ENZYMATIC REMOVAL OF THE 5′-TERMINAL METHYLATED BLOCKED STRUCTURE OF TOBACCO MOSAIC VIRUS RNA AND ITS EFFECTS ON INFECTIVITY AND RECONSTITUTION WITH COAT PROTEIN

Takeshi OHNO, Yoshimi OKADA, Kunitada SHIMOTOHNO*, Kin-ichiro MIURA*, Hideaki SHINSHI**, Masanao MIWA** and Takashi SUGIMURA**

*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, *National Institute of Genetics, Mishima 411, Japan, and **National Cancer Center Research Institute, Chuo-ku, Tokyo 104, Japan*

Received 24 May 1976

1. Introduction

The 5′-terminus of tobacco mosaic virus (TMV) RNA, long thought to be an unphosphorylated A residue [1,2], was recently identified as $m^7G^{5'}ppp^{5'}Gp$ [3,4]. The assembly reaction of TMV RNA with TMV protein is thought to start at the 5′ end and proceed to the 3′ end along the RNA chain [5–7]. It would be interesting to know whether the odd structure at the 5′-terminus is related to the assembly reaction. On the other hand, elimination of the 3′-terminal base of TMV RNA by a chemical procedure (periodate oxidation and subsequent cleavage by aniline) was reported to cause marked loss of infectivity [8]. However, this chemical procedure probably eliminated both the 5′ and 3′ end of TMV RNA, so it is uncertain which elimination reaction was responsible for loss of infectivity.

These considerations prompted us to investigate the removal of the 5′-terminal blocked structure of TMV RNA and its effects on the assembly reaction and infectivity. Recently, a novel phosphodiesterase was purified from cultured tobacco cells [9]. This enzyme released pm^7G from the 5′-terminal blocked structure of cytoplasmic polyhedrosis virus mRNA, but did not attack the polynucleotide chain of the mRNA [10]. We used this enzyme to digest TMV RNA. We found that it preferentially cleaved the 5′-blocked structure of TMV RNA and that TMV RNA lacking the 5′-blocked structure showed unchanged ability to assemble with the coat protein, but no infectivity.

2. Materials and methods

TMV RNA and protein were prepared from purified TMV (Japanese common strain OM) by the acetic acid method [11] and extraction with phenol–bentonite [12], respectively.

TMV RNA labeled with tritium at both termini was prepared by periodate oxidation and subsequent reduction with tritium labeled sodium borohydride, as described before [5]. On this treatment, the base moiety of m^7G, and the ribosyl residues of the 3′ and 5′ ends, were all reduced with tritium borohydride. However, 7-methyl-8-hydro[^{3}H]guanosine was reoxidized very quickly in aqueous solution [13], and the C-8 tritium proton of m^7G rapidly exchanged with a proton of the solvent [14]. Analysis by phosphocellulose (P-cellulose) column chromatography after digestion of tritium labeled TMV RNA with ribonuclease T2 showed that about half the radioactivity was present as $m^7G'pppGp$, arising from the 5′-terminal structure, and the other half as A′, derived from the 3′-terminus (A′ and G′ represent the nucleoside trialcohols of A and G, respectively, as described RajBhandary [15]).

Tobacco phosphodiesterase was purified from cultured tobacco cells. The purification procedure is described elsewhere [9]. The enzyme was incubated with substrate at 30°C in 0.05 M sodium acetate buffer (pH 5.9) containing 1 mM EDTA [10] and the products were analysed by column chromatography on Dowex 1 [16] and on P-cellulose [17].

Experiments on reconstitution of TMV were performed by the method of Fraenkel-Conrat and Singer [18] except that TMV protein was preincubated in 0.1 M phosphate buffer (pH 7.2) at 20°C for 24 h.

The infectivity of the preparations was assayed on *Nicotiana tabacum* var. Xanthi nc by the half-leaf comparison method.

3. Results

3.1. *Enzymatic removal of the 5′-terminal blocked structure of tritium-labeled TMV RNA*

When tobacco phosphodiesterase was incubated with tritium-labeled TMV RNA about 50% of the radioactivity was released into the acid-soluble fraction. This fractions was analysed by Dowex 1 column chromatography (fig.1). The radioactivity was eluted in two fractions: one in the void volume (peak I) and the other in the position close to CMP (peak II). On P-cellulose column chromatography peak I was eluted in a position very close to pm^7G (fig.2, A) and in the same position of m^7G' after digestion with bacterial alkaline phosphatase (fig.2, B). Digestion of peak II with alkaline phosphatase yielded only one product which was eluted in the same position as m^7G' from a P-cellulose column (fig.2, C). Scarcely any radioactivity in the acid-soluble fraction, corresponded to pA′ or A′. These results indicate that the 5′-terminal blocked structure of tritium labeled TMV RNA was quantitatively digested with tobacco phosphodiesterase and released as pm^7G' and ppm^7G'. By the prolonged digestion ppm^7G' was converted into pm^7G' (data not shown).

