REVIEW ARTICLE

The Current Picture of the Structure and Assembly of Tobacco Mosaic Virus

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INTRODUCTION

Tobacco mosaic virus (TMV) has long been a favourite object for studies on both the structure and assembly of rod-shaped viruses. Soon after its initial purification by Stanley (1935) investigations of its structure were begun using both chemical and X-ray diffraction techniques (Bawden & Pirie, 1937; Bernal & Fankuchen, 1941). Moreover, the problems in solving such a large structure led to many developments in techniques. Since the virus has a helical structure (Watson, 1954) and forms an extremely well oriented gel rather than crystals, the three-dimensional structural information is convoluted into two dimensions because of the azimuthal disorder of the particles in the gel. Despite this difficulty, techniques have been evolved allowing the virus structure to be solved to a resolution approaching 0.4 nm (Stubbs et al., 1977). The protein alone will form true crystals as one of its aggregates (the disk; see below) the structure of which has been solved to a resolution of 0.3 nm (Champness et al., 1976; Bloomer et al., 1978), allowing a detailed atomic model to be built.

In parallel with the investigations on its structure, the reassembly of TMV was also studied, with Fraenkel-Conrat & Williams (1955) showing that the isolated protein and RNA could reassemble to give infective virus. This was the first time that such assembly had been demonstrated in vitro and this led to the concept of the 'self-assembly' of viruses, with the protein and nucleic acid containing all the information necessary to assemble the infectious virion without any requirement for other possible 'morphopoetic' factors. This concept has been confirmed and, as will be discussed, extended for TMV although it is now known that the assembly of some other viruses may require additional factors (for a comparative review, see Butler, 1979). Structural studies have also been extended to the polymorphic aggregation of TMV protein and the thermodynamics of its interactions (for reviews, see Caspar, 1963; Lauffer, 1975; Butler & Durham, 1977).

Despite the detailed knowledge now available on the structure and assembly of TMV, its biology and the details of its behaviour in vivo are little understood. These aspects of TMV have been reviewed recently (Hirth & Richards, 1981) and the main development since then has been the determination of the complete RNA sequence of TMV Vulgare (Golet et al., 1982) and the partial sequences at the 3'-ends of the RNA from a number of other TMV strains: TMV-OM, cucumber green mottle mosaic virus (watermelon strain) and TMV-L (a tomato strain) (Meshi et al., 1982, 1983; Takamatsu et al., 1983). These sequences show the total protein-coding capacity and confirm the map of the polycistronic viral genome. Further progress has also been made in identifying possible subgenomic messenger RNAs in both single- and double-stranded forms (Zelcer et al., 1981; Golet & Karn, 1982).

With so much information available on TMV, it is not possible in any review of reasonable length to cover all aspects or even to consider all of the results just on the limited topics of structure and assembly. Therefore, while dealing with these topics, only limited coverage of the earlier literature will be attempted, citing specifically the seminal papers. Fuller references to the earlier work can be found in a number of other reviews (e.g., Caspar, 1963; Lauffer, 1975; Butler & Durham, 1977; Hirth & Richards, 1981).
Fig. 1. Schematic picture of TMV particle, showing arrangement of protein subunits and RNA. Adapted from Caspar (1963).

STRUCTURE

Overall

TMV is a rod-shaped virus (Fig. 1) of length 300 nm and radius 10 nm, with a central hole of radius about 2 nm (Caspar, 1963). The subunits are arranged in a single helix (Watson, 1954), which is right-handed (Finch, 1972) and has $16\frac{1}{2}$ subunits per turn. The single-stranded RNA is intercalated between adjacent turns of the protein helix, binding in fairly extended configuration at a radius of about 4 nm and with three nucleotides per protein subunit. The length of the rod is determined by that of the RNA, which is fully coated, and there is probably an extra turn of protein at the end, since the RNA is protected from nuclease attack in the intact virion. The RNA shows slight sequence polymorphism, including nucleotide insertions or deletions near the 5'-terminus, and the lengths appear to be either 6395 or 6398 nucleotides (Goelet et al., 1982), corresponding to approximately 2140 protein subunits.

Protein aggregates

The aggregation of TMV protein is largely driven by hydrophobic interactions, with larger aggregates at higher temperatures (for review, see Lauffer, 1975). However, the aggregation is highly polymorphic and many different forms have been described, with the control between them largely influenced by conditions of pH and ionic strength (Durham et al., 1971; Durham, 1972; Durham & Klug, 1972). The regions of occurrence of some of the better characterized aggregates are shown in Fig. 2. The dominant variable affecting the mode of aggregation is the pH, with helical polymers occurring at lower pH while at neutral or higher pH the dominant
aggregation is into 'two-layer' aggregates, together with some smaller aggregates. This control is brought about by the partial protonation of two groups with abnormal pK values around 7.1 in the helical aggregates (including the virus) but not in the two-layer aggregates (Caspar, 1963; Butler et al., 1972). Increasing ionic strength causes higher degrees of polymerization, without affecting the basic mode.

The helical aggregates of TMV protein are very similar to the virus, but without the RNA, with the major difference being that the helices may have either 16½ or 17½ subunits per turn (Mandelkow et al., 1976, 1981), whereas only 16½ subunits per turn have been found in the virus. The main form of the protein at high pH values and low ionic strength is a mixture of monomer and small aggregates, collectively known as 'A-protein'. From a theoretical analysis, Caspar (1963) had predicted that this mixture might contain both two- and three-layered aggregates, but the earlier analyses of the equilibrium distribution of sizes in this mixture suggested that it was composed largely of two-layer aggregates (Durham & Klug, 1972). A more recent analysis, carried out at higher protein concentrations and employing spectroscopic and circular dichroism measurements as well as sedimentation velocity and equilibrium, has shown that there may be the two families of aggregates, with the two-layer ones starting from 4S material and a suggested three-layer series from 8S material (Vogel et al., 1979). One critical difference is that while the two-layer aggregation is capable of leading, at temperatures above about 15 °C, to a closed aggregate – the two-ring 'disk' in which each ring contains 17 protein subunits – such a large three-layer aggregate has not been observed. Moreover, at pH values near 7, where virus assembly occurs, the polymerization into disks begins at protein concentrations too low for significant formation of 8S aggregates. It is thus likely that while three-layer aggregates of TMV protein can be found, they are not significant in the virus assembly reactions and they have not been characterized in any detail.
The TMV protein disk is central among the protein aggregates. It occurs at temperatures above about 15 °C at neutral pH and most ionic strengths, and also at higher pH values and ionic strengths. At high ionic strengths, disks stack on each other, either in a polar fashion to give 'limited stacks of disks' or with dyad symmetry as in the crystals of TMV protein. These crystals have proved suitable for crystallographic analysis and the structure has been solved to high resolution and an atomic model built (Bloomer et al., 1978). Disks are also essential for the nucleation of assembly of TMV (Butler & Klug, 1971) as discussed below. Like any reversibly equilibrating aggregate, disks cannot occur alone in the absence of an equilibrium concentration of smaller aggregates. The proportions of disks and A-protein in a 'disk preparation' have been measured over a range of concentrations (Durham, 1972) and the aggregation is found to be a quasi-crystallization, showing a critical concentration of A-protein above which further protein assemblies into disks.

