Preferential Binding of 3′-Terminal Fragments of Alfalfa Mosaic Virus RNA 4 to Virions†

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ABSTRACT: 3′-Terminal fragments present in a partial ribonuclease T1 digest of RNA 4 of alfalfa mosaic virus were selectively bound to virions of the same virus by incubation in 10 mM sodium phosphate and 1 mM EDTA, pH 7.0. The virions with the associated fragments were separated from the remainder of the digest by velocity gradient centrifugation. When the incubation was performed in 27.7 mM Na_2HPO_4 and 6.2 mM citric acid, pH 6.8, plus 10% glycerol, much more, but less specific, binding was obtained. However, in this case the terminal fragments present in unbound material of the digest were almost exclusively 5′ termini. The four RNA species of alfalfa mosaic virus have an extensive 3′-terminal homology. Therefore, it is expected that specific virion binding can be used to obtain 3′-terminal fragments of different lengths of the three genome RNA species (RNAs 1, 2, and 3) of alfalfa mosaic virus and, possibly, of related viruses. Such fragments are of great importance for the study of the specific 3′-terminal interaction with coat protein subunits which is thought to be responsible for the activation of the viral genome.

Each of the four RNA species of alfalfa mosaic virus, when incubated with virions of the same virus, is able to withdraw coat protein subunits from them (Van Boxsel, 1976; Verhagen et al., 1976). The smallest AMV[1]-RNA is RNA 4. It is the subgenomic messenger for the coat protein. When large fragments of this RNA were tested for interaction with virions, it was found that the high-affinity binding sites for coat protein are located close to the 3′ terminus (Houwing & Jaspars, 1978). Because of the extensive 3′-terminal homology of all four AMV-RNAs (Pinck & Pinck, 1979; Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980), the same location of the high-affinity sites is to be expected in the other three RNA species. We think that the preferential binding of the coat protein at the 3′ termini of the RNAs has to do with the recognition by the viral replicase (Houwing & Jaspars, 1978). The fact that the coat protein has to be present on all three genome RNAs for maximum infectivity to occur (Smit & Jaspars, 1980) is in favor of this hypothesis. We wish to study physicochemically the interaction of the coat protein with isolated large 3′-terminal fragments of varying length originating from all three genome RNAs. Therefore, we wondered whether it would be possible to isolate such 3′-terminal fragments from a partial ribonuclease digest by binding them selectively to AMV virions. It is known that in the reaction between AMV-RNA and virions, a virion/RNA complex is formed that is sufficiently stable to show up upon gradient centrifugation (Van Boxsel, 1976; Verhagen et al., 1976; Houwing & Jaspars, 1980). In this report we demonstrate that the same holds for RNA fragments with high-affinity binding sites. We made a partial ribonuclease T1 digest of RNA 4 labeled both 3′ and 5′ terminally with [^{3}H]borohydride and found that, under certain conditions, very good separations between 5′- and 3′-terminal fragments were achieved by means of virion binding.

Materials and Methods

Partial Digest of Terminally Labeled RNA 4. RNA 4 of strain 425 was prepared, terminally labeled (40 000 cpm/μg) with NaB^3H_4, and partially digested with ribonuclease T1 as described previously (Houwing & Jaspars, 1978), except that the digestion was carried out for 30 min at a final RNA concentration of 134 μg/mL in order to reduce the amount of residual RNA 4. The RNA fragments were designated in that article by their apparent molecular weights in thousands of daltons. The same nomenclature is used here, though the molecular weights of the larger fragments particularly were somewhat underestimated due to a 10% underestimation at that time of the molecular weight of RNA 4 used as a marker in gel electrophoresis. Very small and very large RNA fragments and some residual RNA 4 were removed from the digest by preparative electrophoresis (Figure 1A). Very small fragments with the labeled 3′ terminus are not likely to bind to virions (Houwing & Jaspars, 1980), whereas very large fragments may contribute labeled 5′ termini to the binding fraction. The material from the preparative electrophoresis run was precipitated with magnesium phosphate and ethanol according to Dessev & Grancharov (1973), except that a five times higher magnesium phosphate concentration was used. The precipitate was dissolved in a minimum amount of 0.1 M EDTA and dialyzed at 0 °C against the desired buffer. The solutions were stored frozen. Samples to be used for incubations with virions were heated for 5 min at 60 °C.

