Identification of the 3′- and 5′-ends of beet necrotic yellow vein virus RNAs

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Received 14 February 1983; revision received 20 March 1983

Beet necrotic yellow vein virus RNAs are characterized by a 5′-end cap structure and polyadenylated 3′-ends. The presence of a poly(A) sequence with major lengths ranging from 65–140 nucleotides could be demonstrated by homochromatography and sequencing methods. Beet necrotic yellow vein virus RNAs could be 5′-end labelled with kinase only after enzymatic or chemical cap elimination followed by dephosphorylation. The 5′-end structure is found to be of cap O type: m^7GpppNp.

Plant virus *Tobamo virus* *RNA* *Cap structure* *Poly(A)*

1. INTRODUCTION

Beet necrotic yellow vein virus (BNYVV) is a rod-shaped multicomponent plant virus [1] with 4 major particles (390, 265, 100 and 85 nm in length), and is considered as a tentative member of the tobamo virus group [2], like the soil-borne wheat mosaic virus. The multipartite genome of BNYVV is composed of 4 single-stranded RNAs with M_r of 2.3×10^6, 1.8×10^6, 0.7×10^6 and 0.6×10^6, estimated by polyacrylamide gel electrophoresis in non-denaturing conditions [3]. We have undertaken a study of the structure and function of the viral RNAs in order to establish if their characteristics are similar to those of tobacco mosaic virus, the type member of the group. In this communication, we report that the BNYVV RNAs contain poly(A) at their 3′-end, and that the 5′-end is blocked by a cap O type structure (m^7GpppNp).

2. MATERIALS AND METHODS

2.1. *Virus purification*

BNYVV was multiplied on *Chenopodium quinoa*. The virus, extracted as in [3], was further purified by zone electrophoresis in a sucrose density gradient [4]. The virus yield was ~0.5 mg/100 g leaves.

2.2. *Nucleic acid extraction and fractionation*

The RNA was isolated from purified BNYVV by the phenol procedure. The nucleic acid was fractionated on 2.8% acrylamide, 0.14% bisacrylamide, 7 M urea, 1 mM EDTA, 50 mM Tris–borate (pH 8.3), preparative slab gels (0.4 cm × 20 cm × 40 cm). Electrophoresis was for 48 h at 300 V; bands were visualized by UV absorption or by autoradiography, cut out and electroeluted from the gel.

2.3. *3′-End-labelling*

Total RNA was labelled by addition of [5′-^{32}P]pCp (500–1000 Ci/mol from Amersham) at its 3′-OH-end by means of T_4 RNA ligase (P.L. Biochemicals) as in [5].

The 3′-end-labelled RNAs were recovered from the incubation mixture by phenol extraction followed by two ether extractions and ethanol precipitation. The labelled RNAs were fractionated on a 2.8% preparative slab gel as above.

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Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

2.4. *Identification of the 3'-end structure*

The determination of the first 15 nucleotides was made on 3'-end-labelled RNA with 10 μg carrier tRNA in 10 μl H_2O heated for 30 min at 100°C in a sealed capillary to get a random hydrolysis. The hydrolysed mixture was fractionated by electrophoresis at pH 3.5 on cellulose acetate strips and by homochromatography on DEAE thin-layers with a 3.8% homomixture [6]. Complementary analyses of the poly(A) tails were made using the sequencing conditions in [7]. The length and the heterogeneity of the poly(A) tails of the purified 3'-end-labelled RNAs was estimated from complete RNase digests obtained under the following enzymatic digestion conditions: for T_1 2×10^{-2} units/μg RNA and for pancreatic RNase 2 ng/μg RNA incubated for 90 min at 37°C in 0.1 M Tris–HCl (pH 7.4) and for U_2 RNase 5×10^{-2} units/μg RNA in 50 mM NH_4 acetate (pH 4.5) incubated for 12 h at 37°C. The samples were analyzed on a 12% polyacrylamide gel containing 7 M urea.

2.5. *Cap elimination and 5'-end-labelling*

The cap structure was eliminated either by chemical modifications or by enzymatic cleavage. The chemical modifications were performed by periodate oxidation and aniline cleavage as described for Brome mosaic virus RNAs [8]. Enzymatic cap elimination was obtained by treatment of the BNYVV RNAs with tobacco acid pyrophosphatase (TAP). The TAP preparation and the decapping conditions were as in [9]. After cap elimination the RNAs were dephosphorylated with alkaline phosphatase [9], the reaction was stopped by phenol extraction. The treated RNAs were 5'-end-labelled with [γ-^{32}P]ATP (2000 Ci/mmol, Amersham) and T_4 polynucleotide kinase (P.L. Biochemicals) [9].

