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Nucleotide sequence of the 3'-terminal tRNA-like structure in barley stripe mosaic virus genome

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ABSTRACT

This paper describes the sequence of 257 nucleotides from the 3' end of RNA 2 of barley stripe mosaic virus (BSMV, strain Argentina Mild) including an internal oligo(A) tract localized at a distance of 236 nucleotides from the 3' end, and the 3' terminal tRNA-like structure accepting tyrosine. This sequence is shown to be the same with RNAs 1,2 and 3 of another BSMV strain, Norwich, for at least the first 106 nucleotides from the 3' end. The 3' extremity of BSMV RNA bears some resemblance to $tRNA^{Tyr}$ from yeast in its primary structure. The possible secondary structures of the tRNA-like sequence in BSMV genome are discussed.

INTRODUCTION

Viruses with positive-sense RNA genome can be divided into three groups with respect to their 3' terminal sequences. RNAs of some plant viruses, e.g. comoviruses, nepoviruses, potyviruses, and potexviruses (1-3), possess a poly(A) tail at their 3' terminus. Many plant viruses such as tymo-, tobamo-, bromo-, and cucumoviruses (4-6) represent a group carrying amino acid-accepting (tRNA-like) structures at the 3' end of their genomic RNAs. On the other hand, there are some examples of viruses with RNA devoid both of 3' poly(A) and of tRNA-like structure (1).

Recently the mixed variants of arrangement of poly(A) and tRNA-like structure in viral genomes have been described. Thus, there are some indications that the 3'-polyadenylated genomic RNAs of encephalomyocarditis virus and mengovirus contain an internal tRNA-like structure which can be aminoacylated *in vitro* after excising it with nucleases (4). Quite the opposite situation - the presence of an internal poly(A) and a 3' terminal tyrosine-accepting sequence- was reported for the RNA

of two plant viruses, brome mosaic virus (BMV) and barley stripe mosaic virus (BSMV) (7-9). The bicistronic RNA 3 of BMV contains a 16-22-unit oligo(A) between the genes (7). The heterogeneous poly(A) tract in BSMV genome is localized in the extracistronic portion of all virion RNA species at a distance of about 200 nucleotides from the 3' end (8,9).

In this paper, we report the sequence of the first 257 nucleotides from the 3' end of BSMV RNA and propose some models for its secondary structure.

MATERIALS AND METHODS

RNAs, enzymes and nucleotides

The three-component (Norwich) and the four-component (Argentina Mild, AM) strains of BSMV were propagated on wheat plants, and the virus was isolated as described previously (1). BSMV RNA was extracted from purified virus by the phenol technique (10). RNA 2 was isolated from total BSMV AM RNA by the method of Dolja *et al.* (11).

E.coli poly(A) polymerase was prepared as in (12) and oligo(dT)$_{10}$ was purchased from Sigma.

DNA polymerase I from *E.coli*, resitriction endonucleases, and bacteriophage T4 RNA ligase were obtained from the Institute of Applied Enzymology, Vilnius, USSR.

Nuclease S1 was from Sankyo and *E.coli* alkaline phosphatase from Worthington. RNAse H was isolated from *E.coli* MRE-600 cells by the method of Darlix (13). ^{32}P- and ^{3}H-labelled nucleotides were from Amersham. *Hind* III linker was from Bethesda Research Laboratories, Inc. AMV reverse transcriptase was kindly supplied by Dr. V.M. Kavsan.

Polyadenylation of BSMV RNA

The incubation mixture (14) contained 50 mM Tris-HCl pH 7.9, 0.5 mM EDTA, 2 mM $MnCl_2$, 12.5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 0.5 mM ATP, 60 µCi/ml ^{3}H-ATP, 200 µg/ml *E.coli* poly(A) polymerase and 150 µg/ml pure RNA 2 of BSMV AM strain. After 8-min incubation at 37°, the reaction mixture was deproteinized with phenol after adding EDTA to 25 mM, and the RNA was twice precipitated with ethanol (-20°C).

cDNA synthesis

Single-stranded DNA was synthesized in the reaction mixtures containing 40 mM Tris-HCl pH 7.5, 30 mM NaCl, 5 mM $MgCl_2$, 6 µg/ml 3' polyadenylated BSMV RNA, 50 µg/ml oligo(dT)$_{12-18}$, 0.5 mM each of the four dNTP, 5 µCi ^{3}H-dCTP, 500 units/ml reverse transcriptase (15). After 45 min at 40°C, the reaction was stopped by adding EDTA and NaOH to 20 mM and 100 mM, respectively, and heating at 65° for 30 min. DNA was extracted with phenol after neutralizing the reaction mixture with 1 N HCl, precipitated with ethanol, and purified on a Sephadex G50 column.

