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Complexes of Alfalfa Mosaic Virus RNA 4 with One and Three Coat Protein Dimers†

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ABSTRACT: RNA 4, the subgenomic coat protein messenger of alfalfa mosaic virus, was loaded with small amounts of coat protein in a reaction in which complete virions were the protein donor. In such a reaction the protein subunits attach to the high-affinity binding sites near the 3′ end of RNA 4 [Houwing, C. J., & Jaspars, E. M. J. (1978) *Biochemistry 17*, 2927–2933]. At a ratio of up to 13 coat protein subunits to 1 mol of RNA 4, complexes with one and three protein dimers, designated complex I and complex III, respectively, were formed. These complexes were isolated by preparative electrophoresis in 4% polyacrylamide gel. At a large excess of the protein donor (280 protein subunits/mol of complex), both complexes I and III were converted into uniform complexes with 10 protein dimers. There were no indications for stable intermediate complexes. A model is suggested for the structure of the complexes which is based on the model proposed for the protein coat of alfalfa mosaic virus [Mellema, J. E., & Van Den Berg, H. J. N. (1974) *J. Supramol. Struct. 2*, 17–31]. The complexes possibly serve as successive stages in virion assembly. More intriguingly, the complexes could be of regulatory significance. Since the four RNA species of alfalfa mosaic virus have an extensive 3′-terminal homology, and since 3′-terminal interaction with coat protein subunits is thought to be a process leading to recognition of the viral genome by the viral replicase and thus to infectivity, complexes analogous to complexes I and III could represent the infectious forms of the genome RNAs.

In no other class of simple RNA viruses is a dual role of the coat protein more prominent than in the class of alfalfa mosaic virus (AMV)[1] and related viruses. With these viruses no infection is possible in the absence of the coat protein or of its messenger, the small subgenomic RNA 4 [see Van Vloten-Doting & Jaspars (1977) for a review], despite the positive strandedness of their tripartite genomes (consisting of RNAs 1, 2, and 3). There is evidence that the coat protein has to attach to all three parts of the genome in order to induce infection (Smit & Jaspars, 1980). All four species of RNA possess high-affinity binding sites for the coat protein, as is shown by their ability to withdraw protein subunits from intact virions (Verhagen et al., 1976). The high-affinity binding sites are likely to be located close to the 3′ ends of the molecules, since they have been found in a 3′-terminal fragment of 88 nucleotides of RNA 4 (Houwing & Jaspars, 1978) that has been sequenced (Koper-Zwarthoff & Bol, 1979) and since virtually the same sequence is present at the 3′ termini of the genome RNAs (Pinck & Pinck, 1979; Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980). Binding of a few coat protein molecules is an endothermic reaction (Srinivasan et al., 1977) and induces a conformational change, at least in RNA 4 (Srinivasan & Jaspars, 1978, and results to be published). It has been postulated that a conformational change at the 3′ ends of the genome RNAs induced by the coat protein would be necessary for RNA replicase recognition, which would explain the essential role of the coat protein at the start of the infection (Houwing & Jaspars, 1978).

Our primary interest now is to see what is the exact nature of the complexes that are formed when small amounts of coat protein attach to AMV-RNAs. The reaction in which coat protein is withdrawn by viral RNA from AMV virions is highly specific in that only the RNAs of AMV and related viruses are active (Van Vloten-Doting & Jaspars, 1972; Van Boxsel, 1976). The reaction has been applied to test fragments of RNA 4 with regard to protein binding (Houwing & Jaspars, 1978). It is used here to obtain RNA 4/protein complexes with the protein exclusively on high-affinity sites. We succeeded in isolating, by means of gel electrophoresis, complexes

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[1] Abbreviations used: AMV, alfalfa mosaic virus; TYMV, turnip yellow mosaic virus; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

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with one and three coat protein dimers. The formation of these complexes under different conditions and their protein-accepting and -donating properties have been investigated.

Materials and Methods

Virus, RNA 4, and TYMV-RNA were prepared, measured, and stored essentially as reported previously [Houwing & Jaspars (1978), and references therein]. Labeling with [^{35}S]sulfate and [^{3}H]uridine was performed as described by Verhagen et al. (1976). Most of the material sedimenting slower than the bottom component was removed from labeled and unlabeled virus preparations by sucrose velocity gradient centrifugation in a swinging bucket rotor. The molecular weight of RNA 4 anion used in a previous article (257×10^3; Houwing & Jaspars, 1978) has to be changed to 283×10^3, as sequence studies (Koper-Zwarthoff et al., 1979) have shown RNA 4 to consist of 882 nucleotide residues instead of 800 (Heijtink et al., 1977). Extinction coefficients and molecular weights of RNA 4/protein complexes were calculated from their composition. Radioactivities were converted to molar quantities with the aid of virus and RNA preparations of precisely known specific activities in the same solvent as the unknown. Under the conditions of the experiments, RNA and protein did not cause a measurable quenching of radioactivity.

