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Isolation and Characterization of Nucleoprotein Assembly Intermediates of Tobacco Mosaic Virus†

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ABSTRACT: During assembly of tobacco mosaic virus from pure RNA and 20S capsid protein aggregates under conditions where protein is limiting, partially assembled intermediates of specific sizes accumulate; these were isolated on sucrose density gradients. The earliest intermediate found in substantial quantity sedimented at 56 S and was shown, by measurement of its buoyant density and of the length of the RNA segment protected by the capsid protein from nuclease digestion, to consist of RNA that is 13% encapsidated (corresponding to a rod length of about 39 nm); the next intermediate sediments at 78 S and is 18% encapsidated (corresponding to a rod length of about 54 nm). Studies of the distribution of intermediates at various input ratios of protein/RNA indicated that their accumulation results from decreases in the rate constants for protein binding that are local to specific points in the course of encapsidation. After extensive nuclease digestion, the RNA still associated with the first intermediate was shown to include a portion that is unencapsidated. This segment of the RNA may be a region of stable secondary structure that confers the nuclease resistance despite the lack of protection by capsid protein. Such RNA secondary structure, if it exists, would also cause the accumulation of intermediates by imposing an energy barrier to subsequent rod elongation.

Since Fraenkel-Conrat and Williams (1955) first demonstrated the cell-free self-assembly of tobacco mosaic virus (TMV)[1] from its constituent single strand of RNA and subunits of its single coat protein (TMVP), there has been considerable interest in both the structural and mechanistic details of the protein-RNA and protein-protein interactions. Extensive solution studies have revealed details of some aspects of the kinetics (Schuster et al., 1979; Shire et al., 1979; Fukuda et al., 1978; Schuster & Scheele, 1974) and mechanism of the self-association of the coat protein, in the absence of RNA, to form cylindrical disk aggregates and viruslike, helical rods

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[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, TMV coat protein; PAR, partially assembled rod; BMV, brome mosaic virus; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; kb, kilobase(s).

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[for reviews of TMVP self-association, see Butler (1984), Stubbs (1984), Hirth & Richards (1981), Butler & Durham (1977), Lauffer & Stevens (1968), and Caspar (1963)].

Indeed, progress in elucidating the mechanism of assembly of the virion has been hampered by the complexity of the coat protein self-association, which results in several aggregation states and polymorphic forms. When it was shown that one of these forms of TMVP, a 20S aggregate, is an obligatory nucleus for efficient virus reconstitution (Butler & Klug, 1971), some key aspects of the mechanism were clarified. The 20S aggregate was postulated to be the 2-layer, 34-subunit, cylindrical disk aggregate that occurs in crystals (formed in ammonium sulfate solutions) of the kind used for determination of the structure of the coat protein subunit (17 530 daltons) to 2.8-Å resolution (Bloomer et al., 1978). However, results from recent sedimentation equilibrium (Correia et al., 1985) and circular dichroism (Raghavendra et al., 1985) measurements have shown the 20S nucleating species to be a short, helical rod of slightly more than two turns (39 ± 2 subunits).

Physical studies of the kinetics and mechanism of self-assembly of the whole virus have concentrated on overall reaction rates [cf. Butler & Lomonossoff (1980), Butler (1984), Shire et al. (1981), Fukuda et al. (1978), and Fukuda & Okada (1982)] and thermodynamics [cf. Caspar (1963), Durham & Klug (1972), and Steckert & Schuster (1982)]. Other studies have focused on verifying the unique role of the 20S aggregate in the nucleation of RNA, the location of the nucleation region of the RNA, the mechanism of threading of that region of the RNA into the protein binding site, and the relative rates of incorporation of the 4S and 20S TMVP aggregates into the growing rod. [For reviews of previous TMV assembly studies, see Lomonossoff & Wilson (1985), Butler (1984), and Hirth & Richards (1981).] An assembly mechanism has been proposed (Lebeurier et al., 1977; Butler et al., 1977) in which there is a single nucleation site about 1 kb from the 3′ end of the RNA. Subsequent elongation, in this model, proceeds first in the 5′ direction and then in the 3′ direction.

Surprisingly, there have been few studies of the course of TMV assembly between the initial nucleation event and the completion of encapsidation, though the observation of a number of relatively discrete segments of RNA that are protected from nuclease action at various stages (Butler & Lomonossoff, 1978; Fukuda & Okada, 1985) suggests the possible occurrence of partially encapsidated intermediates which accumulate preferentially during assembly. In this report, we describe the preparation, isolation, and characterization of some intermediate species in the assembly reaction. We show that both studying the formation of these intermediates and using them in further reconstitution studies reveal new information about TMV assembly.

Materials and Methods

The standard buffer was potassium phosphate, $I = 0.1$ M, pH 7.0.

Biological Materials. TMV (common strain), TMVP, and TMV RNA were prepared, and their concentrations were measured, as previously described (Raghavendra et al., 1985; Shire et al., 1979b; Steckert, 1982). All protein used in the experiments described below was dialyzed exhaustively, at a protein concentration of 13 mg/mL or greater, against standard buffer (containing 1 mM NaN_3) at 4 °C and then converted to 20S protein (90% 20S protein and 10% 4S protein as shown by analytical ultracentrifugation) by equilibration for at least 48 h at 20 °C (Butler & Durham, 1977). RNA was dialyzed against the standard buffer at 4 °C and stored at −50 °C. Unless otherwise indicated, after being thawed, the TMV RNA was diluted in standard buffer to a concentration not exceeding 2 mg/mL, heated in a water bath at 82 ± 2 °C for 15 min, and then rapidly cooled in an ice bath.

