RNA-PROTEIN INTERACTIONS IN THE ASSEMBLY OF TOBACCO MOSAIC VIRUS

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ABSTRACT Assembly of tobacco mosaic virus is initiated by the binding of a specific loop of the RNA into the central hole of the disk aggregate of protein subunits. Since the nucleation loop is located about five-sixths along the RNA molecule, subsequent elongation must be bidirectional. We have now measured the rates of elongation in the two directions by determining the lengths of RNA protected from nuclease digestion at different times and using either intact TMV RNA, or RNA with most of the longer tail removed. Comparison of the rates with the protein supplied as either a mixture of disks with A-protein (a mixture of less aggregated states) or just A-protein, shows that different mechanisms and protein aggregates are used for the most rapid growth. When disks are present, they add more rapidly along the longer RNA tail but do not appear to add directly on the shorter tail. In contrast, smaller aggregates (A-protein) can add at both ends of the rod, but do so more slowly. Mechanisms for these processes are discussed. Preliminary results on the binding of the specific hexanucleotide AAGAAG to the disk are given and compared with the known changes on binding nonspecific hexanucleotides or the trinucleotide AAG.

INTRODUCTION

Tobacco mosaic virus (TMV) is a simple virus having a single type of coat protein subunit and a single-stranded RNA genome ~6,400 nucleotides long. The coat protein forms a helical aggregate, with 16⅓ subunits per turn, and the RNA is packaged by being intercalated between the turns (for review, see reference 1). The overall length of the rod-shaped virion is determined by the length of the RNA, which packs with 3 nucleotides per protein subunit, with the growing rod elongating until the RNA molecule is completely coated to give the 300-nm rod containing ~2,100 protein subunits.

With such a simple structure, assembly might also be quite simple, with single subunits adding onto the "step" at the growing end of the particle where the RNA would protrude, thereby regenerating the step each time until all of the RNA was incorporated (2). This hypothesis received substantial support from the classic experiments of Fraenkel-Conrat and Williams (3) showing that the virus could reassemble from its isolated protein and RNA, thus for the first time demonstrating the process of "self-assembly" in a biological system. Moreover, self-assembly to regenerate faithfully the original structure was found to be both fairly specific for the conditions under which it occurred and highly specific for the RNA which could be incorporated (4,5). This specificity very strongly favors assembly upon the homologous viral RNA, even against RNA from different strains of TMV.

This straightforward picture of the assembly of TMV overlooks a major difficulty — how does the nucleation occur to start the process? To form the smallest stable nucleoprotein helix, about 20 protein subunits would have to align themselves along the RNA, bonding only to a single neighbor on each side until the growing aggregate was large enough to form more than a complete turn of helix, so that interaction in an axial direction could occur to stabilize the

structure. Single subunits, each interacting with only 3 nucleotides, could not give the high specificity for the RNA which is observed.

The isolated coat protein shows polymorphic aggregation driven by entropic effects (reviewed in reference 6), with the mode of aggregation controlled largely by pH (7). Below pH 7, the protein forms a helix which is very similar to that in the virus, while at higher pH's it occurs as a mixture of small aggregates known as "A-protein." In the region around pH 7, however, a specific aggregate is found, known as the "disk"; of which the structure has now been solved to atomic resolution (8, 9). The disk has two rings of 17 subunits, giving a diameter similar to that of the helix, to which it is readily and rapidly converted when the pH is lowered (7). The subunit packing within each of the rings is very similar to that in the helix, although the contacts between rings are quite different (8).

The occurrence of disks as the main (~80%) component of the protein equilibrium around neutral pH and at 20°C — conditions which have been found to favor reassembly (4) — led us to postulate that they might be essential for overcoming the problems of nucleation by interacting with the RNA to form the first turns of the nucleoprotein helix (10). This proved to be the case. Moreover, it was shown that the nucleation reaction is highly specific for the viral RNA (10) and occurs at a unique site upon the RNA which has been isolated (11). This region of the RNA has been sequenced (12, 13) and the smallest "core" binding to the protein disk during nucleation has been shown to be able to form a hairpin loop of ~50 nucleotides, with the special sequence AGAAGAAGUUGUUGAUGA at the open end (12). Since 3 nucleotides bind per subunit, this repeat pattern with G in every third position strongly suggested that this open loop was the actual origin of assembly. Surprisingly, however, the nucleation region was found to be internal in the RNA, ~1,000 nucleotides from the 3'-end (14), a result confirmed recently by sequence analysis (15).

The structure of the disk, as determined by x-ray crystallography (8, 9), shows the two rings of subunits to be in contact at their outer ends, but opened apart onto the central hole, so that there is ready access from this hole to the RNA binding site between the subunits. Taken together with the probable structure for the nucleation region on the RNA, this led us to propose (16) that nucleation might be occurring by the insertion of the RNA loop into the central hole of the disk to reach the binding site between the protein subunits. As the single-stranded loop of the RNA interacted with the protein binding site, the base paired stem of the loop could melt, allowing further RNA binding around the complete ring. The interaction could then trigger the dislocation of the disk, causing it to form a short protohelix containing the first turn of the RNA. Because of the internal location of the nucleation region on the RNA, such a growing helix would necessarily have both RNA tails protruding from the same end of the rod, one of them being looped back down the central hole. Such a structure has been found (17, 18). Furthermore, it has been shown that the longer (5') tail was the one which was looped back, and that this unusual structure appeared necessary for the rapid elongation of the growing particles (17).

The Outstanding Problems

There is widespread agreement on the requirement of disks for the nucleation of assembly of TMV (10, 19, 20) and even on the kinetic order of the reaction, with a single disk interacting with each RNA molecule in the rate limiting step (21, 22). However, the nature of the protein species involved in the subsequent elongation is still a matter of controversy (reviewed with full discussion of the literature in references 23 and 24, and more recently in references 25–28), which has proved difficult to settle experimentally. The major obstacle is the very

nature of the disk preparations. Under optimal conditons for the reassembly (5) the equilibrium mixture of protein aggregates consists of ~80% disks and 20% A-protein (29) with a rapid microequilibrium of subunits between the states, though a somewhat slower bulk equilibrium (see reference 24 for discussion). It is thus possible to obtain preparations of A-protein alone, but the normal equilibrating disk preparations always contain some A-protein as well as the two-layer disks. In an attempt to avoid this problem some experiments have been conducted under conditons where the "disks" are metastable (28, 30). However, these metastable aggregates were not adequately characterized to show that they were really two-turn disks rather than, say, short helical segments, while their metastability itself shows that they are kinetically "locked" in some way, unlike the normal freely-equilibrating disks (see discussion below). Such results cannot therefore be validly compared with those obtained with equilibrium disks, and their usefulness in understanding the normal elongation of TMV is questionable.

Subsidiary complications arise from the inherent problem in measuring the elongation of particles up to sizes of 300 nm. The interpretation of average properties (e.g., turbidity) requires assumptions about the nucleation of the particles and their competence for further elongation, while apparently direct methods (in particular elecron microscopy) may require specimen preparation techniques that perturb the distribution being measured. Moreover, the internal site of nucleation results in bidirectional elongation, and it has been shown that growth occurs in both directions simultaneously (27). Since the same protein source may not be responsible for growth in each direction, the optimum technique for studying the elongation should be capable of resolving which direction is dominant under the conditions employed.