Pretreatment of ^{35}S-Labeled Virus with TYMV-RNA. Virus to be used for uncoating experiments was freed from radioactive material that attached aspecifically to any RNA species as follows. An amount of 4.2 mg of unfractionated virus in 0.3 mL of 10^{-2} M sodium phosphate, 10^{-3} M EDTA, and 10^{-3} M NaN_3, pH 7.0, was mixed with 1.2 mg of TYMV-RNA in 0.2 mL of 10^{-2} M sodium acetate buffer, pH 6.0, which had been preheated for 5 min at 60 °C, and dialyzed for 23 h at 0 °C against 10^{-2} M sodium phosphate and 10^{-3} M EDTA, pH 7.0. A preparation enriched in bottom component and freed from TYMV-RNA was then prepared by gradient centrifugation.

Preparation of a Complex of TYMV-RNA and AMV Protein. Fragments of TYMV-RNA were prepared by heating a preparation of 2 mg in 0.32 mL of 10^{-2} M sodium acetate buffer, pH 6.0, for 5 min at 60 °C and centrifuging it subsequently for 19 h at 25000 rpm and 20 °C in a Beckman SW 27 rotor in one single 5–20% (w/v) sucrose gradient containing 10^{-2} M sodium phosphate, 10^{-3} M EDTA, and 0.05 M NaCl, pH 7.0. Material sedimenting as fast as RNA 4 in a sister tube was collected, precipitated with 2 volumes of ethanol, and dissolved in electrophoresis buffer to a concentration of 16 μg/mL. To prepare the complex with AMV protein, a sample of 28.4 μg of the RNA was heated for 5 min at 60 °C and then incubated in 1.78 mL of buffer with 10.7 μg of ^{35}S-labeled protein for 17 h at 0 °C.

RNA 1 was purified by sucrose velocity gradient centrifugation in a zonal rotor essentially as described by Heijtink et al. (1977).

Coat protein was prepared from gradient purified nucleoproteins according to Kraal et al. (1972), except that the final preparation in distilled water was not lyophilized but stored frozen at −20 °C.

Miscellaneous Materials. All chemicals were reagent grade. Anhydrous glycerol was not used in these studies. The concentrations of glycerol (v/v) refer to glycerol as supplied, with no corrections for water contamination.

Analytical and preparative polyacrylamide gel electrophoresis was performed in cylindrical gels as reported elsewhere [Houwing & Jaspars (1978), and references therein]. Samples containing protein were never frozen or heated. Preparative electrophoresis was performed in a diluted McIlvaine buffer (21.4 mM Na_2HPO_4 and 9.3 mM citric acid) which contained 10% (v/v) glycerol and had a pH of 5.6. During electrophoresis, which took place at 12 mA and 125 V, the pH of the eluate increased to 7.0. When samples in buffer of lower ionic strength than the electrophoresis buffer were loaded, the voltage was kept at 125 V, which implied that the current could be as low as 5 mA. After 20–60 min, the sample layer was then removed by suction to restore the normal current. Samples of up to 3.6 mL could be loaded. Profiles of absorbance at 254 nm were recorded by an LKB Uvicord III. The elution rate was 3.2 mL/h, and fractions of 0.7 mL were collected. When the fractions were merely used for radioactivity measurements, 7 μL of a 10% $NaDodSO_4$ solution was added to each tube of the fraction collector in order to prevent adsorption of protein to the glass.

Velocity gradient analysis of reaction mixtures was performed by centrifugation in 18–48% (v/v) glycerol gradients in a diluted McIlvaine buffer (27.7 mM Na_2HPO_4 and 6.2 mM citric acid, pH 6.8) at 5 °C in a Beckman SW 27.1 or SW 41 rotor. Fractions diluted to about twice their original volume with 0.2% $NaDodSO_4$ were mixed with 10 volumes of scintillation liquid.