TMV Assembly. Reaction mixtures contained the standard buffer, TMV RNA (0.80 mg/mL), and 20S TMV protein (concentrations given for individual experiments). Less than 1 min before addition of the RNA, the protein was diluted in a volume of standard buffer (at 20 °C) equal to 40–60% of the final volume of the reaction mixture; RNA was then added. The RNA had been diluted with standard buffer in such a way that its addition contributed the remaining volume and brought the solution to 20 °C. After rapid mixing, the preparations were incubated for 15 min at 20 °C.

Sucrose Density Gradient Centrifugation. Exponential (van der Zeijst & Bloemers, 1976) gradients (12 mL) containing the standard buffer were prepared by using 12 mL (per gradient) of 10% (w/v) sucrose in the sealed mixing chamber and 32% sucrose in the reservoir. Fifty-microliter samples, containing the standard buffer, were applied, and the gradients were centrifuged in the Beckman/Spinco SW-41Ti rotor at (unless otherwise indicated) 40 000 rpm and 20 °C; centrifugation times are indicated for individual experiments. The gradients were fractionated at room temperature by using an ISCO fractionator (Model 185); the absorbance at 280 or 260 nm was continuously recorded with an ISCO monitor and flow cell (optical path length, 5 mm; illuminated volume, 50 μL).

CsCl Density Gradient Centrifugation. Samples in standard buffer were mixed with CsCl to give a final CsCl concentration (w/w) of 40% for PAR's not treated with nuclease or 32% for nuclease-treated PAR's, TMV, and TMV protein. The preparations (12 mL) were centrifuged for 48–60 h at 40 000 rpm and 20 °C in the Beckman/Spinco 50Ti rotor and fractionated as described for sucrose gradients. Fractions (0.3 mL) were collected and their densities determined by refractometry.

Analytical ultracentrifugation of assembly reaction mixtures was performed as previously described for protein aggregates (Shire et al., 1979a; Correia et al., 1985) and fully assembled TMV (Shire et al., 1979b, 1981).

Nuclease Digestion of PAR's. After incubation of assembly reaction mixtures, $CaCl_2$ (1 mM) and staphylococcal nuclease (1000 units/mL) were added, and the preparation was again incubated for 80 min at 20 °C. EGTA (sodium salt, pH 7; 5 mM) was then added to stop the reaction.

Extraction of Protected RNA from Nuclease-Treated PAR's. After nuclease treatment of the assembly reaction mixture (3.7 mL), the PAR's were pelleted by centrifugation at 75000*g* for 3 h at 4 °C and redissolved in 0.7 mL of 0.02 M sodium phosphate (pH 7.0)–1 mM EGTA. The mixture was extracted with an equal volume of water-saturated phenol and centrifuged; the aqueous phase was again extracted with phenol and centrifuged. RNA was precipitated from the aqueous phase by addition of 0.05 volume of 2 M sodium acetate and 2 volumes of ethanol, followed by storage at 4 °C overnight. The RNA was collected by centrifugation, twice reprecipitated with ethanol, and dissolved in the standard buffer.

Materials. Staphylococcal nuclease (EC 3.1.31.1) was obtained from Pharmacia, BMV RNA from Promega Biotec, ultrapure nucleic acid grade phenol from Bethesda Research Laboratories, and ultrapure nuclease-free sucrose from Schwarz/Mann. Cesium chloride was optical grade from U.S. Biochemicals. All other chemicals were of reagent grade or better; all water was doubly distilled (the second distillation in glass).

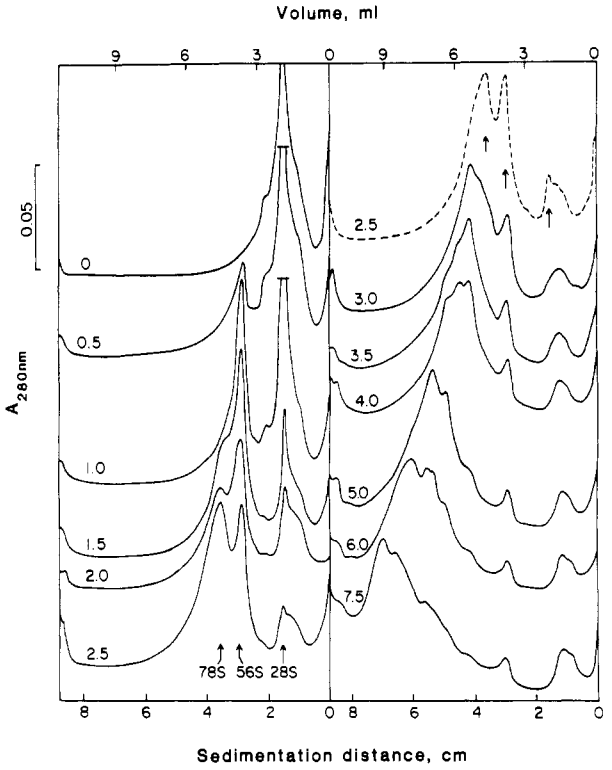

FIGURE 1: Analysis of assembly reaction mixtures by sucrose density gradient sedimentation. The numbers associated with each curve indicate the ratio (w/w) of protein/RNA in the mixture. Centrifugation was for 80 min. The sedimentation constants indicated by the arrows were obtained by analytical ultracentrifugation (see the text); the 28S species is free TMV RNA.