Virions. ^{35}S-Labeled bottom component (229 cpm/μg) was obtained as described by Verhagen et al. (1976).

Miscellaneous Materials. All chemicals were reagent grade. The concentrations of glycerol (v/v) refer to glycerol as supplied, with no correction for water contamination.

Preparative polyacrylamide gel electrophoresis was performed as reported elsewhere (Houwing & Jaspars, 1978, and references cited therein).

Separation of Free and Virion-Associated RNA Fragments by Means of Velocity Gradient Centrifugation. Incubation mixtures were centrifuged in either 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) or 10–40% (w/v) sucrose gradients in 10 mM sodium phosphate and 1 mM EDTA, pH 7.0, in agreement with the buffer in which the incubation had taken place. Radioactivity was determined in 50 μL of each fraction of the gradients. Fractions containing the free and

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

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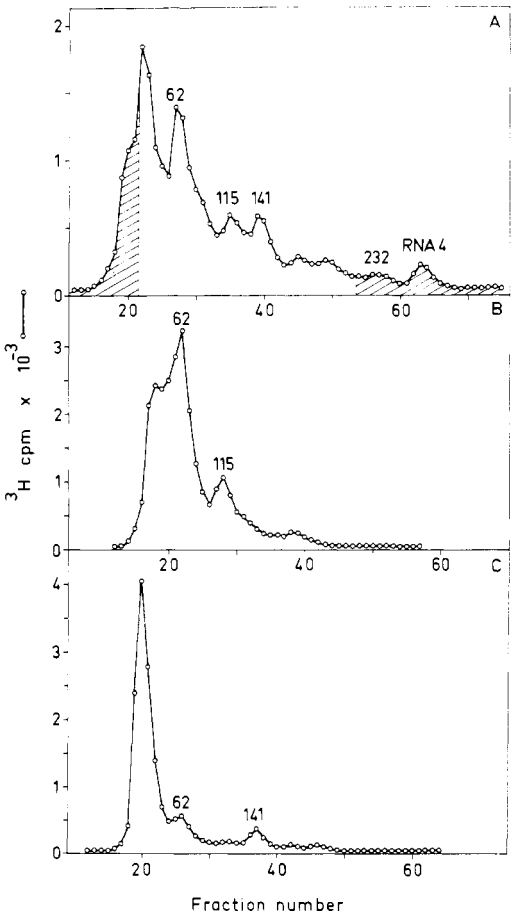

FIGURE 1: Results of separations obtained in a partial ribonuclease T1 digest of RNA 4 by means of virion binding. Preparative electrophoresis in 4% polyacrylamide gel of 40 μg of the digest before incubation with virions (A), of 0.7 μg of the material of the digest not associated with virions when incubated in phosphate–citrate buffer plus 10% glycerol (F in Figure 2A) (B), and of 0.4 μg of the material of the digest associated with virions when incubated in phosphate–EDTA buffer (VA in Figure 2B) (C). The hatched fractions in (A) were omitted from the material used for incubation with virions. Elution was at a rate of 3.2 mL/h. Fractions of 0.70 mL were collected. Radioactivity was determined in 10 (A) or 650 μL (B, C) of the fractions. Peaks in (A) were identified with the aid of electrophoresis patterns from earlier work (Houwing & Jaspars, 1978) and those in (B) and (C) by means of their mobility in relation to the mobility of unlabeled RNA 4, fragment 115, and $tRNA^{Phe}$ from yeast, added as molecular weight markers.