We have recently found that measurements of the RNA protected from nuclease attack provide a reliable method for determining the rate of elongation and, from the protection of oligonucleotides in known locations along the RNA, its main direction. We have already reported some results with this technique, studying reassembly upon intact TMV RNA with the protein as a disk preparation (26, 27). Under these conditions the elongation is mainly in the major (3′ to 5′) direction and much slower in the minor (5′ to 3′) direction (11, 12, 18). However, stripping of TMV under mildly alkaline conditions removes protein specifically from the 5′-end of the particles, so the partially stripped virus (PSV) isolated contains partial RNA molecules with intact 3′-termini but shortened 5′-tails (31). RNA molecules with very short tails to the 5′-side of the nucleation region show much less rapid elongation than intact molecules (32) but, as expected, reaction does still occur (33). Taking advantage of this, we have been able to extend our observations to the rates of elongation specifically along the minor (3′) tail of the RNA and we report these in this paper.

Another problem of continuing interest is the detailed interactions between the RNA and protein. The location of the RNA within the virus has been determined from the 0.4nm resolution electron density map obtained from x-ray diffraction studies on oriented gels of TMV (34), but this can only show an "average nuclotide" in each position and, moreover, is not yet at a resolution to show any detailed contacts. Difference maps of a specific trinucleoside diphosphate (ApApG) from the nucleation region of the RNA, bound into the disks in the crystal, have been obtained to a resolution of 0.5 nm (35) and show clear movements of protein and, possibly, the site of the nucleotide binding, although again not at an adequate resolution to study details. We are currently extending these studies to a resolution higher than 0.3 nm using the oligonuclotide AAGAAG (again corresponding to part of the origin of initiation). The preliminary results are described here.

MATERIALS AND METHODS

TMV, TMV protein (as both A-protein and a disk preparation), and TMV RNA were prepared and their concentrations determined as previously reported (11). Partially stripped virus (PSV) (36) was prepared as previously reported (27), but with dialysis for 24 h, RNA tails being removed with micrococcal nuclease (31), and the RNA prepared as usual. The RNA was fractionated in 15-40% (wt/vol) linear sucrose gradients containing 0.1% (wt/vol) sodium dodecyl sulphate for 24 h at 25,000 r/min and 20°C; the gradients were collected with an Isco gradient fractionator (Isco Manufacturing Co., Inco, Kansas City, Mo.). Fractions containing appropriate length RNA were taken and the RNA was precipitated with ethanol.

All 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 reaction with appropriate amounts of a disk preparation (see below) and, when necessary, fractionated in sucrose step gradients as before (17), except for being in assembly buffer to minimize the time required for subsequent dialysis (4 h).

Assembly was assayed by turbidity increase at 310 nm (10), measured with a Unicam SP 8-100 (Pye-Unicam Ltd., Cambridge, England) or a Gilford 2400-2 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), with an RNA concentration of 0.05 mg/ml. Alternatively, the length of RNA protected from digestion by micrococcal nuclease was determined as before (26, 27), using agarose/acrylamide gel electrophoresis. Densitometry of the negatives from photography of gels stained with ethidium bromide was with a Joyce Loebl densitometer (Joyce Loebl Ltd., Newcastle-upon-Tyre, England). Analysis of coating of specific oligonucleotides was by ribonuclease T_1 digestion and fingerprinting as previously described (27).

RESULTS AND DISCUSSION

Elongation towards the 3'-Terminus

To make a substrate for studies of reassembly in the 3' direction we have partially reassembled PSV RNA preparations with a protein disk preparation to coat completely the 5'-tail but still to leave a substantial 3'-tail. Such rodlets can be used to investigate the reassembly using different protein sources.

A PSV RNA preparation containing molecules 2,200 to 3,200 nucleotides long was reacted with 12.5 times its weight of protein and shown, by ribonuclease T_1 digestion and fingerprinting, to be fully coated to its 5'-end but still to have most of the 3'-tail uncoated. After sedimentation through a sucrose step gradient to remove any unreacted RNA or protein (17), the rodlets were rapidly dialyzed back into reassembly buffer and used to measure the kinetics of elongation, assayed by the rate of turbidity increase. Although the absolute rates of change will depend upon the RNA lengths, the relative rates between different protein species and concentrations will be strictly comparable despite the heterogeneity in original RNA length.

The relative rates of elongation (Fig. 1) show a saturation with increasing concentrations of protein, irrespective of the form in which this was added. The maximum rates are the same when adding either A-protein or a disk preparation, but at lower concentrations, the rate is always faster when adding A-protein rather than a disk preparation (containing ~ 20% A-protein). This suggests that the elongation in this 5' to 3' direction is not occurring directly from disks, but rather from some component of the A-protein in the disk preparation, so that a higher total protein concentration is required to achieve the same rate.

A more sensitive assay for elongation is to measure the length distribution of the RNA at increasing times. To maximize resolution, we employed the shortest PSV RNA which reproducibly contains the nucleation region (1,700–2,200 nucleotides long) and reacted this with a tenfold weight excess of protein to prepare rodlets. This again gave essentially complete coating to the 5'-ends, while leaving the 3'-tails uncoated. Since the measurements were to be of the longest protected fragments, possible unreacted RNA was not removed, as it

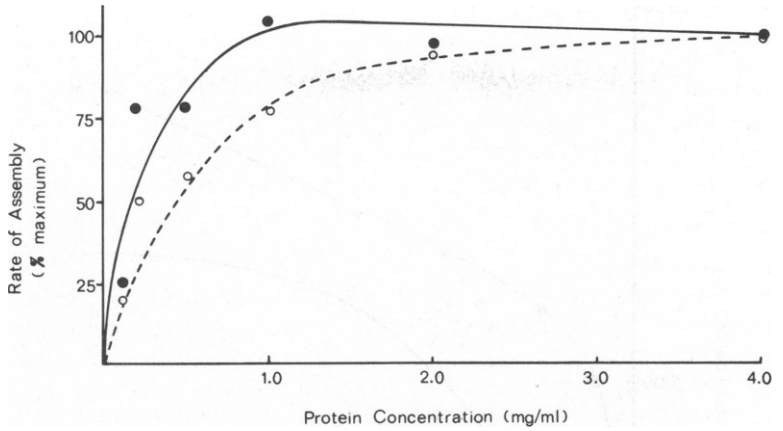

Figure 1 Effects of protein concentration and aggregation state on rate of elongation in 5′ to 3′ direction. Rates are expressed as percent maximum to allow data from different experiments to be shown together. Partially reassembled rodlets with PSV RNA were reacted further with either A-protein (-●-)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

could not affect the measurements and any extra preparative steps might cause some damage to the RNA tails of the rodlets. Such measurements will be dominated by the longest RNA molecules present in the preparation and so will overcome any complicating effect of length heterogeneity at the 5′-ends of the PSV RNA. Elongation was carried out with either a disk preparation or A-protein added to 1 mg/ml, since the turbidity measurements had shown that this gave significant rates of elongation and also a clear difference between the protein species (Fig. 1).

