THE 5'-END GROUPS OF ALFALFA MOSAIC VIRUS RNAs ARE $m^7G^5'ppp^5'Gp$

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Received 29 July 1975

1. Introduction

Recent work on animal virus messenger RNAs [1–4] and mammalian cellular messenger RNAs [5] have shown that in both cases the RNAs carried a methylated blocked 5' terminal end group. It has also been recently shown that the RNA-4 of Bromegrass Mosaic Virus [6] and the RNA of Tobacco Mosaic Virus [7] have a similar blocked endgroup at the 5' position ($m^7GpppGp$). In the present paper we demonstrate the presence of such an 5'-end group in the three heaviest Alfalfa Mosaic Virus RNAs which together with the viral coat protein promote viral infection.

2. Materials and methods

AMV_S was multiplied on *Nicotiana tabacum* var. Xanthi nc. Ten plants were labelled with ^{32}P and the virus extracted 7 days after inoculation according to previously described methods [8]. The purified labelled virus was phenol extracted and fractionated by electrophoresis on polyacrylamide–agarose gels (2.4–0.5%). The purified RNA species were eluted from the gel slices by electrophoresis and the eluted RNA was then phenol extracted and precipitated with 2 vol ethanol. The detailed procedure is given in [8]. [^{32}P]TMV RNA was kindly provided by Dr H. Guilley.

For enzymatic digestion of the RNAs, a mixture of RNase T_1 and T_2 extracted from Takadiastase of *Aspargillus oryzae*, generously provided by Dr G. Keith was used. Pancreatic RNase and snake venom phosphodiesterase were obtained from Worthington Biochemical Corp. and snake venom nucleotide pyrophosphatase from Sigma Biochemicals.

Total AMV RNA or the purified RNA species were digested with a mixture of RNases T_1 and T_2 with an enzyme to substrate ratio of 1 unit per 60 μg RNA and with RNase A with an enzyme to substrate ratio of 1 unit per 40 μg RNA in 50 mM Na acetate buffer pH 4.7, 10 mM EDTA for 3 h at 37°C.

Alkaline phosphatase was used in 0.1 M Tris pH 7.5, 1 mM EDTA, at 0.5 mg/ml. The substrate added to 50 μg of carrier RNA was hydrolyzed for 1 h at 37°C.

Snake venom phosphodiesterase was used at 0.2 mg/ml in 20 mM Tris acetate pH 8.8, 10 mM Mg acetate. The substrate was hydrolyzed for 1 h at 37°C in the presence of 50 μg carrier RNA.

Nucleotide pyrophosphatase digestion was done at 1 unit/ml in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ in the presence of 10 μg NAD and 10 μg ApA for 30 min at 37°C.

The standard methods of electrophoresis and elution according to Sänger et al. were used [9].

Chromatographic separation of nucleotides on cellulose thin layer plates was done by ascending chromatography in two dimensions in the following solvents: 1st dimension isobutyric acid: NH_4OH: water (60:1:33 v/v); 2nd dimension isopropanol: HCl: water (17.6:68:14.4 v/v) A RNases $T_1 + T_2$ digest of tRNA was used as an optical marker.

3. Results

Electrophoresis of small oligonucleotides at pH 3.5 on DEAE paper Whatman (DE_{81}) allows a separation partly according to chain length. Adams et al. [10] used this method to screen R_{17} RNA partial digests for the presence of 5'-terminal pppGp. When a total RNases $T_1 + T_2$ + A digest of AMV RNA was analysed