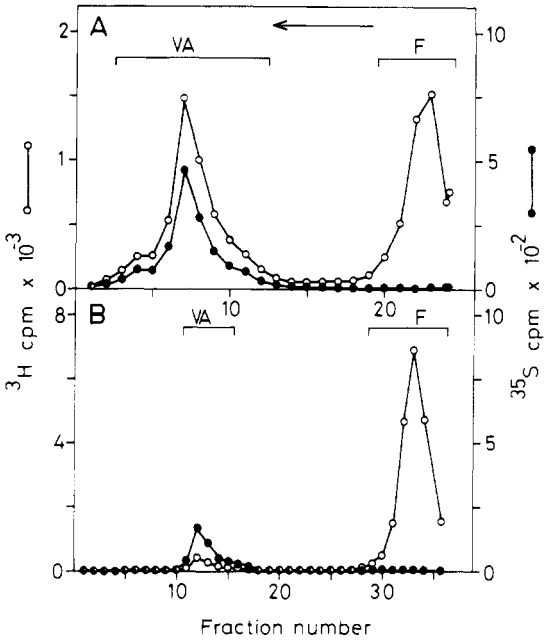

FIGURE 2: Radioactivity profiles of gradients run with incubation mixtures of a partial digest of RNA 4 with ^{3}H-labeled end groups and ^{35}S-labeled virions: (A) 4.0 μg of digest incubated with 112 μg of virions for 4.5 h at 0 °C in 0.62 mL of phosphate–citrate buffer plus 10% glycerol and run in a 18–48% glycerol gradient in an SW 27.1 rotor for 12 h at 25 000 rpm and 5 °C; (B) 12.2 μg of digest incubated with 70 μg of virions for 4.5 h at 0 °C in 1.75 mL of phosphate–EDTA buffer and run in a 10–40% sucrose gradient in an SW 27 rotor for 7 h at 25 000 rpm and 5 °C. Fractions of 0.74 (A) or 1.05 mL (B) were taken. ^{3}H (○) and ^{35}S (●) radioactivities were determined in the fractions as described under Materials and Methods. Fractions containing free (F) and virion-associated (VA) RNA were pooled for phenol extraction.

virion-associated material were pooled, shaken for 5 min at room temperature with an equal volume of water-saturated phenol, and centrifuged. To the virion-associated material from the glycerol gradients was added a volume of distilled water equal to that of the pooled fractions in order to get two phases. The water phases, which were the upper ones except in the case of the virion-associated material from the sucrose gradients, were dialyzed for 3 days at 0 °C against several changes of distilled water and dried under vacuum over P_2O_5 at room temperature.

End-Group Analyses. Alkaline hydrolysis of RNA fragments and chromatography of ^{3}H-labeled termini were performed essentially as described by Ohno et al. (1977).

Radioactivity measurements were performed in a Nuclear Chicago Mark II scintillation counter. The scintillation liquid was Hydroluma from Lumac Systems A.G., Basel, Switzerland.

Results and Conclusion

Under the conditions described in the previous paper (phosphate–citrate buffer, 10% glycerol) (Houwing & Jaspars, 1980), more than 80% of a given RNA 4 preparation could be found sedimenting in a complex with virions, whereas under the conditions of earlier experiments (phosphate–EDTA buffer, no glycerol) (Van Boxsel, 1976), much less RNA was bound to virions. Therefore, we did the incubation first under the conditions of Houwing & Jaspars (1980), viz., at a concentration of about 7 μg of digest/mL with about an equimolar amount of virions (calculated on the basis of 3′ end groups in the digest) in phosphate–citrate buffer plus glycerol. Centrifugation in a glycerol gradient with the same buffer showed that about half of the label was associated with virions (Figure 2A). End-group analysis of the virus-associated material revealed that it had two times more 3′ termini than 5′ termini (Table I; Figure 3B). In this calculation it has been taken into account that in the original digest, for some unknown reason, the 5′ termini have a specific labeling about three times higher than that of the 3′ termini (Table I; Figure 3A). The unequal distribution of label in the original digest is not caused by the electrophoretic removal of very large and very small fragments from the digest (see Materials and Methods), since a similar labeling ratio has been found in intact RNA 4 (Houwing & Jaspars, 1978). From the amount of 3′ termini in the virus-associated material, it could be predicted that few 3′ termini would be present in the fraction of free RNA fragments. End-group analysis of this fraction confirmed the prediction: more than 80% of the fragments are derived from the 5′ terminus (Table I). The electrophoretic pattern of the free RNA fragments shows prominent peaks of the 5′-terminal fragments 62 and 115, whereas the peak of the 3′-terminal fragment 141 has almost disappeared (Figure 1B). Therefore, under these conditions the fraction