RESULTS

Sequence of Formation of Intermediates in TMV Assembly. Figure 1 shows the results of sedimentation, on sucrose gradients, of reaction mixtures containing TMV RNA and various, limiting amounts of TMV protein in the form of 20S aggregates. After the 15-min incubation, no free 20S or 4S protein could be detected in the mixtures by analytical ultracentrifugation; hence, the binding of protein to RNA was complete. Even at the lowest input ratio of protein/RNA (0.5 g/g; the ratio in fully assembled TMV is 20), the smallest partially assembled rod (PAR) found in substantial quantity sedimented at 56 S (analytical ultracentrifugation of appropriate reaction mixtures gave values for $s_{20,w}$ of 55.8 ± 0.3 S and 57.4 ± 0.4 S in two independent determinations). As the ratio of protein/RNA was increased to about 1.5 g/g, more of this intermediate accumulated with little formation of larger PAR's. Under these conditions, substantial quantities of free (28S) RNA remained in the mixtures. As the relative amount of protein was increased above 1.5 g/g of RNA, a second PAR was found, sedimenting at 78 S ($s_{20,w}$ = 78.5 ± 0.4 S, from analytical ultracentrifugation). Further increase of the protein/RNA ratio led to the formation of a sequence of more or less discrete intermediates of increasing size; eventually, all of the 28S RNA was recruited into PAR's, leaving only some oligonucleotide fragments sedimenting at less than 28 S. At input ratios approaching 20 g of protein/g of RNA, particles sedimenting at the rate typical of TMV (180 S) were formed (results not shown; TMV would sediment to the bottom of the tube under the conditions of sedimentation used in Figure 1). With excess protein, virtually all of the PAR's could be "chased" into material sedimenting at 180 S.

Size of the Early Intermediates in Assembly. The buoyant density of a PAR should reflect the extent to which the RNA molecule is coated with protein, since the partial specific volumes of the two molecular species are different. Under the conditions we have used, TMV bands in CsCl gradients at a density of 1.314 g/mL (three independent determinations differed by less than 0.001) and TMVP, whether introduced as the 20S aggregate or as longer helices (formed at pH 5; Raghavendra et al., 1985), bands at a density of 1.290 g/mL (with similar reproducibility). (Apparently, the volume change of TMVP which occurs upon self-association is masked at high CsCl concentrations where only the highly associated form is observed. Therefore, in what follows we are considering an additivity formalism that pertains to helical TMVP and RNA even though the protein is added as 20S material.) Since the volumes occupied by protein and RNA appear to be additive when they form ribonucleoproteins (Spirin et al., 1965; Schon & Mundry, 1984), the buoyant densities of particles containing various proportions of protein and RNA can be related by the equations:

$$1/\rho_{\text{particle}} = X_{\text{RNA}}/\rho_{\text{RNA}} + X_{\text{protein}}/\rho_{\text{protein}} \quad (1)$$

$$X_{\text{RNA}} + X_{\text{protein}} = 1 \quad (2)$$

where ρ is the buoyant density of the subscripted species and X is the weight fraction of the subscripted component of the particle. The measured values for TMV (5.2% RNA) and TMVP, when substituted in eq 1 and 2, yield an extrapolated value of 2.0 g/mL for TMV RNA. In turn, this value and that for TMV protein can be used to calculate the composition of a PAR, given its buoyant density.

The 56S assembly intermediate banded at ρ = 1.441 ± 0.003 g/mL in CsCl (mean ± SD of five independent determinations; cf. Figure 2a). This corresponds to a composition of 71% protein–29% RNA, or 2.4 g of protein/g of RNA, or 17 ± 0.4 turns of capsid protein helix/TMV RNA molecule. (The complete virus contains 130 turns of the capsid protein helix, each turn consisting of $16^1/_3$ protein subunits of 17 530 daltons, plus the RNA molecule of 2.0×10^6 daltons.) The RNA in the 56S PAR is therefore about 13% encapsidated. The second intermediate found, sedimenting at 78S, banded at ρ = 1.408 ± 0.001 g/mL in CsCl (five determinations; cf Figure 2b) and, by a similar argument, contains 3.3 g of protein/g of RNA or 23 turns of capsid helix/RNA molecule; the RNA is about 18% encapsidated.

Continuous coating of a region of the RNA with TMV protein should protect that region from attack by nuclease (Zimmern, 1977), whereas the exposed portions of the RNA should be degraded, leaving a particle consisting of a fully coated fragment of RNA and containing the same ratio of protein/RNA as the virion. The 56S PAR—which has a density of 1.441 g/mL in CsCl—was incubated with staphylococcal nuclease under conditions where, we have shown, naked TMV RNA was degraded entirely to fragments soluble in 5% trichloroacetic acid. The resulting "trimmed" particle banded in CsCl at a density of 1.320 g/mL (Figure 3a; two additional, independent determinations gave values of 1.319 and 1.321 g/mL). This value is to be compared with the density we find for TMV (1.314 g/mL; Figure 3b). If the particle contains 17 turns of capsid helix, then (by application of eq 1 and 2) about 95% of the unencapsidated RNA was removed by the nuclease. (The fact that the densities of the virus and the trimmed PAR are not precisely the same is discussed below.) When nuclease treated, the 56S PAR then sedimented at 68 S (Figure 4b; a replicate determination gave a value of 70 S). This represents an *increase* in the sedimentation rate upon nuclease treatment; probably the unencapsidated RNA contributes more to the frictional constant