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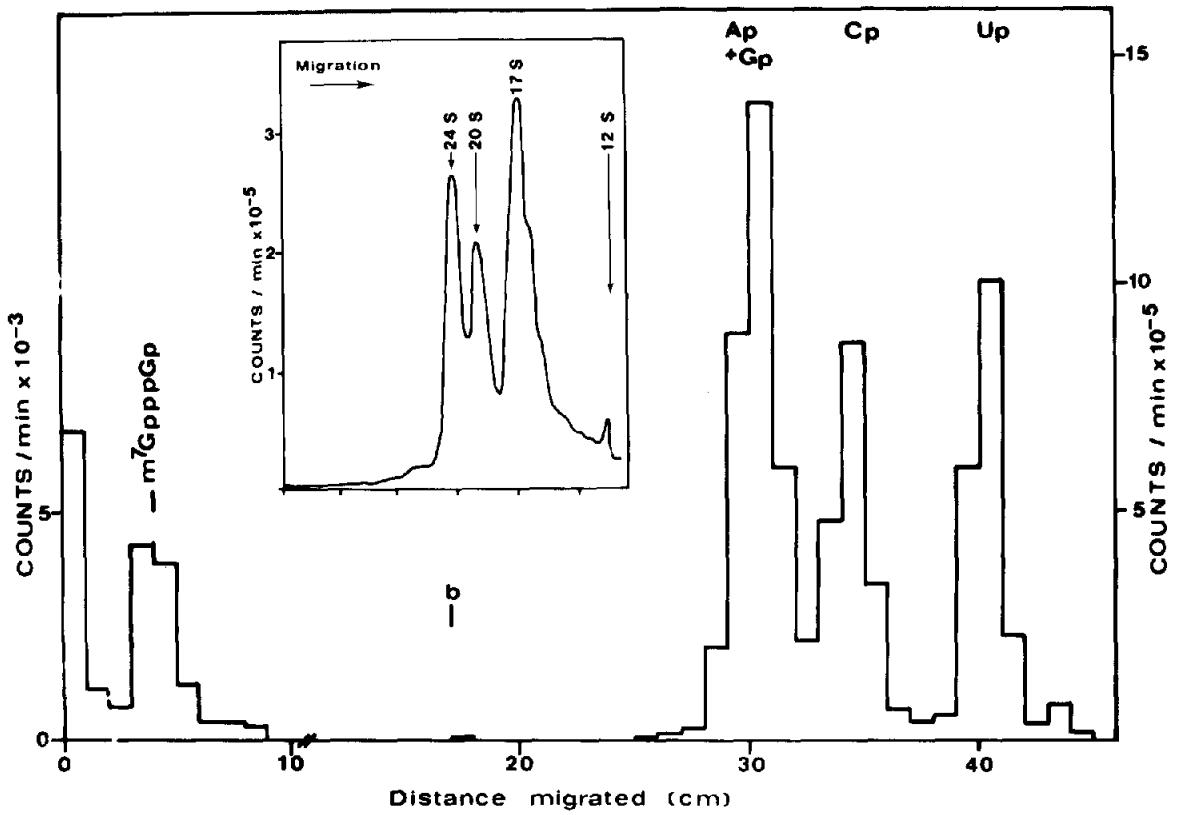

Fig.1. Radioactivity distribution in an electrophoretogram of a RNases T_1 + T_2 + A digest of [^{32}P]AMV RNAs. The separation of the digestion products was made on Whatman DE_{81} paper during 90 min at 2000 V in a pyridine acetate buffer pH 3.5. The dried paper was autoradiographed and then cut into centimeter strips and counted in a toluene-PPO scintillation mixture. The insert shows the radioactivity pattern of the AMV RNA used in these experiments. Polyacrylamide gel electrophoresis 2.4%–agarose 0.5%, migration 2 h at 8 mA. The gel was cut in millimeter slices with a Gilson gel fractionator and the fraction counted as Cerenkov radiation.

by this method at pH 3.5, a product was observed running behind the digested mononucleotides. This spot remained at the same position after a second hydrolysis and migrated at a mobility of 0.25 relative to the blue marker xylene cyanol (fig.1). A resistant spot with the same mobility was observed for TMV RNA analysed in the same way. The relatively high background present in the first electrophoresis was eliminated when the spot was rerun in the same conditions. The material at the origin remains in the same position even after a second digestion. The insert in fig.1 shows the electrophoretic pattern of the total AMV RNA used for this experiment. It should be noticed that this preparation contains little 12 S RNA. This sample of [^{32}P]AMV RNA was also used for isolation of purified 24 S, 20 S and 17 S RNA species.