The mechanism controlling the aggregation of A-protein into disks is still unclear. While the pH dependence would suggest that there must be some proton binding involved, titration experiments have suggested that the two-layer aggregates bound very few protons over the range between pH 8 and 6 (Butler et al., 1972), and more accurate titrations have confirmed this observation (Schuster et al., 1980). However, while there had previously been doubt as to whether the short proto-helix (or 'lockwasher') would exist free in solution, the change in sedimentation coefficient from 19S at pH 7·0 to 20S or above at slightly lower pH values (Durham, 1972) has now been shown to correlate with measurable proton binding and interpreted as a change in aggregation from disks to proto-helices with the same, or a similar, number of subunits (Schuster et al., 1980). Such proto-helices are not closed structures and they could gain, or less probably lose, subunits from their ends.

The rate of formation of disks from A-protein is slow. Thus, at pH 7·0, ionic strength 0·1 M and 20 °C, disks take several hours to form (Durham & Klug, 1971), as they also do at higher pH and ionic strength (Durham, 1972), even though disks are the main component at equilibrium at the protein concentration employed. Commensurate with this, the rate of breakdown is also slow under these conditions (Butler, 1976), although very much faster at lower temperatures or higher pH. However, the micro-equilibrium of protein subunits between disks and A-protein is relatively rapid, with subunits, perhaps individually, rapidly dissociating from the disks and being recaptured without loss of integrity of the disk. This is shown by the rapid equilibration of radioactively labelled subunits between the types of aggregate (Richards & Williams, 1972; Butler & Durham, 1977), but whether this is the direct dissociation of subunits from the rings of the disk or by a rapid equilibration between the disk and proto-helix, with subunit equilibration at the ends of the latter, is not clear.

Changing the conditions can not only cause a rapid breakdown of disks to A-protein, but also their rapid conversion into helix if the pH is reduced. If the change occurs over a period of minutes, long and apparently perfect helices are formed. However, dropping the pH rapidly by mixing the disk preparation directly into a buffer solution at low pH led to the formation of imperfect 'nicked' helices, from which the nicks annealed out over a period of several hours (Durham et al., 1971; Durham & Finch, 1972). Electron micrographs from one such experiment are shown in Fig. 3 together with schematic interpretations of the structures. The initial disks are converted into proto-helices, which are not seen individually but rapidly stack in a poorly ordered fashion. The resulting short rods stack further, but with the individual proto-helices still out of register to produce gaps or nicks in the helices. The separation of these nicks shows that the disks have been converted directly to proto-helices, without dissociation and reassociation of the protein subunits.

A similar picture has also emerged for the nucleation of the helical aggregates when they are assembled from A-protein. If the aggregation is driven by a slow increase in temperature, at constant pH, helices are formed with a length distribution similar to that at equilibrium. However, if the temperature rise is rapid, the lengths of the resulting helices overshoot the equilibrium size. Analysis of the kinetics of cycling the temperature up and down, shows that the critical factor is a limiting supply of nuclei for the helices, with very rapid rod elongation on all available nuclei (Schuster et al., 1979). These nuclei are relatively stable, even at low
Fig. 3. Electron micrographs of TMV protein disk preparation on pH drop. Initial disk preparation was diluted into pH 5 buffer (at 20 °C) and specimens prepared at the times indicated. Bar markers represent 20 nm (right hand micrograph on different scale from other three). A schematic interpretation of the structures is shown underneath. Electron micrographs by courtesy of Dr J. T. Finch, adapted from Durham et al. (1971).

temperature, and they have been shown to sediment around 20S and have been identified as disks or proto-helices (Shire et al., 1979a). On this basis, a fairly detailed description has been given of the rates of interconversion of various of the protein aggregates under the varying conditions employed.

One point which should be noted is that the preceding discussion all relates to reversibly equilibrating protein, even though some of the rates of assembly or disassembly may be slow compared to the change in a variable, hence showing hysteresis in some reactions. TMV protein is readily proteolysed when disaggregated and the resulting damaged protein will aggregate irreversibly into a variety of forms, depending upon the initial conditions (Durham & Finch,
Anomalously stable aggregates have also been observed in mixtures of TMV protein and the protein from the related cucumber virus 3 (Novikov et al., 1974). These aggregates had sedimentation coefficients of 13S and 20S and were suggested to be a single ring and a disk of protein subunits. These stable, mixed disks were incapable of assembling with TMV RNA to give nucleoprotein particles, as might be expected if their structure is locked in some way, giving the anomalous metastability.

**Detailed structures**

**Protein**

The structure of the protein, crystallized as the disk, has been solved to a resolution of 0.28 nm and a detailed atomic model built (Bloomer et al., 1978). The α-carbon backbone, represented as a ribbon, is shown in Fig. 4 looking at a number of subunits viewed down the disk axis (Fig. 4a) and also at a single subunit in each of the two rings of the disk in a vertical section (Fig. 4b).

Between radii of 4 and 7 nm the subunit consists of four α-helices which are held together at their outer ends by a small five-stranded β-sheet. Outside a 7 nm radius, the folding of the polypeptide is less regular and both the amino and carboxy termini are found at the outside of the subunit, on the surface of the aggregate.

The inner ends of the α-helices are joined together into two pairs by loops of the polypeptide chain. One loop, between the upper two helices (residues 34 to 38), is very short and direct and appears to be highly constrained. However, the loop between the lower pair of α-helices (residues 89 to 114) is not visible in the electron density map of the protein. This lack of density could be due either to movement and dynamic disorder or to different folding in the subunits around the rings of the disk and static disorder. However, nuclear magnetic resonance (NMR) studies of a mutant protein, with Thr 107 replaced by Met (which does not occur in the native protein), showed that the region around this residue was in rapid motion in A-protein or disks, but not in the protein helix or the virus (Jardetsky et al., 1978). This is compatible with the interpretation of a dynamic disorder of this inner loop, with the polypeptide chain moving in this region even though the majority of the subunit is rigidly located within the aggregate.

The atomic model also shows the nature of the bonding both within and between protein subunits. The inside of the subunit is largely hydrophobic between the four α-helices and also outside the β-sheet. In this latter region, however, the hydrophobic contacts continue outside the individual subunit to its two neighbours in the same ring, to form a 'hydrophobic girdle' around the circumference of the ring (Fig. 4a). The contacts between α-helices of neighbouring subunits are largely hydrophilic, but with one patch of hydrophobic contact. Protein subunits from different strains of TMV will frequently not co-assemble (e.g. Sarkar, 1960), or else give anomalously behaving aggregates (Novikov et al., 1974). On the assumption that the basic folding of the polypeptide chains is the same in the different strains, these effects can be readily explained by movements in the boundaries between the hydrophilic and hydrophobic patches in the intersubunit contacts, due to amino acid mutations (A. C. Bloomer, personal communication). It therefore appears that the driving energy for the protein aggregation may well come from the hydrophobic contacts between subunits, but the specificity between both different protein species and spatially within an aggregate comes from the hydrogen bonding and charge neutralizations, i.e. the hydrophilic interactions.