2.6. *Labelling of the 5'-terminal cap structure*

The method in [10] based on $NaIO_4$ oxidation and reduction with $NaB[^3H]_4$ (16 Ci/mmol, CEA France) was used to end-label purified BNYVV RNAs.

2.7. *Analysis of the ^{3}H-labelled cap structure*

The procedure used was similar to that described in [11]: The ^{3}H-labelled cap dinucleotide was cleaved off from the ^{3}H-labelled RNAs under the following enzymatic conditions: 7 μg ^{3}H-labelled BNYVV RNAs were digested to completion with either RNases T_1, T_2 or P_1 together with 1 mg unlabelled alfalfa mosaic virus (AlMV) RNA added to produce enough absorbance to monitor elution during DEAE–Sephadex chromatography. This RNA mixture was hydrolysed under the following conditions: for RNase T_1 (100 units) in 1 mM EDTA, 10 mM Tris–HCl (pH 7.4) for 3 h at 37°C. For RNase P_1 (1 μg) the digestion was performed overnight at 37°C in 50 mM NH_4 acetate (pH 5.3). RNase T_2 hydrolysis was performed with 0.5 units in 10 mM EDTA, 50 mM Na-acetate (pH 4.7) for 5 h at 37°C. The resulting labelled cap dinucleotides were separated on a 0.6 × 25 cm DEAE–Sephadex A-25 column eluted with a linear gradient of 500 ml (total vol.) from 0.1–0.6 M NaCl in 7 M urea, 20 mM Tris–HCl (pH 7.5). The flow rate was 40 ml/h; 2.5-ml fractions were collected. For radioactivity measurements 1-ml aliquots were mixed with 9 ml ready-solv. MP scintillation solution (Beckman).

3. RESULTS

3.1. *Analysis of the 3'-end-labelled RNAs*

The evidence for polyadenylated 3'-ends in the 4 viral RNAs was obtained from analysis of randomly hydrolysed 3'-end-labelled RNAs by the electrophoresis-homochromatography method. Fig.1a shows the autoradiogram of the wandering spot analysis done with RNA-1. The results were identical with the other 3 RNAs. The analysis shows repeated identical jumps which most likely correspond to A; however, C jumps cannot be excluded. The first spot is A [^{32}P]Cp. The possibility of a poly(C) tail was ruled out by the sequence data reported in fig.1b since the lanes corresponding to partial RNase U_2 and *Physarum* M nuclease digests give a ladder: all these enzymes cleave ApN bonds but not CpN bonds. The results obtained with T_1 or pancreatic RNase confirm the absence of pyrimidine residues in the last 40 nucleotides from the 3'-end.

To determine the length of the poly(A) tail in each RNA, each 3'-end-labelled RNA was hydrolysed separately to completion with pancreatic RNase, T_1 RNase or U_2 RNase. The hydrolysis products were run on a 12% polyacrylamide gel together with unhydrolysed

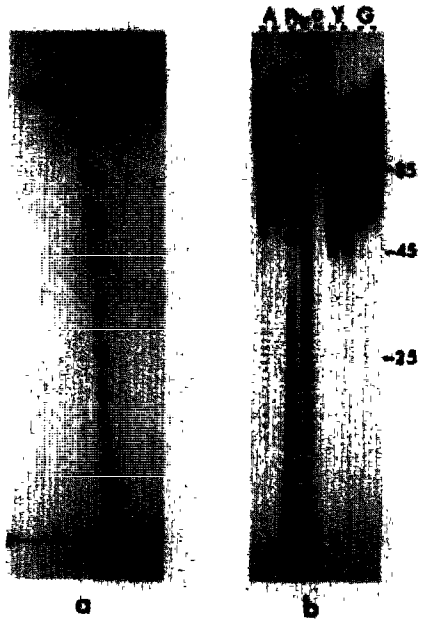

Fig.1a. Autoradiogram of a two-dimensional fractionation of 3'-end-labelled RNA 1 on DEAE thin-layers. The procedure is described in section 2.

