
Southern bean mosaic viral RNA has a 5' -linked protein but lacks 3' terminal poly(A)

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ABSTRACT

Nuclease digestion of SBMV RNA releases a protein of molecular weight approximately 12,000 in addition to the four mononucleotides. The lack of reactivity of SBMV RNA to polynucleotide kinase and the absence of a capping group suggest that the protein is covalently attached to the 5' end of the RNA. RNA sequencing shows that the 3' terminus of SBMV RNA is not polyadenylated.

INTRODUCTION

Southern Bean Mosaic virus¹ is a small spherical virus which has been well characterized both physically and chemically. [For a recent review see Rayment *et al.*²] Its molecular weight is about 7.5×10^6 . It is composed of 180 identical protein subunits of molecular weight 29,000 enclosing an RNA genome of molecular weight 1.4×10^6 . However, very little is known about the structure of the RNA. Such knowledge, especially of the RNA terminal regions, is important for an understanding of several important functions concerned with viral replication viz. initiation of RNA synthesis, ribosome binding, and virion assembly. None of these processes has been characterized for SBMV.

We have undertaken a systematic study of the viral RNA structure and function. In this communication we report that SBMV RNA has a small protein, covalently linked to its 5' end and unlike all other RNAs possessing a genome-linked protein, it lacks a poly(A) at its 3' end.

MATERIALS AND METHODS**Growth and purification of SBMV**

SBMV bean strain was isolated from leaves of *Phaseolus vulgaris* L. bountiful, which had been infected for 21 days.

Radioactively labeled SBMV was obtained from primary leaves treated as follows. Three days after inoculation 20 leaves were cut and the petioles were immersed in 10 ml distilled water containing 30 mCi ³²P

phosphate or 20 mCi ^{35}S sulphate. When most of the liquid was absorbed (about 6 hours) 10 ml of phosphate deficient Hoagland medium, or distilled water was added and the absorption was continued for 3 days.

Virus was purified essentially as described by Hull.³

Extraction and Purification of SBMV RNA

The purified virus was first dissociated in 100 mM NaCl, 10 mM Tris-HCl, pH 7.8, 3 mM EDTA and 2% SDS and the RNA was extracted according to the method of Zimmern.⁴ The RNA thus obtained was purified further by density gradient centrifugation. 2 mg RNA in 1 ml 20 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 was heated to 60° for 5 min and then rapidly chilled to 0°. It was then layered onto a 0.2-0.8 M sucrose gradient containing 100 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl, pH 7.5 and centrifuged for 17 hours at 26,000 rpm in an SW 27 rotor. Thereafter 0.6 ml fractions were collected using an ISCO density gradient fractionator equipped with a recording spectrophotometer. Fractions corresponding to the full length RNA of MW 1.4×10^6 were pooled and precipitated. The RNA pellet was purified further by two cycles of washing and ethanol precipitation. The RNA thus prepared migrated as a single sharp band in a 2.5% polyacrylamide-0.5% agarose gel.⁵

RNA from ^{32}P SBMV had a specific activity of 30-40 x 10^6 Cerenkov counts per min per mg.

RNA from ^{35}S SBMV had a specific activity of 8000 scintillation counts per min per mg.

Enzyme Digestion Conditions

SBMV RNA was digested to completion⁶ with a mixture of 50 units/ml RNase T2 (Calbiochem) + 0.1 mg/ml each of RNases T1 (Calbiochem) and A (Worthington Biochemical Corp.) in 50 mM ammonium acetate buffer, pH 5.2 at an enzyme to substrate ratio of one unit T2 RNase to 100 μg of RNA for 4 hours at 37°C. Proteinase K (Boehringer/Mannheim) digestion was as described by Flanagan *et al.*⁷

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis procedures were as has been described⁸ in 12.5%/1.25% or 15%/0.5% acrylamide/bisacrylamide slab gels. The resolution of the 15% gel was improved by using a spacer gel containing 5% acrylamide, 0.15% bisacrylamide. Like the resolving gel it contained 8 M urea, 1% SDS but the concentration of sodium phosphate, pH 7.0 was lowered to 20 mM. The samples were heated in 8 M urea, 2% SDS, 5% β -mercaptoethanol for 5 min at 100° prior to loading. The gels, 10 cm in

length, were run for 17 hours at 40 V. Fluorography was as described by Laskey and Mills.⁹

Reductive Methylation

Purified RNA was treated with ^{14}C -formaldehyde (NEN, 45.8 mCi/mmol), using the procedures of Rice and Means¹⁰ and Means and Feeney.¹¹ In vitro labelled RNA had a specific activity of about 200,000 scintillation counts per min per mg.