Unlike the RNA protected during reassembly of intact TMV RNA with a disk preparation, which shows discrete bands (26 and below), the RNA protected in the rodlet preparation migrated as a single broad band in gels, due in large measure to the variable lengths at the 5′-ends of the PSV RNA. With the protein concentration below saturation, the spread will increase with time. To eliminate these problems, the length of the longest class of molecules was determined by measuring the position of the trailing edge of the RNA peak in the gel (taking the half-height). After densitometry of photographs of the stained RNA, this trailing edge of the peak was found to be consistent, unlike the edge representing the smaller material, which showed increasing dispersity with time due to damaged RNA molecules which would become fully coated but lack the 3′-terminus.

The time-course of elongation (Fig. 2) again shows the more rapid extension when the protein was added as A-protein. Extrapolation to zero time of elongation gives an estimate that the longest rodlets contained ~ 1,600 protected nucleotides of RNA. The actual elongations with A-protein are ~ 280 nucleotides in the first minute and 130 nucleotides in the second minute; the fall in rate probably reflects the completion of coating on some of the RNA tails, since the longest rods are within the total size range of the PSV RNA by the end of the first minute. With a disk preparation these rates are 40 and 73 bases per minute during the first and second minutes, respectively. The increase is probably due to error in extrapolation to zero time.

The extent of protection of the RNA can also be followed by the recovery of known oligonucleotides (27); since these are specifically located in the sequence, there can be no possible effect of the 5′-terminal heterogeneity. Since the sequence of ~ 1,000 nucleotides at

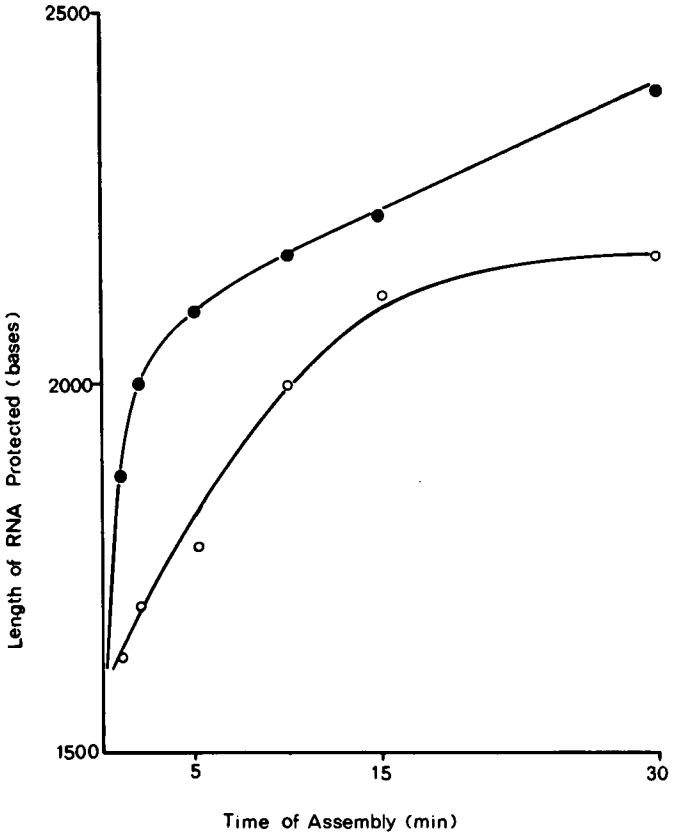

Figure 2 Time course of protection of RNA 3'-tails with different protein aggregates. Lengths of the longest protected RNA molecules were determined (see text) during elongation of partially assembled rodlets containing PSV RNA, with A-protein (-●-) or a disk preparation (O), thus allowing estimation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

the 3'-end of TMV RNA is known (15), we know the location of the characteristic ribonuclease T_1 nucleotides chosen: "spot 4" between 563 and 579 nucleotides in and "spot 5" between 510 and 525 nucleotides in (numbering from ref 27). The time courses for protection of these nucleotides (Fig. 3) again show the more rapid elongation in the 5' to 3' direction from A-protein than from disks, although since the fractional protection will measure the state of the average particle, the actual rates cannot be compared directly with those measured above.

Elongation towards the 5-Terminus

The nucleation region is ~ 1,000 nucleotides from the 3'-end of TMV RNA; hence over 5,000 nuclotides have to be coated in the 3' to 5' direction. Elongation in this direction is much more rapid on intact RNA (11, 18), thus dominating the overall kinetics of assembly, so that most studies apply mainly to this direction. We have found that during assembly with protein supplied as a disk preparation, the protected RNA lengths are "quantized" over the entire range from 470 to 2,750 nucleotides (the limits of resolution of the gels used), with a step size of either 50 or 100 nucleotides, corresponding rather precisely to one or two turns of RNA incorporated, as expected if subunits were adding directly from disks (26). We have now extended this study to include the effects of adding A-protein.

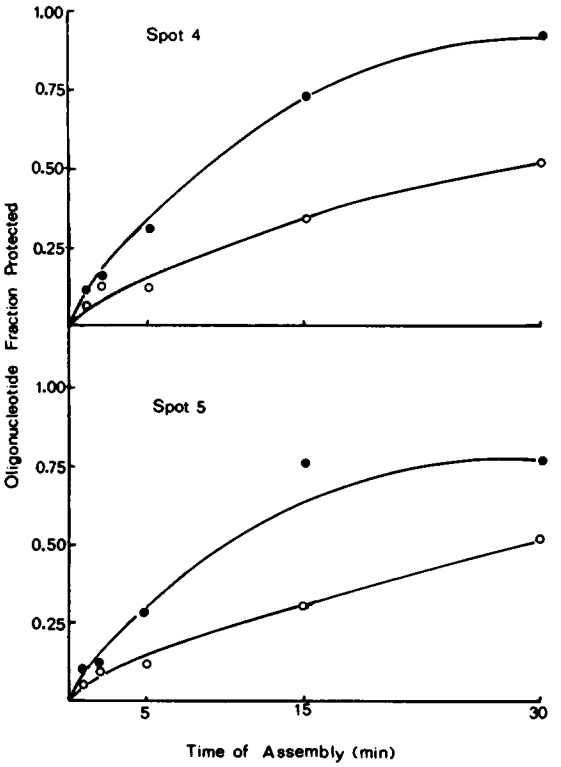

Figure 3 Extent of protection of specific oligonucleotides in the 3'-tail of PSV RNA during elongation with different protein aggregates. The recovery of specific oligonucleotides in the protected RNA was measured during the elongation of partially assembled rodlets containing PSV RNA, with A-protein (-●-) or a disk preparation (-O-), giving a measure of the average extent of coating at each time.