The vertical contacts between subunits in the two rings (Fig. 4b) are all at a radius greater than 6 nm in the disk and are relatively tenous. In the disk the two layers of subunits open apart onto the central hole (Champness et al., 1976; Bloomer et al., 1978) and this structure may well be critical for the roles of the disk in the assembly of the virus (discussed below). Upon conversion of the disk to the helix, the subunits move so that not only do they have the same azimuthal tilt (closing the opening onto the central hole), but they are also displaced circumferentially by approximately half a subunit width (Champness et al., 1976), requiring a completely different axial bonding pattern.

The structure of the helical aggregates of TMV protein has been solved to medium resolution (Mandelkow et al., 1981). Apart from the absence of the RNA, the main difference from the
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(a) c \approx 0

(b) Disk axis

Radius (nm)

Fig. 4. Ribbon drawing of the \( z \)-carbon positions in the protein subunits in the disk. Numbers refer to amino acid position in primary sequence. (a) Four adjacent subunits in one ring viewed from above. Regions of \( \alpha \)-helix (labelled LR and RR for left and right radial and LS and RS for left and right slewed respectively) and \( \beta \)-sheet are indicated on left-hand subunit, and areas of polar (light shading) and hydrophobic (heavy shading) contact are indicated. (b) Side view of subunits in axial section of disk showing vertical contacts between subunits in the two rings (labelled A and B). The flexible loop at inner radius is shown dotted and the RNA-binding site is indicated. Adapted from Bloomer et al. (1978).

virus is in the freedom to form helices with either 16\( \frac{2}{3} \) or 17\( \frac{1}{2} \) subunits per turn, but otherwise the protein appears to have essentially the same structure as in the virus, which is discussed below. In any given gel of the protein helix, all of the helices appear to have the same number of subunits per turn, but the control of this phenomenon is not understood (Mandelkow et al., 1976).
Virus

Despite the technical difficulties involved, the structure of the virus has been solved to a resolution approaching 0.4 nm, although falling off somewhat towards higher radius (Stubbs et al., 1977). Based on the recognition of some bulky amino acid side-chains, an atomic model was built into this electron density map and, in the light of the higher resolution protein structure (Bloomer et al., 1978), subsequently adjusted slightly (Holmes, 1979). The majority of the protein subunit is found to have a structure very similar to that found in the disk, with most of the movement involved in the change between the two aggregation states being a rigid body transformation. Indeed, the atomic model derived from a subunit from one ring of the disk fitted into the electron density maps for the subunits in the other ring of the disk or in the virus almost equally well (A. C. Bloomer, personal communication). The major differences occur at radii less than 4 nm, where the RNA is located and also the inner loop of the polypeptide is now visible.

The two lower α-helices of the protein subunit continue inwards from 4 nm radius for a further one or two turns and then join onto the ends of a vertical feature. This was initially identified as a further α-helix, but the more accurate identification of the locations of amino acids along the polypeptide now suggests that this might be too short to be stable and the actual nature of the folding is now known. Similarly, the early idea that a number of negatively charged groups were clustered together in this region into a ‘carboxyl cage’ (Stubbs et al., 1977) has again not stood up in the light of more accurate identification of the amino acid residues (Bloomer et al., 1978) and while there are several carboxylate residues in this region, a number of them are probably located along the central hole of the virus, and hence neutralizable by solvent ions, so the concentration of charge will not be as great as originally suggested.

The location of the RNA was originally identified by the relatively high electron density of the phosphate groups (Stubbs et al., 1977) and more recently the total density due to RNA has been identified by difference maps between the virus and the protein helix (Mandelkow et al., 1981) and an atomic model of RNA with an ‘average’ nucleotide in each position built into these maps (Stubbs & Stauffacher, 1981). These results confirm the moderately extended configuration of the ribose–phosphate backbone (Fig. 5) and also show some of the possible interactions with the protein. Arginine residues 90, 92 and 113, which are invariant in all sequenced TMV strains, appear likely to interact with and neutralize the phosphate groups, as was predicted from their locations in the protein. The invariant pair of aspartate residues, 115 and 116, appear to hydrogen bond to the 2′-hydroxyl groups of the riboses in the first and third nucleotides of the triplet bound to the protein subunit (Holmes, 1979). The bases themselves lie against the surface of the α-helix between about residues 114 and 123 and, while possible interactions have been suggested, the evidence is not clear cut. It does, however, seem likely that much of this base/protein interaction is hydrophobic with the bases stacked against the hydrophobic surface of the α-helix on three sides. Such stacking does not account for any sequence specificity in the interaction (discussed below) and there is no evidence for the detailed interactions leading to this.

RNA

The physical structure of the RNA as it occurs in the virus (Mandelkow et al., 1981; Stubbs & Stauffacher, 1981) has just been described (Fig. 5). However, while the coat protein must be able to encapsidate all of the RNA and, in the process, is likely to interact with much of it as if it consisted of ‘average’ nucleotides, in practice of course the virus has a distinct nucleotide sequence. Initially, the sequence around the origin of assembly was determined (Zimmern, 1977; Jonard et al., 1977), followed by the 5′-end (Richards et al., 1978; Jonard et al., 1978) and the 3′-end (Guilley et al., 1979). These regions have been joined together with the determination of the complete sequence (Goelet et al., 1982). All of this work was carried out using TMV Vulgare and partial sequences at the 3′-ends of a number of other strains [TMV-OM, cucumber green mottle mosaic virus (watermelon strain) and TMV-L (a tomato strain)] have also been determined (Meshi et al., 1982, 1983; Takamatsu et al., 1983).
One of the most interesting features to emerge from the complete sequence is the polymorphism which exists in the RNA in a bulk preparation of TMV which has not recently been cloned through a local lesion host. With the particular technique employed, sequences were obtained from clones of short lengths of cDNA which were independently copied from RNA preparations made from bulk preparations of TMV and each region was sequenced on average 13 to 14 times, allowing any diversity in the sequence to be observed. In the locations where diversity does occur, typically two or more bases may be found to occur at a single position without altering the coding capacity of the RNA, due to the redundancy of the genetic code. However, towards the 5'-end of the RNA there is a greater concentration of changes and there appear to be two distinct families of sequence (Fig. 6). In the 5'-non-coding region these differ by having nucleotide insertions or deletions and, while the lengths of coding regions are identical, some of the base changes produce conservative changes in the amino acid sequences, i.e. on two occasions each aspartic or glutamic acids and serine or threonine may occur. More typical changes which do not alter the coding are also seen (e.g. around nucleotide 210). In this region of the RNA, the sequence from any particular cDNA appears to have one of the two possible sets of bases, suggesting the co-existence of a pair of ‘paradigm’ sequences, although they may also have further mutations elsewhere in the sequence.