3'-end Labelling of SMBV RNA and the Determination of its 3' Sequence

SMBV RNA was labelled at its 3' end with $[5'\text{-}^{32}\text{P}]\text{pCp}$ (NEN, 2000 Ci/mmol) by use of T4 RNA ligase (PL Biochemicals) essentially as described by Ahlquist and Kaesberg.¹² It was then digested with various nucleases and the products were analyzed on a high resolution sequencing gel.¹²

RESULTS

We investigated the ability of the 5' terminus of SBMV RNA to serve as a substrate for polynucleotide kinase. When the RNA was treated with phosphatase (to remove any possible 5' phosphate groups) followed by enzymatic phosphorylation with polynucleotide kinase and $\gamma\text{-}[^{32}\text{P}]\text{ATP}$, less than .0005% of the input radioactivity was incorporated into the

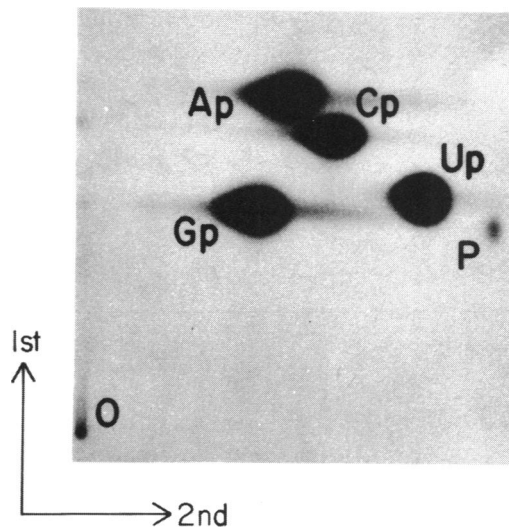


Fig. 1. Two-dimensional thin layer chromatography of the ribonuclease digest of ^{32}P SBMV RNA showing the absence of a capped or phosphorylated structure. Digestion conditions are described in Materials and Methods. Chromatography was according to Nishimura.²¹

RNA (data not shown). Under identical conditions, 6-10% of the label was incorporated into "decapped" BMV RNA4 (our unpublished observation). This suggested that the 5' terminus of SBMV RNA is blocked.

Since blocked structures of the general form $m^7G(5')ppp(5')X(m)p$ are commonly found at the 5'-termini of messenger RNA molecules of eukaryotic cells and their viruses,¹³ a search was made first for the existence of such a 5'-terminal structure in SBMV RNA. This was done by complete hydrolysis of the ^{32}P labeled RNA with a mixture of ribonucleases T2, T1 and A followed by analysis of the products by thin layer chromatography as shown in Fig. 1. In addition to the four major mononucleotides there are two faint spots -- one moving almost with the solvent front in the second dimension which we believe is due to free phosphate produced by a contaminating phosphatase activity in the ribonuclease mixture; the other one is the material left at the origin. There are no additional spots characteristic of a cap structure or a di-, tri-, or tetra-phosphorylated 5'-end. Therefore, the putative block must be other than a cap structure.

Several plant and animal viruses have been shown to contain a protein covalently linked to their genomes.^{7,14-20} The results, below, indicate that the blocking moiety on SBMV RNA is a protein.