Results

Electrophoretic Analysis of Reaction Mixtures Containing RNA 4 and Virions. When RNA 4 is incubated for 2 h at 20 °C with increasing amounts of coat protein in the form of virions and then electrophoresed, more and more material with a lower mobility than RNA 4 is observed (Figure 1A–D). This material consists, presumably, of nucleoprotein complexes of well-defined composition since it migrates as a number of discrete peaks. At 2.2 mol of protein/mol of RNA 4, only a small peak close to RNA 4 and some slower material can be seen. The latter material has developed into another peak at 4.4 and 13.2 mol of protein. Remarkably, at 13.2 mol of protein no peaks with lower mobilities can be seen. There is still some free RNA left. Since we were more interested in the reaction products than in the reaction itself, we have varied the reaction conditions only to find a useful composition of the reaction mixture. It appeared that the reaction is strongly temperature dependent (Figure 1E–H) and that a favorable composition of the reaction mixture with well-detectable concentrations of all the reaction products can be obtained in 30 min at 40 °C (Figure 1G). Even after 5 min at 40 °C the pattern of Figure 1G is almost reached (result not shown). We have designated the different complexes as I, II, and III in order of decreasing mobility. Under the conditions of Figure 1, their electrophoretic migration rate is 94, 87, and 85% of that of RNA 4, respectively. At 60 °C (Figure 1H) three striking features can be seen: complex I is lacking; for the first time material with a lower mobility than that of complex III is observed; a relatively large amount of free RNA 4 is present. The last characteristic may be the result of protein denaturation.

The reaction is strongly speeded up by using free protein subunits instead of virions. Upon addition of 4.4 mol of protein subunits/mol of RNA 4, a pattern similar to that of Figure 1C is found immediately (result not shown). However, since there is no guarantee that free protein will not be bound aspecifically, further work has been done consistently with complexes obtained by incubating RNA 4 with virions.

Composition of Complexes of RNA 4 and Coat Protein. Complexes were made by incubating RNA 4 and ^{35}S-labeled virions for 5–10 min at 40 °C in a protein/RNA molar ratio of 4:1. The mixture was separated on a 4% preparative gel, and the absorbance and radioactivity of the fractions were

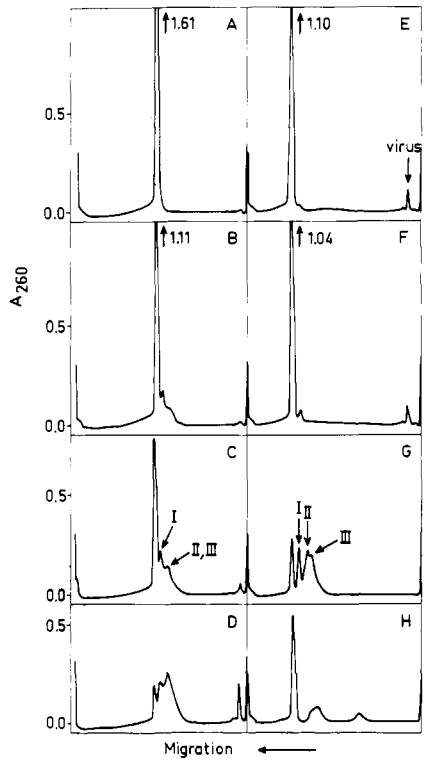

FIGURE 1: Densitometric scans of 3% polyacrylamide gels in which 6 μg of RNA 4 (A) or reaction mixtures of 6 μg of RNA 4 with virions were run (B–H). Reactions were performed in 100 μL of 10^{-2} M sodium phosphate and 10^{-3} M EDTA, pH 7.0, for 2 h at 20 °C with increasing amounts of virions (B–D) or for 30 min at different temperatures with the virion equivalent of 4.4 mol of coat protein/mol of RNA 4 (E–H). Virion equivalents of 2.2, 4.4, and 13.2 mol of coat protein were added in B, C, and D, respectively. Reaction temperatures were 0 (E), 20 (F), 40 (G), and 60 °C (H). Reaction products were designated I, II, and III in order of decreasing mobility. In most cases some of the donor virus or its derivatives can be seen near the top of the gel. Temperature of electrophoresis was about 20 °C in (A–D) and 4 °C in (H–F).

