CUCUMBER MOSAIC VIRUS RNA 5

Partial characterization and evidence for no large sequence homologies with genomic RNAs

Hervé LOT, Gérard JONARD and Kenneth RICHARDS

Station de Pathologie Végétale, 84140 Montfavet et Laboratoire de Virologie, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15, Rue Descartes, 67084 Strasbourg Cédex, France

Received 27 June 1977

1. Introduction

Cucumber mosaic virus (CMV) contains four major RNA species (RNAs 1, 2, 3 and 4), the largest three of which are necessary for infectivity [1,2]. RNA 4 serves as a messenger for synthesis of coat protein in a wheat embryo cell-free system [3]. The nucleotide sequence of RNA 4 must also be present in RNA 3 since it is known that RNA 3 carries the information for coat protein synthesis in vivo [4,5]. In addition to the genome RNAs and RNA 4, a fifth RNA component (RNA 5) of low molecular weight (100 000) has been detected in CMV preparations [1,6]. Interest in RNA 5 was enhanced by the finding that, even though the small RNA is apparently dependent upon the CMV genome RNAs for replication [7], the relative proportions of RNA 5 and RNA 1–4 is tied to the nature of the plant host of viral propagation and, furthermore, the presence of RNA 5 can dramatically alter the response of certain hosts to CMV infection [8,9]. The above findings make it unlikely that RNA 5 is a host RNA normally present in the plant which is entrapped during virus assembly, but they do not in themselves permit us to distinguish between two further possibilities: (1) RNA 5 originates from the CMV RNA but is nonessential for infectivity, i.e., a deletion fragment or partial transcription product of one of the genome RNAs, or (2) RNA 5 is a 'satellite' RNA, that is, a parasite RNA with little or no sequence homology with the helper genome.

As a prelude to a more detailed sequence analysis, we have determined some of the properties of RNA 5. In particular, the 5′ and 3′ extremities of the RNA chain have been identified and the long T_1 RNAase oligonucleotides characterized. We shall show that the sequence of RNA 5 is carried by none of the CMV genomic RNAs, suggesting that RNA 5 is a satellite.

2. Materials and methods

^{32}P CMV, D strain, was prepared by the following method: three days after inoculation 15 young tobacco plants (*Nicotiana tabacum* var. *Xanthi necroticum*) were removed from their pots and their roots were dipped into distilled water containing [^{32}P]phosphate (50 mCi, 2 mCi/ml). Three days later the virus was extracted and purified as described by Lot et al. [10]. The specific activity of the virus was about 30×10^6 cpm/mg. The RNA was extracted by the usual phenol-SDS method, dissolved in water and stored at −20°C. The separation of ^{32}P-labeled RNA components was performed by electrophoresis on 0.5% agarose, 2.4% polyacrylamide gels as described by Mohier et al. [11]. After migration the 5 RNA components of CMV were visualized by staining with *O*-toluidine blue, cut from the gels and eluted from the gel slices by electrophoresis. The close similarity in molecular weights of RNA 1 and RNA 2 did not allow a good separation; the two bands were thus sliced from the gel together and are referred to below as RNA 1 + 2.

Total T_1 RNAase digestion of RNA 1 + 2, RNA 3, RNA 4 and RNA 5 was for 1 h at 37°C in 0.1 M Tris–HCl, 1 mM EDTA (pH 7.2) (1 Unit T_1 RNAase per 50 μg RNA). The T_1 oligonucleotides were separated by

two-dimensional electrophoresis [12]. Preparation of the gel and conditions of migration were the same as previously described [13]. After localization by autoradiography the T_1 oligonucleotides were eluted from the gel electrophoretically onto DEAE paper and characterized by analysis of their total pancreatic RNAase digestion products.

Base composition of each RNA was determined after total alkaline hydrolysis (0.4 N KOH at 37°C for 12 h) and electrophoresis at pH 3.5 on Whatman 3 MM paper. We tested for the existence of abnormal bases in RNA 5 by two dimensional chromatography upon on a thin layer of cellulose after total hydrolysis by T_1, T_2 and pancreatic RNAases [14].

