LOCATION OF THE ORIGIN FOR VIRAL REASSEMBLY ON TOBACCO MOSAIC VIRUS RNA AND ITS RELATION TO STABLE FRAGMENT

D. ZIMMERN and T. M. A. WILSON

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH and *Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, England*

Received 21 September 1976

1. Introduction

It has been thought for some time that the assembly of TMV in vitro was a unidirectional polar process [1] starting at the 5′ end of TMV RNA [2–5]. However this latter conclusion was founded on a variety of chemical evidence dependent on exonuclease digestion or periodate oxidation, whose applicability has become doubtful since the discovery that the 5′ terminus of TMV RNA is blocked with an inverted 7-methyl guanosine residue [6,7]. A more reliable way to characterise the nucleation site, or assembly origin, on the intact RNA is to use the protection towards nuclease attack conferred on TMV RNA by the binding of disks of coat protein to isolate [8] and, using labelled RNA, to sequence [9] the part of the RNA coated in the very early stages of assembly. This has unequivocally shown [8,9] that neither end group of the RNA is encapsidated early in assembly (that is, in particles containing up to 500 nucleotides of RNA), as was noted at the time of the discovery of the cap [6]. Subsequent work indicates that even larger partially reassembled particles do not contain the ends of the RNA molecule [10].

Given these results, it is naturally of interest to discover where the RNA sequence protected early in assembly, which defines the assembly origin, actually is located on the whole RNA. Accordingly, we have screened the 3′ terminal fragments of TMV RNA of progressively increasing size that can be isolated from populations of virus particles partially stripped with alkali [11] for the presence of oligonucleotide fragments known from sequence analysis [9] to be coated very early in assembly. Our results locate the assembly origin between 900 and 1300 nucleotides from the 3′ end of whole TMV RNA.

Abbreviations: TMV, tobacco mosaic virus; EDTA, ethylene diamine tetra-acetic acid; PSV, partially stripped virus; PSV-RNA, RNA extracted from the residually coated portion of PSV particles; SDS, sodium dodecyl sulphate; RF, replicative form; LMC, TMV coat protein messenger RNA

2. Materials and methods

^{32}P-Labelled TMV (*vulgare*) was grown and prepared as described elsewhere [6]. A population of particles partially stripped with alkali was prepared from 700 μg TMV (about 12 × 10^6 cpm) by incubation in 10 mM $NaHCO_3$, pH 9.15, for 15 h at 0°C, and micrococcal nuclease digestion as previously described [11]. The protected (i.e. residually coated) RNA was extracted with phenol, ethanol precipitated, redissolved in sample buffer [12], heated to 130°C in a sealed capillary for 2 min, quenched, and immediately loaded on a 2% acrylamide/0.5% agarose slab gel made and run exactly according to Peacock and Dingman [12] except for addition of 0.1% SDS to all the buffers. The bands of labelled, protected RNA were located by autoradiography of the wet gel, excised with a scalpel and eluted by soaking in 0.5 M NaCl, 0.1 M Tris–Cl, pH 7.4, 10 mM EDTA, 0.1% SDS at 15°C for 6 h. Acrylamide was removed by filtration through glass wool in an Eppendorf tip, followed by perchloric acid precipitation together with 70 μg yeast tRNA, and the RNA was ethanol precipitated and digested with RNAase T_1 [13,14].

North-Holland Publishing Company – Amsterdam

The digests were fingerprinted using standard procedures [13,14]. Oligonucleotides were identified by their fingerprint positions, by characterisation using various standard redigestion procedures and by comparison with the published map of large RNAase T_1 oligonucleotides of TMV (*vulgare*) RNA [15].

Fragments of bacteriophage θX 174 RF DNA produced by digestion with the restriction endonuclease Endo R Hae III were kindly donated by Clyde Hutchison. They were labelled at the 5′ terminus using polynucleotide kinase and [γ-^{32}P]ATP essentially as described by Maniatis et al. [16], denatured by heating as described above, and electrophoresed in parallel with the samples of PSV-RNA.

Enzymes were obtained from the following sources: RNAase T_1 from Sankyo Ltd., Tokyo via Calbiochem; micrococcal nuclease from Boehringer; polynucleotide kinase from P-L Biochemicals Inc. BDH electrophoretic grade agarose, acrylamide and methylene bisacrylamide were used without further treatment for making the slab gel. Radiochemicals were from Amersham.