To see whether the phosphodiester bond of TMV

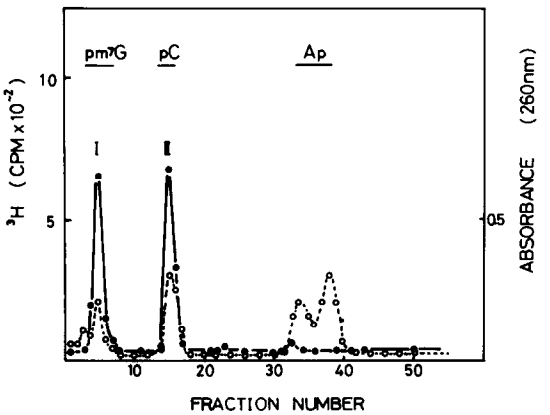

Fig.1. Dowex 1 X 2 column chromatography of cleavage products formed by incubation of ^3H-labeled TMV RNA with tobacco phosphodiesterase. ^3H-labeled TMV RNA (10 μg) was incubated with the enzyme (0.07 units) at 30°C for 10 min under the standard condition and then the mixture was applied to a column (0.5 X 20 cm) of Dowex 1 X 2. The column was washed with water and then eluted from tube number 10 with a linear gradient of 0.005 N HCl to 0.01 N HCl + 0.1 M NaCl. pm^7G, CMP and 2′,3′AMP were included as internal markers. (○) Absorbance at 260 nm, (●) radioactivity.

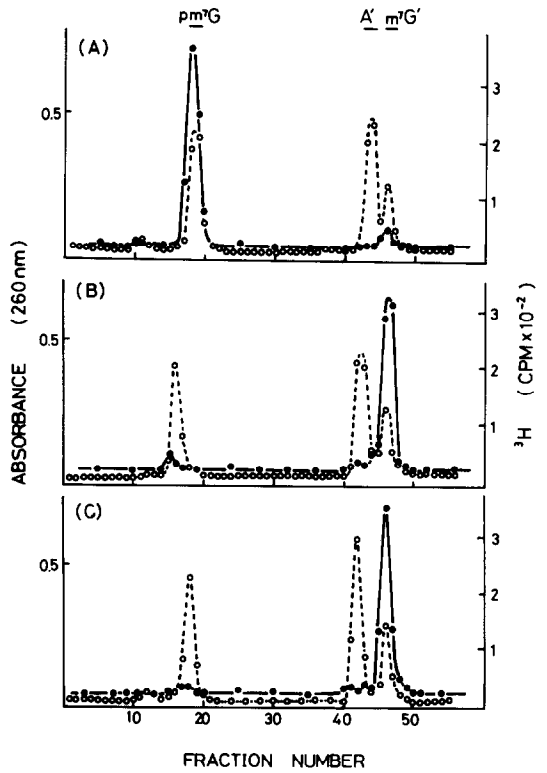

Fig.2. Analysis of cleavage products of TMV RNA on digestion with tobacco phosphodiesterase by column chromatography on phosphocellulose. (A); Peak I in fig.1 was directly applied to a column (0.9 X 35 cm) of phosphocellulose equilibrated with 10 mM $HCOONH_4$ (pH 3.85). The column was washed with the same buffer and then eluted with a linear gradient of 10 mM to 0.4 M $HCOONH_4$ (pH 3.85). (B), (C); Peak I (B) or peak II (C) in fig.1 was incubated with bacterial alkaline phosphatase (0.2 units) in 0.1 M Tris-HCl (pH 7.8) containing 0.1 mM $MgCl_2$ at 37°C for 2 h, and then the mixture was analyzed. (○) Absorbance at 260 nm, (●) radioactivity.