Table 1 gives the percentage of the total counts on DEAE paper represented by the RNases-resistant spot for the total RNA and 20 S RNA. Assuming the presence of one m^7 GpppGp 5′-end group in each RNA, there will be 4 mol of phosphate in the resistant spot per mol of starting RNA. The percentage of counts in the resistant species calculated for total RNA, assuming a total additive chain length of 8900 nucleotides and corrected for the relative amount of each RNA in the preparation used, gives a value in good agreement with the experimental value. The same calculation may be made for 20 S RNA of 3000 nucleotides chain length. The base compositions in the different experiments listed in table 1 are in good agreement with the previously established values [11].

Such a structure migrating in the trinucleotide

Table 1

Material	Counts	$T_1 + T_2$ resistant product				Mononucleotides		
		Back-ground	cpm % of total	Calculated[a] value in %	Molar yield	Ap + Gp %	Cp %	Up %
Total AMV RNA	7860	1400	0.128	0.142	0.90	47.8	23.3	28.5
20 S RNA	5050	1310	0.11	0.133	0.82	48.0	22.1	28.9
Published [11]						48.6	22.3	28.9

[a] Calculated from a formula $m^7GpppGp$ and a chain length of 3900 nucleotides for 24 S RNA (35%), 3000 for 20 S RNA (15%) and 2000 for 17 S RNA (50%) [8], 35%, 15% and 50% refers to the relative amount of each RNA species in the preparation used.

region could result from either a phsophorylated end group or the presence of two adjacent ribose methylated nucleotides [7]. The digestion of this component with *E. coli* alkaline phosphatase liberated about 30% of ^{32}P counts as inorganic phosphate and the spot shifted from the trinucleotide to the dinucleotide region of the electrophoretogram. This action was also observed with TMV RNA digested and run under the same conditions. This result favors the ribose methylated product and rules out phosphorylated groups (fig.2D).

From venom phosphodiesterase digestion (fig.2B) two products were observed, one product near the origin and another spot was observed in the Cp region. The radioactivity distribution between these two spots is in a ratio 1:3. When the spot from the Cp region was run on unmodified Whatman 1 paper at pH 3.5 it migrated very close to the origin. A comparison of the modification of mobility as well as the radioactivity distribution with the observations made on TMV digestion products [7] suggests a m^7Gp structure for this species. The product migrating near the origin on DEAE paper yields only inorganic phosphate after alkaline phosphatase digestion suggesting a mononucleoside polyphosphate structure as an end product of venom phosphodiesterase digestion.

The analysis of the RNases $T_1 + T_2 + A$ resistant spot from AMV RNAs by cellulose thin layer chromatography (fig.3a) together with the same product obtained from TMV RNA shows the same migration pattern and strongly suggests the identity of the digestion product for the three AMV RNAs. The venom phosphodiesterase digestion products of AMV RNAs migrate in the same manner as those of TMV RNA [7] (fig.3c).