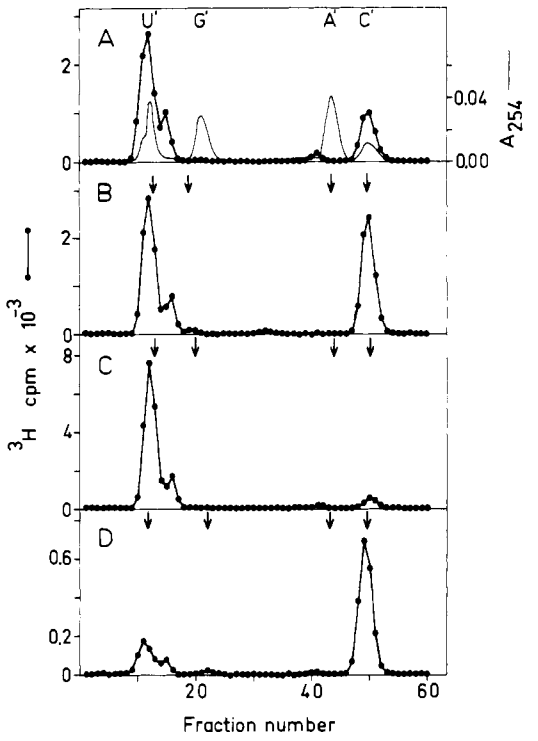

FIGURE 3: Analyses of ^{3}H-labeled end groups of a partial ribonuclease T1 digest of RNA 4, unfractionated (A) and fractionated in virion-associated (B, D) and free material (C) by incubation with virions followed by velocity gradient centrifugation. Incubation and centrifugation took place in phosphate–citrate buffer plus 10% glycerol (see Figure 2A) (B, C) or in phosphate–EDTA buffer (see Figure 2B) (D). Alkaline hydrolysates of the RNAs were chromatographed on a phosphocellulose column. The position of the four unlabeled nucleoside trialcohols used as markers is indicated by the Uvicord III track in A, and by arrows in B, C, and D. The oxidized and reduced cap elutes somewhat faster than the trialcohol of uridine (Ohno et al., 1977).

Table I: Percentage of 3′ End Groups in Virion-Bound and Unbound Fractions of Partial Ribonuclease T1 Digest of End-Labeled RNA 4[a]

buffer of incubation	fraction of digest[b]	^{3}H in 3′ end group (% cytosine)[c]	ratio of 3′/5′ end groups[d]
	unfractionated	25.5 25.8	1.00
phosphate–citrate, 10% glycerol	virus associated	42.1 41.5	2.10 2.06
	free	6.2 6.4	0.19 0.20
phosphate–EDTA	virus associated	67.5[e] 73.8 74.9	6.02 8.18 8.65

[a] RNA 4 was terminally labeled by oxidation with $NaIO_4$ and subsequent reduction with NaB^3H_4. [b] Incubation and fractionation were as described in the legend of Figure 2. [c] Percentage of ^{3}H counts per minute in peak of cytosine derivative related to the total ^{3}H counts per minute in peaks of cytosine and cap derivatives together, in alkaline digest chromatographed as in Figure 3. Duplicate values are from independent incubations. [d] Ratio found in unfractionated digest set at 1.00. [e] Value is from incubation of 1.75 μg of digest with 50 μg of virions in 0.26 mL of buffer.

of free RNA fragments could serve as a source of 5′-terminal fragments that are only slightly contaminated with 3′-terminal fragments. However, the use of these 5′-terminal fragments will be limited because of the contamination with unlabeled fragments.