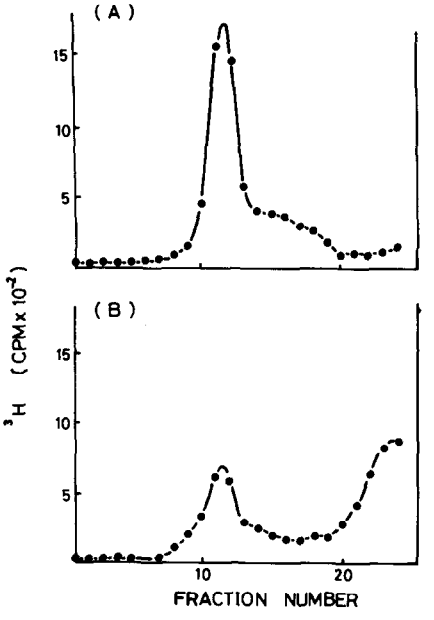

Fig.3. Sucrose density gradient analysis of ^{3}H-labeled TMV RNA after incubation with tobacco phosphodiesterase. ^{3}H-labeled TMV RNA was incubated with (B) or without (A) the enzyme under the conditions described in the legend to fig.1. The RNA was analyzed by centrifugation at 22 000 rev/min in a 5–20% sucrose gradient in 10 mM Tris-HCl buffer (pH 7.4) for 18 h at 4°C in a SW-25-1 swinging bucket rotor of a Hitachi ultracentrifuge. The radioactivity in aliquots of the fractions was counted. Sedimentation is from right to left.

RNA was digested by tobacco phosphodiesterase, tritium-labeled TMV RNA was incubated with the enzyme and then the mixture was directly analysed by sucrose density gradient centrifugation. Approximately half the radioactivity was recovered at the top of the gradient while the other half was found in the position of intact TMV RNA (fig.3). Digestion of TMV RNA with tobacco phosphodiesterase caused no change in the size of RNA.

3.2. *Assembly of TMV protein with TMV RNA lacking the 5′-terminal blocked structure*

A mixture of native TMV RNA and tritium-labeled TMV RNA, was digested with tobacco phosphodiesterase. The reaction mixture was then incubated with TMV protein under the conditions for the reconstitution reaction, and analysed by sucrose density gradient centrifugation. As a control, undigested TMV RNA was incubated with TMV protein and analysed in the same way. As shown in fig.4, the amount of reconstituted particles formed from phosphodiesterase-treated TMV RNA was almost equal to that formed from untreated TMV RNA, although the specific radioactivity of these reconstituted particles was about a half that of the control particles formed from untreated TMV RNA. Radioactive pm^{7}G′pppGp and A′ were both identified in alkaline digests of the control particles whereas only radioactive A′ was found in the digest of particles formed from enzyme treated TMV RNA (data not shown).

From these results, it was concluded that TMV RNA

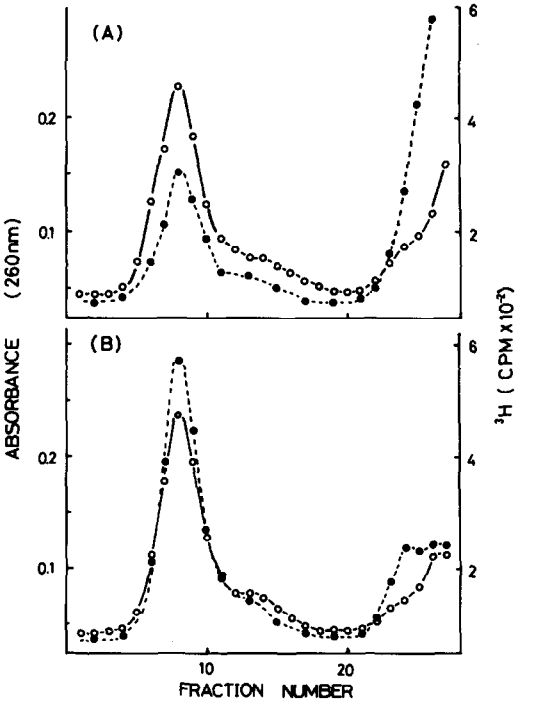

Fig.4. Sucrose density gradient analysis of the particles reconstituted from TMV protein and TMV RNA treated (B) or not treated (A) with tobacco phosphodiesterase. Native TMV RNA containing ^{3}H-labeled TMV RNA was treated with the enzyme as described in the legend to fig.1, and then incubated with 30-fold excess of TMV protein in 0.1 M sodium phosphate buffer (pH 7.2) at 25°C for 60 min. The reaction mixture was analyzed by centrifugation at 24 000 rev/min on a 5–20% sucrose gradient in 10 mM phosphate buffer (pH 7.2) for 2 h at 4°C. Sedimentation is from right to left. (○) Absorbance at 260 nm, (●) radioactivity.