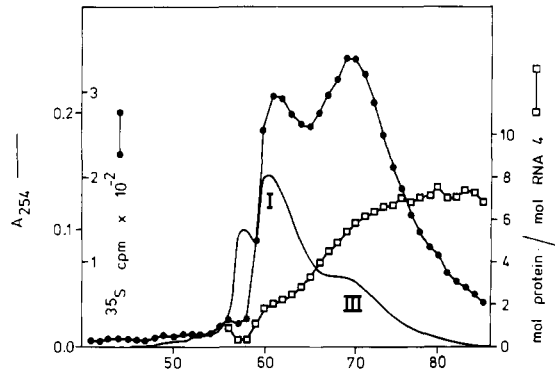

FIGURE 2: Preparative electrophoresis in a 4% polyacrylamide gel of 200 μg of RNA 4 which had reacted for 5 min at 40 °C in 1.0 mL of 10^{-2} M sodium phosphate and 10^{-3} M EDTA, pH 7.0, with 90 μg of ^{35}S-labeled virus (141 cpm/μg of protein; equivalent to 4.4 mol of coat protein/mol of RNA 4). Before the sample was applied to the gel, 0.8 mL of glycerol was added. Absorbance at 254 nm (—) was measured continuously. Radioactivity (●) was determined in samples of 0.65 mL. From the absorbance, not taken from the Uvicord track but measured in a Zeiss spectrophotometer and the radioactivity, the molar ratio of protein and RNA (□) was calculated for all relevant fractions.

measured (Figure 2). Radioactivity peaks were found at the positions of complexes I and III. Complex II must be in between, but is not visible as a separate peak under these conditions. From the radioactivities and absorbances in the

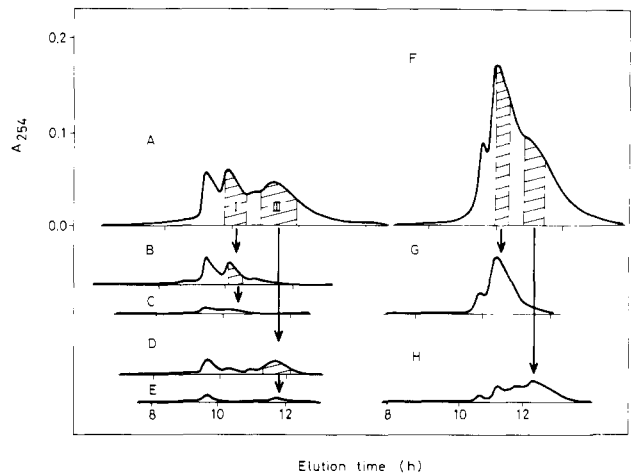

FIGURE 3: Stability of the RNA 4/coat protein complexes I and III upon handling and reelectrophoresis in a buffer according to Loening (1967) (A–E) or in a diluted phosphate–citrate buffer with 10% (v/v) glycerol (see text) (F–H). Preparative electrophoresis was performed in 3.7% polyacrylamide gels. Due to small variations in the applied voltage, the elution times of the complexes varied. For reasons of comparison, in each column of profiles the first eluting peaks (free RNA 4) have been made coinciding. Reaction mixtures shown in (A) and (F) were obtained as described in the legend of Figure 2, except that unlabeled virus was used, the reaction time of A was 15 min, and only half of the reaction mixture was used in A. To the reaction mixtures of A and F were added 125 μL of reaction buffer with 50% (w/v) sucrose and 800 μL of glycerol, respectively, before they were applied to the gel. Hatched fractions were pooled, dialyzed for 1 day in the cold against ten times diluted electrophoresis buffer, made 10% (w/v) in sucrose (B–E) or 50% (v/v) in glycerol (G, H), and rerun.

fractions, it can be calculated that complexes I and III contain 2 and 6 protein molecules, respectively. At the slow side of the peak of complex III, the specific radioactivity levels off at about 7 mol of protein/mol of RNA, suggesting that complexes with a much higher protein occupation than complex III are scarce. It is conceivable that complex II has four protein molecules and reduces the specific radioactivity at the fast side of the peak of complex III.

Isolation and Stability of Complexes I and III. The fact that the reaction products of RNA 4 and virions show up as discrete peaks upon electrophoresis demonstrates that they are rather stable nucleoprotein complexes. However, when isolated, dialyzed, and reelectrophoresed, both complexes I and III degrade (Figure 3A–E). The pattern of Figure 3D suggests that complexes II and I are intermediate stages in the degradation of complex III.

Since we wanted the isolated complexes to be as stable as possible in view of further investigations with them, we have tried to improve the conditions of storage and electrophoresis. Addition of glycerol appeared to have a favorable effect on the stability of the complexes. Of the buffers tested, a diluted phosphate–citrate buffer with a pH of 5.6 appeared to be better than a Loening buffer. Therefore, in all further work the complexes were separated and stored in this buffer with 10% (v/v) glycerol. (F–H) and (A, B, D) of Figure 3 must be compared to see the difference.