3. Results

Discrete viral fragments of various sizes can be prepared from TMV by mild treatment with alkali and nuclease digestion [11]. RNA extracted from them contains the 3′ terminus, and since the virus particle is necessarily colinear with its RNA, such intermediates effectively carry 5′-terminal deletions of various lengths, and can be used for direct sequence analysis or other procedures aimed at locating markers on the genome. Our approach resembles that first used with SDS stripped virus by Mandeles [17], although he did not attempt to isolate discrete products of the stripping process. Earlier genetic studies [18] used a fundamentally similar rationale.

Six size classes of alkali-generated PSV particles (or PSV-RNAs) can be resolved on sucrose gradients [11], although only two of these survive slightly more extreme alkaline conditions [19,20], and the products may also vary according to the strain of TMV used [20]. We separated the PSV RNAs directly on agarose–acrylamide gels [12] since fractions taken from sucrose gradients were too

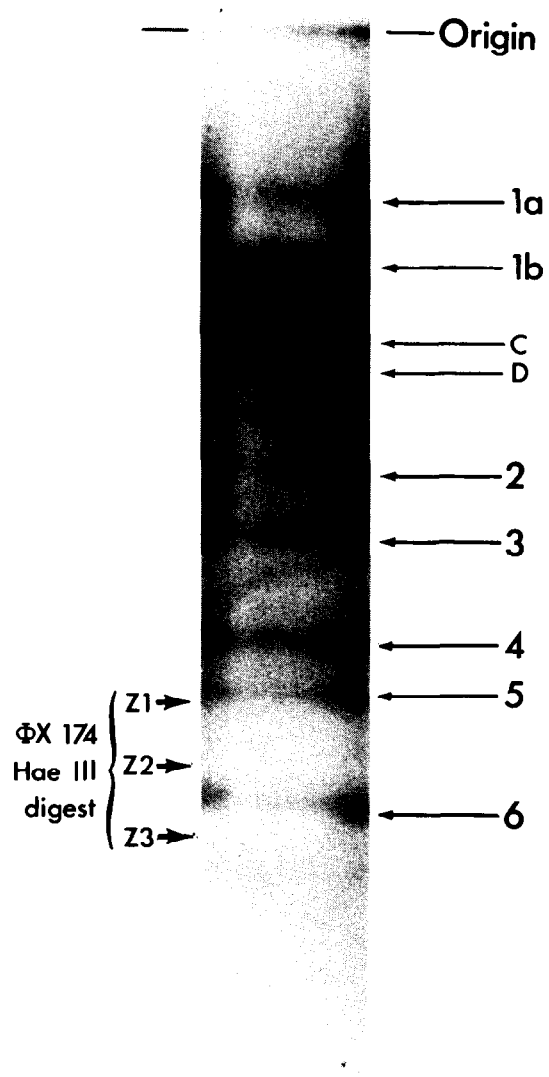

Fig.1. Separation of PSV-RNAs on a 2% acrylamide/0.5% agarose slab gel. Migration was at 10 V/cm for 3.5 h. C and D denote two of five irregularly observed bands. Bands 1a and 1b are probably conformational isomers of TMV RNA, although this is not yet certain. The positions of denatured restriction fragments of θX 174 RF DNA are shown on the left. Their chain lengths are 1300 (Z1), 1100 (Z2) and 870 (Z3) residues [24], giving interpolated chain lengths for PSV5 and 6 RNAs of 1330 and 920 residues. Except for 5 and 6, the numbering of bands does not necessarily correspond to that used earlier for PSVs [11].

cross-contaminated for mapping purposes (T. Hunter and T.M.A.W., unplublished results). An autoradiograph of one gel using ^{32}P-labelled TMV is shown in fig.1. The sizes of the fragments produced were estimated from their electrophoretic mobilities compared with those of denatured, polynucleotide kinase labelled restriction fragments of θX 174 RF DNA run on the same slab gel (fig.1). These sizes were then compared to those predicted on the basis of the rod lengths of their parent PSV particles, as measured in the electron microscope and by ultracentrifugation (unpublished results of R. N. Perham and T.M.A.W. quoted in ref. [11]). The agreement is excellent in the cases of PSV5 and 6 RNAs with which this paper is mainly concerned, when allowance is made for the probable turn of protein at each end of the PSV helices.