Since disks are needed for nucleation, it is not possible to observe assembly simply from A-protein. We therefore compared the lengths protected during reassembly of RNA (0.2 mg/ml) with (*a*) a disk preparation (4 mg/ml); (*b*) with a disk preparation plus A-protein (each at 4 mg/ml); (*c*) A-protein (4 mg/ml) after 10 min reaction with a disk preparation (0.2 mg/ml) to allow nucleation (average length of rodlet 1/20th that of TMV). The results (Fig. 4) show the more rapid increase in length of protected RNA whenever disks are present, although, not surprisingly, A-protein can add in this direction. By following the longest molecules, any possible complication of partial nucleation in the initial stages is eliminated. (The RNA markers show the expected log/linear relationship from 870 to 3,375 nucleotides, but, as is usual in acrylamide gel electrophoresis (37), this relationship ceases to be linear at the highest molecular weights, in this case between 3,375 and 6,400 nucleotides long.)

The addition of extra A-protein together with the disk preparation has little effect; if anything, it slows the elongation compared with addition of disk preparation alone. However, elongation from A-protein alone, after nucleation with a disk preparation, is markedly slower. As shown in Fig. 4 and other time courses, the first full length RNA molecules are protected within 6 min with a disk preparation and within 15 min with A-protein, corresponding to elongation rates of 1,067 and 427 nucleotides per minute, respectively. Measurements made during the second minute (to offset any effect of nucleation; see Fig. 4) give rates of 1,200 and 400 nucleotides per minute for the disk preparation and A-protein, respectively. We therefore conclude that the overall rate of elongation is two-and-a-half to three times faster from a disk

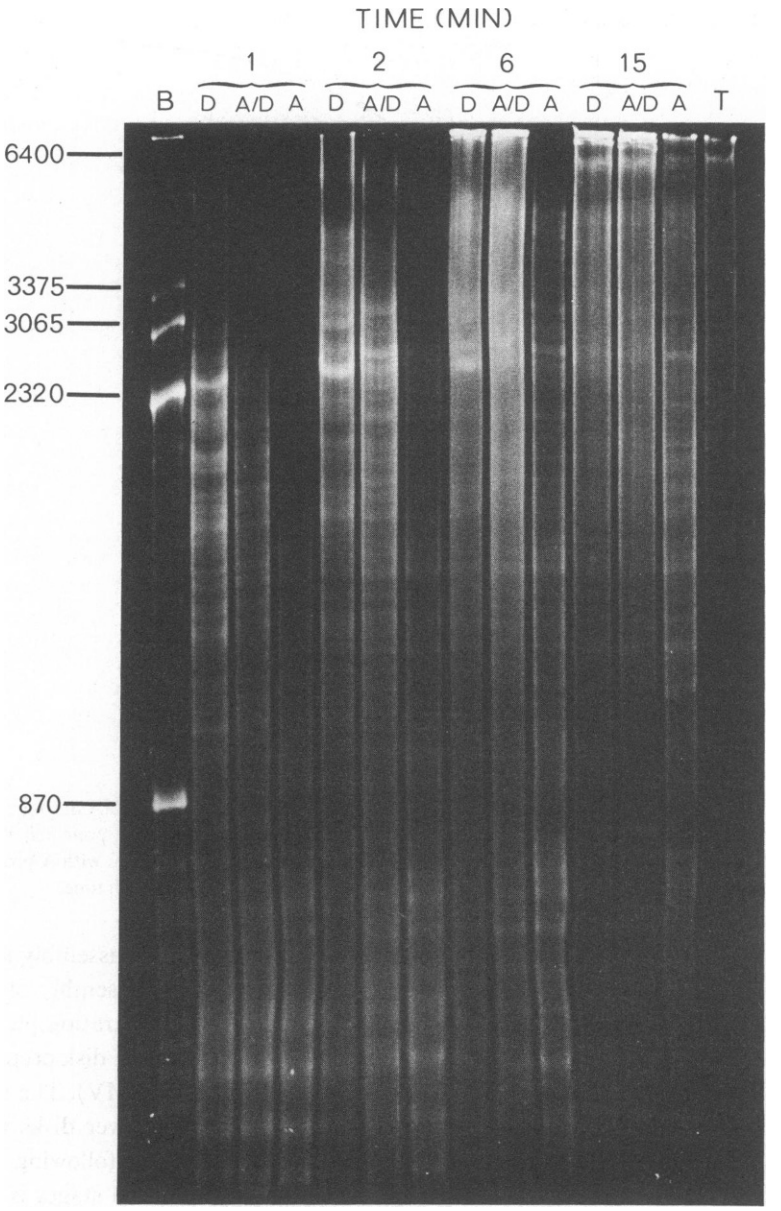

Figure 4 Agarose/acrylamide gel to determine sizes of protected RNA during assembly with different protein aggregates. Assembly was carried out with TMV RNA and either a disk preparation (D), A-protein together with a disk preparation (A/D), or A-protein after nucleation with limited amounts of a disk preparation (A) (see text for details). Uncoated RNA tails were removed by digestion with micrococcal nuclease and the protected RNA extracted after inactivation of the nuclease. The lengths of markers of brome mosaic virus RNA (track B) and the original TMV RNA (track T) are shown in nucleotides.

preparation than from A-protein. Furthermore, since we have shown by the same method that elongation in the minor (5′ to 3′) direction is faster from A-protein, growth in the major (3′ to 5′) direction must be favored from disks by more than this factor.

The one feature which might be in any way discordant with this picture is the occurrence of a banding pattern during elongation from A-protein. For the shorter bands this is the direct

result of the nucleation and initial elongation from the disk preparation giving such a banded pattern, and the pattern after 1 min elongation from A-protein is almost indistinguishable from that of just the nucleated RNA (data not shown). The persistence of some specific bands when only A-protein is present suggests that their structure presents some barrier to addition of subunits from the A-protein, a result consistent with the earlier observations of the lack of effect of A-protein upon the rate of turbidity increase from a disk preparation (22, 38). The longer bands which appear only during the extension do not correlate in detail with those from a disk preparation and, in particular, do not occur at the spacing of 50 or 100 nucleotides. Thus it is likely that they are a consequence of regions of the RNA with sequences unfavorable for coating either because of unfavored RNA-protein interactions or strong base pairing of the uncoated RNA. Such sequences would have much less effect upon elongation from disks because the cooperative addition of many subunits would more readily overcome such inhibition (10), although a number of bands do still persist in an anomalous fashion.

Comparison of Rates of Elongation

The most efficient elongation of the TMV particle is clearly that which occurs most rapidly. Changes in conditions or in the form in which protein is supplied, which result in slowing down the rate, may well be achieving this effect by altering the mechanism of assembly; in particular, they might possibly be preventing more complex assembly directly from disks while still allowing elongation by the simpler mechanisms from A-protein. Since these experiments are frequently intended to distinguish between these mechanisms, any experimental technique that requires or involves a lowering of the rates must be of doubtful value.

