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Blocked 5′ Termini in Brome Mosaic Virus RNA

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All four components of brome mosaic virus RNA have $m^7G^{5'}$ ppp $^{5'}$Gp as their 5′ terminus. The m^7G can be removed by β-elimination, resulting in the conversion to pppGp.

Brome mosaic virus (BMV) is a multicomponent plant virus belonging to the bromovirus group (15). Its genetic information is divided among four RNAs, designated 1 to 4 in order of decreasing size. The RNAs are contained in three virions: a heavy virion H containing RNA1, a light virion L containing RNA2, and an intermediate-density virion M containing RNAs 3 and 4. Infectivity studies with isolated RNAs show that RNAs 1, 2, and 3 are essential for infection, whereas RNA4 is dispensable. However, RNA4 appears in progeny virus (14). Nucleotide sequence analyses showed that RNA4 originates from RNA3 (23).

Translation studies with BMV RNAs have been carried out in a cell-free system derived from wheat embryo. All four RNAs have been found to be active messengers, inducing the synthesis of four different proteins. RNA4 is highly efficient as a monocistronic messenger for the viral coat protein, of mol wt about 20,000 (3, 22). Although RNA3 also contains the coat protein cistron, it is translated into coat protein at very low efficiency; RNA3 induces the synthesis, largely, of a protein of mol wt 35,000. RNA1 induces the synthesis of a protein of mol wt 110,000, and RNA2 induces the synthesis of a protein of mol wt 105,000 (12, 22).

In the light of the recent findings that a methylated, blocked 5′-nucleotide terminus occurs in a wide variety of RNAs in eukaryotic systems (2, 6, 9–11, 13, 19, 20, 24, 27), it was of interest to identify the 5′ termini of the BMV RNAs. We have analyzed the 5′ termini of the four RNA components of BMV. The results show that, in all of them, the 5′ terminal base is a modified guanosine attached to the penultimate base through a 5′ p-p-p 5′ link.

MATERIALS AND METHODS

Preparation of ^{32}P-labeled RNA. The procedure used to grow BMV and to obtain ^{32}P-labeled RNA has been described before (4). Briefly, 30 mCi of carrier-free ^{32}P was used to label 10 secondary leaves of barley plants infected in their primary leaves. Virus was isolated by homogenization of the plants, polyethylene glycol precipitation, and differential centrifugation. Approximately 5 to 8 mg of virus of specific activity 10^8 counts/min per mg was obtained. RNA was isolated from the virus by extraction with phenol. RNA4 and RNA3 were obtained by fractionation of whole BMV RNA on a 5 to 20% sucrose density gradient centrifuged in a Spinco SW27 rotor at 26,000 rpm for 20 h. To obtain pure RNA1 and RNA2 it was necessary to separate the virus into light- and heavy-virion fractions prior to RNA extraction and sucrose density gradient centrifugation. This was done by a rubidium chloride density centrifugation (14).

Analysis of the T1 RNase digestion products. ^{32}P-labeled BMV RNA1, RNA2, RNA3, and RNA4 (10^6 to 10^7 counts/min) were digested with T1 RNase for 30 min at 37 C using 1/10 the weight of enzyme to that of RNA, and the resulting oligonucleotides were fractionated by two-dimensional electrophoresis as described by Sanger et al. (21).

Periodate oxidation and β-elimination of RNA4. To a solution of 20 μg of ^{32}P RNA in 20 μl of 0.1 M sodium acetate (pH 5) was added 2 μl of 0.01 M $NaIO_4$. After incubation for 1 h at 21 C in the dark, 50 μl of ethanol was added, and the RNA was allowed to precipitate for at least 4 h at −20 C and was recovered by centrifugation. The RNA was washed by dissolving it in 0.1 ml of 0.1 M NaOAc and reprecipitating it with 0.2 ml of ethanol. The RNA precipitate was then suspended in 10 μl of 0.33 M redistilled aniline (adjusted to pH 5.0 with HCl), and 2 μl of 0.1 M NaOAc was added to it. After incubation for 3 h at 25 C in the dark, the RNA was precipitated with 50 μl of ethanol. To remove all aniline, the RNA was washed twice by dissolving in 0.1 M NaCl and reprecipitating with ethanol. T1 ribonuclease digestion and two-dimensional electrophoresis of this RNA were as described for the untreated RNA.