The separated bands of PSV RNA were excised from the gel and eluted and were then digested with RNAase T_1 and fingerprinted. The fingerprints of the two

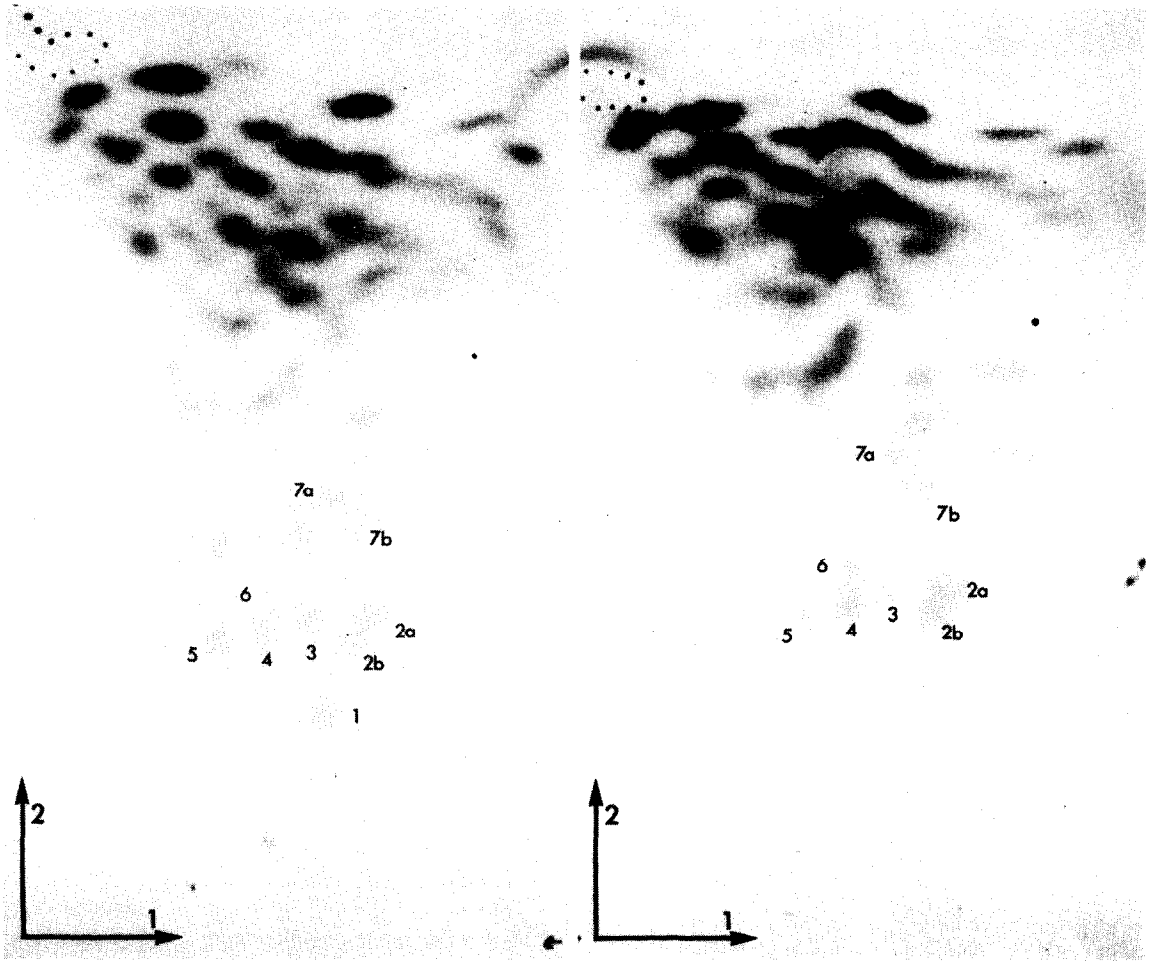

Fig.2. Fingerprints of RNAase T_1 digests of PSV5 (left panel) and 6 (right panel) RNAs. Fractionation was by electrophoresis on cellulose acetate, at pH 3.5, in 8 M urea (first dimension) and homochromatography using 5% unhydrolysed homomixture (second dimension). Spots are numbered as described in the text and in a previous communication [21]. Spots 1, 2a, 2b and 5 correspond to the large oligonucleotides ϕ, ϵ, ρ, and τ isolated by Garfin and Mandeles [15]; spots 2b, 3, 4 and 5 code for portions of the coat protein and spot 7a derives from a location 70 nucleotides from the 3′ terminus of whole TMV RNA ([21], and D. Z. and H. Guilley, unpublished).