The 5'-end of TMV RNA is 'capped', having the sequence m<sup>7</sup>G<sup>4</sup>ppp<sup>5</sup>Gp (Zimmern, 1975; Keith & Fraenkel-Conrat, 1975). This capping is typical for a eukaryotic mRNA, which is an essential early function of TMV RNA, and it is necessary for infectivity, with removal of the cap leading to a loss of infectivity (Ohno et al., 1976). This removal did not, however, have any major effect on the reassembly of the RNA with TMV coat protein, although clearly the packaging at the extreme 5'-end of the rods may have been different.
Fig. 6. Nucleotide sequence of the 5'-end of TMV RNA. The sequences of approximately 290 nucleotides are shown for each of the two paradigm RNAs, aligned to show the insertions or deletions and with asterisks under nucleotide differences. The 5'-non-translated region is underlined and the codon translation is given above the RNA sequence in the single-letter code. From Goelet et al. (1982).

**ASSEMBLY**

**Nucleation**

In their classic demonstration of the assembly of TMV from its isolated RNA and protein, Fraenkel-Conrat & Williams (1955) incubated the reassembly mixtures “at 3° for at least 24 hours” under conditions of undefined ionic strength and about pH 6 to 7. In a later series of experiments (Fraenkel-Conrat & Singer, 1959) conditions were varied in order to seek the optimum. Starting with the protein as A-protein it was found that 0.1 M-pyrophosphate buffer (ionic strength about 0.5 M) was preferable to 0.1 M-phosphate buffer, with the optimum pH about 7.3. The reassembly then approached completion in about 6 h at 23 to 25°C and in about 3 h at 30°C.

This rate seemed very slow for the assembly of a virus, but it is similar to the rate of formation of disks from A-protein. A major problem in the assembly of a helix such as that of TMV is the nucleation step. For this, at least 17 protein subunits would have to align themselves along a strand of RNA before a complete turn could be formed and any axial interaction could occur to supplement the side-to-side bonding; this might well be kinetically very slow. The structure of the disk, however, makes it an ideal candidate to act as a former or ‘jig’ upon whose surface protein subunits and RNA might aggregate to overcome this kinetic block. Butler & Klug (1971) tested this by observing the effect of adding a disk preparation on the rate of reassembly of A-protein with RNA.

The effect of adding disks to the reassembly mixture was dramatic. The rate of assembly, monitored by the increase in turbidity due to rod formation, was increased by more than an order of magnitude. After 1 min, electron micrographs showed many growing rods, but no full length ones, suggesting that the nucleation was occurring relatively rapidly and that the turbidity rise was due to elongation of a population of partially assembled particles, rather than increasing numbers of particles which rapidly attained full length. The surprising feature was
that the disk preparation had to be added at concentrations similar to those of the A-protein and that omitting the extra A-protein had no effect upon the rate. A-protein was, of course, still present as it represents about 20% by weight of the equilibrium disk preparation, but the reaction proceeded to such an extent that significant amounts of disks must have been involved and measurements of the residual free protein in the analytical ultracentrifuge showed depletion of the 20S boundary, but little change in the amount of 4S material.

From these results, Butler & Klug (1971) concluded that not only were disks essential for the nucleation reaction, but that they were also incorporated during this step rather than merely acting as a former. Moreover, it appeared that substantial elongation could occur with disks as the main protein source although whether acting simply as a 'delivery package' was not at this time clear. The requirement for disks for the nucleation reaction was confirmed by other groups (Okada & Ohno, 1972; Richards & Williams, 1972), although they questioned their involvement in any subsequent elongation (discussed below). The slower rates of assembly found in the earliest reconstitution reactions were probably due to the protein being supplied purely as A-protein, with the consequent delay before sufficient disks would form for the nucleation.

**Specificity of interaction with RNA**

The assembly of TMV protein into nucleoprotein rods was found to be selective for the RNA species supplied (Fraenkel-Conrat & Singer, 1959, 1964). Thus, the homologous RNA was found to reassemble most efficiently, as would the homopolymers poly(A) and poly(I), while heterologous RNAs from other TMV strains assembled less efficiently, if at all, and other homopolymers and yeast RNA failed to assemble. These results were explained and placed on a quantitative basis when Butler & Klug (1971) supplied the protein directly in its active form, i.e. as a disk preparation, and found that homologous TMV RNA nucleated assembly more than an order of magnitude faster than poly(A) or poly(I), while no nucleation could be detected with yeast RNA or other homopolymers. Moreover, nucleation on poly(A) was found to show second order dependence upon the protein concentration, suggesting two disks interacting per RNA molecule (Butler, 1972), while TMV RNA nucleates with first order kinetics, requiring only a single disk in the rate-limiting step (Butler, 1974a).

Selectivity for RNA at the stage of nucleation is not only the most efficient point, since protein will not be used up abortively coating RNA molecules non-specifically, but is also essential even between sites on the TMV RNA if particles are not to be produced with defects due to faulty packing together of helices elongating towards each other after nucleation at two sites on the same RNA.

The site for nucleation of assembly on TMV (Vulgare) RNA was identified by Zimmern & Butler (1977). They reassembled very limited amounts of a disk preparation with TMV RNA and removed the uncoated, and unprotected, RNA tails by nuclease digestion and then re-isolated the protected RNA fragments. These consisted of a family of overlapping fragments, all containing a common core sequence but extended to different extents at the two ends. The fragments would re-bind to disks rapidly, quantitatively and with high affinity, becoming more nuclease-resistant in the process. Such re-binding would not occur with A-protein and the properties of the fragments suggested that they all contained the origin of assembly, which must be in the common core.

The nucleotide sequence of these fragments was determined (Zimmern, 1977) and the minimum fragment protected was found to be about 55 to 65 nucleotides long, consisting of a sequence capable of forming a weakly base-paired stem with a loop containing the sequence AGAAGAAGUUGUUGAUGA. In view of the binding of three nucleotides per protein subunit, this repetition of G in every third position looks significant. By looking for the presence of characteristic nucleotides in a series of 3'-fragments of TMV RNA, Zimmern & Wilson (1976) located the nucleation region between 900 and 1300 nucleotides from the 3'-end of the RNA, and the sequencing has since confirmed that it is just over 900 nucleotides (Guilley et al., 1979; Goelet et al., 1982).

Despite the very high specificity of nucleation on intact TMV RNA, this is partially lost if the RNA is degraded into shorter fragments. Thus, if TMV RNA is digested with ribonuclease A
into fragments of about 3S, many of them will bind to disks (Tyulkina et al., 1975), although with only half the RNA/protein ratio found in the normal nucleoprotein. A similar low ratio is found for some of the fragments generated with ribonuclease T1 which appear to be showing a degree of specificity in binding (Jonard et al., 1975). Sequencing of these fragments (Guilley et al., 1975a, b) showed them to be derived from within the coat protein cistron and, when the true nucleation region was sequenced, they were found to have some homology with this.

The structure of the nucleoprotein complexes with these less-specific fragments of the RNA is not clear. From their low RNA/protein ratios it is not possible for them to have the same structure as the virus, unless there are significant amounts of protein at the ends of the rods beyond the RNA. This is in contrast to the nucleoprotein complexes from the nucleation region, which have a normal RNA/protein ratio. From the limited evidence available, it is likely that the non-specific RNA is bound into short stacks of disks, but no details are known.