The synthesis of the double-stranded cDNA (dsDNA) was carried out in a mixture containing 120 mM potassium phosphate buffer pH 6.9, 10 mM $MgCl_2$, 10 mM DTT, 1 mM of each of the four dNTP, 1 mCi/ml α-^{32}P-dCTP, and 200 units/ml DNA polymerase I. After 16 h of incubation at 15°C, the reaction was terminated and the dsDNA was recovered as indicated above except that NaOH was not added.

Nuclease S1 digestion

One to two micrograms/ml dsDNA and 50 µg/ml yeast tRNA carrier in 26 µl 200 mM NaCl, 50 mM sodium acetate pH 4.5, 2 mM $ZnCl_2$ were incubated with 200 units/ml nuclease S1 for 30 min at 37°C. The digestion was stopped by adding EDTA and phenol, and DNA was precipitated twice with ethanol.

Ligation and phosphatase treatment

HindIII linker 5'-labelled with γ-^{32}P-ATP (16) was ligated to the dsDNA in a mixture containing 66 mM Tris-HCl pH 7.5, 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 1 mM dNTP, 100 units/ml *E.coli* DNA polymerase I, and 100 units/ml T4 DNA ligase. DNA polymerase I was included for repairing any damaged DNA. Incubation was for 12 h at 15°C. The reaction mixture was then supplemented with 100 units/ml restrictase HindIII and incubation continued for 4 h at 37°C. DNA was extracted with phenol and precipitated with ethanol followed by the Sephadex G75 chromatography to remove the unbound linker.

pBR322 was digested with HindIII and then treated with alkaline phosphatase for 24 h at 60°C (1 µg of enzyme was added per µg of DNA). HindIII-linearized pBR322 was ligated with lin-

ked cDNA in the same reaction mixture as above except that dNTP and DNA polymerase I were omitted, and incubation time at 15°C was decreased to 4 h.

Transformation of E.coli and selection of recombinant plasmids

Transformation of E.coli HB 101 cells was carried out by a standard technique (15). The clones containing the 3'-terminal sequences of the BSMV genome were selected by colony hybridization on nitrocellulose filters (17). The 3'-^{32}P-labelled BSMV RNA fragments used as a probe were obtained as follows. Total virion RNA from BSMV AM was labelled at the 3' end in the presence of ^{32}P-cytidine 5',3'-biphosphate (pCp) and T4 RNA ligase (18) and recovered by precipitation with ethanol after phenol extraction. The labelled RNA was subjected to mild alkaline hydrolysis, and the resulting fragments were fractionated by electrophoresis in a 5% polyacrylamide gel (PAG). Fragments not longer than 200 nucleotides were eluted from the gel (19) and used for hybridization.

Sequencing of the cDNA insert

The plasmid DNA of the clone pBS 2/2 was labelled at its 5' or 3' end after EcoRI, HindIII, and EcoRV digestion. The incubation mixture contained 66 mM Tris-HCl, pH 7.5, 6.6 mM $MgCl_2$, 10 mM DTT, 500 µCi/ml α-^{32}P-dATP or dGTP (410 Ci/mmol), 1 mM of corresponding nonlabelled dNTP, 300 µg/ml plasmid DNA, 100 units/ml DNA polymerase. After 10 min of incubation at 20°C, the labelled DNA fragments were subjected to electrophoresis in 4% PAG, eluted from the gel and sequenced (16).

Cleavage of BSMV RNA with RNAse H and isolation of the 3'-terminal fragment

This procedure was detailed in our previous paper (9). Briefly, 200 µg of total virion RNA of BSMV Norwich were incubated with 4µg of oligo(dT)$_{10}$ and RNAse H for 4 h at 4°C. The cleavage products were separated by centrifugation in linear sucrose density gradients (10-40%). The 3'-terminal fragment about 200 nucleotides long (Sh fragment) was precipitated from the peak fractions with ethanol. The RNA pellet was washed with ethanol and dissolved in distilled water at a concentration of 1 mg/ml.