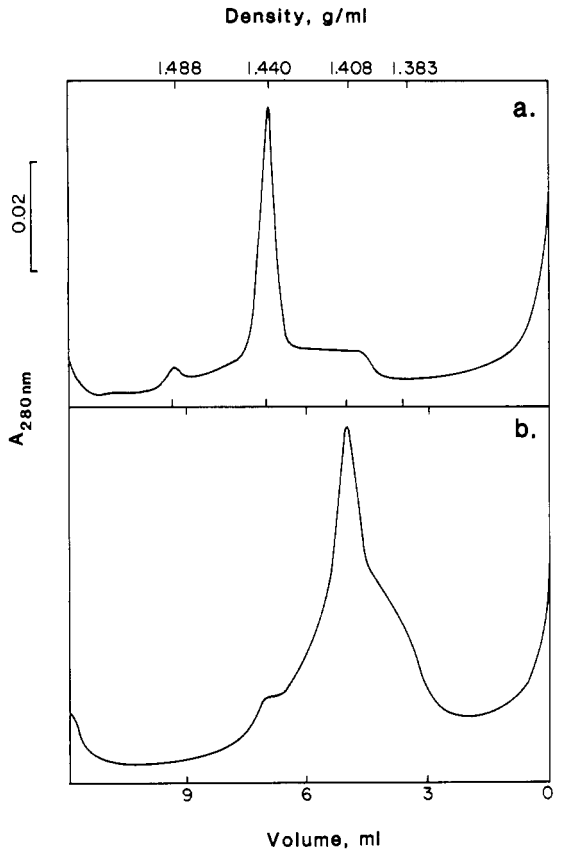

FIGURE 2: Buoyant densities of PAR's in CsCl. Cuts containing the 56S PAR (a), from three gradients similar to that labeled 1.0 in Figure 1, or the 78S PAR (b), from three gradients similar to that labeled 2.5 in Figure 1, were pooled, dialyzed overnight against standard buffer at 4 °C, and analyzed by CsCl density gradient centrifugation.

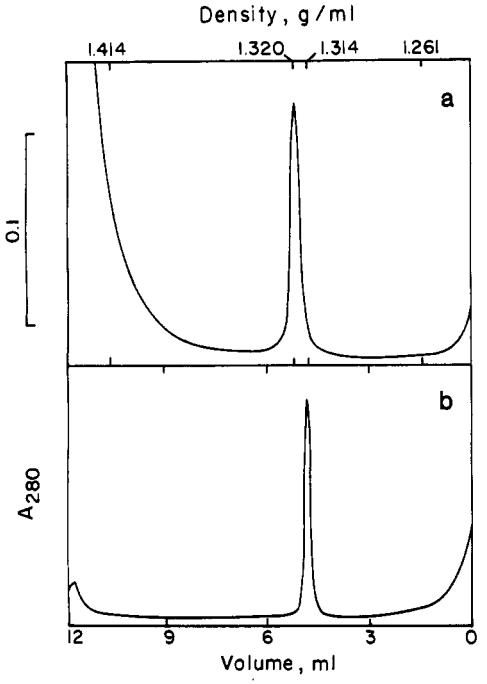

FIGURE 3: Buoyant densities of the 56S PAR after nuclease digestion (a) and of TMV (b). The CsCl gradients contained (a) an assembly reaction mixture (0.2 mL) that had been constituted with 1.0 g of protein/g of RNA (cf. Figure 1, curve 1.0), incubated, and treated with nuclease; (b) TMV (16 μg).

of the untreated PAR than it does to the mass and density. Since the RNA in the trimmed PAR contributes only about 7% of its mass (based on the buoyant density determination),