The occurrence of a region in the coat protein cistron with favourable binding to the disk and some homology to the nucleation region on TMV *Vulgare* RNA led to the suggestion (Zimmern, 1977) that this might act as an alternative origin of assembly in some strains, particularly the cowpea strain. This hypothesis has since been confirmed for both the cowpea strain (Fukuda et al., 1980) and cucumber green mottle mosaic virus (Fukuda et al., 1981) and in both cases nucleation occurs about 300 nucleotides from the end of the RNA. This selectivity probably depends upon the coat protein as well as the sequences of the possible assembly origins.

Direct measurements have also been made of the binding of short oligonucleotides to TMV protein. The trinucleoside diphosphate AAG occurs several times in the origin of assembly loop, and the hexanucleoside pentaphosphate fraction from a combined ribonuclease A and phosphatase digest of RNA will contain oligonucleotides with five A or G residues and a single U or C. Both produced differences when soaked into crystals of the disk and difference maps from the native protein, calculated to 0.5 nm resolution (Graham & Butler, 1979), showed that these different nucleotides were producing similar effects. At high concentrations, both caused the crystals to disintegrate, perhaps by mimicking the nucleation and causing the disks to dislocate into proto-helices, while at lower concentrations the main effect was to cause changes in the two α-helices projecting in towards the centre of the disk. However, a single peak of density could be seen which might be the bound nucleotide. These studies are being continued with the specific hexanucleoside pentaphosphate, AAGAAG, and while the binding looks reasonable in projection along the disk axis (Butler & Lomonossoff, 1980), further work is still needed to solve the position in three dimensions.

Studies of the binding constants for a series of trinucleoside diphosphates to the protein at neutral or acid pH have been made (Schuster et al., 1980; Steckert & Schuster, 1982). While weak binding was observed for AAG, CAG and UAG at pH 7, much stronger binding was found in the range pH 5 to 6 and binding constants were calculated for this pH, where the protein will be in the helical state. All the trimers which bound contained a G residue and binding was strongest if this was in the 3' position. The binding constants did not simply depend upon addition of binding for the bases involved in each position, but rather seemed to depend upon the specific sequence and no simple rule could be discerned. Although an attempt was made to suggest the ‘phase’ for binding the nucleation region of TMV RNA to the protein subunits (Steckert & Schuster, 1982), the conclusions are logically suspect and, anyway, are derived from binding to helical protein while nucleation occurs with the disk and it is not known if the same relationships would hold in the two forms.

**Mechanism**

The structure of the disk, with the two rings of subunits opened apart onto the central hole (Fig. 4b), and also the possible secondary structure of the origin of assembly on TMV RNA (Fig. 7), led to the hypothesis that nucleation might occur by the insertion of this hairpin loop into the central hole of the nucleating disk (Butler et al., 1976). A picture of such a mode of nucleation is shown in Fig. 8. In this, after the nucleation loop inserts into the hole of the disk, the single-stranded RNA at the end of the loop binds into the RNA-binding site between the rings of the disk, with the weakly based-paired loop melting to allow binding of a complete turn of RNA.
Some feature of the interaction could then cause dislocation of the disk into a proto-helix, with consequent closing together of the subunits and entrapment of the RNA. Further RNA adjacent to the entrapped turn could then bind onto the outer surfaces of the proto-helix and more protein could add, to start the elongation reaction.

This hypothesis leads to the prediction that both tails of the RNA should be at the same end of the growing rods, despite nucleation occurring internally on the RNA. This was found to be the case and, moreover, it was shown that it was the longer 5'-tail which was looped back down the length of the rods (Butler et al., 1977; Lebeurier et al., 1977). This latter observation implies the elongation in the major direction may well involve some special configuration of the RNA, rather than simply a tail protruding from the partially formed rod, and this point is considered below.

During the nucleation there is a strong tendency for rods to grow rapidly until about 300 nucleotides are encapsidated and, if only limited amounts of protein are supplied, such rods tend to dominate even at the expense of leaving unencapsidated RNA molecules (Zimmern & Butler, 1977; Zimmern, 1977). This suggests that there may be further special features of the RNA in the nucleation region in addition to the loop of the actual origin of assembly, which enhance the rate of interaction with further disks over a limited stretch after the rate-limiting step involving a single disk (see above). An indication of what this might be comes from the observation by Zimmern (1983) that in the nucleation regions of all of the TMV strains where the sequence is known, two further weakly base-paired stems and loops occur to the 5'-side of the origin of assembly, spaced by 75 to 80 nucleotides. A potentially similar structure can also be predicted for
Fig. 8. Picture of possible mechanism for nucleation of assembly of TMV. The protein and RNA are drawn diagrammatically and also a diagram is given for the expected configuration of the RNA backbone at each stage. Details are discussed in the text.
the region in the coat protein cistron which can bind specifically. Moreover, the TMV mutant Ni 2519 is temperature-sensitive for assembly and at non-permissive temperatures nucleation has been shown to occur simultaneously at both the normal origin and also this extra location (Talianisky et al., 1982; Kaplan et al., 1982). Sequence analysis of the nucleation region of Ni 2519 RNA shows a mutation in the third base-paired stem which back-mutates to give reversion to the wild-type behaviour (Zimmern & Hunter, 1983). The obvious effect of this mutation in Ni 2519 RNA would be to weaken the base-pairing in this third stem until it might well no longer have any stability.

It thus appears that loss of the third stem can lower the selectivity for the nucleation region until other, admittedly favourable, regions can compete equally with it, with lethal consequences for the virus assembly. An obvious suggestion (Zimmern, 1983) is that these equally spaced loops may play a role in guiding one or two further disks into interaction with that already bound to the origin of assembly. The possible mechanism for this is not clear. It can hardly be by an interaction of the single-stranded loop with the protein subunits in a strictly analogous fashion to the nucleation, as the packaging would then become topologically impossible without subsequent dissociation of this binding. One possibility would be for these loops simply to bind loosely, holding the disks in a stack which might even be responsible for facilitating the dislocation of the nucleating disk. If this were a kinetic effect, it could explain the role of the loops in providing specificity without contradicting the observed rebinding of RNA fragments lacking one or both of these loops (Zimmern & Butler, 1977).

**Elongation**

*Bidirectional growth*

The internal location of the nucleation region (Zimmern & Wilson, 1976) requires that elongation occurs in both directions along the RNA. The nucleation region is around 900 nucleotides from the 3'-end of the RNA and 5500 nucleotides from the 5'-end and hence there is more than five times as much RNA to be encapsidated in the 5'-direction as in the 3'. When reassembly was carried out with a disk preparation present in substantial excess, rods in the size class from 260 to 300 nm were observed after about 6 min (Butler & Finch, 1973). More recent experiments have been reported from Okada's laboratory (Fukuda et al., 1978) in which only rods up to 260 nm were seen after 5 to 7 min and full-length rods were seen only after 30 to 50 min. The difference in results could be explained by the relatively low resolution of the earlier histogram of rod lengths, which might fail to show such an apparent stop to rapid elongation. However, other obvious differences are the use of TMV OM and, possibly most significantly, of a high ionic strength (about 0.25 M) by the Tokyo group, rather than TMV Vulgare and ionic strength 0.1 M as used by all other workers (the significance of these differences is discussed below). In agreement with the earlier observation, the RNA protected after 6 min reassembly contained a fraction which was indistinguishable in gel electrophoresis from full-length viral RNA (Butler & Lomonossoff, 1978), and it is unlikely that a 13% difference in lengths would not have been seen. Despite these differences (discussed further below), all these results show that the 5'-tail is substantially coated within about 6 min and hence that about 15 nucleotides per second must be encapsidated in the major direction under optimal conditions.