Ability of Complexes I and III To Interact with Virions. In principle it is possible that three dimers or even a single protein dimer saturates the specific site(s) with a high affinity for the coat protein, so that no further interaction of complexes with virions takes place. Though the formation of complex III in reaction mixtures where much uncomplexed RNA 4 is still present suggests that complex I readily withdraws protein from virions, it is quite conceivable that this interaction only

Table I: Number of Protein Molecules in RNA 4/Protein Complexes Formed upon Incubation of RNA 4, Complex I, and Complex III with Equimolar Amounts of Virions[a]

material reacting with virions	molar ratio of protein/RNA in complex[b] at			
	0 h[c]	11 h	22 h	33 h
RNA 4		17 (15, 21, 11)	20 (19, 23, 15)	19 (8, 24, 21, 9)
complex I	1.4 (1.6, 1.3)	20 (20, 23, 14)	21 (23, 23, 16)	21 (13, 24, 21, 9)
complex III	3.7 (3.6, 4.5, 1.7)	21 (20, 23, 16)	21 (23, 23, 14)	23 (19, 27, 23, 12)

[a] Reaction conditions and isolation of complexes as described in the legend of Figure 4. [b] Calculated from ^{35}S and ^{3}H radioactivities in slowly sedimenting peak (compare Figure 4C,D) and known specific radioactivities of coat protein and RNA 4. Values of individual fractions are given between parentheses in order of fraction number. [c] Reaction prevented by addition of formaldehyde to the reactants before they were mixed (Figure 4A,B).

occurs with virions that have already lost most of their quaternary structure. At the ratio of 4.4 mol protein/mol of RNA 4 there is only 1 virion per 55 RNA 4 molecules in the solution (Heijtink et al., 1977).

In order to investigate the interaction with virions, equal amounts of the complexes, ^{3}H labeled in their RNA and ^{35}S labeled in their protein, were incubated at 0 °C with ^{35}S-labeled virions at approximately a 1:1 virion/RNA molar ratio. ^{3}H-Labeled RNA 4 and unlabeled TYMV-RNA were incubated with virions under the same conditions. At 11, 22, and 33 h, samples were taken, fixed with 1% formaldehyde, and analyzed on glycerol gradients. From previous work it is known that reaction of RNA and virions takes place in two steps: first, a virion/RNA complex is formed, which is sufficiently stable to manifest itself upon gradient centrifugation; subsequently, the virion/RNA complex dissociates, and the RNA takes some protein with it (Verhagen et al., 1976; Van Boxsel, 1976). Since we made use of ^{3}H-labeled RNA 4 and RNA 4/protein complexes, the amount of virion-associated material could be measured as well as the number of protein molecules in the RNA/protein complexes near the meniscus. From Figure 4 it is evident that the ability of complex I to associate with virions and to withdraw protein subunits from them is similar to that of RNA 4. In comparison with complex I and RNA 4, complex III is found less in association with virions but causes them to lose about the same amount of protein subunits in the same time.

From Table I it appears that the number of protein molecules per RNA 4 molecule in the newly formed complexes is mostly 20 or 21, irrespective of the starting material and the incubation time. We have not investigated the homogeneity of these complexes, but if the molar ratio protein/RNA 4 is calculated for the fractions of one peak, no clear correlation with sedimentation is found. The measurements at zero time show that the complexes with which the incubations were started were not pure, probably as a result of protein losses. The preparation of complex I contained 30% free RNA, whereas that of complex III probably contained both complexes II and I, and possibly also some free RNA 4. Though these contaminations may have influenced the results, it is unlikely that they have obscured essential differences between the complexes. In comparable experiments, it was found that preparations of complexes I and III, which were not used for incubation with virions but were standing for 33 h at 0 °C and then centrifuged for 16 h in a glycerol gradient at 5 °C, still had protein/RNA molar ratios of 1.4 and 4.5, respectively.