Analysis of the 5′-terminal nucleotide composition and sequence. After two-dimensional electrophoresis, the oligonucleotides were located on the electropherograms by autoradiography. The 5′-terminal oligonucleotide was selected and characterized by further digestion with enzymes and subsequent high-voltage paper electrophoresis as follows. Digestion with pancreatic RNase was carried out for 1 h at 37 C with 0.1 mg of enzyme per ml containing 1 mg of unlabeled yeast RNA per ml as carrier.

RNase T2 was used at 500 U/ml with 20 μg of carrier RNA, and the digestion was carried out for 18 h at 37 C. For alkaline phosphatase and nucleotide pyrophosphatase digestions, samples were incubated with enzyme at a concentration of 0.1 mg/ml for 1 h at 37 C. Snake venom phosphodiesterase was used at 0.2 mg/ml, and the hydrolysis was carried out for 2 h at 37 C. Alkaline digestion of oligonucleotides was for 18 h at 37 C in 0.2 N NaOH. In all cases the digestion was carried out in a total volume of 5 to 10 μl, the hydrolyzed samples were applied as thin lines of 1-cm length on Whatman 540 paper or DE-81 paper, and the products were separated by electrophoresis at 5,000 V (100 V/cm) for 40 min in pyridine acetate buffer, pH 3.5.

Removal of m^7G from the T1 oligonucleotide after dephosphorylation with alkaline phosphatase was carried out using the procedure of Wintermeyer and Zachau (26). The oligonucleotide was first treated with alkaline phosphatase, and the enzyme and free phosphate were removed by a short electrophoresis on Whatman 540 paper. The dephosphorylated product was then eluted from the paper with water and treated with 0.1 M $NaHCO_3$, pH 9.5, for 4 h at 45 C. The mixture was again subjected to a short electrophoresis to remove $NaHCO_3$, and the product was eluted and treated with 0.3 M aniline-HCl, pH 3.5, for 4 h at 45 C. The mixture was then applied to DE-81 paper along with unlabeled markers such as pG, ppG, pppG, and pppGp and electrophoresed for 2 h at 1,500 V in pH 3.5 buffer.

Two-dimensional thin-layer chromatography of the snake venom phosphodiesterase digest of the T1 oligonucleotide was done according to the method of Nishimura (18). The enzyme was used at a concentration of 0.5 mg/ml, and the hydrolysis was carried out for 2 to 3 h at 37 C. Digestion with the enzyme was also carried out after treatment of the oligonucleotide with 0.3 M NaOH for 30 min. In the latter case, the alkali was removed by chromatography of the reaction mixture on Whatman 540 paper using isobutyric acid-0.5 M NH_3, 5:3 (vol/vol), as solvent. The product was then eluted from the paper with water, digested with the enzyme, and subjected to thin-layer chromatography. Unlabeled pm^7G and alkali-treated pm^7G were used as markers.

For the preparation of markers like ^{32}P-labeled pm^7G and pGp, *Escherichia coli* $tRNA^{lys}$ was digested with pancreatic ribonuclease, and the oligonucleotides Gpm^7GpUp and pGpGpUp were isolated from the fingerprint. Further digestion of these with snake venom phosphodiesterase and T1 ribonuclease yielded pm^7G and pGp. Unlabeled pm^7G was prepared by DEAE-Sephadex column chromatography of the pancreatic RNase digest of beef liver tRNA and subsequent digestion of the m^7G-containing fraction by alkaline phosphatase and snake venom phosphodiesterase. It was located on the thin-layer plate by absorbance under UV light.

Sources of enzymes and radioisotopes. T1 and T2 RNase were purchased from Calbiochem (Sankyo). Pancreatic RNase, alkaline phosphatase, and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp., Freehold, N.J. Nucleotide pyrophosphatase was purchased from Sigma Chemical Co., St. Louis, Mo. Carrier-free (^{32}P) *o*-phosphate was obtained from International Chemical and Nuclear Corp.