Isolation and characterization of the 5′-terminal group of the RNA 5 component were performed as described by Klein et al. [15]. ^{32}P-labeled m^7GpppGp prepared from TMV RNA was used as a reference material.

Identification of the 3′-terminal T_1 oligonucleotide of RNA 5 was according to the procedure of Minson and Darby [14,16,17].

3. Results and discussion

Uniformly ^{32}P-labeled CMV RNAs 1–4 and RNA 5 were extracted from CMV, strain D, prepared as described in the Materials and methods and the RNA components were separated from one another by electrophoresis in polyacrylamide-agarose gels [11]. Further electrophoresis of the purified components on a polyacrylamide-agarose gel containing urea showed that RNA 5 so prepared was pure; gel fractionated RNA 4 contained no intact RNA 3 but may be slightly contaminated with degradation products of the larger RNAs, and RNA 3 contained a trace of RNA 4 as well as several minor components migrating between RNAs 3 and 4. RNA 1 + 2 was judged to be free of contamination by the other RNAs.

In order to test for abnormal bases, ^{32}P-labeled RNA 5 was digested exhaustively with a mixture of T_1, T_2 and pancreatic RNAases and the products were separated by two-dimensional chromatography. In addition to the 4 normal bases, a faint spot can be detected not far from the origin of migration (fig.1). The mobility of this product resembles that of an

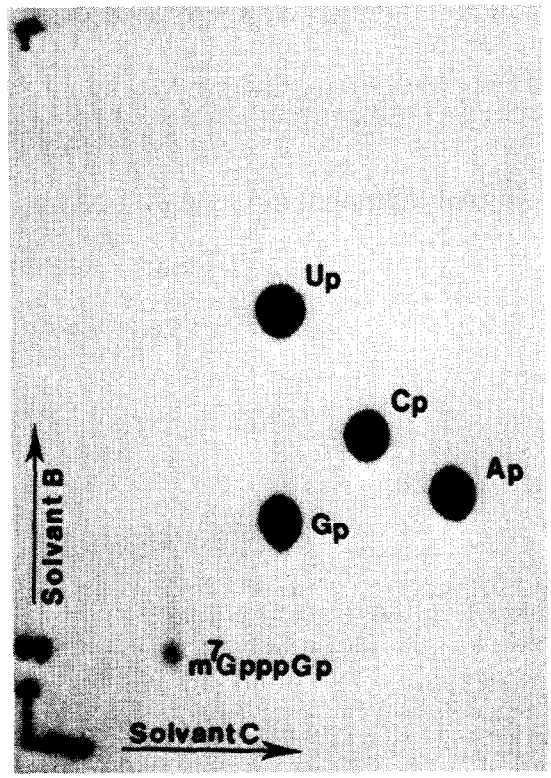

Fig.1. Thin-layer chromatography of ^{32}P-labeled RNA 5 after total hydrolysis by a mixture of T_1, T_2 and pancreatic RNAases. ^{32}P-Labeled RNA 5 was added to 100 μg of carrier yeast tRNA in 50 μl of 0.05 M ammonium acetate buffer at pH 4.5 and incubated for 12 h at 37°C with 2.5 units of a mixture of T_1, T_2 RNAases and 1 μg of pancreatic RNAase. Solvent C (isobutyric acid–NH_4OH–water 66 : 1 : 33) was used in the first dimension; solvent B for the second dimension was concentrated hydrochloric acid–isopropanol–H_2O 17.6 : 68 : 14.4. Relative proportions of Ap : Gp : Cp : Up were 0.19 : 0.28 : 0.23 : 0.30.

endonuclease-resistant 5′-terminal 'cap' of the type m^7 GpppNp. It is well known that such a moiety occurs at the 5′-terminus of most mRNAs from eucaryotic cells and viruses. The existence of such a group at the 5′-end of RNA 5 is consistent with the finding that RNA 5 is an efficient messenger in a cell-free system and that messenger activity is inhibited by m^7G 5′-monophosphate [18].

