ASSEMBLY OF TOBACCO MOSAIC VIRUS: ELONGATION TOWARDS THE 3′-HYDROXYL TERMINUS OF THE RNA

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1. Introduction

The assembly of Tobacco Mosaic Virus (TMV) from its isolated coat protein and RNA is initiated at a single site on the RNA [1] located ~1000 nucleotides from the 3′-hydroxyl terminus [2]. A consequence of this internal nucleation site is that subsequent elongation must be bidirectional and while the kinetics and mechanism of growth in the major (3′ to 5′) direction have been extensively studied [3], little is known about growth in the reverse (minor) direction except that it occurs at a considerably slower rate [4–7]. We have taken advantage of the possibility of isolating fragments of TMV RNA containing the 3′ terminal region to investigate the kinetics of elongation in the 5′ to 3′ direction.

Stripping of TMV under mildly alkaline conditions removes protein specifically from the 5′ end of the particle. Thus nuclease-treated Partially Stripped Virus (PSV) contains fragments of the viral RNA with intact 3′-termini but shortened 5′-tails [8]. RNA molecules with very short tails to the 5′ side of the nucleation region show much less rapid elongation than intact molecules [9] but the reaction does still occur [10]. Taking advantage of this we have examined the kinetics of elongation towards the 3′ terminus using RNA isolated from nuclease-treated PSV rods. The results indicate that assembly in this direction is more rapid when the protein is supplied in the form of small aggregates (A-protein) than when a disk preparation is used.

2. Materials and methods

TMV (strain vulgare) was grown and isolated as previously described [11]. The coat protein (as both A-protein and a disk preparation) was isolated and its concentration determined as before [1]. A PSV preparation was produced by treating the virus (10 mg/ml) with 10 mM $NaHCO_3$ buffer, pH 9.5, for 24 h at 0°C, and removing the exposed RNA tail with micrococcal nuclease [8]. The protected RNA was then isolated [12] and centrifuged through a 15–40% (w/v) linear sucrose gradient in 100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, containing 0.1% SDS for 24 h at 25 000 rev./min and 20°C. The gradients were fractionated using an Isco gradient fractionator and fractions containing the appropriate length RNA were pooled and the RNA precipitated with ethanol.

Reassembly experiments were carried out in sodium phosphate buffer, pH 7.0, ionic strength 0.1 M at 20°C. Partially assembled rodlets were prepared by reacting PSV RNA with an appropriate amount of a disk preparation and, when necessary, fractionated in sucrose step gradients buffered in assembly buffer [13]. The sucrose concentration of the rodlet preparation was reduced by a brief (4 h) dialysis against assembly buffer.

Assembly was assayed by following increase in turbidity at 310 nm [14] with a final RNA concentration of 0.05 mg/ml. Alternatively the lengths of RNA protected were measured in agarose/acrylamide gels as previously described [15]. Densitometry of the negatives from photographs taken under ultraviolet light of the gels stained with ethidium bromide was carried out using a Joyce-Loebl densitometer and the length of the longest protected RNA estimated by determining the position of the trailing edge of the RNA peak (taking the half-height). Analysis of the coating of specific oligonucleotides was carried out as

previously described [7] except that intact TMV RNA was used to correct for differences in labelling efficiency.

3. Results

Reaction to completion of a PSV RNA preparation containing molecules 2200 to 3200 bases long with 12.5 times its weight of a disk preparation was shown by fingerprint analysis of the nuclease-resistant RNA to result in the complete encapsidation of the 5′ region but to leave most of the 3′ tail uncoated. Following sedimentation through a step gradient and dialysis, rodlets produced in this way were used to follow the kinetics of elongation along this uncoated 3′ tail using turbidity change.

The rates of elongation (fig.1) show saturation with increasing protein concentration irrespective of whether the protein is added in the form of a disk preparation or A-protein. Moreover the maximum rates obtained were the same but at non-saturating concentrations the rate was always greater when all the protein was added as A-protein than when a disk preparation (containing ~20% of the less aggregated A-protein) was used. This suggests that elongation along the 3′ tail does not occur directly from disks and that the rate of assembly found with a disk preparation is due to its A-protein content. Thus a higher total protein content is required to achieve saturation.

A more sensitive assay for elongation is to measure the length distribution of nuclease-resistant RNA at increasing times of reassembly [15]. To maximise resolution a shorter, more homogeneous PSV RNA preparation, containing molecules 1700–2200 nucleotides long, was used. This was reacted with a 10-fold excess (by weight) of a protein disk preparation which again resulted in the 5′ tail alone being substantially coated (data not shown). Since measurements were to be of the longest protected fragments, it was unnecessary to remove any unreacted RNA by sucrose gradient centrifugation since it could not affect the measurements and any extra handling tends to cause further degradation. Elongation was carried out with a final protein concentration of 1.0 mg/ml, since the turbidity measurements indicated that significant differences in the rate of assembly with different protein aggregates occur at this concentration (fig.1).