In the earliest measurements where the two tails could be distinguished (Lebeurier et al., 1977), no shortening of the shorter tail was observed while the longer tail was substantially reduced, and it was therefore suggested that only one direction of elongation was occurring. However, the measurement of lengths of single-stranded RNA in the electron microscope is extremely difficult and the error in the measurements, due to both random errors and possible partial stripping under the denaturing conditions (Butler et al., 1977), means that this result is not reliable.

The conclusion that no elongation occurs towards the 3'-end, until the 5'-tail has been completely coated, was also reached by other workers (Otsuki et al., 1977; Fukuda et al., 1978). Unfortunately, the high ionic strength and TMV-OM were used in both of these sets of experiments and the behaviour may not be the same under these different conditions. There must also be some doubt whether a relatively slow elongation towards the 3'-end would have been detected in either case. In the earlier work the elongation was observed by electron microscopic
serology', which the authors state is not very precise for quantitative measurements (Otsuki et al., 1977), while Fukuda et al. (1978) used various methods but, as has been pointed out before, there are internal inconsistencies in the results which must cast doubt on their precision (Lomonossoff & Butler, 1979). Most of the experiments measure 'finishing events', such as nuclease-resistant infectivity or protection of the extreme termini from nuclease, and will thus be susceptible to any slow steps for even the final turns of the nucleoprotein helix formation. The other measurements were length measurements of rods in the electron microscope in which the rods only reached a length of 260 nm within 5 to 7 min and then 'the elongation of rods beyond this length apparently stopped for about 15 min', which was interpreted as slow elongation towards the 3'-tail only occurring after completion to the 5'-end, although this is speculation. A rod length of 260 nm would correspond to uncoated RNA about 850 nucleotides long and, if all of this were located at the 3'-end, the protection in this direction would only have reached less than 50 nucleotides beyond the origin of assembly in this direction, a conclusion contradicted by the results of Lomonossoff and Butler who measured the coating of the 3'-tail directly.

To do this they measured the protection against nuclease of characteristic nucleotides, distributed each side of the nucleation region, during a time course of elongation (Lomonossoff & Butler, 1979). This showed that, although elongation in the 5'-direction proceeded substantially faster than that in the 3'-direction, nucleotides on the 3'-side were being protected before those on the 5'-side were totally coated and that several hundred nucleotides of the 3'-tail were protected within 5 min. It therefore seems likely that elongation can occur in both directions simultaneously, but faster in the 5'-direction. This is, of course, compatible with the relative lengths of the RNA tails and, for greatest efficiency of RNA packaging, one might expect approximately a fivefold difference in rates. Moreover, it does mean that any measurement of the overall rate of elongation will be dominated by that in the 5'-direction and that measurements of growth in the 3'-direction will only be possible with specially designed techniques.

5'-direction

Unlike the wide agreement on the requirement for disks in nucleation, their possible role in elongation has been subject to considerable controversy. Thus, when Butler & Klug (1971) first showed the involvement of disks in assembly and observed that these were used up in stoichiometric fashion, they concluded that disks must be active in the elongation reactions. In their discussion of elongation, they emphasized this rather surprising point, to the exclusion of any comment about the possible role of A-protein. It was, however, always considered likely that under appropriate conditions A-protein could add directly (e.g. Butler, 1972), although the fact that it may do so in no way precludes addition from disks, if present, either as a protein source or as a delivery package which only breaks down at the site of, or even during, the actual incorporation into the growing helix. The initial omission of any discussion of A-protein was taken by some other workers as implying that Butler and Klug thought that A-protein could not participate in the elongation (Richards & Williams, 1972; Okada & Ohno, 1972; Ohno et al., 1972) and these groups demonstrated that elongation could occur with A-protein as sole source, concluding that it was the sole source. Recognition that A-protein being able to add does not mean that disks cannot add came from Richards & Williams (1973) when they again confirmed the addition of A-protein, but some other workers have continued with this argument, showing that A-protein can add and concluding that disks are therefore not involved (Ohno et al., 1977; Fukuda et al., 1978; Fukuda & Okada, 1982). In contrast, others have repeatedly shown that elongation can be more rapid in the presence of disks than of A-protein alone (Butler & Finch, 1973; Butler, 1974a, b) and that the resulting rods are more infective and nuclease-resistant (Lebeurier & Hirth, 1973), hence concluding that disks do play a part in elongation.

Part at least of the disagreement may arise from the choice of different starting materials, conditions and assays for the reassembly. Thus, while most groups have standardized conditions using TMV Vulgare and working at pH 7.0, ionic strength 0.1 M and 20 °C, the group of Okada use TMV-OM as a 'common strain' and also carry out heterologous reactions with protein from cucumber green mottle mosaic virus. Moreover, they frequently work in ionic strength between
0.25 and 0.5 M at 25 °C. The choice of TMV strain could be significant since it is known that a single mutation can render the coat protein non-functional and dramatically alter its aggregation properties (for example, PM6 protein discussed by Hubert et al., 1976) or can change the interaction of the RNA even with *Vulgare* protein (discussed above for the nucleation). Even more important is the fact that both temperature and ionic strength markedly affect the aggregation of TMV protein. Thus, raising the temperature or ionic strength causes the 20S disks to stack into 28S aggregates and these have been shown to interconvert into protein helix only with difficulty, forming many anomalous structures in the process (Durham & Finch, 1972). The effect of such aggregates on the rate of assembly (and probably also its pathway) was pointed out early on, when it was found that they assembled much more slowly (Butler & Klug, 1973), but this has been ignored by Okada and his colleagues. These differences in material and conditions therefore render it difficult to assess the relationship between the different results and conclusions. An additional complication is that Okada and his colleagues have often followed the assembly by measurements of infectivity or protection of the RNA termini after nuclease treatment. As already discussed, while these are excellent assays for the completion of assembly, they will be very strongly influenced by any ‘finishing events’ which might occur in packaging the ends of the RNA, and may well not be measuring the main processes of elongation reliably. Even measurements of full-length RNA protection by electrophoresis in polyacrylamide gels is complicated by the observation of an anomalous behaviour with increasing concentrations of RNA (Asselin & Zaitlin, 1978).

Measurement of the kinetics of assembly (Butler, 1972, 1974a), showed that the rate of reaction saturated with increasing disk preparation concentration in a manner analogous to an enzyme-substrate interaction. The protein concentration for half-maximal rate was about 1 mg/ml disk preparation while the maximum rate corresponded to approximately 5.4 subunits/s. With the demonstration of the complex structure of the growing particles (Butler et al., 1977; Lebeurier et al., 1977) it became obvious that the main RNA tail might have a special, and possibly labile, structure. For this reason it is probably desirable to consider only kinetic measurements which have been made under conditions where rapid growth is occurring, so as not to allow time for loss of any special structure. A compilation of such measurements (Butler & Lomonossoff, 1980) showed average rates of elongation of 6.5 subunits/s and 2.1 subunits/s from disk preparations and A-protein, respectively. These measurements were made with several different techniques, at different times, and the standard deviations are both less than 15% of the rate, suggesting that they are reasonable estimates of the true rates. The measurements with A-protein were made on pre-nucleated rods; thus, these results suggest that elongation under optimal conditions is about three times faster from a disk preparation than from A-protein alone.