RESULTS

Identification of the 5′ termini by fingerprint analysis. BMV RNAs 1, 2, 3, and 4 were hydrolyzed with T1 RNase, and the products were separated by two-dimensional electrophoresis. Fig. 1a shows the pattern with BMV RNA4. When these oligonucleotides were eluted and further digested with pancreatic RNase or T2 RNase, only one (designated t-5′) was found to be completely resistant to both enzymes. Treatment of this oligonucleotide with alkali changed its electrophoretic mobility only slightly but did not convert it into products migrating as mononucleotides. The oligonucleotide moves close to UUG in the fingerprint, indicating a net charge of −4, and its resistance to endonucleases and alkali indicates an unusual structure. The same oligonucleotide occurs in the T1 RNase fingerprint of BMV RNAs 1, 2, and 3 (not shown).

To determine the structure of the oligonucleotide it was digested with several exonucleases and subjected to paper electrophoresis. The results are shown in Fig. 2. Treatment with snake venom phosphodiesterase was carried out without prior treatment with alkaline phosphatase so as to achieve a limited digestion. This produced two major products; one has the same mobility as the authentic marker pm^7G, and the other moved near inorganic phosphate and on further analysis was found to be ppGp. In addition small amounts of pGp and Pi were also produced. Thus the reaction produces ppGp and pm^7G, as would the scission $m^7G^{5'}$ p/pp $^{5'}Gp$, and then ppGp was cleaved further to pGp and Pi. The appearance of pm^7G indicates that the m^7G has a free 3′ hydroxyl and is linked through its 5′ phosphate. Treatment of the oligonucleotide with alkaline phosphatase released only 25% of its radioactivity as inorganic phosphate. The remainder was in a phosphatase-resistant single compound. When the digestion was done with nucleotide pyrophosphatase, the oligonucleotide was converted into pm^7G, pGp, and Pi. A small amount of ppGp was also produced as a partial digestion product. This indicates that m^7G is joined to the adjacent G by a pyrophosphate linkage.

To determine the number of phosphates between m^7G and the penultimate G, the T1 oligonucleotide was dephosphorylated with alkaline phosphatase to remove 3′ phosphate. Elimination of m^7G was then done by the procedure of Wintermeyer and Zachau (26), which