We consider only experiments under optimal conditions and have compared the rates of elongation we have measured by various techniques (Table I). For the sake of comparability, these rates have been taken either from experiments where a high protein concentration was used (at or above 4 mg/ml), or extrapolated to the "maximum rate" from the data available at various protein concentrations and assuming that saturation kinetics will apply (as observed, 39, 41), thus obtaining rates with nonlimiting protein concentrations. All rates are expressed in common units (subunits per second), converting turbidity changes as previously described (39), length changes by taking 7.1 subunits/nm (i.e., 2,130 subunits in a 300nm rod), and RNA lengths by using 3 nucleotides bound per subunit. It has not been possible to make a detailed comparison with the results of other workers because the necessary details for the conversions are often not available to us, and also because of differing conditions for the experiments, which in some cases cause large effects, or changes in the overall effect being measured (e.g. infectivity rather than elongation) (see reference 24 for discussion).

Measurements 1 to 5 were made either by following the elongation of the most rapidly growing rods (2, 4, and 5) or during the initial stages of growth when the effects of any damaged RNA will be minimal (1 and 3), and will therefore give estimates of the maximum rate under these conditions. The average rates are 6.5 subunits/s (SD 0.9) and 2.1 subunits/s (SD 0.3) from disk preparations and A-protein, respectively. In each case the standard deviation of the measurements is less than 15% of the rate, despite the different measurement techniques employed, suggesting that all of them are giving reasonable estimates of the true rate. Since experiments 1 and 3 involved the nucleation of assembly, no measurement was possible with A-protein alone (i.e., in the absence of a disk preparation). In all of the measurements upon the longest particle (2, 4, and 5), there can be no possible effect due to continuing nucleation, as has been suggested for the average rates (19, 20), for the reason already discussed.

TABLE I

COMPARISON OF THE RATES OF ASSEMBLY OBSERVED WITH DIFFERENT TECHNIQUES AT NONLIMITING PROTEIN CONCENTRATIONS

Technique employed	Rate		Reference
	Disk preparation	A-protein	
	(*subunits per second*)		
1 Turbidity with free RNA	5.4	—	39
2 Full length rods in e/m	7.1	1.8	40
3 Pulse-chase of labeled protein	7.6	—	22
4 Protection of full-length RNA	5.9	2.4	—
5 Increase in protected RNA length	6.7	2.2	—
6 Average growth rate in e/m	2.9	0.62	40
7 Turbidity with prenucleated rodlets*			
a	3.6	0.67	41
b	0.85	0.37	—
8 Rate of protection of 3′-tails of RNA with prenucleated rodlets‡			
a	0.22	1.6	—
b	0.41	(0.70)§	—

When available, separate rates are shown for elongation with a disk preparation or A-protein as the protein source. In the latter case, nucleation had to be carried out beforehand with a disk preparation. Assembly is at 20°C in sodium phosphate buffer, pH 7.0, ionic strength 0.1 M.

*Absolute rates vary between preparations, depending upon the free RNA tails still available (see text), but comparisons between protein sources (across line) are valid.

‡Assembly was prenucleated with limited amounts of a disk preparation upon RNA lacking most of the tail to the 5′-side of the nucleation region (see text). Elongation was then measured on the sole (3′) tail. The rates estimated during the first and second minutes are given.

§Value in second minute is unreliable, as rods are running out of RNA (see text).

Experiments 6 and 7 measure quantities which depend upon all the particles present in the solution during the experiment and are therefore rather slower and more variable than the measurements upon the fastest growing particles, probably due to differing degrees of damage to the RNA tails, particularly when partially assembled rodlets are used (cf. 7*a* and *b*). However, the comparative picture is again similar, with elongation from a disk preparation 3–4 times faster than that from A-protein.

An opposite effect is seen when the rates of elongation along the minor RNA tail are compared (line 8). In this case A-protein gives a rate significantly higher than that from the disk preparation, suggesting that it alone can add in this direction (see above). The rapid fall-off in the rate with A-protein between the first and second minutes (a and b) is probably due to the RNA tails on the longest rods, which are those being followed, becoming fully coated. Comparison of the fastest rates obtained in this direction with those for overall growth shows that the two rates are similar from A-protein. Since the overall rate includes elongation in both directions, this suggests that the 5′ to 3′ elongation is somewhat inhibited during the overall reaction, probably because the "doubled back" tail down the central hole gets in the way.

RNA/Protein Interaction in the Disk

To date, very little information is available about the detailed atomic interactions between any protein and nucleic acid chain. We have found that specific oligonucleotides can be bound into