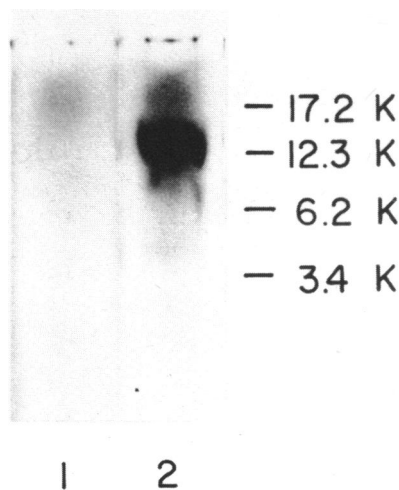


Fig. 2. SDS gel electrophoresis of ribonuclease digested ^{32}P SBMV RNA. The autoradiograph is an analysis on a 12.5% polyacrylamide, 1.25% bisacrylamide slab gel. Lane 1, RNA treated with proteinase K. Lane 2, RNA not treated with proteinase K. Molecular weights of stained markers are given on the right-hand side. Mononucleotides migrated out of the gel.

Isolation of a ^{32}P -labelled Protein Associated with SBMV RNA

Purified ^{32}P SBMV RNA was digested to completion with ribonucleases. The products were then treated with SDS, urea, and β -mercaptoethanol to remove any non-covalently associated protein. The resulting material was then analyzed on a 12.5% polyacrylamide gel as shown in Fig. 2. A single band of ^{32}P -labeled material was found (lane 2). The band was absent (lane 1) when the RNA was pretreated with proteinase K prior to the nuclease digestion. Use of protein markers showed that the band migrated with a mobility slightly greater than that of cytochrome C ($M = 12,300$). These data are consistent with the existence of a small protein covalently linked to SBMV RNA through a phosphate group.

Isolation of a ^{35}S -labelled Protein Associated with SBMV RNA

SBMV virions labelled in vivo with ^{35}S -sulphate were extracted with phenol in the presence of SDS and EDTA, to isolate SBMV RNA. When the RNA was analyzed on a sucrose gradient (.2 M to .8 M), a peak of ^{35}S label coincided with the absorbance profile of the RNA (Fig. 3). When

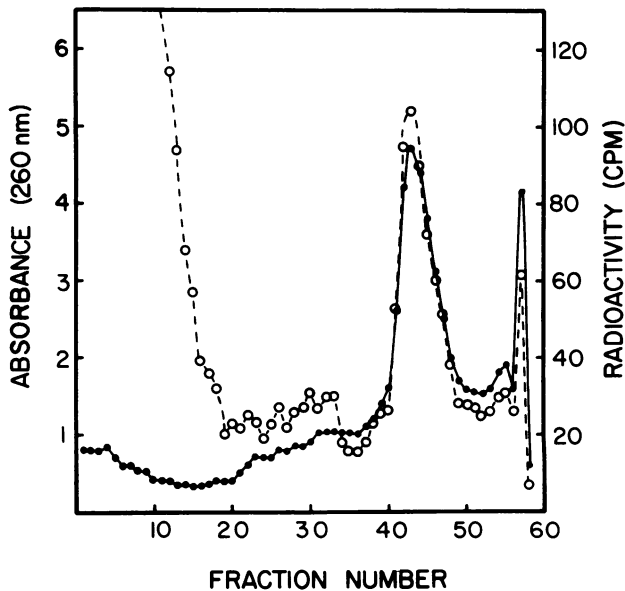


Fig. 3. Gradient centrifugation of SBMV RNA isolated from ^{35}S -labelled virions on 0.2-0.8 M sucrose gradients. Sedimentation was from left to right. (●) absorbance; (○) radioactivity. Radioactivity in the fractions was determined by counting 60 μl aliquots for 20 min.

this ^{35}S -labelled RNA was pooled, precipitated, and an aliquot analyzed on an RNA gel, a band of radioactivity comigrating with stained full-length RNA was seen. When the RNA was pretreated with proteinase K before the gel analysis, the RNA was no longer labelled (data not shown). These data suggest that a protein remains associated with SBMV RNA even after repeated phenol extractions and heating in denaturants. Because there is substantial radioactivity at the top of the gradient, it was possible that the protein associated with SBMV RNA might be residual coat protein. To discount this, the ^{35}S -labeled RNA was digested to completion with ribonucleases with and without prior proteinase K treatment. The products were analyzed on a 15% gel together with ^{14}C -labelled molecular weight standards as shown in Figure 4. Lane 2 shows a single band migrating like a protein of about 12,000 molecular weight significantly smaller than the 29,000 molecular weight SBMV coat protein. The absence