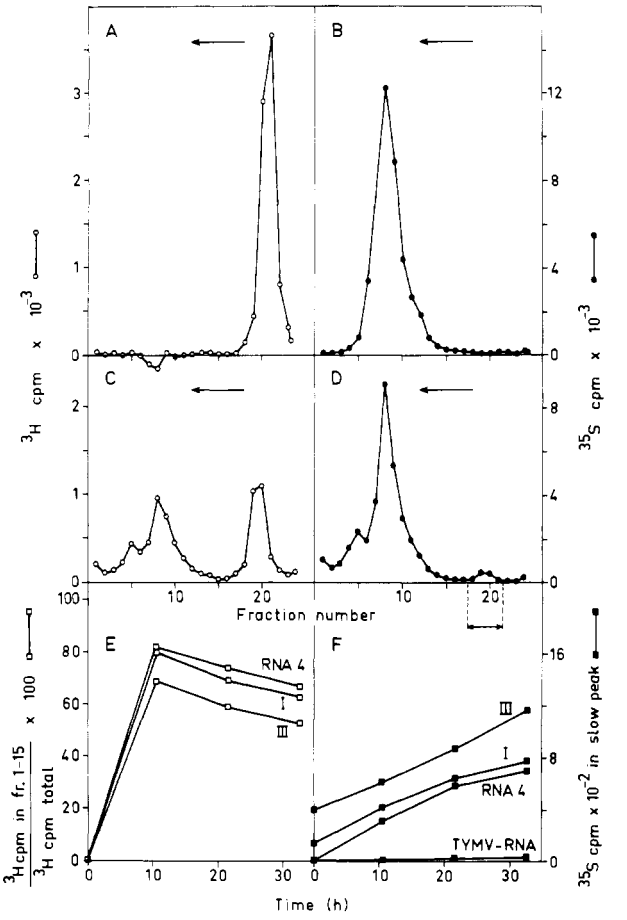

FIGURE 4: Reaction of complexes I and III with virions as compared to those of RNA 4 and TYMV-RNA. Amounts of 12.3 and 15.9 μg of complexes I and III, respectively, obtained as the material from the hatched regions of Figure 3F, ^{3}H labeled in their RNA (2648 cpm/μg) and ^{35}S labeled in their protein (446 cpm/μg), were incubated immediately after isolation, at 0 °C in 1.65 mL of the electrophoresis buffer with 300 μg of ^{35}S-labeled virus (446 cpm/μg of protein), which was pretreated with TYMV-RNA as described under Materials and Methods. At 11, 22, and 33 h, samples of 0.55 mL of the reaction mixtures were taken, made to 1% in formaldehyde, and run in glycerol gradients in an SW 27.1 rotor for 12 h at 25 000 rpm and 5 °C. The patterns of ^{3}H (○) and ^{35}S radioactivity (●) in 0.74-mL fractions of the gradient of the 22-h sample of complex I is shown in (C) and (D), respectively. In (A) and (B) the same material is analyzed, but here the formaldehyde was added before complex I and virus were mixed. The same incubations were performed with 10.5 μg of ^{3}H-labeled RNA 4 (2648 cpm/μg) and unlabeled TYMV-RNA. The percentage of the ^{3}H radioactivity sedimenting in fractions 1–15 (□) and the total ^{35}S radioactivity sedimenting in a slow peak near the meniscus (■) are represented in (E) and (F), respectively.

Do Complexes I and III Yield Protein to RNA 1? In order to investigate whether the protein in complex I is bound tighter to the RNA than the protein in complex III, ^{35}S-labeled complexes were incubated for 34 h at 0 °C with RNA 1. Analysis on glycerol gradients showed that no shift of ^{35}S label whatsoever had occurred in the case of complex I, whereas 19% of the label was found at the RNA 1 position in the case of complex III (Figure 5A,B). However, in view of the contamination of complex III with complex II, it is not certain that complex III itself takes part in this protein exchange. Under the same conditions, an artificial complex of TYMV-RNA fragments and ^{35}S-labeled AMV coat protein yielded almost all protein to RNA 1 (Figure 5C,D).

Discussion

The interaction between nucleic acid and coat protein of a virus is likely to start with the attachment of one or a few