Two different approaches have been adopted to try to avoid the difficulties of arguing from kinetics. In one the RNA was added to mixtures of A-protein and a disk preparation, and after allowing reaction the residual free protein species were measured (Shire et al., 1979b, 1981; Schuster et al., 1980). In all of these experiments the protein was simply described as ‘4S’ or ‘20S’ and no attempt was made to distinguish (even as far as might be possible) between the different species which sediment around the same rate, e.g. disks and proto-helices both sediment around 20S (Schuster et al., 1980) but it is unlikely that proto-helices, where the subunits are already in the ‘final’ positions, could participate directly in any nucleoprotein assembly reaction. The earliest experiments were carried out at pH 6.5, ionic strength 0.1 M and 6.5 °C, conditions where it is unlikely that disks would exist and where any 20S material is probably proto-helices, and indeed preferential addition of A-protein was found (Shire et al., 1979b). However, when the reaction was carried out at pH 7.0 and 20 °C, where disks will be a significant component of the 20S material, almost all of the protein could come from this source. Measurements were made of the fraction of protein removed from each size class at different starting ratios of the classes, but in view of the known mixture of species sedimenting around 20S, it is not clear what the actual amount of any potentially active species may be. This is the more important as only a 20% excess of protein was employed and so at lower ratios of 20S/4S there may not be sufficient of any active species in the 20S material to supply very much protein for the elongation.
Despite this problem of quantification, two clear observations emerge. Firstly, the protein source for greater than 90% of the reassembly can be from the 20S peak, showing that disks or some similar aggregate can take part in elongation, although possibly breaking down to an A-protein-like state in the process. Secondly, a minimum of about 25% of the protein must come from the 20S material, corresponding to coating of over 1500 nucleotides and showing that here disks must act as a delivery package, only breaking down, if at all, during the actual interaction with the growing rod. While Schuster and his colleagues chose to interpret all of this coating as nucleation (Schuster et al., 1980; Shire et al., 1981), this seems to be becoming a semantic argument and to owe much to a residual view that disks are only involved in nucleation. Given the first order dependence of the nucleation rate on the disk concentration (Butler, 1974a), the argument that disks are not involved in elongation becomes rather strained.

The other alternative to kinetic measurements has been to investigate the lengths of RNA protected from nuclease attack during reassembly (Butler & Lomonossoff, 1978). In these experiments, RNA was reacted with a substantial excess of a disk preparation at high concentration, thus ensuring rapid growth, and samples were taken at various times, the reaction stopped and the tails removed by nuclease digestion. After extraction and electrophoresis in polyacrylamide gels, the RNA appeared as a series of bands (Fig. 9a), suggesting some quantization of the elongation and protection. The sizes of these bands were estimated, by comparison with markers, and they appeared to be relatively equally spaced although certain bands in the series were either very weak or not visible. Over the range from 450 to 1250 nucleotides, the spacing was about 54 nucleotides, while in a separate experiment, it was 99 nucleotides over the range from 1260 to 2750 nucleotides. These results compare closely with the possible addition of 17 subunits per ring of a disk, which would coat 51 or 102 nucleotides depending on whether one or both rings were incorporated at once. Such precision is clearly fortuitous, both because of errors in length measurements and as a few subunits would be expected to be lost or gained during any such reaction and, moreover, no account is being taken of possible growth in the minor 3'-direction (discussed below).

Two main objections can be raised to the interpretation that this quantization arises because of the direct addition of protein from disks. Counting the number of steps is complicated by the weak or absent bands, about which assumptions have to be made, while an alternative explanation could be the presence of some feature in the sequence recurring with the spacing of the bands. Both of these can be investigated in a totally objective way by the use of Fourier transformation to analyse for any repeating feature of the gel pattern and the RNA sequence. Photographic negatives from the gels of protected RNA fragments were scanned with a densitometer (e.g. Fig. 9b) and the sections of the traces covering the regions of the gels where banding had been seen were analysed by one-dimensional Fourier transformation. A typical transform is shown in Fig. 10(a) for the trace shown in Fig. 9(b). The vertical scale is arbitrary, since it depends on the photographic exposure and densitometer sensitivity, but the horizontal scale is in number of nucleotides, estimated from the marker RNAs, and has a maximum estimated error of ± 10%. Because of the limited range of the data to be analysed (800 nucleotides, from 450 to 1250) and the restricted number of sampling points (81, corresponding to 10 nucleotide intervals), the transform will inevitably show strong low-order terms (i.e. long repeat) and a falling background towards higher order, and therefore the peaks around 150 nucleotides or longer are not significant. However, the striking feature is the very strong repeat around 53 nucleotides, in good agreement with the earlier estimate of around 54. The analysis of the longer RNA fragments (not shown) is not as clear-cut and is much noisier, but again there is a significant peak around 90 nucleotides. These results therefore confirm the conclusion that the RNA fragments form a series of bands with spacings in the region of 50 or 100 nucleotides, as we had previously argued on a qualitative basis (Butler & Lomonossoff, 1978).

A similar analysis is not technically feasible for elongation from A-protein alone. In order to obtain almost complete nucleation, such an excess of disk preparation must be used and allowed to react to completion, that the banding pattern already extends out beyond 1000 nucleotides in the nucleated rodlet preparation. During subsequent elongation after addition of A-protein, this pattern is not effaced as all bands tend to elongate similarly and so the pattern simply shifts up in
Fig. 9. Protection of RNA during TMV elongation with disk preparation as protein source. (a) Time course of elongation showing banding pattern. Reassembly was carried out for the indicated times (min) and the uncoated tails were removed by micrococcal nuclease digestion for 30 min. Lane R, *E. coli* ribosomal RNA used as a marker; lane T, TMV RNA preparation used to examine elongation. Dashed markers indicate positions of weak bands. From Butler & Lomonossoff (1978). (b) Densitometer scan of the 1 min time point on negative of (a). Also shown is the enlarged scan of the region where bands are most clearly visible, as used for Fourier analysis.
Fig. 10. Fourier analysis of TMV RNA banding pattern. (a) Transform of densitometer tracing of negative of gel electrophoresis of protected RNA (Fig. 9b) between 450 and 1250 nucleotides long. Vertical scale is in arbitrary units, as it depends upon photographic exposure and densitometer sensitivity. Horizontal scale is calibrated from marker RNAs. (b) Transform of purine repeats (and, by complement, pyrimidines) in RNA sequences over 2000 nucleotides to 5'-side of nucleation region and from nucleation region to 3'-terminus. Vertical scale represents probability of observed distribution occurring in random sequence RNA.
size while maintaining the banding. Hence any analysis continues to be dominated by the initial growth from disks and it is not possible to determine the pattern which A-protein alone might have given.