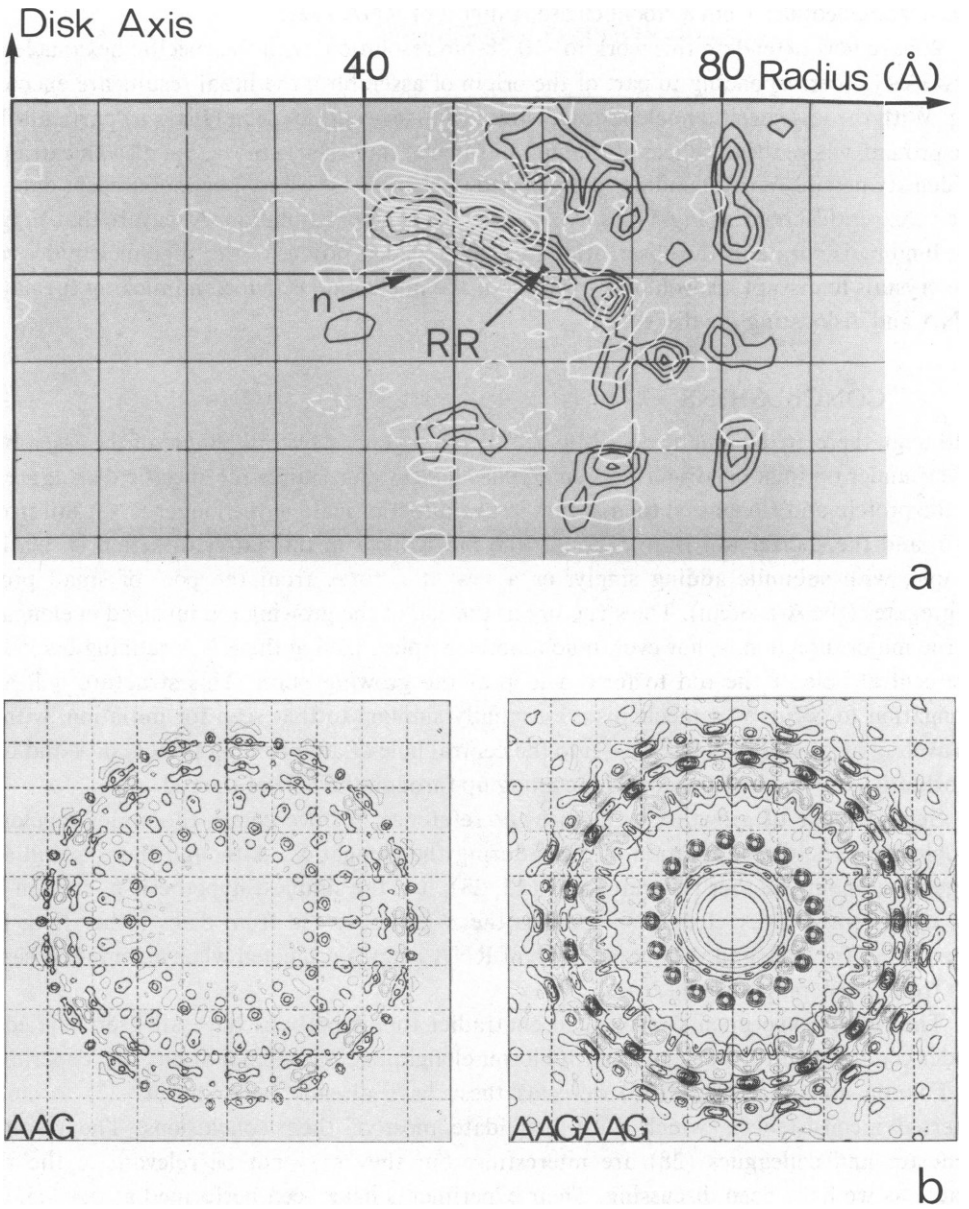

Figure 5 Electron density difference maps with nucleotides bound to TMV protein disks. (*a*) Vertical section containing the radius passing through the right radial helix (marked RR; nomenclature of reference 8). The native electron density is shown in black and the difference density due to binding of mixed hexanucleotide in white, with negative contours dotted. The upward movement of the helix is visible, together with a positive peak (marked n) without any corresponding negative peak, which is tentatively identified as the bound nucleotide (from reference 31). (*b*) Projection down the disk axis, comparing the effects of the trinucleotide AAG (similar to mixed hexanucleotides as in *a*) and the specific hexanucleotide AAGAAG. Positive differences are shown by heavy contour lines and negative differences by faint contour lines.

the protein disk of TMV in the crystal, producing differences in the x-ray diffraction pattern, and have obtained electron density difference maps to ~ 0.5-nm resolution with both AAG and hexanucleotides from a ribonuclease A digest of RNA (32).

We are now extending this work to ~ 0.28-nm resolution, with the specific hexanucleotide AAGAAG (corresponding to part of the origin of assembly); the intial results are encouraging. With the less specific nucleotides, distinct changes could be seen (Fig 5 *a*) particularly in the protein, where the α-helices of the subunits in one ring clearly move, but also an extra peak of density possibly corresponding to the bound nucleotide is visible. The more recent data give even clearer differences (Fig.5 *b*) and also extend to higher resolution. A feature that suggests the binding is normal is the observation that high concentrations of the oligonucleotides cause the crystals to disrupt, as would be expected if the nucleotide is indeed mimicking the natural RNA and dislocating the disks.

CONCLUSIONS

Although there are still many details to be filled in, a general overall picture of the assembly of TMV under optimal conditions has emerged. Nucleation requires the specific disk aggregate of the protein and elongation then occurs in two directions: along the longer RNA tail from 3′ to 5′ and the shorter tail from 5′ to 3′. The mechanism in this latter direction is relatively simple, with subunits adding singly, or a few at a time, from the pool of small protein aggregates (the A-protein). The structure at the end of the growing rod involved in elongation in the major direction is, however, much more complex, having the RNA running back down the central hole of the rod to form a loop at the growing point. This structure will allow elongation to occur by a mechanism essentially similar to that seen for initiation, with the "traveling loop" of RNA inserting into the central hole of an incoming protein disk and being constantly renewed by further RNA coming up through the hole of the rod.

Such a mode of growth (for picture see reference 42) overcomes the major topological problem for the direct addition of disks during the elongation. Although there is still some argument about whether this can occur (25, 28), it is the simplest hypothesis to explain both the consistently faster elongation towards the 5′-end observed from disks rather than from A-protein (Table I), and the quantization of RNA lengths protected when assembly is carried out from a disk preparation (26).

The recent experiments invoked to contradict this hypothesis have all been carried out under conditions involving significantly slower elongation. We will not discuss the experiments of Fukuda and co-workers (25) here, as these have already been shown (27) to contain internal inconsistencies which must invalidate most of their conclusions. The results of Schuster and colleagues (28) are interesting, but they may not be relevant to the rapid reactions we have been discussing. Their experiments have been performed at pH 6.5, ionic strength 0.1 M, and 6.5°C, conditions which were deliberately chosen to give metastable 20-S protein aggregates. Unfortunately, these are also conditions in which the protein has been shown to start forming short helical nuclei which have sedimentation coefficients about 20-S (43) and so are impossible to distinguish from disks on the basis of sedimentation analysis. The assumption that the metastable 20-S aggregates are two-layer disks is therefore unsafe. Moreover, the metastability of the aggregates invalidates their use in drawing conclusions about the possible involvement of the freely equilibrating aggregates, since their half-life of 12 d (cf. normal disks in reference 44) shows that their structure is "locked." Interestingly, the actual rate found is only ~ 6% of that under more usual conditions (pH 7, 20°C), and therefore well within the rate observed for A-protein alone. It is thus highly probable that the

metastable aggregates take no part in the reaction, not because disks cannot participate (as concluded), but rather because of their specific metastability, while elongation from A-protein alone simply occurs at a rather slow rate.

The major outstanding problems concern the structure of the virus and the interactions between the RNA and protein. While the structure of the disk is known at atomic resolution (9), work is still in hand to solve the virus structure in such detail and to elucidate the interactions involved both in causing the disk to dislocate into a short helix and in stabilizing the resulting helix. Despite the magnitude of these problems, considerable progress is being made and one can hope to see soon the atomic details of the interaction between a protein and an RNA chain to complement our knowledge of their kinetic behavior.

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DISCUSSION

Session Chairman: Victor Bloomfield *Scribe*: Jill Taylor

J. KING: The curves of assembly vs protein concentration (Fig. 1 in the text) look like Michaelis-Menten curves. Asakura interpreted similar data with flagellin assembly to mean that the rate-limiting step in assembly was the

transformation of the newly bound subunit on the growing face of the flagellin into a form which could now bind another subunit. Why shouldn't this data be interpreted similarly, i.e., the Michaelis-Menten enzyme substrate complex being represented by the growing rodlet, with one disk bound but not yet competent to bind another disk?

BUTLER: The actual curve shown in the text is for addition in the 5′ to 3′ direction where it is probably single subunits going on. At the same time the rate is of the order of 2 subunits per second. However, none of the rates is very precise; Table I is only meant to show that the rates of assembly are similar by different measurement techniques.

Earlier on we did observe saturation by adding the disk preparation to total RNA, elongating 3′ to 5′. We followed the same analysis as Asakura, so I accept your point. We estimate for the major growth a rate nearer 200 ms per protein subunit. If it is disk that is adding, it may not be the same for a subunit in each of the rings. The complexity of the system precludes any simple-minded interpretation. I'm sure our old interpretation was rather too simple-minded.

