THE POLARITY OF STRIPPING OF COAT PROTEIN SUBUNITS FROM THE RNA IN TOBACCO MOSAIC VIRUS UNDER ALKALINE CONDITIONS

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1. Introduction

The coat protein of TMV* can be partly or wholly removed from the RNA by a number of different treatments. The degradation in alkali [1–3], in detergent [4–6] and in urea [7] is thought to occur by progressive loss of protein subunits predominantly, if not exclusively, from one end of the virus particle. This polar loss of protein subunits in the presence of SDS has been shown to be initiated at the 3′-end of the RNA [6] and it has been reported that the alkaline degradation of TMV proceeds in the same direction [8].

A detailed investigation of the breakdown of TMV under alkaline conditions has revealed five, well-defined, stable intermediates that form during the stripping process [3,9]. These intermediates are in addition to the "stable fraction", approx. 25%, of the virus population that resists degradation [1,3]. We show here that, contrary to expectation, the direction of the polar breakdown of TMV under alkaline conditions is from the 5′- to the 3′-end of the RNA. Our conclusions have been reported briefly elsewhere [10].

2. Materials and methods

^{32}P-labelled TMV (*vulgare*) was produced in *Nicotiana tabacum* var.Sansum [11] and isolated by conventional means [12]. The alkaline degradation of ^{32}P-labelled TMV was carried out as described previously, RNA 'tails' exposed during the stripping process being removed by digestion with pancreatic ribonuclease [3]. Unlabelled TMV was treated similarly except that the RNA tails were removed by digestion with micrococcal nuclease (final concentrations 2 μg/ml) at 20°C for 30 min in the presence of 1 mM $CaCl_2$. The nuclease activity was terminated by adding 0.1 M EGTA (as the K_4 salt) to a final concentration of 5 mM. Nucleoprotein fragments were separated by sucrose density gradient centrifugation as before [3]. ^{32}P-labelled RNA was extracted from the virus and its fragments by phenol extraction, digested with ribonuclease T1 and the oligonucleotides mapped and characterized by the methods of Sanger et al. [13,14].

A mixture of aminoacyl tRNA synthetases was prepared from yeast [15] and the ability of TMV RNA species to form aminoacyl derivatives with ^{3}H-labelled histidine (55 mCi/μmol, The Radiochemical Centre, Amersham, Bucks, UK) was tested as described elsewhere [16] except that the aminoacylated RNA was precipitated for counting by diluting 10 μl of the assay mixture into 1 ml of 2% cetyltrimethylammonium bromide in 20 mM sodium acetate buffer, pH 5.2. To this was added 1 ml of 0.5 M sodium acetate, pH 5.2, containing 0.25 mg/ml yeast RNA as 'cold' carrier. The precipitate was filtered on Whatman GF/C glass filters, dried, and counted in a Packard Tricarb scintillation counter using toluene containing 2,5-diphenyloxazole (5 g/l) as scintillant.

Micrococcal nuclease was a gift from Dr R. T. Hunt and ribonuclease T1 and snake venom phosphodiesterase were gifts from Mr B. G. Barrell.

Abbreviations: TMV, tobacco mosaic virus; SDS, sodium dodecyl sulphate.