With the complete RNA sequence (Goelet et al., 1982) it has been possible to analyse for any repeats in the RNA which depart from a random base sequence. Such Fourier analysis was carried out on a stretch of 2000 nucleotides to the 5'-side of the nucleation region and, as a control, on the 900 nucleotides to the 3'-side (both stretches taken to include the origin of assembly). The results of an analysis for the presence of purine residues (and, by complement, pyrimidines) is shown in Fig. 10(b). The scale is an absolute one of the probability of the distribution observed occurring in a random sequence and no significant repeat is observed on either tail in the region of 50 or 100 nucleotides. Similar analyses looking for triplets with 3'-terminal G and any combination of A or U in the other places were equally negative. These results eliminate any regular distribution of particularly favourably bound sequences, but there was still a possibility of regularly spaced hairpin loops which might obstruct elongation. Potentially stable hairpin loops were located by looking for all loops with stems containing at least six A/U or G/C base pairs (and allowing G/U pairing) and single-stranded loops between two and 20 nucleotides. The distribution was again tested by Fourier analysis and no significant regularity was found.

These Fourier analyses thus show that, under favourable conditions for reassembly, the RNA tends to be protected in discrete lengths of one or two turns for lengths over the range from 450 to 2750 nucleotides and that this effect is not due to sequence features of the RNA either favouring protein binding or obstructing assembly. It is therefore most reasonable to suggest that this result arises due to the protein being pre-packaged into units which add one or two turns at a time, and the most obvious such package is the disk.

One hypothesis for such a mode of elongation along the major 5'-tail is pictured in Fig. 11. On this hypothesis, the tail, which is folded back down the central hole of the growing rod, forms a 'travelling loop' after binding to the upper surface of the rod, which can then interact with an incoming disk in a fashion analogous to that of the nucleation loop, with the travelling loop inserting into the central hole and binding round between the two turns of protein. The interaction with the RNA and the end of the existing nucleoprotein helix causes the disk to dislocate, entrapping the RNA. More RNA might then move up through the central hole, binding to the upper surface and regenerating the travelling loop. The size of this loop may well be critical for its interaction with an incoming disk: if it were too large it would not be able to insert and the disk might no longer be able to interact. The effect of random movements of the RNA along the central hole of the rod is essentially linear diffusion with a boundary (i.e. the RNA will not be pulled away from its interaction with the protein) and the result is that the size of the loop will grow with time. Hence, the special structure required for elongation from disks may well be labile and be lost either under conditions of slow elongation or else on sitting around or during any adverse handling. This could well explain some of the apparent differences in experimental results, and hence conclusions, on the involvement of disks, depending upon how the elongation is carried out.

3'-direction

Few experiments have been performed on elongation specifically towards the 3'-end and, because of the more rapid elongation in the opposite direction, little is known about it apart from one report (Lomonossoff & Butler, 1980). In these studies the authors partially stripped TMV with alkaline conditions, to expose the 5'-tails of the RNA (Perham & Wilson, 1976). These were then removed by nuclease digestion, the RNA isolated and fractionated and RNA fragments taken which contained the nucleation region and only a short tail to the 5'-side but with a complete 3'-tail. After nucleation with small amounts of a disk preparation elongation would be very rapid in the 5'-direction, to give rodlets coated to their 5'-ends but with residual uncoated 3'-tails upon which further elongation could then be observed.

Unlike elongation upon intact RNA, the rodlets grew more rapidly with A-protein than with a disk preparation, irrespective of whether this was measured by the turbidity increase, the length
Fig. 11. Picture of possible mechanism of elongation along 5'-tail from protein disk. The protein and RNA are drawn diagrammatically and also a diagram is given for the expected configuration of the RNA and its movement up through the central hole of the rod. Details are discussed in the text.
of the longest protected RNA or the protection of specific oligonucleotides. The kinetics of the reaction showed saturation with increasing protein concentration, but with a disk preparation having to be present at about fourfold greater concentration to reach saturation than A-protein, it is almost certainly this component which is adding onto the 3'-tails. This hypothesis is also supported by the absence of any banding pattern in the RNA protected from nuclease digestion, with the RNA migrating as a single broad band when run in gels. Although no details are available, it is likely that the protein will be adding directly from the A-protein, either as single subunits or a few subunits at a time. The protruding RNA at the ‘step’ at the end of the nucleoprotein helix would provide a favourable site for such an interaction, as originally suggested by Watson (1954) for all TMV assembly.

OVERALL CONCLUSIONS

The assembly of TMV initiates in a unique region on the viral RNA, located in *Vulgare* about 900 nucleotides from the 3'-end. The actual origin of assembly is probably a specific hairpin loop which inserts into the central hole of a disk aggregate of the coat protein and binds between the two rings of subunits. The interaction then causes the disk to dislocate into a proto-helix, entrapping the RNA to start the formation of the nucleoprotein rod. Two further hairpin loops, on the 5'-side of the origin of assembly, enhance the initial growth in that direction, possibly either by interacting similarly to the origin or even by interacting simultaneously and triggering off a concerted reaction. Elongation then occurs in the two directions.

Only protein from small aggregates, collectively known as A-protein, appears to add along the 3'-tail of the RNA, which protrudes directly from one end of the growing rod. However, the longer 5'-tail is doubled back down the central hole of the rod and may well form a special structure, a ‘travelling loop’, which is essential for the most rapid elongation in this direction. As long as this structure is undamaged, elongation towards the 5'-end appears to occur most rapidly from subunits in disks (or some similar component in the 20S mixture), although A-protein subunits can add at a lower rate and certainly do so alone if the structure of this travelling loop is lost for any reason. Nothing is known about the finishing events in which the final turns of the RNA will be encapsidated and the processes may become different as these ends are approached.

The nucleation reaction shows a high specificity for the most favourable RNA/protein interaction, which appears to be with sequences containing G in every third position and A (or less often U) in the other two positions. Depending upon whether the coat proteins are capable of co-aggregating, phenotypically mixed particles may be formed during double infections (Taliansky et al., 1977; Otsuki & Takebe, 1978), but in general homologous RNA and protein assemble most readily. Evidence for some lowering of specificity also comes from the presence, at about one copy per virion, of H protein which has the amino- and carboxy-termini of coat protein, but also another polypeptide, possibly as a branched fusion product (Collmer et al., 1983). While small amounts of H protein will reconstitute with the coat protein and RNA, particles containing it do not show any differences in stability or infectivity (Collmer & Zaitlin, 1983), and its function is unknown.

The polymorphic forms shown by the aggregates of TMV protein thus closely reflect its role in the virus assembly. The most stable is the protein helix, which is essentially the protein component of the virion, but is unable to participate directly in any assembly reaction. Central for the assembly is the protein disk, which is structurally related to the helix and is essential for nucleation of assembly and also participates in the most rapid elongation in the major direction. Also required are the smaller protein aggregates, the A-protein, although few details are known directly about their structures. During assembly both disks and A-protein must be converted into nucleoprotein helix and so the fine balance between the three polymorphic forms is probably essential for the most efficient growth of TMV.

REFERENCES