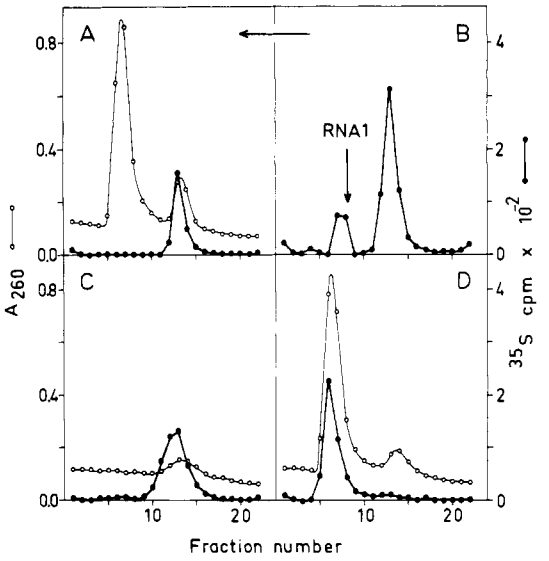

FIGURE 5: Reactions of complexes I and III and of an artificial complex of TYMV-RNA fragments with AMV protein with RNA 1. The reaction mixtures contained 40 μg of RNA 1 and 10.0 μg of complex I (1.4 mol of protein/mol of RNA; 568 ^{35}S cpm/μg of protein), 5.5 μg of complex III (4.5 mol of protein/mol of RNA; 1108 ^{35}S cpm/μg of protein), or 11.0 μg of TYMV-RNA/AMV protein complex (4.4 mol of protein/mol of RNA; 568 ^{35}S cpm/μg of protein) in 520 μL of electrophoresis buffer. Complexes I and III were obtained as the material from the hatched regions of Figure 3F, and the TYMV-RNA/AMV protein complex was obtained as described under Materials and Methods. After an incubation of 34 h at 0 °C, the mixtures were run in glycerol gradients in an SW 41 rotor for 16 h at 37 000 rpm and 5 °C. Absorbance at 260 nm (○) and ^{35}S radioactivity (●) were measured in 0.55 mL fractions of gradients with mixtures containing complex I (A), complex III (B), and the TYMV-RNA/AMV protein complex (D). An identical sample of the last complex to which no RNA 1 was added is shown in (C). In (B) no absorbance was measured; the position of the peak of RNA 1 in a sister gradient is indicated.

protein subunits to a defined site of the nucleic acid molecule (Caspar & Klug, 1962). Thus far, unique nucleation sites have been identified as sequences protected by small amounts of protein against ribonuclease degradation in the case of the helical RNA virus tobacco mosaic virus (Zimmern & Butler, 1977; Zimmern, 1977) and the icosahedral RNA bacteriophages (Bernardi & Spahr, 1972; Berzin et al., 1978; Jansone et al., 1979). Interestingly, in both cases nucleation takes place at an internal site of the RNA and possibly plays a role both in virion assembly and in regulation. With tobacco mosaic virus, it is evident that assembly starts from the identified site (see Butler & Durham (1977) for a review). Lomonossoff & Butler (1979) have suggested that nucleation followed by a short elongation in the direction of the 3′-hydroxyl end of the RNA could prevent processing of the adjacent part of the RNA, which contains the coat protein cistron, into a subgenomic messenger. With the RNA bacteriophages, it is well established that the coat protein acts as a translational repressor of the replicase cistron (Bernardi & Spahr, 1972; Berzin et al., 1978), but it is questionable whether the regulatory site is also the nucleation site for virion assembly (Zagórska et al., 1975).

Unfortunately, neither with tobacco mosaic virus nor with RNA bacteriophages has it been possible to detect and isolate nucleation complexes as separate and well-defined entities. This hampers the analysis of the subsequent steps in the interaction of RNA and protein subunits.

In this study we succeeded in isolating nucleation complexes of RNA 4 and coat protein of AMV by means of preparative gel electrophoresis, which enabled us to draw the following

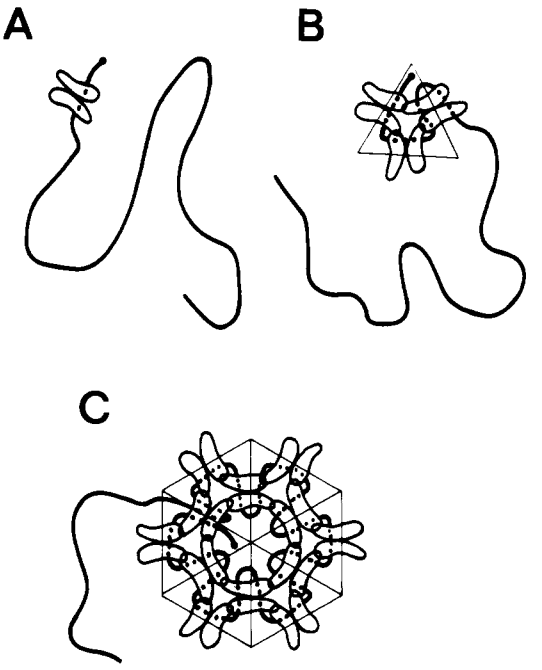

FIGURE 6: Tentative representation of nucleation complexes of RNA 4 with 2 (A, complex I), 6 (B, complex III), and 24 coat protein molecules (C). The protein molecules have been drawn in dimer clusters at the dyad positions of a hexagonal lattice, as is proposed by Mellema & Van Den Berg (1974) for the coat of AMV virions. The lattice is filled up so as to have the maximum number of protein–protein contacts in each case. The 3′ end of the RNA is indicated by a dot. RNA–protein contacts and shape of the protein molecules are arbitrary.

tentative picture of the successive steps of the interaction.