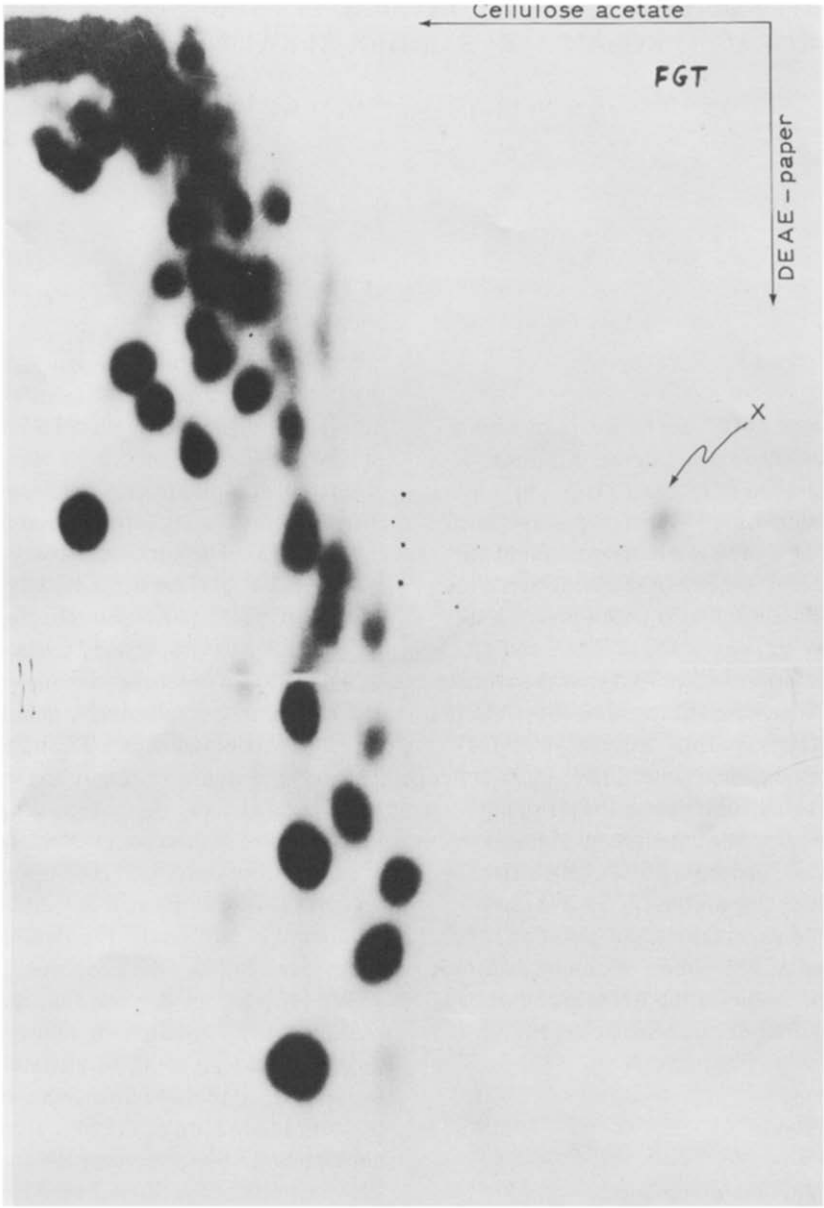

Fig. 1. An autoradiograph of the oligonucleotides produced by digestion with ribonuclease T1 of ^{32}P-labelled RNA from the stable fragment (49 nm long) of TMV. The oligonucleotides were separated by electrophoresis on cellulose acetate at pH 3.5 and on DEAE-paper in 7% formic acid [13], as shown.

3. Results

3.1. *3′-Terminal sequences of RNA in partly stripped TMV*

The most stable intermediate in the alkaline degradation of TMV is a fragment of approx. 50 nm in length and this is easily purified by sucrose density gradient centrifugation after the exposed RNA tail is removed by digestion with pancreatic ribonuclease [3]. This nucleoprotein fragment and the stable fraction of the virus that resists alkaline degradation [1,3] were isolated from ^{32}P-labelled virus. The RNA was extracted from the stable fragment, the stable fraction and from undegraded ^{32}P-labelled TMV and digested with ribonuclease T1. The oligonucleotides produced were separated by two-dimensional electrophoresis [13] and autoradiographs were compared.

Fig.1 shows the map of the oligonucleotides from the stable fragment. This piece of RNA has a length of about 1000 nucleotides (one-sixth that of the intact viral RNA [3]) and, for our present purpose, the most significant spot is that marked 'X'. After elution and alkaline hydrolysis of spot X only Cp could be detected when the hydrolysate was examined by electrophoresis at pH 3.5. On the other hand, after digestion of spot X with snake venom phosphodiesterase, pC and pA were resolved by electrophoresis at pH 3.5: scintillation counting of the nucleotides showed them to be in the molar ratio of approx. 2:1. These facts, together with the characteristic position of spot X in the map consistent with it being an oligonucleotide lacking a 3′-phosphate group, are sufficient to identify spot X as CpCpCpA-OH. The yield of spot X was estimated to be about 0.8 mol/mol RNA applied to the mapping procedure.

The same spot was present in the comparable maps of the ribonuclease T1 digest of the RNA (approx. 6400 nucleotides) extracted from untreated TMV and from the stable fraction of TMV. The yields were again about 0.8 mol/mol RNA applied to the maps. The 3′-terminal nucleotide sequence of TMV RNA is known to be GpCpCpCpA-OH [17,18]. Our results therefore indicate that the 3′-end of the viral RNA is to be found in both the stable fraction and the stable fragment of the virus produced by alkaline degradation. We conclude therefore that the direction of stripping must be at least principally from the 5′- to the 3′-end of the RNA.