The time-course of elongation (fig.2) again shows more rapid elongation occurring when all the protein

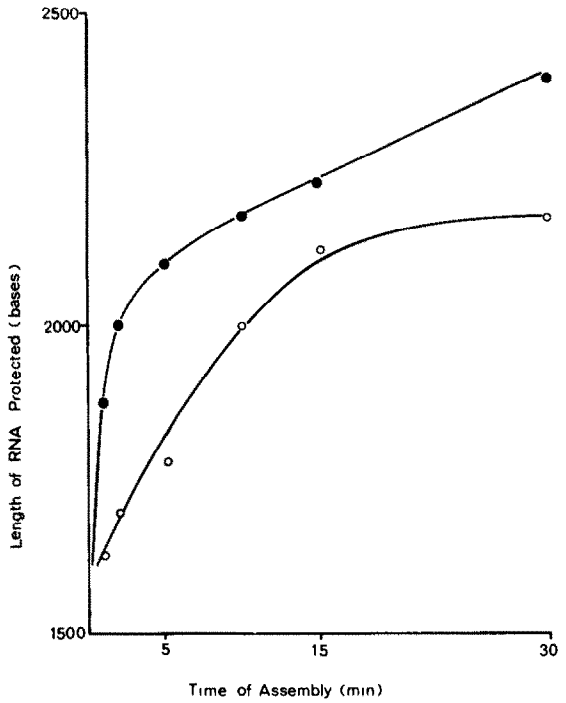

Fig.1. Effects of protein concentration and aggregation state on rate of elongation in 5′ to 3′ direction. Rates are expressed as % maximum to allow data from different experiments to be shown together. Partially reassembled rodlets with PSV RNA were reacted further with either A-protein (- • -) or a disk preparation (- -o- -).

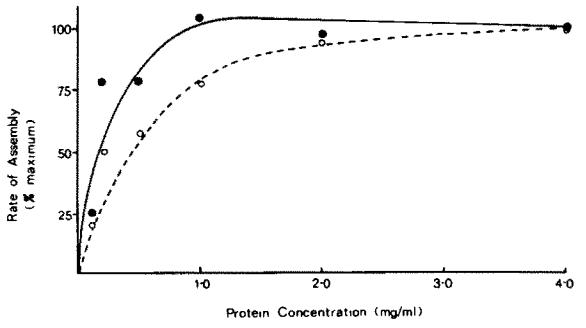

Fig.2. Time course of protection of RNA 3′-tails with different protein aggregates. Lengths of the longest protected RNA molecules were determined (see text) during elongation of partially assembled rodlets containing PSV RNA, with A-protein (•) or a disk preparation (o), thus allowing estimation of maximal rates of reassembly.

is added as A-protein. Unlike the RNA protected during reassembly of intact TMV RNA with a disk preparation, which shows a distinct banding pattern [15], the RNA protected in the rodlet preparation migrated as a single, broad band when run in gels. By following the change in position of the trailing (high MW) edge of the peak an estimate of the maximum rates of elongation can be obtained. With A-protein this is about 280 nucleotides in the first min and 130 in the second compared to 40 and 73 for a disk preparation. The fall in rate with A-protein probably reflects the completion of coating of some of the particles.

The protection of oligonucleotides characteristic of the 3′ tail can also be used to follow 5′ to 3′ elongation [7]. Since the sequence of the 3′-tail is now known [16], the precise locations of the T_1 oligonucleotides chosen can be deduced. Oligonucleotide 4 lies between 563 and 579 nucleotides and oligonucleotide 5 between 510 and 525 nucleotides from the 3′-hydroxyl terminus (numbering from [7]). Samples of the RNA isolated for examination by gel electrophoresis were digested with ribonuclease T_1, kinase-labelled and fingerprinted by 2-dimensional electrophoresis/homochromatography. The time courses of protection of oligonucleotides 4 and 5 (fig.3) again show that more rapid 5′ to 3′ elongation occurs with A-protein than with a disk preparation.