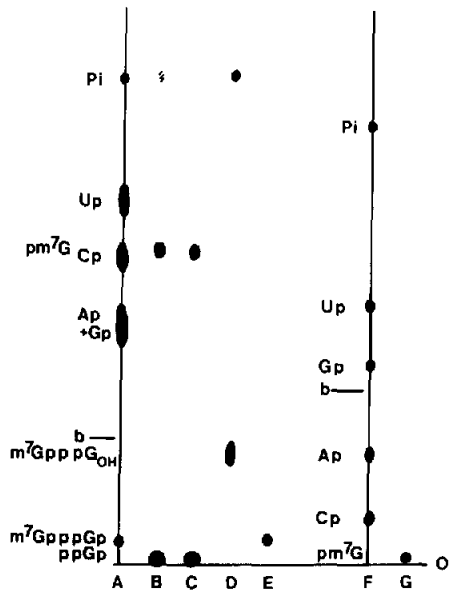

Fig.2. Analysis of the RNases $T_1 + T_2 + A$ resistant nucleotide and its various digestion products by electrophoresis at pH 3.5 on DE_{81} paper (on the left A–E) and on Whatman 1 paper (on the right F–G). (A) RNAases $T_1 + T_2 + A$ digestion products of AMV RNAs, also used in fig.1. (B) RNases $T_1 + T_2 + A$ resistant spot snake venom phosphodiesterase digest (low spot = 1980 cpm, upper spot 750 cpm). (C) Snake venom nucleotide pyrophosphatase digest (lower spot = 15 020 cpm, upper spot 3890 cpm). (D) Alkaline phosphatase digest (lower spot 6150 cpm, Pi = 2100 cpm). (E) RNases $T_1 + T_2 + A$ resistant spot from 20 S RNA second run the same migration position is observed with TMV RNA and AMV RNA 24 S and 17 S. (F) Migration of marker nucleotides on Whatman 1 paper. (G) Migration of the upper spot obtained in B or C (pm^7G) on Whatman 1 paper. The position of the blue marker is indicated by b.