SCHUSTER: I want to clarify the question of which may be the adding species in the elongation phase of the assembly reaction. There is fairly good agreement in different laboratories that an aggregate of ~20S is required for the nucleation reaction, but there have been conflicting results obtained concerning the elongation phase. What you have shown here is evidence from RNA protection experiments suggesting that the disk aggregate adds preferentially. I have measured your large original photos for Fig 4 of the new gels of your experiments with A-protein, and plotted these results as Fig. A. This is a plot of the length distribution of protected RNA after A-protein has been added to the already nucleated species.

In Fig. A we have plotted the log of the number of kilobases protected vs migration distance in the gel for two sets of data: (1) The open circles are the 1 and 2 min reconstitution data after which the reaction was stopped and the amount of protected RNA determined. These data go up to just under one kilobase. (2) The closed circles are the protected RNA data after longer times (15 min) and there are longer protected pieces. The triangles indicate marker RNA's.

What I would like to point out is that the difference in these values is almost always a multiple of 50 nucleotides. 49 nucleotides is what you would expect for protection of only one-turn of the helix, twice that number if a disk were

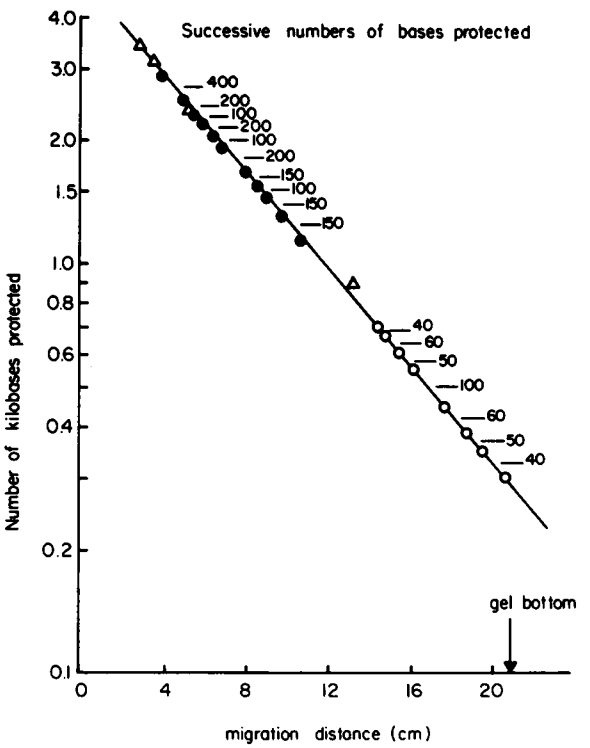

Figure A Analysis of the RNA banding pattern produced when TMV A-protein is supplied as the protein source for virus reconstitution in the protection experiments of Butler and Lomonossoff. Data were obtained from the original print used for their Fig. 4. Δ, marker RNAs; O, 1 and 2 min reconstitution times; ●, 15 min reconstitution time; all data from A-protein gel tracks.

added and the RNA were protected. As far as I can tell, this is exactly the same incremental number of protected nucleotides that you obtained in the previous publication and in this work in which you used disk preparations as the source of protein for reconstitution. Please comment.

J. KING: I would like to interrupt to make sure that we understand the point. You are saying that in the absence of disk, where the small subunit is adding you still have quantized RNA protection. You are implying that it is some property of the RNA itself.

BUTLER: I think there are two factors here that complicate the issue. We have done similar plots of our data.

The first problem is that to get nucleation at all you have to add the disk preparation; at one min after you've added the A-protein you have virtually not changed the length distribution from the original nucleated mixture. This is now shown in the paper. It is a point you raised as a reviewer, and we realized we had previously forgotten to say it. So we wrote it into the text. The fact is that, at these early time points, the step length is almost entirely determined by what happened during nucleation and not by the subsequent elongation. The thing hasn't grown enough to have changed the picture significantly. I agree it hasn't smeared it off completely, but it has certainly not changed significantly.

SCHUSTER: Then just for clarification, these data at short times represent the distribution of starting sizes.

BUTLER: That represents your plot from our figure rather than the original data, which you did not have. We have plotted this out and where we have compared elongation from a disk to A-protein the band pattern is not at the same places, i.e. the bands are not parallel across the gels. There certainly are some bands which come up and which persist long beyond the time you would expect them to disappear from the pattern. I think this is almost certainly due to some sort of interruption of elongation, because there is a kinetic barrier to overcome. I would point out some very early results of Stussi, Lebeurier and Hirth who showed that when you assembled by adding RNA to a rather unspecified protein mixture (but probably mostly 4S) you get incomplete rods of a very defined length which were stable for long periods but could be isolated and then further elongated to give complete rods. Another factor is that even if this banding were true and you were getting a repeat of one or two turns (although I don't accept this), it is an odd fact that you should have this constancy throughout the evolution of the virus when you are adding subunits singly or from small aggregates. We are dealing with a single-stranded RNA virus. These have very high mutational frequencies—it is almost 10^{-5} per base pair copied. The result of this is that in the average TMV preparation, the RNA of a typical particle will contain three changed bases compared to the paradigm RNA sequence. This is derived from data of Dr. Weissman in Zurich for phage ϕ_β. The constancy of the RNA sequence of TMV, of ϕ_β in which he showed it, and of any other single-stranded RNA virus is solely due to back selection holding the sequence at an optimum. Therefore you cannot compare mutational drift and mutational rates with those you are more familar with in eucaryotic systems or even in procaryotes or anything that is DNA coded rather than RNA coded.

Any feature that has been held constant through time must be beneficial, otherwise it would have gone with mutational drift. We have direct evidence that TMV derived from the same stock seven years before but grown separately thereafter, and compared later, showed changes in its RNA. There were only a small number of changes, but we found them. We now have some data that even in the same preparation at certain sites you may have roughly 50/50 of a particular nucleotide. We do not yet know what form it was in for protein coding, but it clearly cannot be deleterious, or it wouldn't be in the mixture.

In our plotting of the data we did not get this precision of length between different channels. The bands we saw after adding A-protein to nucleated rods did not match exactly with the bands we get from the disk preparation. I think it is mostly a hangover from nucleation, but we could argue about that further.

SCHUSTER: I would like to offer an alternate interpretation to this experiment. If the lower part of Fig. A represents the initial distribution of sizes of the partially assembled rods that are used for subsequent elongation, then it can be seen that there are quantized lengths of RNA being protected during elongation. One interpretation is that disks are adding. However if disks add by incorporating less than the full two turns, we would expect to see some free protein coming off into solution. We know from analytical ultracentrifugation studies of reconstitution that this does not happen. Rather, if there is some minimum cooperative length for the RNA as the RNA comes up through the center of the rod, and if it is protected only when a full turn of subunits covers it, then you would expect to see this type of quantized protection. On a partially assembled rod, there will be two tails of RNA coming out of the base of the rod, the short 3′ tail and the long 5′ tail. At the top there would be a loop of RNA which goes down through the center. I would remind you that the binding site for the RNA is composed of half of the upper subunit and half of the lower subunit. Therefore the exposed RNA on the top of the growing rod could be susceptible to digestion. If one starts with lengths that are quantized on the basis of starting the nucleation with disks, and if the binding of the RNA goes in some minimum cooperative length, i.e. one turn, and is then protected as protein subunits add to the growing end, then you would expect to get exactly the results shown in this gel pattern. In other words, these quantized gel patterns may represent a cooperative binding of the RNA loop to the growing rod and not a quantized addition of protein.