To more firmly establish the identity of the putative cap moiety, a total T_1 + T_2 + pancreatic RNAase hydrolysate of ^{32}P-labeled RNA 5 was fractionated by

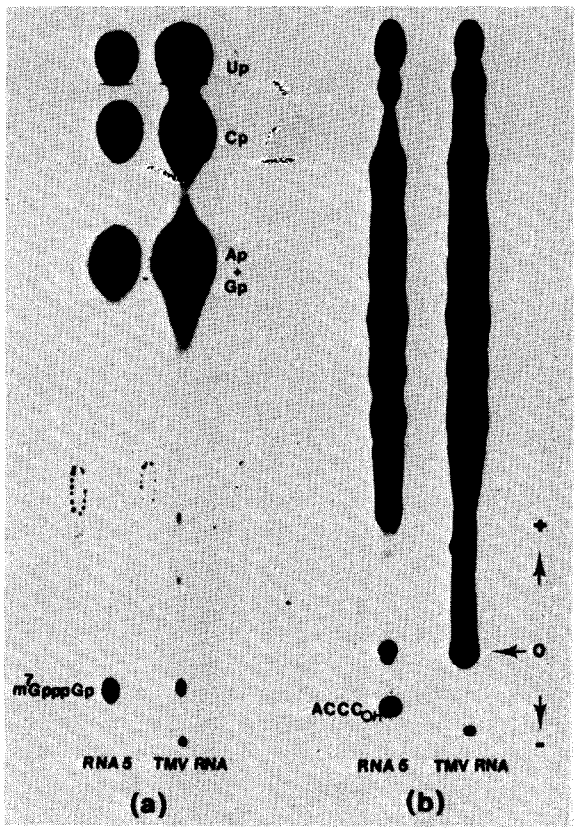

Fig.2. (a) Electrophoresis of a total T_1 RNAase + T_2 RNAase + pancreatic RNAase digest of ^{32}P-labeled RNA 5 on DEAE paper at pH 3.5. An hydrolysate of TMV RNA was used as marker. (b) Electrophoresis on Whatman 3 MM paper at pH.2.5 of ^{32}P-labeled RNA 5 digested to completion with T_1 RNAase (1 Unit enzyme per 20 μg of RNA 5). As a control, reverse migration was also performed on a total T_1 RNAase digest of TMV RNA which is known to end with $CCCA_{OH}$.

electrophoresis on DEAE paper at pH 3.5. A product comigrating with m^7 GpppGp from TMV RNA is present in the digest (fig.2a). Assuming that the nuclease resistant species contains 4 phosphates and that RNA 5 has a chain length of 350 nucleotides it can be calculated that at least 90% of the molecules possess such a group. Phosphatase digestion of the nuclease resistant moiety causes the release of an inorganic phosphate and shifts the mobility of the spot relative to the xylene cyanol marker from 0.2 to about 0.8, behavior which again parallels that of m^7 GpppGp from TMV RNA upon dephosphorylation. Total venom phosphodiesterase digestion of the dephosphorylated species gave rise to pm^7G, pm^7G* (2-amino-4 hydroxy-5-*N*-methylformamide 6-ribosyl-aminopyridine, an alkaline breakdown product of pm^7G), pG, some ppG, and inorganic phosphate, proving the structure of the cap to be m^7GpppGp. It should be noted that CMV RNAs 1–4 have an identical sequence at their 5′-extremities ([19] and Lot, H., personal observations).