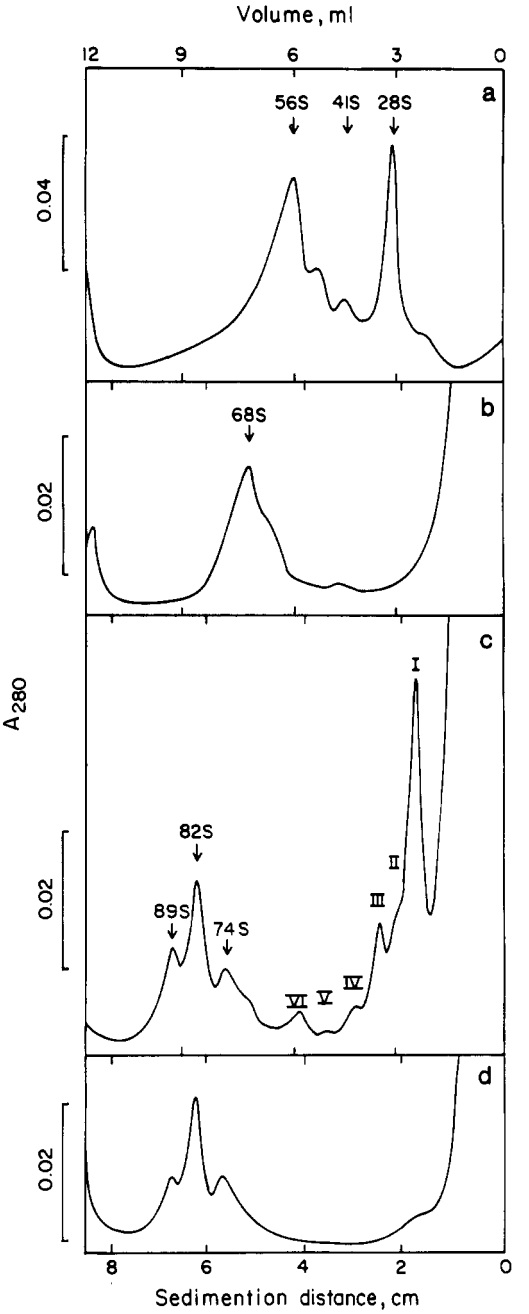

FIGURE 4: Sucrose density gradient centrifugation of PAR's. Centrifugation time was 120 min. (a) An assembly reaction mixture containing 1.0 g of protein/g of RNA (as in Figure 1, curve 1.0). (b) As in (a) but nuclease treated after assembly. (c) as in (b) but 20S protein (1.2 mg/mL) was added after nuclease treatment, and the preparation was then incubated 15 min at 20 °C. (d) An assembly reaction mixture (minus TMV RNA) containing 3.3 μg of the RNA extracted from the nuclease-treated PAR's of a preparation identical with that described for (b), plus 20S TMVP (1.8 mg/mL). The sedimentation constants indicated by the arrows in frames b and c were obtained by calibration of the gradients using the positions of the 28S, 56S, and 78S species that had been characterized by analytical ultracentrifugation (the preparation containing the 78S intermediate is not shown but was similar to that represented in Figure 1, curve 2.5). When the distances sedimented by these species in the gradients were plotted against $s_{20,w}$, a straight line was obtained.

the relation between length and sedimentation constant for nuclease-trimmed PAR's should be similar to that for protein helices. Helices of three turns sediment at 24 S (Schuster et al., 1980; Correia et al., 1985); recent results from sedimentation and electron microscopy in this laboratory (Raghavendra et al., 1986) indicate that six-layer TMVP rods sediment at 36–38 S. Extrapolation of these values on the basis of the

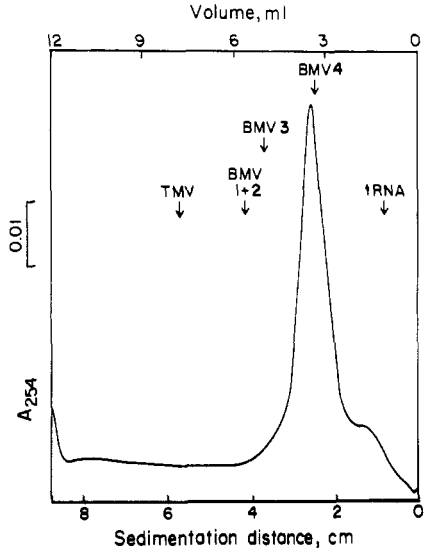

FIGURE 5: Sedimentation of the RNA fragment protected from nuclease in the 56S assembly intermediate. An assembly reaction mixture containing 1.0 g of protein/g of RNA (cf. Figure 1, curve 1.0) was treated with nuclease (cf. Figure 4b), and RNA was extracted from the PAR's. Ten micrograms of the RNA was centrifuged on a sucrose density gradient (34 000 rpm at 4 °C for 20 h). The arrows show the positions of standards (centrifuged in replicate gradients in the remaining positions of the rotor): TMV RNA, 6300 nucleotides; BMV RNA 1 and 2, 3400 and 3100 nucleotides (these did not separate); BMV RNA 2, 2350 nucleotides; BMV RNA 4, 877 nucleotides; tRNA, approximately 75 nucleotides.

known geometry of the aggregates yields values consistent with the length of 17 turns estimated from the buoyant density of the 78–80S trimmed PAR.

The size of the region of RNA that is protected from nuclease digestion in a PAR is another indication of the extent of encapsidation [cf. Butler & Lomonossoff (1980)]. After treatment of the 56S PAR with nuclease, the RNA from the resulting trimmed particle was extracted with phenol and analyzed by sedimentation in sucrose gradients; its size was estimated by comparison with standards (Figure 5). The protected fragment sedimented at nearly the same rate as brome mosaic virus RNA 4, which contains 877 nucleotides (Lane & Kaesberg, 1961). One turn of TMV capsid helix covers 49 nucleotides ($16^1/_3$ subunits of protein × 3 nucleotides per subunit; Caspar, 1963); this result, too, is consistent with our estimate of about 17 turns of capsid helix (which would cover about 840 nucleotides) in the 56S PAR.

Structure of the 56S Intermediate. The buoyant densities (1.19–1.21 g/mL) measured for the nuclease-treated PAR are higher than that of TMV (Figure 3), indicating that some uncoated RNA remains in the PAR after nuclease treatment. Substitution of the relevant values in eq 1 and 2 leads to the conclusion that the uncoated region amounts to 20–30% of the RNA present. An increase in the time of incubation with nuclease or the amount of enzyme did not remove this uncoated, but resistant, fraction. However, this region of RNA could be encapsidated by addition of excess protein to the trimmed PAR. Recall that the 56S PAR (Figure 4a) sedimented at 68–70 S after nuclease treatment (Figure 4b); the results of addition of excess protein to this trimmed PAR are shown in Figure 4c. In this experiment, in order to avoid the risks of PAR aggregation and possible structural alterations attendant upon sedimenting the PAR to form a pellet and then redissolving it, the free RNA remaining after the assembly reaction and the products of nuclease digestion were not removed after each step. The peaks numbered I–VI in Figure 4a are small protein aggregates stabilized by RNA fragments produced during the digestion. All of them were found in approximately the same amounts and proportions in control preparations (not shown) in which pure, protein-free TMV RNA was first digested with nuclease and then mixed with protein. The faster sedimenting peaks (74–89 S) shown in Figure 4b were absent from these control preparations and therefore represent the products formed from the trimmed PAR and the added protein. This effect is further illustrated by the fact that when the protected RNA fragment extracted from the trimmed PAR was mixed with protein, the 74–89S complexes were formed, but peaks I–VI were absent (Figure 4d). In this case, the PAR had been pelleted by ultracentrifugation before extraction in order to remove the digestion products.