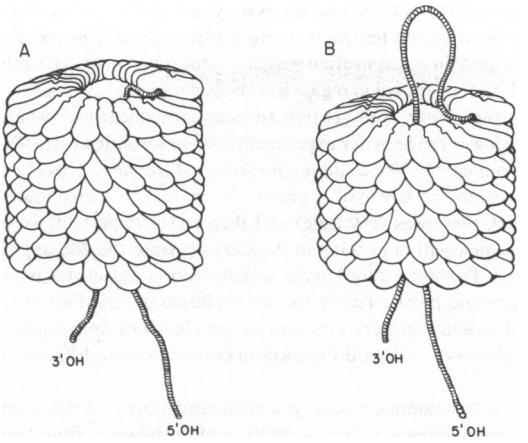

Figure B Schematic representation of partially assembled nucleoprotein rods (PARs) of TMV. These two structures, six and seven turns of the protein helix, are taken as two representatives of "most probable states" during RNA incorporation which could account for the banding pattern observed by Butler and Lomonossoff.

BUTLER: I quite agree with the possibility that RNA lying on the surface might or might not be clipped. I make no comment either way on whether it is. I think this could explain why some of the steps are 50 and some are 100. Don't forget you also have RNA lying on the surface at the bottom end. I am not so sure about the structural interpretation of your hypothesis where subunits start to bind at the top surface covering part of the RNA, e.g. to the opposite side of the ring so the interface is now shifted across. I do not see why the RNA strand should not be around on the newly made underlying surface. I cannot see that there is any mechanism to stop it coming up, and binding, until after the complete turn is coated. It seems to be a very complicated hypothesis. I cannot eliminate it, but it seems not to be the simplest hypothesis to explain this observation.

I think that where we differ on this is in what we regard as the simplest hypothesis. My way is to deliver a package of subunits at once. We know that such a package of a turn or two exists in solution; the simplest hypothesis is to use it. k with this. I obviously can't rule out the other hypothesis, but I don't see what gating mechanism you are going to propose that really says that when you are half-way around in adding the protein subunits, you can't put a further half turn of RNA on. I don't see why the RNA should not bind to the surface of each additional subunit as it is put on, but rather, as you require, when the subunits have reached half-way around the RNA should still be waiting to bind down till the interface has gone a whole turn round. I don't see what easy mechanism you are going to put forward to explain that.

FULLER: I have a speculative mechanism which might explain how a unit length of RNA could be protected even if the protein added as small aggregates instead of as disks. The actual binding site for the RNA is between two jaws, one from the lower level and one from the upper level, and there is a closure on binding of the RNA—is this correct?

BUTLER: That is correct when it is the disk. There are no jaws in the helix.

FULLER: If you propose that a disk adds, then presumably that disk is in the open jawed structure just as when it acts as an initiator. Then you would have to propose that the disk comes down, the RNA lies in the jaws, and then the jaws close.

BUTLER: In our model, the first turn is probably lying on the surface of the nucleoprotein helix and is trapped under the incoming disk. The second turn probably does go into the jaws and is trapped between the two rings as they merge into a helix.

FULLER: Do you have direct evidence that in the growing structure, the RNA lies exposed on top of a disk, bound to only half of its normal binding site? Perhaps you first have to bind protein to create a channel containing both sides of the binding site before the RNA can enter the channel. In the second case you could postulate that a whole disk or a whole turn's worth of subunits must bind before the RNA will lie in the channel and be protected by the conformational change which clamps the jaws shirt and holds the RNA.

Such a model would result in a protected unit length without differentiating between either Butler's or Schuster's models.

BUTLER: Unfortunately there is no direct evidence for exactly how the RNA lies on the surface. I frankly don't see what the experiment would be. I have spent ten years trying to think of one. One piece of evidence we do have is that when we measure a rate against protein concentration we get a saturation curve. This refers back to the original effect that Jonathan King mentioned. In the saturation region it is the packing event which has become rate limiting. As you drop the protein concentration sufficiently, you get into an essentially linear part where it is the collision frequency that is limiting. Working in the lower range in our experiments, when we added extra 4S protein to a disk preparation we did not change the latter significantly. We could not measure a difference. Therefore in these experiments, which are quite old, we were not getting any increment of A-protein. If we used nucleated rodlets which were separated from any unreached protein (of which there was very little) and then added A-protein, we could get growth. The sort of model we put forward is that some event in packing in the subunits from the 20S aggregate and from the disk, may present single subunits adding in. To some extent I agree with the sort of model you are suggesting but the completion of the addition may not be clean and precise before the next collision occurs. Part of the discrepancy in some cases may be due to the fact that if disk addition were very slow to complete, but were much quicker if nudged by the next disk to come along, it could explain some of our differences in observations at different times.

MAKOWSKI: First, in drawing these simple models you must remember that these are not monomers adding to the growing helix, but in fact are small agregates that probably contain subunits from two turns. We know that under some conditions, smaller units can add to TMV. I don't want to speculate on which of these mechanisms might be working here, but it is not as simple as has been drawn.

The other comment I want to make is with regard to your statement that elongation by addition of disks is more favorable than addition of A-protein. I think we have to consider that an even more favorable situation would be a virus particle which had a choice of two or more assembly pathways, so that it could use the disks and the small subunits in the addition. We do not know what is happening in vivo, but it is possible that if there were more than one assembly pathway, it would give the system much more adaptability.

BUTLER: I agree with you. Our data say that going in the other direction, i.e. 5′ to 3′, you certainly use small aggregates. We also quite agree that you can use small aggregates to go in the major direction. To a large extent we have been concerned with establishing what is possible for this protein system. We think studying the most rapid events gives a measure of the capabilities. I wish we knew how to establish what actually happens in vivo. I have a feeling none of us knows how to get into the in vivo system at the moment. We think the 20S addition, i.e., the disk addition, is a possible mechanism in the 5′ direction, and I would say from our measurements of rates all the way along that it may well occur throughout the whole of the 5′ tail.

KALLENBACH: If you start at one-sixth along the length of the RNA and you propagate in both directions, one of the ends will run down and stop and you will be growing exclusively in one direction. Do you see that reflected by increasing sharpness in the gel pattern?

BUTLER: I'm afraid that what I didn't make clear enough is that the rates in the two directions are very different. The rate along the longer tail towards the 5′ end is several times faster than the rate towards the 3′ end (see Table I). There is some discussion between the different groups as to whether the 3′ end goes at all until the 5′ tail is pulled in. In our opinion it does, but there are different interpretations of the data. It is very hard to make precise measurements. Basically, it looks as though completing the two ends occurs at about the same time. I cannot be precise here. There is a big rate difference. The mechanisms are totally different. The nature of the ends are so different that there is really no reason to expect the same mechanism or the same rate even in the two directions.