It has been shown elsewhere [14,17] that the 3′-OH terminal T_1 oligonucleotide of an RNA molecule (provided that it contains no G or U) is the only species in a total T_1 RNAase hydrolysate which is positively charged at acid pH and so may migrate toward the cathode during electrophoresis at pH 2.5. When [^{32}P] RNA 5 was subjected to such treatment a spot was found to move backward from the origin upon electrophoresis (fig.2b). Alkaline hydrolysis of the spot produced Cp and Ap in the ratio 2 : 1 and pancreatic RNAase released 1 AC and 1 C; the absence guanosine 3′-phosphate confirms that the backward-migrating species is not a normal T_1 RNAase end product and hence must derive from the 3′-OH terminus. Total venom phosphodiesterase hydrolysis of the 3′-terminal oligonucleotide released only pC. The 3′-terminal sequence must therefore be $(G)ACCC_{OH}$.

As noted in the Introduction, the evidence suggests that RNA 5 is either (1) a satellite RNA or (2) part of the CMV genome RNA which has lost its ability to replicate independently and is unessential for infectivity In the latter case RNA 5 could be likened either to the defective interfering RNAs which accompany many animal viruses or to the subgenomic mRNA fragments produced in vivo by partial transcription or cleavage of the genome RNA of numerous plant viruses [20]. As a mean of distinguishing between the above two alternatives we have compared the T_1 RNAase catalogue of RNA 5 to that of CMV RNAs 1–4. Figure 3d shows a T_1 RNAase fingerprint of [^{32}P] RNA 5 in which the oligonucleotides have been separated from one another by two-dimensional gel electrophoresis. Fingerprints of ^{32}P CMV RNAs 1 + 2, 3 and 4 are shown in figs.3a–c; some of the larger oligonucleotides of RNAs 3, 4 and 5 are identified in table 1. The fingerprints show clearly that all the large oligonucleotides of RNA 4 are also present in RNA 3, the result expected in view of the genetic evidence that the RNA 4 sequence is contained in RNA 3. On the other hand, comparison