smallest PSV-RNAs, whose size corresponded to that expected from the lengths of PSVs 5 and 6 in the nomenclature of Perham and Wilson [11], are shown in fig.2. The fingerprint of PSV6-RNA, the smallest RNA species, appears nearly identical to that previously obtained by others (B. G. Barrell and R. N. Perham, unpublished observations) of RNA extracted from the particularly alkali stable fragment one-sixth the length of a complete TMV particle [19,20]. A characteristic row of six spots, representing the largest RNAase T_1 products in the digest, runs horizontally along the lower part of both fingerprints (spots 2a–6 in fig.2). PSV5-RNA also gives rise to these six RNAase T_1 products, and an additional oligonucleotide of yet lower homochromatographic mobility (spot 1 in fig.2). The close similarity of the fingerprints arises since PSV5 and 6 RNAs are overlapping fragments of the TMV genome containing the 3′ terminus [11]. Spot 1, appearing only in PSV5-RNA, must accordingly be located between the end points of PSVs 5 and 6, or 920–1330 nucleotides from the 3′ end (fig.1). This location of spot 1 was initially less certain than the others. It had been found in quite high yield on the fingerprint of the sucrose gradient fraction (PSV6 RNA) which corresponded in size to stable fragment RNA but which was isolated after only a limited alkaline incubation (see [21]), but not on that of stable fragment prepared by more extensive alkaline incubation (B. G. Barrell and R. N. Perham, unpublished results). However, the present results show that this was almost certainly due to cross contamination of PSV5 and 6 RNAs on the sucrose gradients used. In particular, the close overall similarity of the fingerprints of PSV5 and 6 RNA rules out the possibility that spot 1 derives from a minor mode of alkaline stripping, for example, from rarely occurring particles in which stripping proceeded from the 3′ end of the rod. Spot 1 is important because, in another experiment with mixed PSV5 and 6 RNAs from sucrose gradients, it was found to give rise to the pancreatic RNAase products (U,AU,AAU,AAAU,AAAAU)AG, which together with the fingerprint position of the spot ([15] - see below) uniquely define the oligonucleotide AAAUAAUAUAAAAUUAG, which is found to derive from the assembly nucleation site of TMV RNA, about 60 nucleotides to the 5′ side of the probably assembly origin [9]. On this evidence the origin for viral reassembly in vitro must be located between 900 and 1300 nucleotides from the 3′ end of the TMV RNA molecule.

Most of the larger RNAs were also fingerprinted. None of the fingerprints we examined lacked the characteristic large oligonucleotides of PSV6-RNA, confirming that all the major products of alkaline stripping overlap at the 3′ end [11]. The oligonucleotides denoted ω, ψ_1, ψ_2 and χ by Garfin and Mandeles [15] are missing in PSV RNAs up to at least 2800 nucleotides long, consistent with the location of ω, ψ_1 and ψ_2 within the half of TMV stripped first by SDS [17], which is now known to be the 5′ end as it is with alkali [22].

4. Discussion

We have shown that the part of TMV RNA coated at the outset of TMV reassembly in vitro lies internally on the RNA molecule at least 900 nucleotides from the 3′ end and therefore more than 5000 nucleotides from the 5′ terminus where assembly was previously supposed to start [2–5]. One immediately obvious consequence is that assembly elongation following nucleation must be bidirectional, in apparent conflict with numerous electron microscopic studies of partially assembled TMV particles (e.g.[1]) which have consistently shown free RNA at only one end of the partially assembled rods, leading to the suggestion that assembly was unidirectional. This discrepancy has recently been resolved by the observation that the longer RNA tail is doubled back along the growing rod [23], resulting in both RNA tails being at the same end.

Although the origin for assembly is internal, it is much closer to one end than the other, and one might expect differences in the rates of elongation in opposite directions, or perhaps in the aggregation state of the coat protein required. Interestingly, at least for the first 400 nucleotides to be coated after initiation by protein predominantly in the disk form, elongation is extremely asymmetric, with 3′ to 5′ elongation being preferred to the reverse direction by a factor of approximately 10 [9].

The present results do not exactly locate the assembly origin within PSV5-RNA but there are indications that it forms the end point of PSV6-RNA.