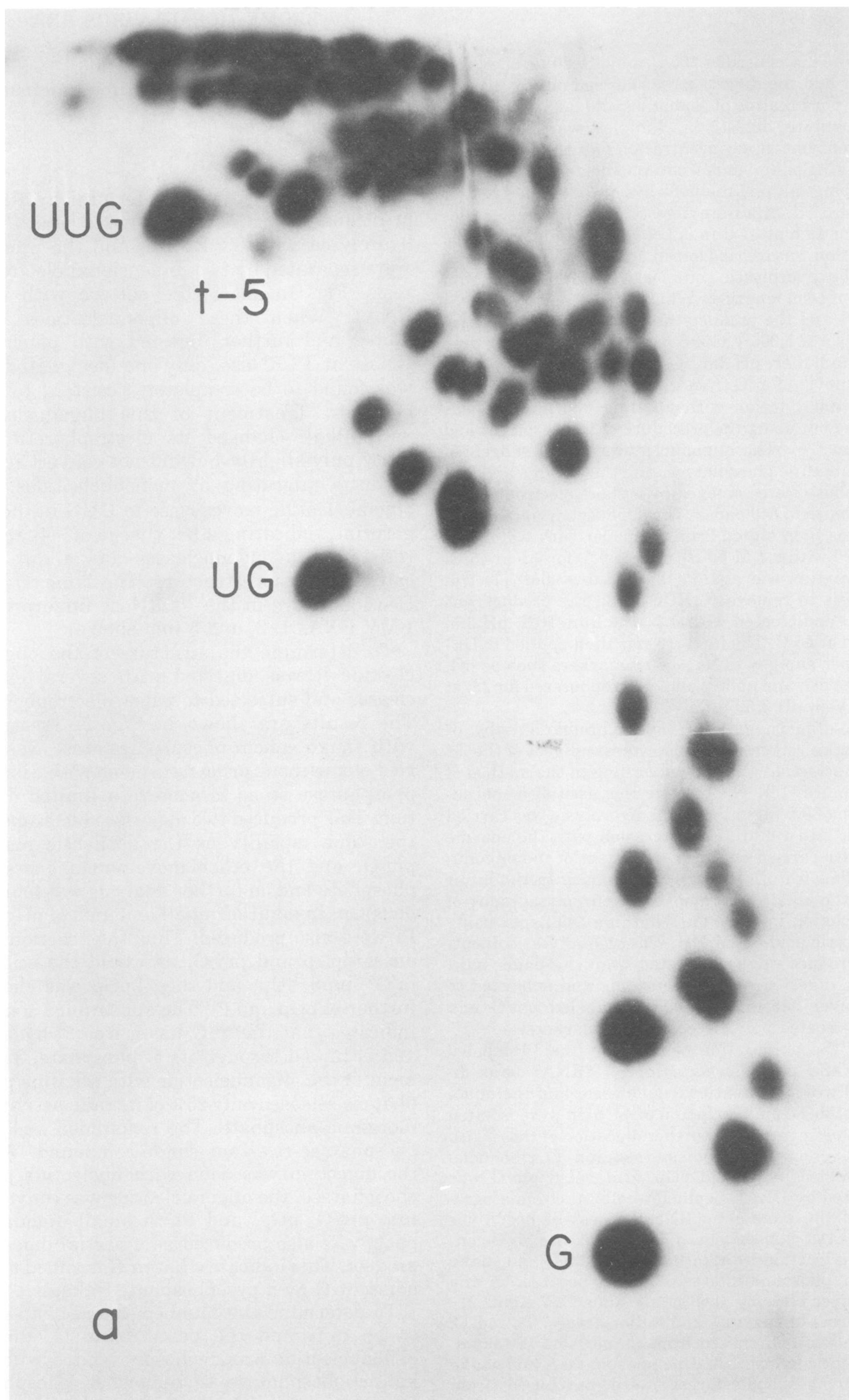

FIG. 1. *Oligonucleotides produced by hydrolysis of BMV RNA4 with T1 RNase. (a) Electropherogram of BMV RNA4. (b) Electropherogram of BMV RNA4 after periodate oxidation and β-elimination. The 5'-oligonucleotide is designated t-5.*