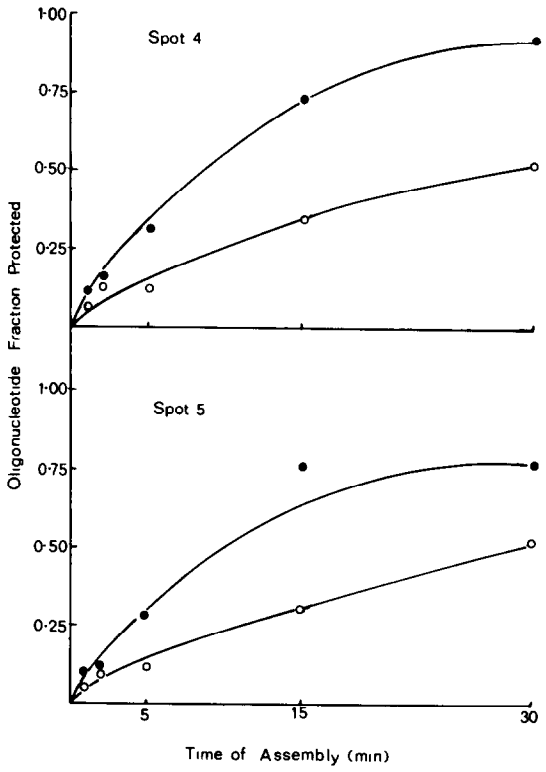

Fig.3. Extent of protection of specific oligonucleotides in the 3′-tail of PSV RNA during elongation with different protein aggregates. The recovery of specific oligonucleotides, spots 4 and 5, in the protected RNA was measured during the elongation of partially assembled rodlets containing PSV RNA, with A-protein (•) or a disk preparation (○), giving a measure of the average extent of coating at each time.

4. Discussion

The results reported here strongly suggest that the assembly of TMV in the minor 5′- to 3′-hydroxyl direction occurs by the addition of individual subunits or small aggregates thereof rather than by the direct addition of disks. The direct addition of disks cannot be ruled out entirely but seems unlikely in view of the conformation of the 3′ tail [5,13]. This is in contrast to the situation found for the major (5′) tail where a special 'travelling loop' structure makes disk addition easy to envisage (for a picture see [17], although there is still some argument as to whether disk addition does occur, e.g. [18]).

Fully protected PSV RNA first appears after ~5 min of reassembly with A-protein (as judged by gel electrophoresis) – a time similar to that taken for the appearance of full-length TMV when overall assembly is followed [15,19]. This similarity in the time for encapsidation of the 3′-tail indicates that the measurements on the kinetics of assembly of PSV are indeed relevant to assembly of intact TMV RNA and that both tails are probably fully coated at about the same time, a result consistent with our previous studies on the overall assembly of the virus [7].

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References

[1] Zimmern, D. and Butler, P. J. G. (1977) Cell 11, 455–462.

[2] Zimmern, D. and Wilson, T. M. A. (1976) FEBS Lett. 71, 294–298.
[3] Butler, P. J. G. and Durham, A. C. H. (1977) Adv. Protein Chem. 31, 187–251.
[4] Zimmern, D. (1977) Cell 11, 463–482.
[5] Lebeurier, G., Nicolaieff, A. and Richards, K. E. (1977) Proc. Natl. Acad. Sci. USA 74, 149–153.
[6] Otsuki, Y., Takebe, I., Ohno, T., Fukuda, M. and Okada, Y. (1977) Proc. Natl. Acad. Sci. USA 74, 1913–1917.
[7] Lomonossoff, G. P. and Butler, P. J. G. (1979) Eur. J. Biochem. 93, 157–164.
[8] Perham, R. N. and Wilson, T. M. A. (1976) FEBS Lett. 62, 11–15.
[9] Wilson, T. M. A., Perham, R. N. and Butler, P. J. G. (1978) Virology 89, 475–483.
[10] Pelcher, L. E. and Halasa, M. C. (1979) Virology 97, 488–491.
[11] Leberman, R. (1966) Virology 30, 341–347.
[12] Zimmern, D. (1975) Nucl. Acids Res. 2, 1189–1201.
[13] Butler, P. J. G., Finch, J. T. and Zimmern, D. (1977) Nature (Lond.) 265, 217–219.
[14] Butler, P. J. G. and Klug, A. (1971) Nature New Biol. 229, 47–50.
[15] Butler, P. J. G. and Lomonossoff, G. P. (1978) J. Mol. Biol. 126, 877–882.
[16] Guilley, H., Jonard, G., Kukla, B. and Richards, K. E. (1979) Nucl. Acids Res. 6, 1287–1308.
[17] Butler, P. J. G. and Klug, A. (1978) Sci. Am. 239, No.5, pp. 62–69.
[18] Shire, S. J., Steckert, J. J., Adams, M. L. and Schuster, T. M. (1979) Proc. Natl. Acad. Sci. USA 76, 2745–2749.
[19] Butler, P. J. G. and Finch, J. T. (1973) J. Mol. Biol. 78, 637–649.