The first protein dimer will attach to a site near the 3′ end of the RNA (Figure 6A). This site is likely to be in the last 88 nucleotides since a 3′-terminal fragment of this size, called fragment 29B, interacts with virions (Houwing & Jaspars, 1978; Koper-Zwarthoff & Bol, 1979). However, the site is not located at the extreme end of RNA 4 since a fragment comprising the 58 5′-terminal nucleotides of fragment 29C was found recently to bind specifically to virions (Stoker et al., 1980), whereas a 3′-terminal fragment of 36 nucleotides is inactive in this respect (C. J. Houwing, unpublished results). The complex of RNA with the first protein dimer, called complex I, is still able to withdraw protein from virions. In fact, virions donate protein to complex I in the presence of naked RNA 4. In a cooperative reaction with two more protein dimers, complex III is formed. Strong cooperativity is also likely to play a role in RNA bacteriophages (Chroboczek & Zagórski, 1975) and tobacco mosaic virus (Zimmern & Butler, 1977; Zimmern, 1977) when the first three dimers or three 34-mers, respectively, of the coat protein are bound by the RNA. Complex I is stable in that it does not lose protein to one of the genome RNAs upon prolonged incubation at 0 °C. Complex III (or complex II) is less stable in this respect. From this it may be concluded that the degradation of complex I and possibly also of complex III to RNA 4 or lower RNA 4/protein complexes, as observed upon handling and storing, is caused by denaturation of the protein at glass and solvent surfaces, rather than by dissociation of the complexes. At relatively low concentrations of the protein donor (see Figure 1), complex III does not easily accept more protein and therefore probably corresponds with the titration end point of the high-affinity sites of RNA 4, estimated to be between 3 and 6 monomer sites (Van Boxsel, 1976).

If the protein interactions in complex III are of the same

nature as those in the coat of complete virions, the model of Mellema & Van Den Berg (1974) may apply. In this model the coat protein subunits are clustered as dimers around the dyad positions of a hexagonal lattice, avoiding the sixfold and threefold positions (Mellema, 1975). The first three dimers could occupy three adjacent positions of the lattice and in that way form a relatively stable structure in which 50% of the interdimer binding sites are involved in interdimer bonds (Figure 6B).

At relatively high concentrations of the protein donor (see Figure 4), complex III will associate with a virion and accept much more protein. After a certain protein exchange has taken place, the virion/complex III structure will fall apart. The relatively stable complex formed could be represented by the hexagonal structure of Figure 6C in which 12 positions of the lattice are occupied by protein dimers. In such a structure, 75% of the potential binding sites are involved in bonds. A small change in the bond angles permits the construction of a comparable pentagonal structure with ten twofold positions occupied. Such a structure could serve as the start of the assembly of a bacilliform particle with the 3′ end of the RNA in one of the rounded caps, which are thought to have a pentagonal structure (Hull et al., 1969). The number of coat protein monomers experimentally found in these complexes is mostly 20 or 21 (Table I), which is in favor of the hypothetical pentagonal structure. However, structures of 24 subunits could be contaminated with starting material that has not yet taken part in the reaction.

The contacts of the RNA chains with the protein subunits in Figure 6 are purely speculative, as is the precise outline of the subunits, but obviously there will be at least two potential RNA–protein interaction sites per dimer. It is of much interest to know whether, in the small complexes I and III, some of these sites are empty or whether they are used for making contacts with distant sites on the RNA chain that exist only in these complexes, which could make them of regulatory importance. Physicochemical studies suggest that the first protein molecules that attach to RNA 4 interfere with its tertiary structure (Srinivasan & Jaspars, 1979, and unpublished results). Also it has been found that the capacity of RNA 4 to interact with virions is not lost after removal of about 10% of the molecule at the 3′ end (Houwing & Jaspars, 1978). Since we have now well-defined nucleation complexes at our disposal, it will be possible to detect the RNA–protein contacts in each of them by means of ribonuclease protection studies. Recently, it has been found that the four major AMV-RNAs have a sequence homology of at least 80% in the last 150 nucleotides at their 3′ ends (Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980). Thus, the findings with RNA 4 will hopefully contribute to the understanding of the activation of the AMV genome by small amounts of coat protein.

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