sgRNA, the beginning of the coat protein gene coding sequence (initiation codon underlined), and the region of complete homology (boxed) with the potato aucuba mosaic virus RNA [14].

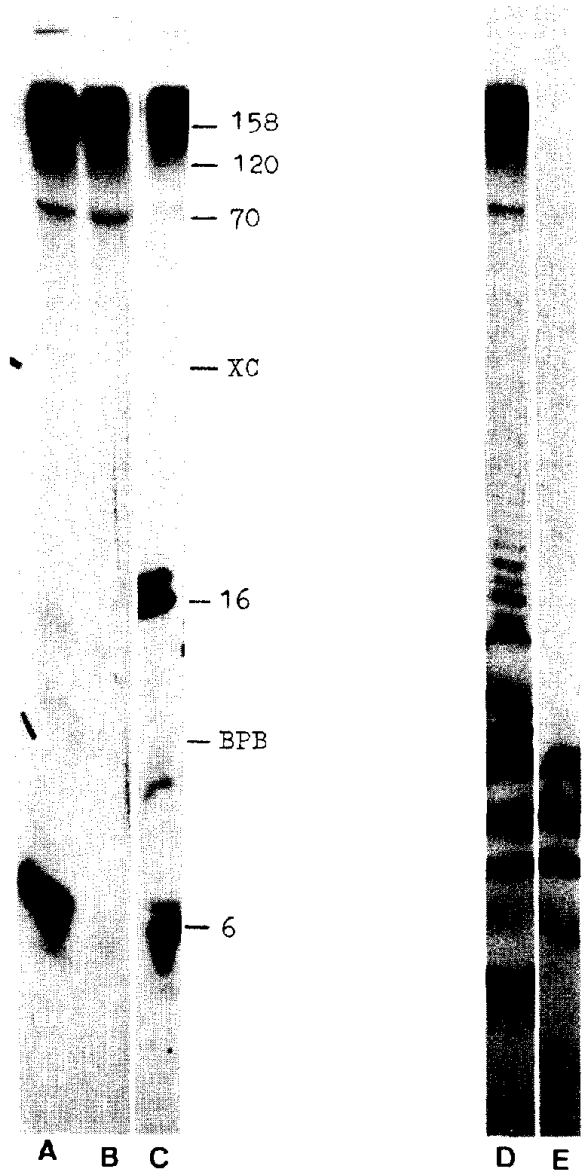

Fig.4. (A–C) 15% PAG of RNase T_1 digests of pCp-labeled and gel-purified PVX RNAs. (A) Ds RNA of genomic size, (B) virion RNA, (C) ds RNA of 2.1 kbp. The positions of markers (5.8 S RNA, 158 nucleotides; 5 S RNA, 120 nucleotides; tRNA, 70 nucleotides) and major labeled T_1-oligonucleotides are indicated. (D,E) 15% PAG of RNase T_1 (D) or RNase A (E) digests of [γ-^{32}P]ATP-labeled and gel-purified PVX genomic size ds RNA.

(−) chain that is unpaired. The terminal structures of the PVX RF are presented in the following scheme, where the sequences in ordinary type are taken from [2] and those determined in the present study are given in boldface:

(+) 5′ p_xGAAAAC ... UUAUUU(**A**)$_n$ OH 3′

(−) 3′ HO GCUUUUG ... AAUAAA(**U**)$_n$ p_x 5′

To date, the extra G has already been found at the 3′-termini of the RF (−) chains in two groups of plant viruses: cucumo- [15], and hordeiviruses [16].

We have also made an attempt to characterize the terminal structures of the ds forms of PVX sgRNAs. The 3′-ends of the (+) chains of ds sgRNAs of 0.9 and 2.1 kbp were found to contain the poly(A) tail typical for PVX (see, e.g. fig.4c). The (−) chains of these RNAs carry 3′-terminal T_1-oligonucleotides of 9 (0.9 kbp sgRNA) or 16 (2.1 kbp sgRNA) units. In both cases the ultimate nucleotide is C, i.e. most of the (−) chains in the ds forms of these sgRNAs lack the unpaired G residue. It must be noted that the length of the 3′-terminal T_1-oligonucleotide from the (−) chain of the coat protein sgRNA agrees well with that predicted from the 5′-terminal sequence of its (+) chain (see fig.3). Thus, the 3′-end of the (−) chain in the ds form of the genomic RNA carries an unpaired G residue whereas that of the sgRNA does not. This observation indirectly indicates that (−) chains of sgRNAs can no longer be templates for making new copies of sgRNAs.

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REFERENCES

[1] Sonenberg, N., Shatkin, A.J., Riccardi, R.P., Rubin, M. and Goodman, R.M. (1978) Nucleic Acids Res. 5, 2501–2512.

[2] Morozov, S.Y., Gorbulev, V.G., Novikov, V.K., Agranovsky, A.A., Kozlov, Y.V., Atabekov, J.G. and Bayev, A.A. (1981) Dokl. Akad. Nauk SSSR 259, 723–725.

[3] Wodnar-Filipowicz, A., Skrzeczkovsky, L.J. and Filipowich, W. (1980) FEBS Lett. 109, 151–155.
[4] Bendena, W.G. and Mackie, G.A. (1986) Virology 153, 220–229.
[5] Morozov, S.Y., Zakharyev, V.M., Chernov, B.K., Prasolov, V.S., Kozlov, Y.V., Atabekov, J.G. and Skryabin, K.G. (1983) Dokl. Akad. Nauk SSSR 271, 211–215.
[6] Grama, D.P. and Mashkovsky, N.N. (1986) Biopolim. Kletka 2, 328–334.
[7] Guilford, P.J. and Forster, R.L.S. (1986) J. Gen. Virol. 67, 83–90.
[8] Short, M.N. and Davies, J.W. (1983) Biosci. Rep. 3, 837–846.
[9] Garnier, M., Mamoun, R. and Bove, J.M. (1980) Virology 104, 357–374.
[10] Dolja, V.V., Lunina, N.A., Chumakov, K.M., Ziegler, A. and Atabekov, J.G. (1986) Dokl. Akad. Nauk SSSR 288, 999–1001.
[11] Carlson, J. and Messing, J. (1984) J. Biotechnology 1, 253–264.
[12] Looney, J.E., Han, J.H. and Harding, J.D. (1984) Gene 27, 67–73.
[13] Simpson, A.M., Neckelmann, N., De la Cruz, V.F., Muhich, M.L. and Simpson, L. (1985) Nucleic Acids Res. 13, 5977–5993.
[14] Bundin, V.S., Vishnyakova, O.A., Zakharyev, V.M., Morozov, S.Y., Atabekov, J.G. and Skryabin, K.G. (1986) Dokl. Akad. Nauk SSSR 290, 728–733.
[15] Collmer, C.W. and Kaper, J.M. (1985) Virology 145, 249–259.
[16] Dolja, V.V. and Atabekov, J.G. (1987) FEBS Lett. 214, 313–316.