Since 3′-terminal fragments could be enriched with respect to, but not freed from, 5′-terminal fragments by incubation of the digest with virions in phosphate–citrate buffer plus glycerol, we tested whether incubation in phosphate–EDTA buffer without glycerol would yield a more specific binding. These incubations were performed for the same length of time and at the same RNA concentrations as the previous incubations. The amount of virions was varied; besides an equimolar amount, one-fourth of this amount (calculated on the basis of 3′ end groups in the digest) was also tested. It appeared that the binding of 3′-terminal fragments was strongly favored in comparison with incubations in phosphate–citrate buffer plus 10% glycerol. However, only 15 and 5% of the label, respectively, was found in association with virions (Figure 2B).

In the latter case (5% of the label associated), 90% of the termini were 3′ termini (Table I; Figure 3D). Even this figure is an underestimate since some of the label at the position of the cap derivative in the eluate of the phosphocellulose column originates from a contamination in the NaB^3H_4 preparation. The electrophoretic pattern of the associated RNA fragments shows that the 5′-terminal fragments 62 and 115 have almost disappeared and that the 3′-terminal fragment 141 is clearly present, as well as a large peak at the position of the 3′-terminal fragments 29B and 29C (Figure 1C). Since the resolving power of a 4% gel is poor in this region, the latter peak may also contain 3′-terminal fragments somewhat larger and smaller than fragments 29B and 29C.

When the incubation was performed in phosphate–EDTA buffer with an equimolar amount of virions (15% of the label associated), the enrichment of 3′ termini over 5′ termini was less (Table I).

In conclusion, it can be stated that incubation with virions can be applied to select 3′-terminal fragments from a partial digest of the AMV-RNA species number 4. It has proved possible to find conditions under which only 3′ termini and almost no 5′ termini of the original RNA molecule were present in the virion-associated material. This makes it very likely that under these conditions the virions bind only RNA fragments on which high-affinity binding sites for coat protein are located. Thus, use of this method looks promising as a first step in the isolation of protein-binding fragments of different lengths from digests of all RNA species of AMV. These could then be further purified by preparative gel electrophoresis, sequenced, and used in comparative studies of the interaction of the coat protein with isolated binding sites of varying length and sequence. Since the RNAs of other heterocapsidic viruses of the group of RNA viruses with tripartite genomes also interact with AMV virions [see Van Vloten-Doting & Jaspars (1977) for a review], the method can probably also be applied to digests of these RNAs.

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Phosphorus Nuclear Magnetic Resonance Studies on the Lipid-Containing Bacteriophage PM2†

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ABSTRACT: ^{31}P NMR spectra of intact bacteriophage PM2 virus were obtained in the presence of 60% sucrose. The spectrum is composed of two major components. One is a powder pattern typical of an axially symmetrical motion, and this was assigned to the phospholipid bilayer of PM2 by comparison with the spectrum of the extracted lipid from PM2 and the known structure of this virus. The chemical shift anisotropy was about −47 ppm at 6 °C. The other component was much broader. This was assigned to the packaged DNA of PM2 by comparison with the ^{31}P NMR spectrum of bacteriophage T4, a virus which has no lipids. A powder pattern spectrum of the PM2 nucleocapsid was also obtained in the presence of 6 M urea and 50% sucrose. The spectrum was quite similar to that of PM2. This fact clearly shows that the nucleocapsid still contains a phospholipid bilayer, in contradiction to an earlier model, and that the structural arrangement of the lipid bilayer and DNA in the nucleocapsid is similar to that in the intact PM2. In the temperature-shift experiments, a spectral change of PM2 was observed in the region from 15 to 22 °C for both the phospholipid and DNA components and above 34 °C for the phospholipid component. The temperature dependence of the spectrum of the extracted bulk phospholipid was found to be different from that of the virus. Furthermore, the effect of temperature on the infectivity of this virus in an early stage of the growth cycle was examined. After exposure to a given temperature for 5 min at the start of infection, the yield increased in a stepwise fashion in the range from 15 to 22 °C and had a maximum at 22.5 °C, suggesting a correlation between the viral yield and a structural change of the virion.