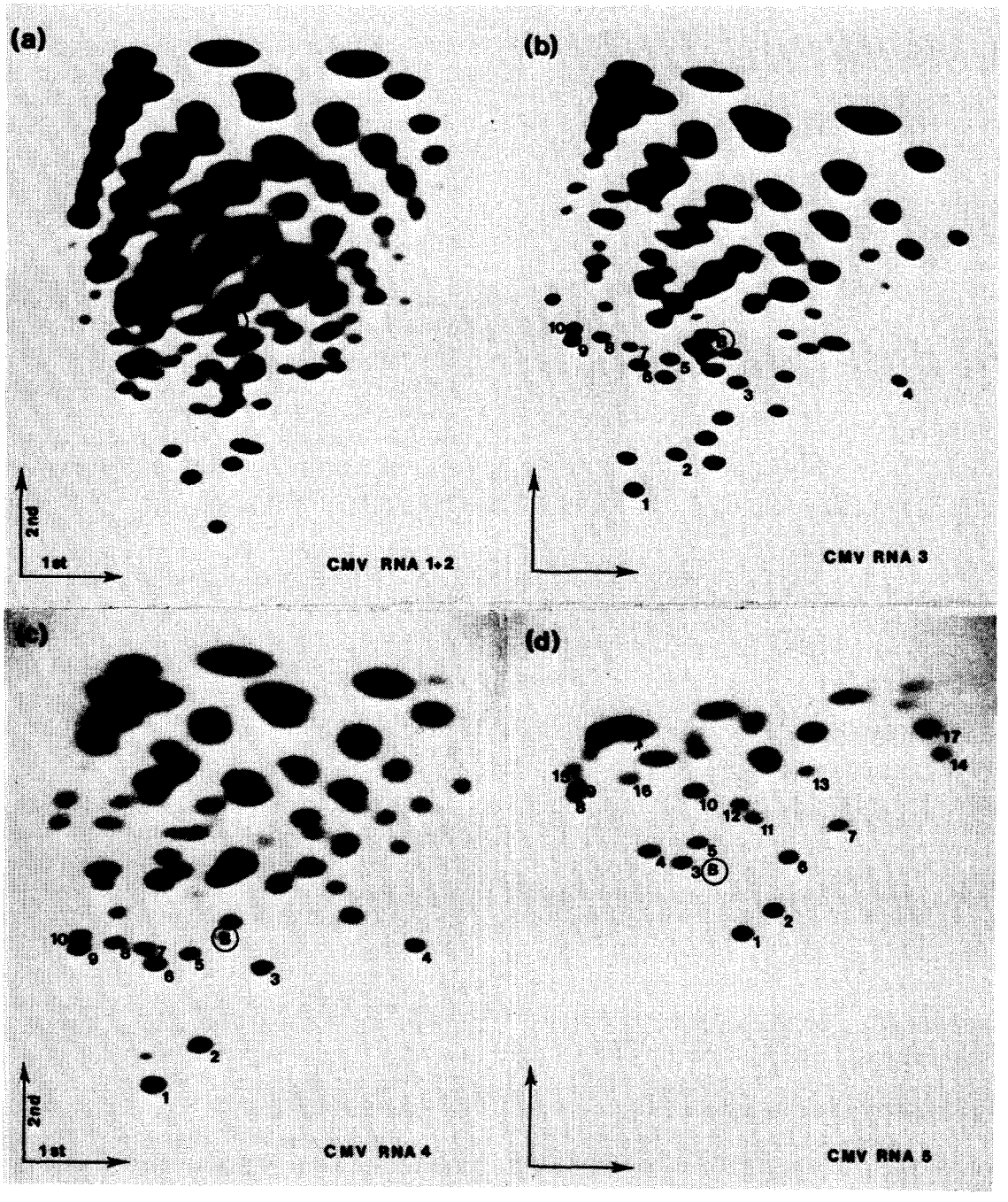

Fig.3. Two dimensional polyacrylamide gel electrophoresis of total T_1 ribonuclease products of the CMV RNA components. (a) RNA 1 + 2 (b) RNA 3 (c) RNA 4 and (d) RNA 5. Electrophoresis was from left to right in a 10% polyacrylamide (bisacrylamide–acrylamide 1 : 30, w/w) gel slab (40 × 20 × 0.4 cm) in 6 M urea, 0.025 M citric acid (pH 3.5), for the first dimension. The second dimension was from bottom to top in a 20% polyacrylamide gel (40 × 30 × 0.4 cm) in 0.1 M Tris borate, 0.0025 M EDTA (pH 8.3).

Table 1

	Large T_1 oligonucleotides common to RNAs 3 and 4			Principal T_1 oligonucleotides of RNA 5	
Spot	Pancreatic RNAase products	Chain length	Spot	Pancreatic RNAase products	Chain length
1	3 AAC, 1 AU, 1 AC, 6-7 C, 6-7 U, 1 G	≃ 27	1	1 AU, 3 AC, 2 C, 5-6 U, 1 G	17
2	2 AU, 2 AC, 4-5 C, 6 U, 1 AAAG	23	2	2 AU, 6 C, 5 U, 1 G	16
3	1 AAAU, 1 AU, 1 AC, 2-3 C, 4 U, 1 G	16	3	1 AU, 1 AC, 4-5 C, 2 U, 1 G	12
4	≃ 9 U, 2 C, 1 G	12	4	1 AAAC, 1 AC, 1 C, 2 U, 1 G	10
5	2 AU, 1 AC, 5 C, 2 U, 1 G	14	5	1 AC, 3-4 C, 3 U, 1 AG	11
6	1 AAAC, 1 AU, 4-5 C, 2 U, 1 AG	15	6	1 AU, 1 C, 3 U, 1 AAAG	10
7	1 AC, ≃ 8 C, 3 U, 1 G	14	7	2 AU, 1 C, 3 U, 1 G	9
8	1 AAC, 7 C, 1-2 U, 1 G	13	8	2 AC, 1 C, 1 AG	7
9	1 AAAC, 2 AAC, 2 C, 1 U, 1 G	14	9	1 AAC, 2 C, 1 G	6
10	1 AAAAU, 1 AC, 3 C, 1 AG	12	10	1 AU, 3 C, 1 U, 1 G + 1 AC, 2 C, 2 U, 1 G	7 + 7
			11	2 AU, 1 AC, 1 U, 1 G	8
			12	3 C, 2 U, 1 G	6
			13	1 AU, 1 C, 2 U, 1 G	6
			14	4 U, 1 G	5
			15	4-5 C, G	6
			16	1 AU, 1 AC, 1 C, 1 G	6
			17	3 U, 1 G	4