Fig.1b. Sequencing gel of 3'-end-labelled BNYVV RNA 3. The different lanes correspond to: 0.6 and 0.3 $\times 10^{-3}$ units U_2 RNase/μg RNA (A), 0.2 and 0.1 units *Physarum* M nuclease/μg RNA (Phy), control (O), 0.1 and 0.05 ng pancreatic RNase/μg RNA (Y) and 2 and 1 $\times 10^{-3}$ units T_1 RNase/μg RNA (G) partial digests incubated for 15 min at 50°C in 7 M urea and analyzed on a 12% sequencing gel. Numbers indicate fragment sizes.

RNAs and two labelled markers of known length $n = 53$ and $n = 178$ (RNase T_1 fragments of AlMV RNA 3) to allow size estimation. Fig.2 shows the autoradiogram of this gel; it appears clearly that only the T_1 and pancreatic RNase reactions gave a broad zone of labelled material corresponding to poly(A) stretches heterogenous in length. The positions of the bromophenol blue and xylene cyanol blue correspond to 12 and 45 nucleotides, respectively. The U_2 RNase digest resulted in a major spot at the bottom of the 20% gel corresponding to labelled Ap as expected for a complete hydrolysis of a poly(A) tail since only this enzyme is able to remove the final nucleotide (Cp) of the labelled RNA. The ladder in the pancreatic digest lanes results from non-specific cleavage under the strong

Fig.2. Length estimation of the poly(A) tails of 3'-end-labelled BNYVV RNAs. The experiment is described in the text. The different lanes correspond to the control (O), the pancreatic RNase (Y), the T_1 RNase (G) and the U_2 RNase complete digests (A) or RNAs 1, 2, 3 and 4, 3'-end-labelled with [5'-^{32}P]pCp. BB and XC indicate the position of bromophenol blue and xylene cyanol markers. Lane N contains a 53 nucleotide-long fragment used as marker. The position of a 178 nucleotide-long fragment is indicated by an arrow.

hydrolysis conditions used here. The amount of radioactivity distributed in the different fragments indicate a heterogeneity in length (lanes Y and G) but 3 sets of fragments, with mean lengths of 65, 120 and 140 nucleotides respectively, are dominant as indicated by a, b, c in fig.2. The maximum length of the poly(A) sequences could be estimated as 140 nucleotides from longer migration times (not shown).

3.2. *Analysis of the 5'-end-labelled RNAs*

We first investigated the susceptibility of BNYVV RNAs labelling by [γ-^{32}P]ATP and polynucleotide kinase. These attempts were carried out in parallel with AlMV RNAs which are known to be capped at their 5'-end [12]. Attempts at labelling untreated or alkaline phosphatase-treated RNAs were unsuccessful (fig.3, lanes 1,4). Tobacco acid pyrophosphatase (TAP) treatment followed by dephosphorylation was a prerequisite for