Addition of excess protein increased the modal sedimentation constant of the trimmed PAR from about 68–70 S to about 82 S, an increase quite consistent with the addition of 20–30% more protein helix, as would be predicted from our estimate that this portion of the RNA in the trimmed PAR was uncoated. Increasing the amount of protein added to the preparation represented in Figure 4c did not change the sedimentation behavior of the 74–89S components (results not shown); hence, coating of the RNA most likely was complete. In addition to the increase in modal—and average—sedimentation rate, the complete coating of the RNA caused the broad, 68S peak of the trimmed PAR (Figure 4b) to be resolved into three components, a major one at 82 S and minor ones at 74 and 89 S, each of which is represented by a narrower distribution of sizes (Figure 4c). The explanation for this increased resolution is not clear.

Effect of Heat Denaturation of RNA. In all the aforementioned experiments, the RNA used, after extraction from the virus with phenol, had been first heated at 80 °C and then rapidly chilled. The effects on the sedimentation behavior of the RNA (Figure 6) are twofold. Components sedimenting faster than 28 S, and presumably representing aggregates of RNA [cf. Jaspars (1985)], were removed by the heating, and there was an increase in the amount of material sedimenting more slowly than 28 S. This smaller material appears as a shoulder to the 28S peak in Figure 6. When RNA that had not been heat denatured was used in experiments similar to that shown in Figure 1, the results (not shown) differed from those obtained with heated RNA (and shown in the figure) in two ways. The sedimentation pattern of assembly intermediates was somewhat more complex, presumably because of partial assembly on aggregated RNA, though the early intermediates described above were still the major ones found. In addition, there was a fraction (20–30%) of the RNA sedimenting at 28 S that could never be recruited into PAR's even when excess protein was added. This second effect could have resulted if small fragments of RNA had formed duplexes with the intact polynucleotide strands in positions where they blocked the nucleation site for assembly. This interpretation is consistent with the increase in small RNA species when the TMV RNA was heated (Figure 6).

Regardless of the exact cause of these effects, they point to the need to consider the consequences of the methods used in preparation of viral RNA for studies of self-assembly. The commonly used RNA preparative method, phenol extraction followed by ethanol precipitation and solution at high RNA concentration, may promote intermolecular interactions that persist and influence the extent or rate of reconstitution.

Discussion

The experimental conditions under which the 56S and 78S assembly intermediates accumulate make it evident that their

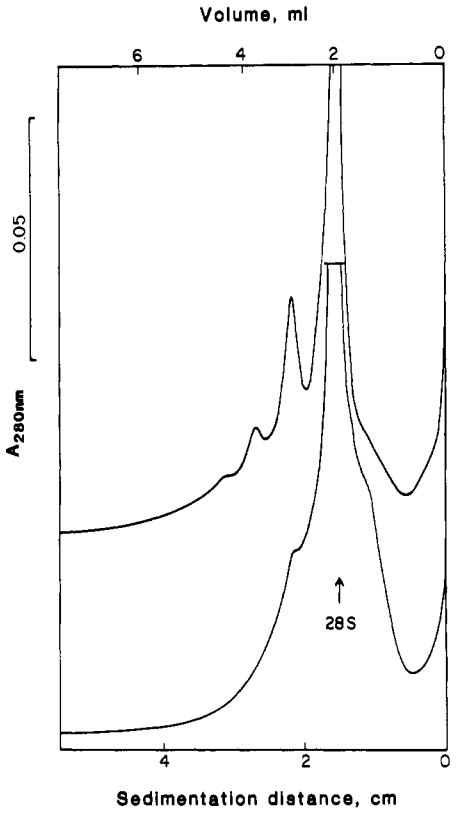

FIGURE 6: Sucrose gradient centrifugation of TMV RNA. Centrifugation time was 80 min (the bottom portion of the gradient contained no bands and is not shown); 40 μg of RNA was applied. Top, RNA not heated; bottom, RNA heated and rapidly cooled (as described under Materials and Methods).