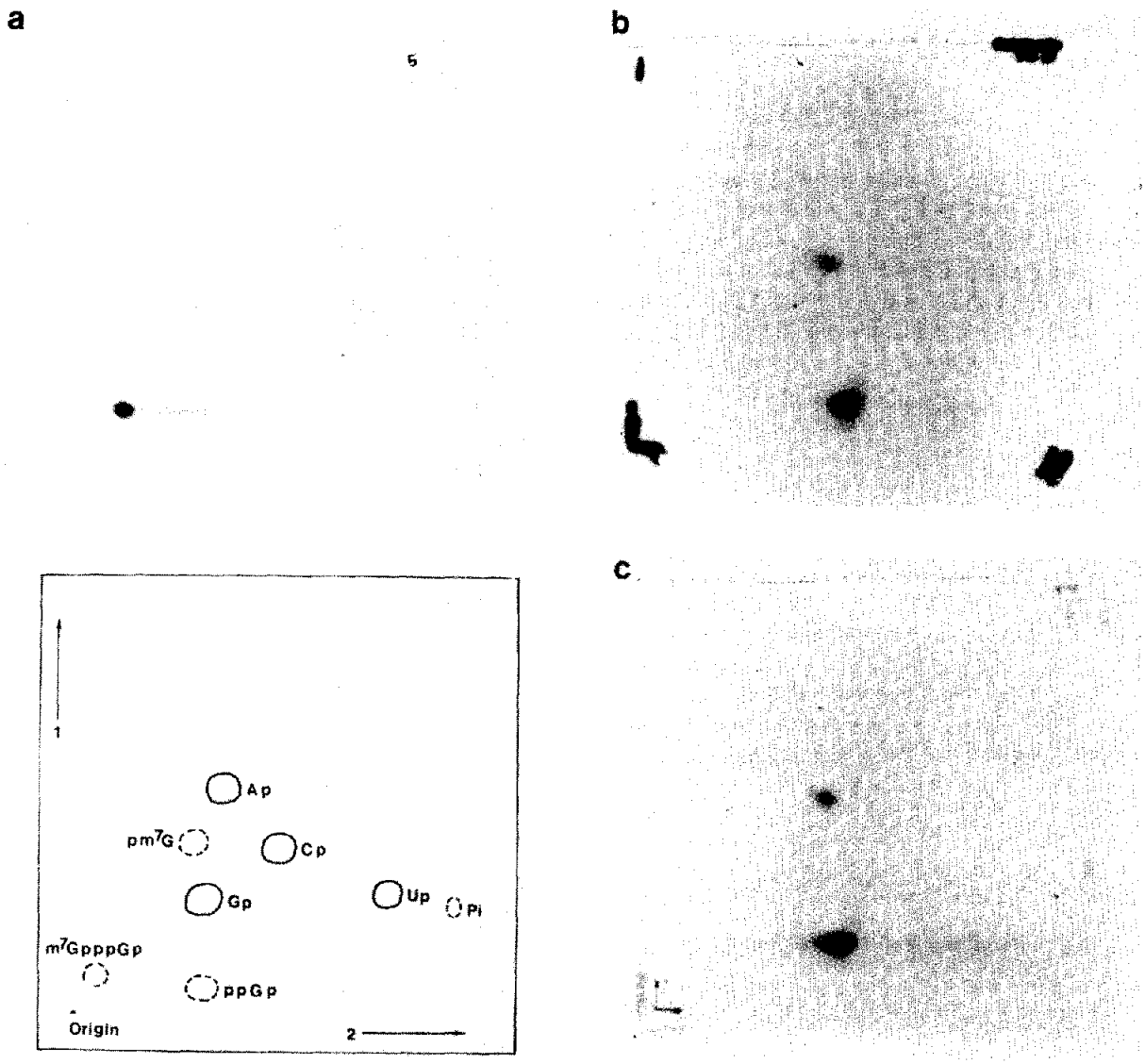

Fig.3. Two dimensional cellulose thin layer chromatography of the RNases $T_1 + T_2$ resistant nucleotide and its digestion products. (a) Control, the total AMV RNAs or the three purified RNAs yield the same spot after $T_1 + T_2$ digestion; (b) nucleotide pyrophosphatase digest; (c) snake venom phosphodiesterase digest. The position of the different digestion products relative to the optical markers used in each analysis ($T_1 + T_2$ hydrolysate of tRNA) is summarized in the diagram on the bottom left. Chromatographic conditions are as indicated in Material and methods.

4. Discussion

The major digestion products of the resistant spot by venom phosphodiesterase are a phosphorylated end group plus a 7-methylguanosine monophosphate. The mobility on DEAE paper and the chromatographic behaviour suggest a structure of the form $m^7G^5ppp^{5'}Gp$ similar to those proposed for TMV [7], BMV [6] and animal viruses, in which the inverted 5′ to 5′ linkage of the m^7G with a free 2′, 3′ hydroxyl results in it being a good substrate for venom phosphodiesterase while the phosphates between the G bridge

are resistant to alkaline phosphatase [7,10].

The existence of such a structure of the form $m^7G^{5'}ppp^{5'}Gp$ was confirmed after digestion with nucleotide pyrophosphatase. This enzyme gives hydrolysis products of the RNase $T_1 + T_2 + A$ resistant nucleotides identical to those of venom phosphodiesterase, both in their mobility on DE_{81} paper (fig.2C) and cellulose thin layer chromatography (fig.3b).

The results reported above for AMV RNAs are similar to those obtained for TMV RNA [7]. The molar yield of the $T_1 + T_2$ resistant nucleotide, as indicated from the values obtained in table 1, is in good agreement with the presence of one 5'-blocked end per RNA species, each RNA giving a resistant nucleotide with the same migration on DE_{81} paper (fig.2E) and on thin layer cellulose plates (fig.3a). Moreover, each type of AMV RNA gives the same digestion products for the $T_1 + T_2$ resistant spot as those shown for total AMV RNA in fig.3 (results not shown). From this it may be concluded that each AMV RNA has the same 5'-end structure in the form of $m^7G^{5'}ppp^{5'}Gp$. Such a structure also found in the case of BMV RNA 4 may be a general feature of plant virus RNAs. The 5'-blocked terminus may be required for the translation of these RNAs in vitro as has been observed in the case of animal virus mRNA [12–13].

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