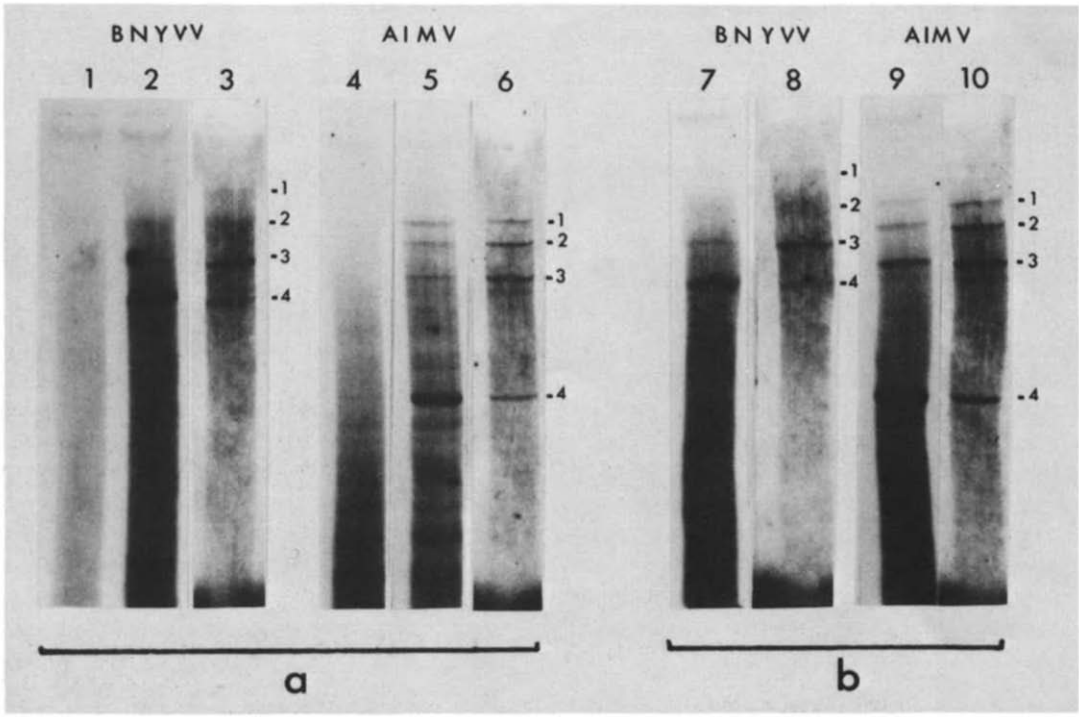

Fig.3 Polyacrylamide gel analysis of 5′-end [γ-^{32}P]ATP-labelled BNYVV and AlMV RNAs after enzymatic (a) or chemical cap elimination (b). A composite acrylamide–agarose (2.2–0.5%) slab gel (90 × 150 × 0.5 mm) prepared in 6 M urea, 2 mM EDTA, 40 mM Tris–acetate (pH 7.8) was loaded with 2–2.5 μg RNA/lane and electrophoresed for 3.5 h at 120 V. The autoradiogram shows the labelling obtained with untreated RNA (lanes 1,4) and with TAP and phosphatase-treated RNA (lanes 2,5) with BNYVV and AlMV RNAs, respectively. The corresponding *O*-toluidine stained gels are shown in lane 3 and 6. (b) Labelling (lanes 7,9) and staining (lanes 8,10) of the chemically decapped BNYVV and AlMV RNAs.

labelling with polynucleotide kinase (fig.3, lanes 2,5). Staining intensity indicates that RNA 3 and RNA 4 are the major RNAs in BNYVV preparations (lane 3). The low level of label observed for RNA 1 and 2 reflects both the low amount of these RNAs in the preparation and the difficulty in labelling high-M_r RNAs in the presence of shorter RNA species as often observed in similar conditions. Accordingly, the amount of label in BNYVV RNA 4 is predominant over that of RNA 3. Fig.3b shows the results obtained under the same labelling conditions but with chemically decapped and phosphatase-treated AlMV and BNYVV RNAs: The labelling intensities are comparable to TAP decapped RNAs except that for BNYVV RNA 1 or 2 and AlMV RNA 1 the chemical treatment produces some degradation as noticed from the staining and labelling of these RNAs.

3.3. *Identification of cap dinucleotides*

The characterization of the cap structure was done using ^{3}H-labelled BNYVV RNAs. Tritium-labelled total RNA of BNYVV (and AlMV used as control) were hydrolysed with RNase P_1. Upon separation of the products on DEAE–Sephadex A-25 the patterns shown in fig.4 were obtained. The first labelled peak (a) is due to residual by-products of the borohydride reaction. The second peak (b) corresponds to the terminal nucleotide with an open ribose ring p[^{3}H]A′ from 3′ poly(A) of BNYVV RNA digests. Together, both peaks contain most of the radioactivity eluting from the column.

The radioactive material eluting with a -2.8 charge corresponds to the dinucleotide expected for a m^{7}GpppN structure [11]. In a control experiment run with ^{3}H-labelled AlMV RNAs digested

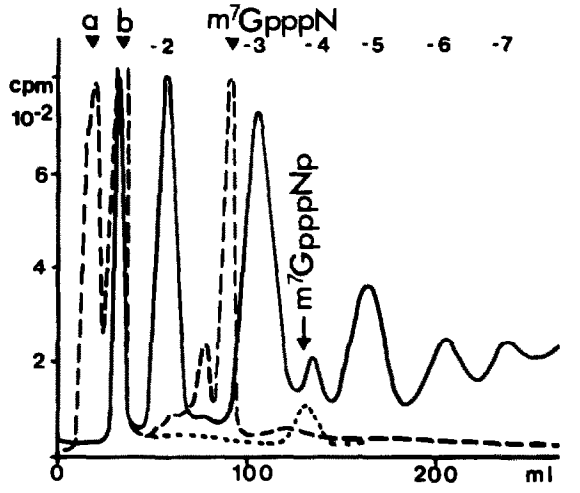

Fig.4. Separation of total P_1 or T_2 digests of [^{3}H]borohydride-labelled BNYVV RNAs on DEAE–Sephadex A-25. Digestions were as in the text. (——) Absorbance profile obtained from a T_1 RNase digest of AlMV RNA indicates the elution positions of mono- to hexa-nucleotides. Their charges (−2 to −7) are listed on top. The radioactivity patterns obtained from P_1 RNase (– – –) and T_2 RNase digests (···) of ^{3}H-labelled BNYVV RNAs are superimposed.