3.2. *Aminoacylation of TMV RNA and its fragments produced by partial stripping*

TMV was partly stripped of protein by treatment at pH 9.2 and 2°C for 15 h [3] and the exposed RNA tails were removed by treatment with micrococcal nuclease. Analytical sucrose density centrifugation showed the presence of five well-defined intermediates whose rod lengths have been measured [3,9] and are listed in table 1. The residual RNA in these fragments was extracted with phenol and purified by centrifugation in sucrose density gradients (10–40%) buffered at pH 7.5 with 0.01 M Tris–HCl/0.1 M NaCl/0.001 M EDTA. The same procedure was used to prepare RNA from an undegraded virus sample and from the stable fraction remaining in a degraded virus sample. TMV RNA from intact TMV can be charged with histidine in the presence of yeast histidyl-tRNA synthetase [15,16], which reaction requires the presence of the intact 3′-terminal nucleotide sequence CpCpCpA-OH [15].

The ability of each RNA species we had prepared to be charged with histidine was therefore tested and the results are given in the table 1. There was little or no difference observed for the various RNA species and, in all cases, acceptor activity was destroyed by prior treatment with snake venom phosphodiesterase. From the specific radioactivity of the histidine used, we can calculate that the RNA species have an acceptor activity of approx. 0.32 mol histidine/mol RNA, in reasonable agreement with the value of 0.37 mol/mol observed for intact TMV RNA by others [15].

4. Discussion

The results of 3′-terminal nucleotide sequence analysis and of the tests of histidine-acceptor activity in aminoacylation reactions are both consistent with the direction of protein stripping from TMV under alkaline conditions being principally, if not exclusively, in the direction of 5′- to 3′- along the RNA. This is the reverse of what has been reported earlier [8]. However, since that report depends on the assumption that protein stripping in the presence of SDS takes place from the 3′-end of the RNA [6], our results suggest that the direction of SDS-stripping might bear re-investigation. In support of this view, it should be added that attempts to locate the long ribonuclease T1-resistant

Table 1
Histidine-acceptor activity of TMV RNA and its fragments

Nucleoprotein source	Length of rod (nm)	Histidine-acceptor activity of RNA extracted from nucleoprotein (cpm ^{3}H-labelled histidine/pmol RNA)	Histidine-acceptor activity after treatment of RNA with snake venom phosphodiesterase (cpm ^{3}H-labelled histidine/pmol RNA)
Native TMV	300	4200	350
Stable fraction (fragment 1)	300	4200	–
Fragment 2	197	4200	400
Fragment 3	115	4300	400
Fragment 4	90	4300	350
Fragment 5	67	4200	450
Fragment 6	49	4400	–
Controls			
1. Without RNA	–	300	350
2. Without tRNA-synthetase	–	300	300

The various species of RNA were incubated for 30 min at 30°C with the aminoacyl-tRNA synthetase system described in the text. The lengths of the nucleoprotein rods were determined in a separate study [3,9].

polynucleotides described by Mandeles [19] within the approx. 1000 nucleotides of the shortest stable fragment (fig.1 and table 1) have proved unsuccessful (B. G. Barrell, R. N. Perham, T. M. A. Wilson and D. Zimmern, unpublished work). Yet these nucleotides have been ascribed positions close to the 3′-terminus of the viral RNA [19] on the assumption that SDS-stripping proceeds from the 3′-end of the RNA.

The existence of stable intermediates in the alkaline degradation of TMV has been attributed to variability of interaction between the base sequence of the RNA and the protein subunits along the rod [3]. We can now calculate, knowing the length of the intermediate rods (table 1), how far from the 3′-end of the RNA these regions of high interaction are to be found and it will be of interest to determine the nucleotide sequences responsible for them. One would expect them to be rich in adenine [20]. The region of high interaction about 1000 nucleotides from the 3′-end which determines the existence of the most stable fragment, that approx. 50 nm in length [3], will be of especial interest. These fragments of viral RNA of defined structure should also prove useful in locating other interesting nucleotide sequences within the viral RNA. For example, it has already been shown that the coat protein cistron is found in the RNA of the shortest fragment i.e. is within 1000 nucleotides of the 3′-end of the viral RNA (A. R. Hunter, J. Knowland, R. T. Hunt and D. Zimmern, unpublished work), in agreement with the report of Richards et al. [21] who place it within 2000 nucleotides of the 3′-end.

Finally, since the RNA from the stable fraction terminates at the 3′-end in the usual nucleotide sequence, GpCpCpCpA-OH, any difference in the RNA-protein interaction that causes the enhanced stability must reside in the 5′-terminal end. In this connexion it is significant that the 5′-end of that RNA appears to lack (T. M. A. Wilson and D. Zimmern, unpublished work) the structure m^7G5′ppp5′G-found at the 5′-terminus of normal TMV RNA [22,23].

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