occurrence results from discontinuities in the assembly process and not simply from the limitation of the amount of protein available. The results must be interpreted in light of the facts that (i) there is only one demonstrated nucleation site in the RNA (Butler et al., 1977), and, therefore, increments in the size of PAR's represent elongation of a single region of nascent nucleocapsid, and (ii) the accumulation of the 56S and 78S species could not have resulted from the complete encapsidation of RNA fragments, since these PAR's were found in large quantities at low, but not at high, input ratios of protein/RNA (Figure 1). In the preparation (Figure 1) containing 1.0 g of protein/g of RNA, the amount of protein added is 5% of the amount that would be required to encapsidate the full length of all the RNA molecules. If nucleation of RNA molecules proceeds with a rate much greater than that for elongation of the nascent nucleocapsids thus initiated, one would expect that virtually all of the RNA molecules in this mixture would have been nucleated, and since the protein reacts quantitatively, the RNA molecules would be, on the average, encapsidated over 5% of their length. In fact, the protein nucleated only about 30% of the RNA molecules, and the resulting nascent nucleocapsids were elongated until virtually all of the PAR's had been converted to the 56S species (in which, as demonstrated above, there are about 17 turns of capsid protein helix and 13% of the length of the RNA is encapsidated). There was little material sedimenting between this PAR and the 28S, free RNA band (Figure 1, curve 1.0). Apparently, elongation of the coated region of nucleated RNA molecules is favored over the nucleation of new ones—at least until the nascent nucleocapsid reaches a length of about 17 turns of helix. An alternative hypothesis—that all PAR's containing less than 17 turns of capsid helix are unstable when centrifuged into the density gradients—seems less likely in view of the apparent stability of some even smaller complexes formed when protein is mixed with oligonucleotides derived by nuclease digestion of the RNA (see Figure 4 and the associated text). As the protein input was increased from 0.5 to 1.5 g/g of RNA (Figure 1), more of the 56S, 17-turn intermediate was formed, but there was very little formation of larger PAR's. It seems, therefore, that once the nascent nucleocapsid has reached a length of about 17 turns of its helix, the probability of further elongation decreases markedly. As the input ratio of protein/RNA was further increased to 2.5 g/g (Figure 1), a second intermediate (78 S, containing 23 turns of capsid helix, i.e., with the RNA 18% encapsidated) appeared; its accumulation suggests that, once elongation of the capsid helix proceeds beyond 17 turns, protein binding is again relatively rapid until another local decrease in the rate is encountered at a capsid length of about 23 turns.

TMV assembly, then, appears to occur in "jumps". Our results obtained by direct examination of intermediates in density gradients are entirely consistent with those obtained indirectly (Butler & Lomonossoff, 1978; Fukuda & Okada, 1985) by examination of the RNA segments protected from nuclease digestion in assembly reactions limited by incubation time rather than by availability of protein. At any given time, the aforementioned investigators found a sequence of more or less discrete RNA fragments, reflecting the accumulation of PAR's of particular lengths.

Our studies of the structure and properties of the 56S PAR provide a potential explanation for the kinetic inhomogeneities in the early stages of TMV nucleocapsid elongation. After exhaustive nuclease digestion of the 56S PAR, the 68S, trimmed PAR which results still contains some RNA—20–30% of the amount present—that is unencapsidated, as evidenced by the facts that the buoyant density of the PAR is significantly higher than that of TMV (Figure 3) and that additional protein can be incorporated (Figure 4). The nuclease resistance of this unencapsidated region of the RNA results either from a physical association formed by the polynucleotide chain or from an inability of the enzyme to reach some portions of the RNA, or from both of these possible causes. Although we have not yet determined the sites of nuclease cleavage, we think it likely that the protection of the nuclease-resistant, unencapsidated region of RNA is caused by the formation, at one or both ends of the PAR, of RNA secondary structure resulting from internal RNA base pairing. If the region(s) of base pairing was (were) sufficiently long, this alone could account for the protection. The fact that a large number (180–270) of unencapsidated nucleotides are protected raises the possibility that another factor is involved. According to the assembly mechanism proposed by Lebeurier et al. (1977) and by Butler et al. (1977), the RNA from the encapsidated region to the 5′ end loops back through the central 4-nm diameter hole as it is incorporated into the growing rod. If nuclease digestion of the 5′ portion occurred at the growing end of the PAR, there would be no protected region of RNA since the 5′ strand would be free to diffuse out of the central hole. However, if the RNA were protected at the growing end by a region of extensive RNA base pairing and if the portion of RNA inside the hole were inaccessible to the nuclease, a section of RNA about the length of the PAR would remain covalently bound to the PAR. Only the two distal portions of the RNA, the 5′ tail that extends outside of the hole and the 3′ tail, would be digested. Other mechanisms for protection of the RNA, such as interaction with the surface of the capsid helix, are also conceivable. The presence of regions of RNA duplex could also explain the observed discontinuities in as-

sembly (Butler & Lomonossoff, 1980). Binding of protein aggregates would take place easily in random-coil regions of the RNA, but if a region with a high proportion of paired bases were encountered, the effective activation energy of protein binding would be increased. Therefore, a larger number of protein subunits would have to bind in order to provide enough binding energy to melt the RNA secondary structure and result in formation of a stable protein–RNA complex. This energy barrier would produce a local decrease in the rate of capsid elongation. Once the local secondary structure were degraded and elongation continued through the base-paired region, the assembly rate would again increase. The unencapsidated RNA found in the nuclease-treated PAR may result from such base-paired regions that are encountered during encapsidation and are responsible for accumulation of the assembly intermediate. It appears that the discontinuities at specific points in TMV assembly, "punctuated assembly", may result from RNA secondary structure and therefore be a function of the base sequence of the RNA. The possible analogy between the function of TMVP as an "RNA-melting protein" and that of DNA-melting proteins (von Hippel et al., 1984) is worth noting.