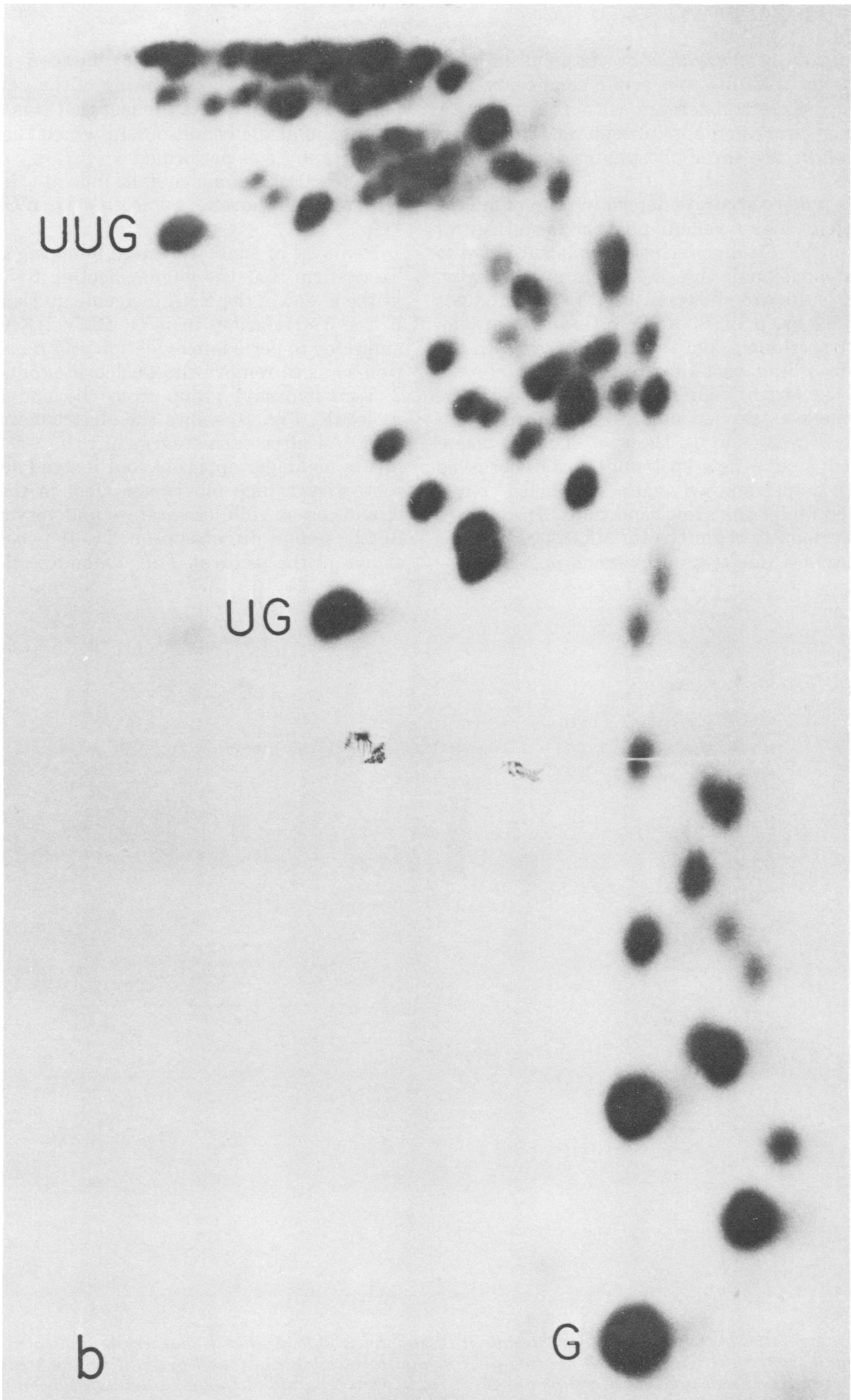

Fig. 1 B

specifically removes m^7G by virtue of its lability under alkaline and acidic conditions. The product coelectrophoresed with the authentic marker guanosine 5′-triphosphate. This shows that there are three phosphates between m^7G and G.

For a more accurate determination of m^7G, a complete snake venom phosphodiesterase digest of the T1 oligonucleotide was subjected to two-dimensional thin-layer chromatography. The results are shown in Fig. 3. The products obtained are pGp, Pi, and pm^7G, and the radioactive spot due to pm^7G superimposed with the UV-absorbing spot of pm^7G used as marker (Fig. 3a and b). When the digestion with the enzyme was carried out after alkaline treatment (Fig. 3c and d), the spot of pm^7G disappeared, and a new spot pm^7G^* was observed which superimposed with unlabeled pm^7G treated under the same conditions. The change in the mobility of pm^7G after alkali treatment is presumably due to its conversion to 2-amino-4-hydroxy-5-(*N*-methyl)carboxamide-6-ribosyl pyrimidine (16). The streaking of the new spot pm^7G^* might be due to the fact that it is unstable in the acidic conditions in which the chromatography was performed.

Thus all of the above data indicate that the structure of the oligonucleotide is $m^7G^{5'}$ ppp $^{5'}$Gp.