of the oligonucleotide contents of RNA 5 to those of RNAs 3 and 4 reveals a total lack of correspondance (table 1). Due to the complexity of the RNA 1 + 2 fingerprint, not all the spots were analyzed. Instead, we concentrated on those large oligonucleotides in the fingerprint of RNA 1 + 2 with mobilities similar to large oligonucleotides 1–7 of RNA 5. None of the oligonucleotides analyzed had a composition similar to that of the like-migrating spot of RNA 5. We conclude that the sequence of RNA 5 is present on none of the CMV genome RNAs and that, consequently, RNA 5 is most probably a satellite. We have recently learned (Kaper, personal communication) that competition hybridization experiments reveal at most only 10% homology between RNA 5 of the 5 strain of CMV and viral RNAs 1–4 [21] a finding which is consistent with our observations and which reinforces the above conclusion.

Acknowledgements

We wish to thank Professor L. Hirth, in whose laboratory this work was carried out, for his interest and support and Dr J. M. Kaper for communicating results to us prior to publication.

References

[1] Peden, K. W. C. and Symons, R. H. (1973) Virology 53, 487–492.
[2] Lot, H., Marchoux, G., Marrou, J., Kaper, J. M., West, C. K., Van Vloten-Doting, L. and Hull, R. (1974) J. Gen. Virol. 22, 81–93.
[3] Schinghamer, M. W. and Symons, R. H. (1975) Virology 63, 252–262.
[4] Marchoux, G., Devergne, J. C., Marrou, J., Douine, L. and Lot, H. (1974) C.R. Acad. Sci. Paris Série D 279, 2165–2167.
[5] Habili, N. and Francki, R. I. B. (1974) Virology 61, 443–449.
[6] Kaper, J. M. and West, C. K. (1972) Prep. Biochem. 2, 251–263.
[7] Kaper, J. M., Toussignant, M. E. and Lot, H. (1976) Biochem. Biophys. Res. Commun. 72, 1237–1243.
[8] Kaper, J. M. and Toussignant, M. E. (1977) Virology, in press.
[9] Kaper, J. M. and Waterworth, H. E. (1977) Science (in press).
[10] Lot, H., Marrou, J., Quiot, J. B. and Esvan, C. (1972) Ann. Phytopathol. 4, 25–38.
[11] Mohier, E., Pinck, L. and Hirth, L. (1974) Virology 58, 9–15.
[12] De Wachter, R. and Fiers, W. (1972) Anal. Biochem. 49, 184–197.
[13] Richards, K. E., Briand, J. P., Klein, C. and Jonard, G. (1977) FEBS Lett. 74, 279–282.

[14] Guilley, H., Jonard, G. and Hirth, L. (1975) Proc. Natl. Acad. Sci. USA 72, 864–868.
[15] Klein, C., Fritsch, C., Briand, J. P., Richards, K. E., Jonard, G. and Hirth, L. (1976) Nucleic Acids Res. 3, 3043–3061.
[16] Minson, T. and Darby, G. (1973) J. Mol. Biol. 77, 337–340.
[17] Briand, J. P., Jonard, G., Guilley, H., Richards, K. E. and Hirth, L. (1977) Eur. J. Biochem. 72, 453–463.
[18] Owens, R. A. and Kaper, J. M. (1977) Virology, in press.
[19] Symons, R. H. (1975) Mol. Biol. Rep. 2 (4), 277–285.
[20] Jaspars, E. M. J. (1974) Advan. Virus Res. 19, 37–149.
[21] Diaz-Ruiz, J. R. and Kaper, J. M. (1977) Virology, in press.