Table 1
Infectivity of TMV RNA lacking the 5′-terminal blocked structure

	Lesions per half-leaf			
	RNA		Reconstitution mixture	
Enzyme treatment	+	−	+	−
No. of leaves				
1.	7	592	11	470
2.	1	487	3	456
3.	5	375	3	324
4.	0	231	3	188
5.			0	176

TMV RNA lacking the 5′-terminal blocked structure and its reconstituted complex with TMV protein were prepared as described in the legend to fig.4 and their infectivities were assayed at concentrations of native RNA of 1.57 μg/ml and 1.14×10^{-3} μg/ml, respectively.

lacking the 5′-terminal blocked structure assembled with coat protein as well as native TMV RNA.

3.3. *Infectivity of TMV RNA lacking the 5′-terminal blocked structure*

The infectivity of TMV RNA which had been treated with tobacco phosphodiesterase was assayed on tobacco plants as free RNA or after reconstitution with TMV protein. As shown in table 1, TMV RNA lost its infectivity almost completely on removal of the 5′-terminal blocked structure.

4. Discussion

In this work it was found that tobacco phosphodiesterase cleaved tritium labeled TMV RNA releasing pm^7G' and ppm^7G'. Results indicated that the enzyme preferentially cleaved the pyrophosphate bond of the 5′-terminal methylated blocked structure of TMV RNA, even when the terminal nucleoside was modified to a nucleoside dialcohol derivative by periodate oxidation and subsequent reduction with borohydride.

It has been thought that reconstitution of TMV starts at the 5′-end of the RNA [5–7]. However, the present work showed that, the 5′-terminal blocking structure of TMV RNA is not necessary for initiation of the reconstitution reaction. Previously we found that almost all of the radioactivity of partially reconstituted RNA prepared from tritium labeled TMV RNA was liberated into the acid-soluble fraction on digestion with ribonuclease [5]. This indicates that the 5′ and 3′ ends of partially reconstituted RNA are both attacked by ribonuclease, at least, during the early stage of the reconstitution reaction. That is, assembly does not seem to start from the 5′ end of TMV RNA, although it may start close to the 5′ end. The present results support this idea.

TMV RNA lacking the 5′-terminal blocking structure has no infectivity, although the biological meaning of the removal of the blocking structure is not clear yet. This is the first report of direct evidence that the blocking structure of virus RNA is essential for infectivity.

Acknowledgments

We thank Drs H. Inoue and Y. Nozu, Institute for Plant Virus Research, for kindly supplying TMV. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and a grant from the Toray Science Foundation.

References

[1] Sugiyama, T. and Fraenkel-Conrat, H. (1963) Biochemistry 2, 332–334.
[2] Fraenkel-Conrat, H. and Fowlks, E. (1972) Biochemistry 11, 1733–1736.
[3] Zimmern, D. (1975) Nucleic Acid Res. 2, 1189–1201.
[4] Keith, J. and Fraenkel-Conrat, H. (1975) FEBS Lett. 57, 31–33.
[5] Ohno, T., Nozu, Y. and Okada, Y. (1971) Virology 44, 510–516.
[6] Butler, P. J. G. and Klug, A. (1971) Nature New Biology 229, 47–50.
[7] Thouvenel, J. C., Guilley, H., Stussi, C. and Hirth, L. (1971) FEBS Lett. 16, 204–206.
[8] Steinschneider, A. and Fraenkel-Conrat, H. (1966) Biochemistry 5, 2735–2743.
[9] Shinshi, H., Miwa, M., Kato, K., Noguchi, M., Matsushima, T. and Sugimura, T. (1976) Biochemistry, in the press.
[10] Shinshi, H., Miwa, M., Sugimura, T., Shimotohno, K. and Miura, K. (1976) FEBS Lett. in the press.
[11] Fraenkel-Conrat, H. (1957) Virology 4, 1–4.

[12] Fraenkel-Conrat, H., Singer, B. and Tsugita, A. (1961) Virology 14, 54–58.
[13] Pochon, F., Pascal, Y., Pitha, P. and Michelson, A. M. (1970) Biochem. Biophys. Acta 213, 273–281.
[14] Tomasz, M. (1970) Biochem. Biophys. Acta 199, 18–28.
[15] RajBhandary, U. L. (1968) J. Biol. Chem. 243, 556–564.
[16] Shimotohno, K. and Miura, K. (1976) FEBS Lett. in the press.
[17] Lewandowski, L. J., Content, J. and Leppla, S. H. (1971) J. Virol. 8, 701–707.
[18] Fraenkel-Conrat, H. and Singer, B. (1964) Virology 23, 354–362.