The existence of a 'stable fragment' of this size was reported over 20 years ago by Schramm [19] so the reason for its stability is a long standing problem [19,20]. To form PSV6, stripping must terminate at a point where RNA–protein interactions are particularly strong [20], and it is tempting simply to equate this with the assembly origin. (This supposes that the factors determining binding of free RNA to coat protein disks and those governing strength of binding within the mature particle are either the same or are both present at the assembly origin.) The observed size of reverse transcripts primed from within the nucleation site using sucrose gradient purified PSV6-RNA as a template also supports this possibility (D. Z. and H. Guilley, unpublished). If it is true, then the assembly origin can be located rather more precisely at about 925 nucleotides from the 3′ end of TMV RNA, from the size of PSV6-RNA measured independently by electron microscopy [11] and by gel electrophoresis (fig.1), results which agree closely. It is interesting that this position is quite close to the 5′ end of the sequence in the TMV genome from which the independent small coat protein messenger RNA('LMC') derives [21], about 750 nucleotides from the 3′ end of the genome. One possible mechanism by which LMC could be made is by cleavage of progeny full length TMV RNA molecules, and the assembly origin could be close enough to the putative cleavage site to act as a feedback regulator of LMC production by ensuring immediate blockage of the cleavage site on initiation of assembly. Taken together, these ideas enable the otherwise somewhat magical resemblance between stable fragment RNA and LMC [21] to be plausibly explained, and suggest that internal nucleation of assembly may have arisen partly in order to integrate viral functions efficiently.

Acknowledgements

We are grateful to St John's College, Cambridge for the award of the Hutchinson Research Studentship to T.M.A.W.

References

[1] Stussi, C., Lebeurier, G. and Hirth, L. (1969) Virology 38, 16–25.
[2] Butler, P. J. G. and Klug, A. (1971) Nature New Biol. 229, 47–50.
[3] Guilley, H., Stussi, C. and Hirth, L. (1971) C. R. Acad. Sci. Ser. D, 272, 1181–1184.
[4] Thouvenel, J. C., Guilley, H., Stussi, C. and Hirth, L. (1971) FEBS Lett. 16, 204–206.
[5] Ohno, T., Nozu, Y. and Okada, Y. (1971) Virology 44, 510–516.
[6] Zimmern, D. (1975) Nucleic Acids Res. 2, 1189–1201.
[7] Keith, J. and Fraenkel-Conrat, H. (1975) FEBS Lett. 57, 31–33.
[8] Zimmern, D. (1976) Phil. Trans. Roy. Soc. London B (in the press). Zimmern, D. and Butler, P. J. G. Manuscript submitted to Cell.
[9] Zimmern, D. Manuscript submitted to Cell.
[10] Jonard, G., Richards, K. E., Guilley, H. and Hirth. L. Manuscript submitted to Cell.
[11] Perham, R. N. and Wilson, T. M. A. (1976) FEBS Lett. 62, 11–15.
[12] Peacock, A. C. and Dingman, C. W. (1968) Biochemistry 7, 668–674.
[13] Barrell, B. G. (1971) in: Procedures in Nucleic Acid Research, (Cantoni, G. L. and Davies, D. R., ed) vol. 2, pp. 751–779, Harper and Row, New York.
[14] Brownlee, G. G. (1972) Determination of Sequences in RNA, North-Holland, Amsterdam.
[15] Garfin, D. E. (1972) PhD Dissertation, Univ. of California. Garfin, D. E. and Mandeles, S. (1975) Virology 64, 388–399.
[16] Maniatis, T., Jeffrey, A. and Kleid, D. G. (1975) Proc. Natl. Acad. Sci. USA 72, 1184–1188.
[17] Mandeles, S. (1968) J. Biol. Chem. 242, 3103–3107.
[18] Kado, C. I. and Knight, C. A. (1966) Proc. Natl. Acad. Sci. USA 55, 1276–1283.
[19] Schramm, G., Schumacher, G. and Zillig, W. (1955) Z. Natur. 10b, 481–492.
[20] Perham, R. N. (1969) J. Molec. Biol. 45, 439–441.
[21] Hunter, T. R., Hunt, T., Knowland, J. and Zimmern, D. (1976) Nature (London) 260, 759–764.
[22] Wilson, T. M. A., Perham, R. N., Finch, J. T. and Butler, P. J. G. (1976) FEBS Lett. 64, 285–289.
[23] Butler, P. J. G., Finch, J. T. and Zimmern, D. Manuscript submitted to Nature (London).
[24] Jeppesen, P. G. N., Sanders, L. and Slocombe, P. M. (1976) Nucleic Acids Res. 3, 1323–1339.