Sequencing of the Sh grafment and total virion BSMV RNA

The Sh fragment of BSMV Norwich RNA was labelled with ^{32}P-pCp (18). The 3'-labelled fragment was purified by electrophoresis in 8% PAG (0.5 mm-thick slab) containing 7 M urea, eluted and subjected to direct chemical sequencing (19). The same method (19) was used for sequencing of the 3'-terminal region in RNA2 from BSMV AM.

RESULTS AND DISCUSSION

Two approaches were used in this study to sequence the 3'-terminal region of the BSMV genome. Firstly, the collection of bacterial clones containing the DNA copies of BSMV RNA 2 (strain AM) were obtained and the primary structure of the inserts was determined. Secondly, a 3'-terminal Sh fragment derived from total virion RNA (Norwich strain) was sequenced by a direct chemical method. The synthesis of cDNA was carried out with oligo(dT)$_{10}$ as a primer and the 3'-polyadenylated BSMV RNA2 as a template. All the individual BSMV RNA species have an internal oligo(A) tract (8,9) and thus can themselves serve as natural templates for reverse transcription with oligo(dT) (10). Hence, only a portion of the cDNA clones possessed the sequence localized exactly at the 3' end of the BSMV genome. The clone pBS 2/2 containing this sequence was selected using the fragments of the 3'-labelled BSMV RNA as probes, and its DNA insert was sequenced by the method of Maxam and Gilbert (16). The cloning manipulations may lead to the loss of a part of the 3'-terminal RNA sequence. The direct sequencing (19) of the 24-unit tract from the 3' end of RNA2 (strain AM) was carried out to test this possibility. The results of this experiment show that the selected clone pBS 2/2 lacks the decanucleotide sequence present in native BSMV RNA. The resulting sequence, containing the 3'-terminal CCA$_{OH}$ and the 5'-terminal 21-nucleotides-long oligo(A), is represented in Fig. 1.

The internal 8-40-unit oligo(A) sequence was used in this study as a site for addressed fragmentation of BSMV RNA (9) to obtain a homogeneous 3'-terminal fragment suitable for direct chemical sequencing. Total virion RNA of another BSMV strain, Norwich, was cleaved with RNAse H in the presence of

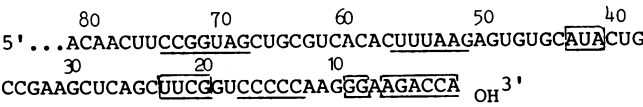

Fig. 1. The primary structure of the 3'-terminal region of BSMV RNA established by sequencing of the cDNA clone.

The sequences common to $tRNA^{Tyr}$ from yeast and localized exactly at the same positions as in tRNA are in boxes. The underlined sequences are displaced from their location in tRNA by 1-3 nucleotide residues.

oligo(dT). The 3'-terminal Sh fragment of total viral RNA, like genuine tRNAs, proved to be a very efficient substrate for ligation with ^{32}P-pCp. The electrophoregram of the labelled Sh fragment reveals one major hand, however slightly heterogeneous (data not shown). This band was excised from the gel and sequenced according to the procedure of Peattie (19). The sequence of the 3'-terminal 106 nucleotides, presumably covering the tRNA-like structure of BSMV RNA, was virtually in exact correspondence with that of the cloned cDNA insert (see Fig. 1) except G vs U at 36 and U vs A at 87. These discrepancies, if not a sequencing artifact, may be attributed to the minor differences in RNA sequences between these BSMV strains. One should bear in mind that the Sh fragment sequenced in this study originates not from an individual RNA, but from the mixture of three different RNA species of the Norwich strain. The clear sequencing pattern suggests that all these RNA species have an identical primary structure at their 3' termini, at least within the 106-nucleotides-long region.

Taken together, these sequencing data suggest that the individual RNA species of different BSMV strains possess a universal sequence at the 3' end. The high degree of nucleotide sequence conservation of the 3'-terminal region has also been reported for the four virion RNAs of bromo- and cucumoviruses (6).