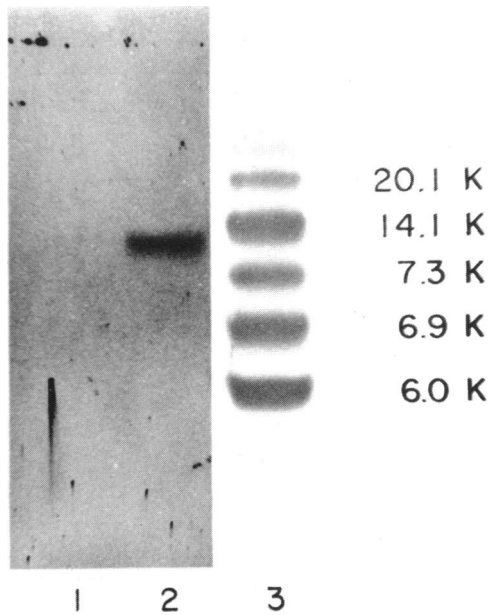


Fig. 4. SDS gel electrophoresis of nuclease digested ^{35}S SBMV RNA. The fluorograph is an analysis on a 15% polyacrylamide .5% bisacrylamide slab gel. Lane 1, RNA treated with proteinase K; Lane 2, RNA not treated with proteinase K; Lane 3, ^{14}C -Leu CNBr peptides of BMV coat protein. Their molecular weights are given on the right hand side. Lanes 1 and 2 are printed more darkly than Lane 3.

of the band in lane 1 (the proteinase K-treated RNA) confirms the conclusion that the label is in a protein. Presence of substantial radioactivity at the top of the gradient also suggests that, under these conditions, non-covalently bound protein would be expected to dissociate from the RNA.

In vitro Labelling of SBMV RNA-associated Proteins

The procedures, above, result in rather weak labelling of the putative SBMV RNA-associated protein. Substantially more label can be introduced into the protein by in vitro labelling with formaldehyde and sodium borohydride.^{10,11} Such a reductive methylation using ¹⁴C-formaldehyde was applied to purified SBMV RNA. The RNA was then digested to completion with ribonucleases and the digest analyzed on a gel as before (Fig. 5). The majority of the label appears in a band that migrates like a protein of about 12,000 molecular weight, i.e., in the same position as the in vivo labelled protein. A minor band of radioactive material, migrating like a protein of about 7000 molecular weight, was also visible. We have

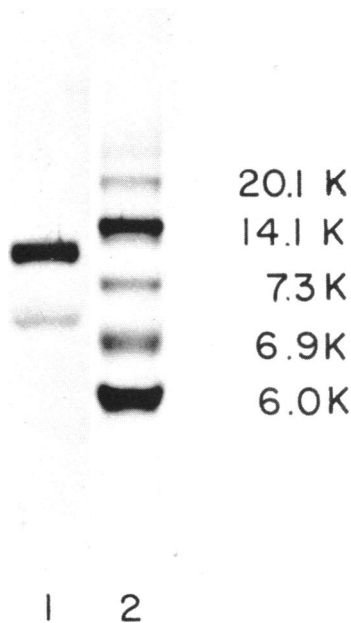


Fig. 5. SDS polyacrylamide gel analysis of in vitro labelled proteins associated with SBMV RNA. The fluorograph is an analysis on a 15% polyacrylamide-0.5% bisacrylamide slab gel. Lane 1, ribonuclease digest of SBMV RNA labelled with ¹⁴C-formaldehyde; Lane 2, ¹⁴C-Leu labelled CNBr peptides of BMV coat protein.

not analyzed the latter material further.