with RNase P_1, the m^{7}GpppG 5′-end structure is indeed found in this position. Accordingly, the labelled material at −2.8 found in ^{3}H-labelled BNYVV RNA digests may be tentatively assigned a m^{7}GpppN structure. This assumption is strengthened by the behaviour of the terminal dinucleotide after T_2 RNase digestion of ^{3}H-labelled BNYVV RNA. T_2 RNase does not hydrolyse the phosphodiester bond adjacent to the cap structure and therefore a shift of the elution position at −3.9 is observed in agreement with a m^{7}GpppNp structure. A similar elution position is found for m^{7}GpppGp from AlMV used as control and indicated by an arrow in fig.4. The significance of the minor peak at −2.5, which is also observed with AlMV RNA digests is unknown.

Additional experiments not reported here indicate that in an RNase T_1 digest of BNYVV RNA the 5′-terminal structure is eluted in the heptanucleotide region; therefore the cap structure does not contain a G in position 1. This residue was characterized in RNAs 3 and 4 by the following experiments: 5′-end-labelled BNYVV RNAs 3 and 4 were cut out of the gel of fig.3a, digested overnight at 37°C with 0.1 μg P_1 nuclease in 50 mM NH_4 acetate (pH 5.3) and the [5′-^{32}P]nucleotide identified as pA by electrophoresis at pH 3.5 [13] (not shown). These results suggest that the 5′-end is blocked by a cap structure m^{7}GpppA in RNAs 3 and 4.

4. DISCUSSION

This report is the first on the end structures of BNYVV RNA. We show that each of these RNAs has a poly(A) tract at its 3′-end. The length distribution of these tracts is very heterogeneous, with maxima at 65, 120 and 140 nucleotides. Such heterogeneity has also been reported for animal viruses [14]. A bimodal length distribution, with maxima of 40 and 60 residues has been described for mouse globin mRNA [15] but the significance of this observation is so far unknown.

By analogy with the RNAs of the como- and nepoviruses, which also possess a 3′-poly(A), the possibility was considered first that the 5′-end of BNYVV RNA would be linked to a 'Vpg-like' protein. This possibility was ruled out since attempts at Vpg iodination by the chloramine T method gave an insignificant level of labelling in comparison with tobacco black ring, a nepovirus used as control (C. Fritsch, unpublished). Attempts to label directly the 5′-end using [γ-^{32}P]ATP and polynucleotide kinase were also unsuccessful. We were able to 5′-label all 4 RNA species only after the RNAs were treated with tobacco acid pyrophosphatase or chemically decapped and phosphatase-treated, albeit at low yield for the larger RNAs. Therefore, we conclude that all 4 RNAs are capped.

Further analysis of the cap structure was done after labelling with tritiated borohydride and nuclease treatment. Labelling of the BNYVV RNAs with borohydride was more efficient than with polynucleotide kinase since this chemical method is less sensitive to the length and secondary structure of the RNA and needs no cap removal. Borohydride labelling is therefore the choice method when dealing with an RNA such as that of BNYVV and most soil borne viruses, which can only be obtained in minute amounts. The cap structure in BNYVV RNA is of type O (m^{7}GpppN); i.e., the first nucleotide (N) is not

methylated since P_1 or T_2 RNase cleavage occur after this nucleotide. In the case of RNA 3 and 4 the first nucleotide was identified as pA: their cap structure is therefore $m^7GpppAp$.

The simultaneous presence of a 5′-cap and of a 3′-poly(A) end structure has not yet been reported for the members of the tobamo virus group; therefore, the classification of BNYVV among the members of this group [2] is questionable. Some similarities exist between BNYVV RNAs and barley stripe mosaic virus (BSMV) RNAs [16]. However, in BSMV RNA a 3′-tyrosine accepting structure has been reported [17] in addition to the poly(A) sequence located near the 3′-end. Our data exclude such a possibility in the case of BNYVV RNAs.

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor L. Hirth's continuous interest and advice. We also wish to thank Dr B. Rhoads for helpful discussions and Dr A. Durr for providing us with the tobacco callus cultures used to prepare the acid pyrophosphatase. Part of this work was supported by the CNRS, contract 2380: ATP Phytopathologie, INRA-CNRS, 1980.

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