Removal of the 5′-terminal blocking group. To confirm that the oligonucleotide t-5′ exists at the 5′ end of the RNA molecule and contains a free 3′-OH group in m^7G, BMV RNA4 was subjected to periodate oxidation and β-elimination so as to remove the nucleoside-containing 2′,3′-*cis*-hydroxyl group from the end of the molecule. Fig. 1b shows the electropherogram of RNA4 after such treatment. The t-5′ oligomer is no longer apparent, but instead another spot exists which moves very fast in the first dimension on cellulose acetate and very slowly in the second dimension on DE-81 paper (not shown in the picture). This oligonucleotide co-

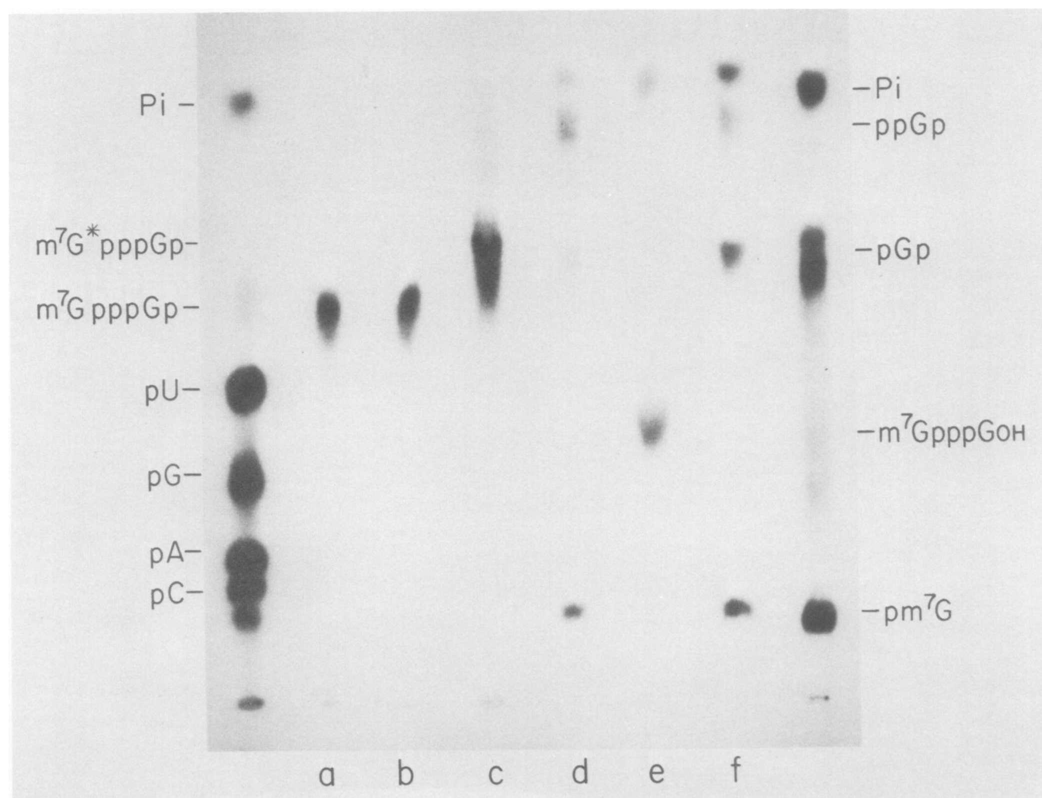

FIG. 2. *Paper electrophoresis of the 5′ terminal T1 oligonucleotide. Authentic markers are indicated on the autoradiogram. m^7G^* is 2-amino-4-hydroxy-5-(N-methyl)carboxamide-6-ribosyl pyrimidine, the breakdown product of m^7G. Conditions for digestion are described in Materials and Methods. (a) undigested control; (b) T2 ribonuclease digest; (c) alkaline digest; (d) snake venom-phosphodiesterase digest; (e) alkaline phosphatase digest; (f) nucleotide pyrophosphate digest.*