Absence of Poly(A) at the 3' End of SBMV RNA

We examined the 3' end of the SBMV RNA by labelling the 3' terminus

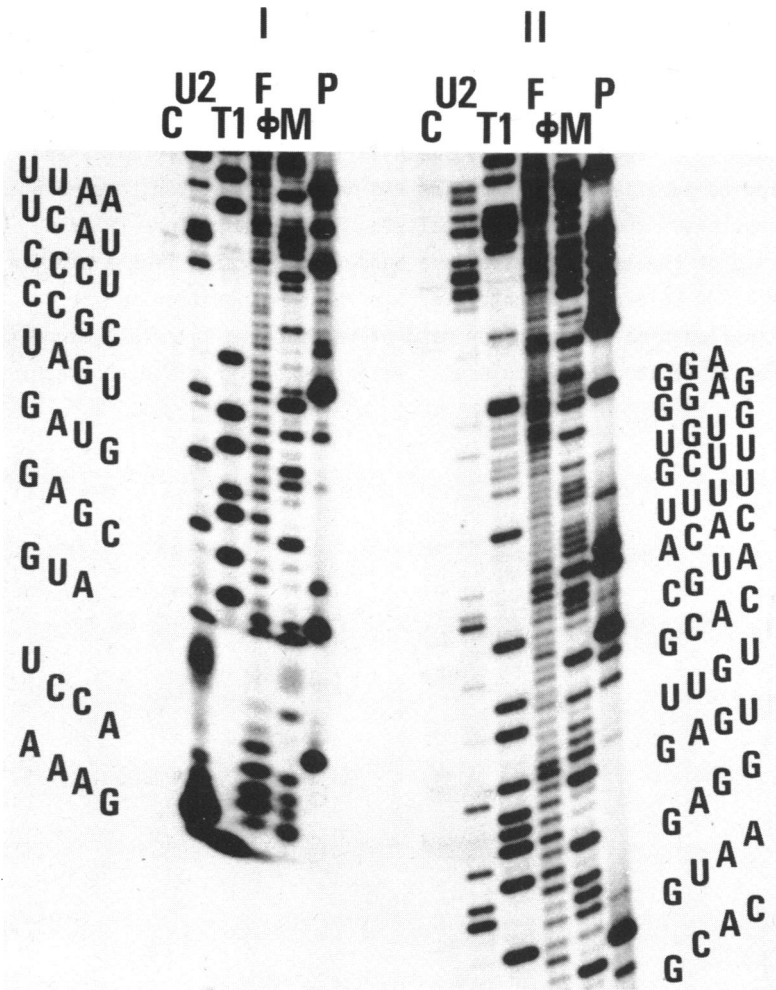


Fig. 6. Sequencing gel pattern showing 3'-end sequence of SBMV RNA. The lanes are labelled according to treatment of the 3'-end labelled RNA : C : control, no nuclease; U2 ribonuclease U2; T1 ribonuclease T1; F Formamide; ϕM Ribonuclease Phy M; P Pancreatic Ribonuclease. Two separate loadings of the sample were made. Panel I is the shorter run showing bases from the 3' end. Panel II is the longer run.

The sequence is to be read GAAA upwards in Panel I and after reaching AAUU, from GCAC upwards in Panel II.

with ^{32}P using T4 RNA ligase and $[\text{5}'\text{-}^{32}\text{P}]\text{pCp}$ and then analyzing it on a high resolution nucleic acid sequencing gel.

The sequence at the 3' terminus includes all four bases with no evidence for a poly(A) region (Fig. 6).

DISCUSSION

The results in this paper indicate that a protein of molecular weight of approximately 12,000 is linked to the genome of the SBMV RNA. The bond linking the protein to the RNA is probably covalent because we were unable to break it by treatments that normally disrupt non-covalent protein-nucleic acid interactions. Our results are in accord with the work of O. P. Sehgal, who found that treatment with a protease destroys the infectivity of SBMV RNA (personal communication).

All the RNA viruses, with a genome linked protein, discovered so far have been found to be polyadenylated at their 3' end.^{19,22-25} SBMV RNA is an exception in having a protein attached to it but no poly(A) tail.

For some of these viruses, notably polio, EMC and cowpea mosaic virus, the RNA-associated protein is not required either for efficient translation or infection.^{18,26} On the other hand its presence has been shown to be an absolute requirement for infection by calici and nepo viruses^{20,27} and now with the work of Professor Sehgal, by SBMV. The lack of a poly(A) tail on SBMV RNA shows that the function of the protein and poly(A) are not obligatorily related.

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Abbreviations: BMV, brome mosaic virus; EMC, encephalomyocarditis virus; SBMV, southern bean mosaic virus; T1, T2 and A, takadiastase ribonuclease 1, takadiastase ribonuclease 2 and pancreatic ribonuclease A, respectively.

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