The foregoing interpretation is supported by the results of Fukuda and Okada (1985), who observed, in cross-assembly experiments using RNA from one strain of TMV and protein from another, that the modal sizes of RNA fragments protected from nuclease digestion after partial assembly—and therefore, the nascent nucleocapsid lengths of the PAR's that accumulated—depended upon the source of the RNA and not upon the source of the protein. An alternative hypothesis for discontinuous TMV assembly was advanced by Butler and Lomonossoff (1980), who observed that the nuclease-resistant RNA fragments contained integral multiples of about 100 nucleotides (the approximate number that would be protected by one protein "disk", that is, 2 turns of helix) and concluded that the RNA banding pattern resulted from the fact that protein is incorporated in the form of 20S "disks". This, alone, cannot explain the results of the present study: the difference in nascent nucleocapsid lengths between successive major intermediates (Figure 1) is greater than two turns. It is also significant that intermediates of certain sizes accumulate during stripping of the protein from TMV by treatment with mild alkali or low concentrations of urea which results in polar stripping of subunits, predominantly from the 5′ terminus (Perham & Wilson, 1978; Pelcher & Halasa, 1979a,b; Hogue & Asselin, 1984a,b). It has been proposed that these "pauses" in the disassembly process result from different strengths of protein–RNA binding along the RNA (Perham & Wilson, 1978) or from reversibility of subunit binding (Pelcher et al., 1980). However, the relationship of these disassembly intermediates in denaturing solvents to the assembly intermediates in reconstitution buffer reported here is not clear since the latter are 39 (17 helical turns) and 53 (23 helical turns) nm in length and the closest disassembly intermediates are in the ranges of 44–49 and 57–69 nm in length. Since previous direct binding studies have demonstrated a nucleotide sequence dependence of the binding of the capsid protein (Steckert & Schuster, 1982), it is expected that such effects would be manifested in the sequential kinetic events of assembly and disassembly. However, the results reported here cannot be explained solely by nucleotide sequence dependent differences in protein binding since the 56S intermediate contains a nuclease-resistant but unencapsidated region of RNA. It is unlikely that this unencapsidated region results from subunit dissociation of a protected region after RNA cleavage since the protected RNA region can be encapsidated (see Figure 4).

In assembly reactions carried out in sodium pyrophosphate buffer at pH 7.25 and 0.675 M ionic strength, containing 22 g of TMVP/g of RNA (an excess of protein), Stussi et al. (1972) observed the accumulation of large amounts of an assembly intermediate in which the nascent nucleocapsid was 70 nm long (whereas the fully assembled virus measures 300 nm). The 70-nm species could be further encapsidated after transfer to lower ionic strength. As Stussi et al. suggested (though without direct evidence), its accumulation even in the presence of excess protein may have resulted from the stabilization of a region of RNA secondary structure by the high ionic strength of their assembly reaction mixtures.

Zimmern (1977; Zimmern & Butler, 1977) postulated the existence of a nucleation complex consisting of six turns of capsid helix bound to RNA, on the basis of length measurements and sequence studies of the RNA segments protected from nuclease in protein-limited assembly reaction mixtures. However, direct observation of the nucleation complex was not reported. Even at the lowest protein inputs we used, which were comparable to theirs, no substantial quantities of PAR's this small were observed. The reason for this difference in results is unknown. However, the methods used in the present study may be sufficiently different from those used by Zimmern and Butler to preclude comparisons.

These combined preparative and analytical sedimentation methods have led to evidence that suggests the role of RNA secondary structure in governing intermediate phases in TMV assembly. The physical and chemical characterization of other assembly intermediates will further facilitate investigation of the structural determinants of the rate of virus assembly.

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Monoclonal Antibodies to Epitopes in both C-Terminal and N-Terminal Domains of *Escherichia coli* Ribosomal Protein L7/L12 Inhibit Elongation Factor Binding but Not Peptidyl Transferase Activity†

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ABSTRACT: Two monoclonal antibodies against different epitopes in *Escherichia coli* ribosomal protein L7/L12, one within residues 74–120 and the other within residues 1–73, shown before to inhibit the binding of EF-G, have been tested for their effects on the binding to *E. coli* ribosomes of EF-Tu–aminoacyl-tRNA–GTP ternary complex and on peptidyl transferase activity. Both antibodies inhibit the binding of ternary complex and EF-Tu-dependent GTPase but have no inhibitory effect on peptidyl transferase activity. The inhibition of binding of both elongation factors is indicative of overlapping binding sites for EF-G and EF-Tu. The inhibition by both antibodies implies the contribution of both domains of L7/L12 to this binding site. This implies the location of one or more of the C-terminal domains of L7/L12 on the body of the 50S subunit. The absence of any inhibition of peptidyl transferase activity shows distinct separation of this site from the factor binding site.

The elongation cycle of protein synthesis involves the successive binding to the 70S ribosome–mRNA complex of the two elongation factors: EF-Tu, in a ternary complex with aminoacyl-tRNA and GTP, brings the cognate aminoacyl-tRNA to the ribosomal A site; EF-G is involved in the translocation of the peptidyl tRNA to the ribosomal P site. The action of both factors is accompanied by GTP hydrolysis. The interaction between each of the two elongation factors and the ribosome has been studied extensively [reviewed in Miller and Weisbach (1977), Brot (1977), Liljas (1982), and Liljas et al. (1986)]. An important question has been whether the ribosomal binding sites for the two factors are separate or overlapping, possibly identical. Major conclusions support the existence of overlapping sites: EF-Tu and EF-G do not bind to the ribosome concurrently; they bind poorly to ribo-

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