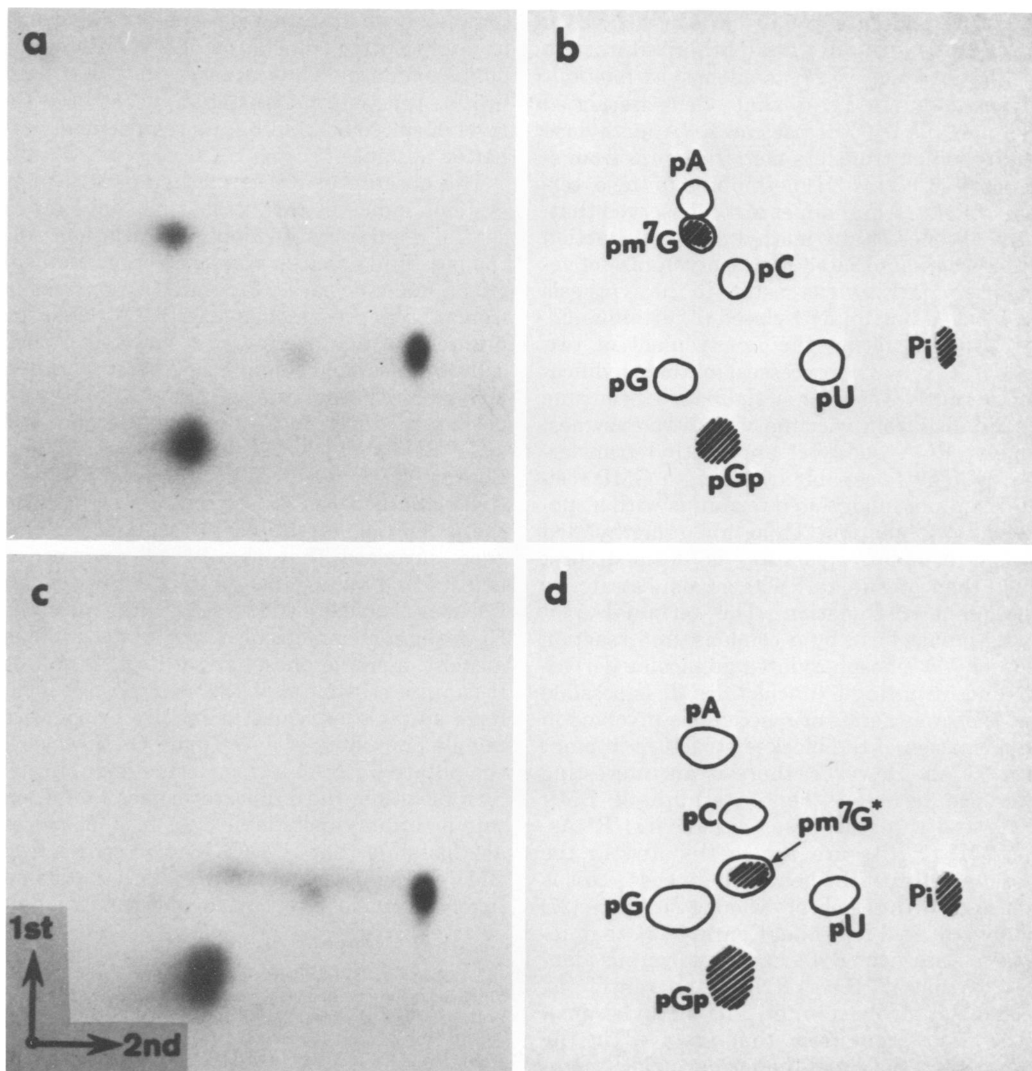

FIG. 3. *Two-dimensional thin-layer chromatography of the digestion products obtained by hydrolysis of the 5' T1 oligonucleotide by venom phosphodiesterase. (a) and (c) illustrate autoradiographs of the chromatograms. (a) Oligonucleotide digested with snake venom phosphodiesterase. (c) Oligonucleotide first treated with alkali and then digested with snake venom phosphodiesterase. (b) and (d) are schematic diagrams of the chromatograms (a) and (c), respectively. The positions of the unlabeled markers are indicated by circles and the radioactive spots by hatched circles. A faint spot of pU is due to a contamination of the T1 oligonucleotide with UUG.*

electrophoresed with an authentic marker pppGp. Digestion with alkaline phosphatase converted it quantitatively into Pi.

DISCUSSION

Evidence presented in the preceding section shows that the 5′ termini of the four RNA components of BMV are $m^7G^{5'}$ ppp $^{5'}$Gp. Recently, this type of structure has been detected in a number of cellular and viral mRNAs. These include mRNA's from reovirus (6, 10), vaccinia virus (24, 25), cytoplasmic polyhedrosis virus (9), vesicular stomatitis virus (1), tobacco mosaic virus (13, 27), mouse myeloma cells (2), HeLa cells (11), and rabbit reticulocyte globin mRNA (17). In addition, several low-mol-wt RNAs isolated from Novikoff hepatoma cell nuclei have also been found to have this type of structure (19).