Lipid-containing viruses are suitable models for the investigation of the structure and function of biomembranes because of their simple composition. Bacteriophage PM2 (PM2)[1] is one of the best candidates for such studies. PM2 grows on the marine bacterium *Alteromonas espejiana*. Biochemical and physicochemical studies on it have been thoroughly carried out (Franklin, 1974, 1977). The shape of the virus is spherical, and it has a lipid bilayer. The major phospholipids are phosphatidylglycerol (PG), ~60%, and phosphatidylethanolamine (PE), ~40%. PG is located predominantly in the outer leaflet of the viral lipid bilayer, whereas PE predominates in the inner leaflet (Schäfer et al., 1974). The virus contains only four proteins, namely, proteins I, II, III, and IV. Protein I forms small spikes at the vertexes of the outer shell, which is composed of protein II (Hinnen et al., 1974; Schäfer et al., 1974). Protein IV interacts specifically with DNA (Marcoli et al., 1979). The DNA of the virus is a closed supertwisted double helix. In the presence of 4–6 M urea, the nucleocapsid was isolated and was reported to be a lipid-free complex of DNA, protein III, protein IV, and 10% protein II (Schäfer et al., 1978). Recently a detailed model of the structure of PM2 was proposed on the basis of neutron-scattering experiments (Schneider et al., 1978).

In spite of these studies, relationships between the structure and the biological function are not yet clear. In this respect, nuclear magnetic resonance (NMR) offers an attractive possibility, because the energetic perturbation of the sample is small enough to keep it intact and no additional probe molecule needs to be incorporated. Attempts have been made to look at the membranes of some intact viruses or cells. High-resolution ^{13}C NMR of ^{13}C-enriched vescular stomatitis virus (VSV) was measured (Stoffel & Bister, 1975), followed by ^{31}P NMR of VSV (Moore et al., 1977). ^{1}H NMR of chick embryo cells in the absence and presence of myxoviruses or RNA tumor viruses suggested a change in the cell membrane induced by the virus (Nicolau et al., 1978). Recently ^{2}H NMR of intact *Escherichia coli* incorporated with specifically deuterated fatty acids was reported (Gally et al., 1979). There was a good qualitative agreement in the order parameter profile of hydrocarbon chains in the intact membrane and in artificial phospholipid bilayers, indicating the promising future of this method. It can be said from these studies that in order to get useful information by the use of NMR, a particular molecule of a particular system should be labeled for an unambiguous assignment of the spectrum. Furthermore, since the sensitivity of the NMR method is generally very low, a large amount of pure sample is necessary for the measurements. We have chosen to work with PM2 because the virus

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[1] Abbreviations used: ^{31}P NMR, phosphorus nuclear magnetic resonance; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PM2, bacteriophage PM2; $NaDodSO_4$, sodium dodecyl sulfate; buffer A, 1.0 M NaCl, 0.02 M Tris, 0.01 M $CaCl_2$, and 0.1% β-mercaptoethanol, pH 7.5 at 20 °C; buffer B, 0.1 M Tris, 85 mM NaCl, 2 mM NH_4Cl, and 1 mM $MgCl_2$, pH 7.5 at 20 °C.

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