The comparison of the primary structure of the 78-nucleotides-long region at the 3' end of BSMV RNA with those of $tRNA^{Tyr}$ reveals some similarity. Thus, there are several sequences common to both RNAs (see Fig. 1). It is noteworthy that

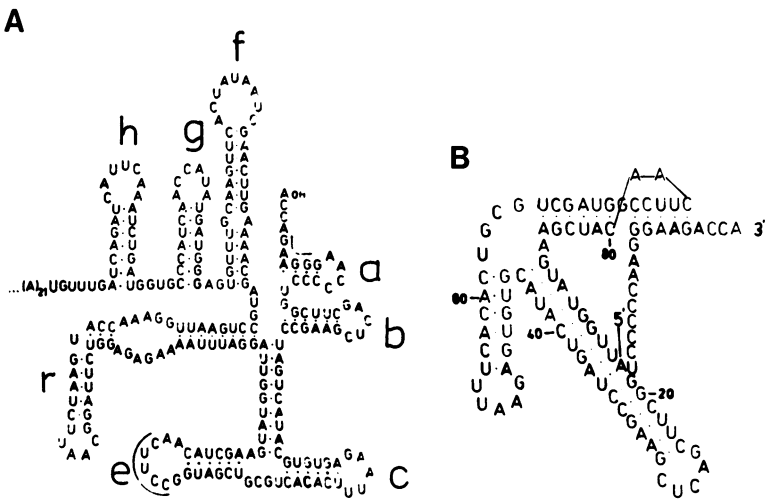

Fig. 2. Possible secondary structures of the 3'-terminal region of BSMV RNA. The stems and loops (A) are designated with lower-case letters in accordance with Ahlquist et al. (6) The sequences of the stem "a" and the loop "e" involved in the secondary structure of the other type (B) are indicated by lines.

the potential tyrosine anticodon and the UUCG analog of TΨCG in BSMV RNA are localized exactly at the same positions as in genuine tRNA (Fig. 1).

Although the 3'-terminal sequence of BSMV genome resembles $tRNA^{Tyr}$ in some points, the former cannot be folded into the orthodox clover-leaf structure. With all this, when the 3'-terminal region of BSMV RNA is packed into a secondary structure according to Ahlquist et al. (6) it acquires clear semblance to those of bromo- and cucumoviruses which also accept tyrosine (Fig. 2A). The absence of a "d" loop between loops "c" and "e" is a characteristic feature of tRNA-like structures in the genomes of BSMV and of a member of bromovirus group, broad mean mosaic virus (6). On the other hand, loop "r" is a characteristic feature of BSMV RNA.

Another possible configuration of tRNA-like structures in bromo- and cucumoviruses implies base pairing between the sequences in loops "a" and "e" (6,20,21). In this case, the "amino acyl arm" is formed by interactions between the 3' and 5'

regions of the tRNA-like sequence as well as by folding of its 5' region (20). BSMV RNA can be similarly folded into an L-shaped structure (Fig. 2B), assuming base pairing between nucleotides 73-77 and 5-9 (shown in Fig. 2A). Such configuration of the tRNA-like structure in bromo- and cucumovirus genome is strongly supported by the data on its sensitivity to enzymic digestion and by the analysis of the minimal length requirements for adenylation and aminoacylation (6,20,21). The structure of the first type (Fig. 2A) can also exist if RNA is partially denatured (21). These models allow one to predict the minimal size of a 3'-terminal fragment of BSMV RNA capable of being tyrosylated. According to Joshi et al. (20), the minimal length of an aminoacylatable sequence is set by the 5'-proximal nucleotide forming the stem between loops "e" and "c" (Fig. 2). Thus, the size of this sequence in BSMV RNA can be estimated at about 95 nucleotides (Fig. 2B) - the smallest among the tyrosine-accepting sequences studied to date (6,20).

The ability of the tRNA-like structures to interact with tRNA-specific enzymes both *in vivo* and *in vitro* is now well established (4,22,23), pointing to a probable function of these structures in virus reproduction. On the other hand, it has been shown that plant viral RNAs are contained within the virion in a de-acylated form (4). Different virus functions, i.e. encapsidation, replication, and translation, probably need some conformational changes in its RNA. The existence of two or more alternative foldings of a tRNA-like structure may provide for switching over between some of these functions. Extensive structural studies of amino acid-accepting sequences in viral genomes are likely to shed some light on this problem.

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