The reactions involved in formation of the

blocked 5′ ends are yet to be established. In vitro studies with mRNA's synthesized in vitro in the presence of s-adenosyl-L-[*methyl*-^{3}H]methionine suggest that virus-associated RNA polymerase possesses an RNA methylase activity which transfers methyl groups from s-adenosyl-L-[*methyl*-^{3}H]methionine to the 5′ termini of RNA. Abraham et al. (1) observed that, in the absence of any methyl donor, the action of the virion-associated RNA polymerase of vesicular stomatitis virus results in the synthesis of a blocked but unmethylated 5′ terminus $G^{5'}$ ppp $^{5'}$Ap, indicating the involvement of two distinct enzymatic processes in the modification. Recently Ensinger et al. (8) have been able to solubilize from vaccinia virus two enzymes, namely, RNA guanylyl and methyl transferases, which are capable of adding a GMP residue to a diphosphorylated terminus with a subsequent methylation. Thus, an unmethylated blocked-terminal structure is formed first which then presumably acts as a substrate for subsequent methylation. The terminal 5′-5′ linkage must form by a condensation reaction between a 5′-phosphorylated guanosine derivative and initiating 5′-nucleotides. It is possible that the same series of reactions is involved in the formation of the blocked structure in plant viral RNAs. However, there is an interesting difference between the 5′ termini of BMV RNAs and those of most animal viral RNAs. The BMV RNAs are not methylated in the ribose moieties of the penultimate base. This is consistent with the observation of Zimmern (27) and Keith and Fraenkel-Conrat (13) that tobacco mosaic virus RNA has a 5′ terminus identical to that of BMV RNA. Apparently the mechanism involved in the 2′-O-methylation of ribose is different from that involved in the base methylation, and the former is lacking in the plant systems. When reovirus and VSV RNAs are translated in vitro by wheat germ extracts in the presence of s-adenosyl-L-[*methyl*-^{3}H]methionine, methylation occurs exclusively at the 5′ ends, yielding m^7GpppG and m^7GpppA, respectively (17), indicating that wheat germ extracts lack the ribose methylation activity.

Detailed studies concerning the possible implication and biological function of the methylated, blocked 5′ termini are being carried out in several laboratories. It has been shown that in vitro translation of reovirus and vesicular stomatitis virus mRNA requires the presence of m^7G (17). In a previous report, we have shown that the 5′ end of RNA4 is included in the ribosome-binding site (7). Removal of m^7G by β-elimination has been found to reduce but not completely abolish the ribosome-binding capacity and in vitro translation of BMV RNA (unpublished data). Thus, whereas m^7G has a definitive function in translational control at the level of ribosome binding, its requirement may not be absolute.

The absence of ribose methylation in plant systems indicates that it does not have an essential regulatory function in translation. Also the possibility that it acts as a recognition site for an endonuclease when mRNA is processed from a large precursor nuclear RNA (11) seems unlikely for plants. However, since such plant cellular messengers have not been shown to lack ribose methyl groups, this possibility cannot be ruled out. It is interesting to note that BMV RNA3 and RNA4 have the same 5′ end. Because BMV RNA4 is not required for infectivity and because its sequence is identical to the 3′ portion of the RNA3 sequence, it has been surmised that the RNA4 sequence is transmitted to progeny via RNA3. Presumably RNA3 is cleaved to yield a RNA4-like molecule. However such a molecule lacks a m^7G and has, at most, a single phosphate at its 5′ terminus. It is quite possible that the viral host does not have suitable enzymes to readily provide the middle phosphate of $m^7G^{5'}$ ppp $^{5'}$G. However if the putative RNA4-like molecule is duplicated by replication, the duplicated copies would contain a suitably modifiable end. It is, of course, also possible that BMV RNA4 is copied from BMV RNA3 by enzymatic processes that do not involve a nucleolytic cleavage of RNA3.

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