Attachment of Avidin-Coupled Spheres to Linear and Circular Forms of Mengovirus Double-Stranded RNA

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Picornavirus-infected cells contain a double-stranded RNA (replicative form [RF] RNA), composed of viral genomic RNA hydrogen bonded to cRNA of similar nucleotide length. Mengovirus RF RNA reacted with a succinimide ester of biotin was shown by electron microscopy to bind avidin-coupled polymethacrylate spheres. These binding sites are taken to indicate the presence of VPg protein molecules by methods previously applied to poliovirus RF RNA (Richards et al., *Proc. Natl. Acad. Sci. U.S.A.* **76**:676-680, 1979). One sphere was bound at or very near one terminus of linear RF molecules while a second sphere was bound at a site which was 1 to 4% of the genome length from the other terminus. Assignment of VPg positions was limited by the physical dimension (ca. 60-nm diameter) of the heavy metal-contrasted sphere observed by electron microscopy. A third site of sphere binding was detected at a lower frequency of occurrence at a site which was 10 to 20% of the genome length from one or the other terminus. Circular RF RNA molecules were also detected with two spheres attached at juxtaposed sites. The termini of the linear RF RNA were located within the circular structures by sphere attachment at sites which were very close to and obscured a nucleic acid projection which we have described to occur on circular mengovirus RF RNA (D. L. Robberson, M. V. Marshall, G. B. Thornton, and R. B. Arlinghaus, manuscript submitted for publication). CsCl gradient fractions of RNA reacted with avidin-coupled spheres were highly enriched in circular structures.

The genomic RNA of picornaviruses contains a small basic protein covalently attached to its 5' terminus (6-8, 10, 14, 16, 19, 20). This protein, VPg, is also attached to the 5' ends of intracellular forms of viral RNA involved in viral RNA replication. Double-stranded poliovirus RNA (replicative form [RF] RNA) contains VPg at the 5' end of the plus and minus strands (6, 13). One way to detect protein bound to such structures is by use of electron-opaque avidin-coupled spheres (16). In such experiments, biotin is chemically linked to the VPg-RNA complex. The high affinity of avidin for biotin is then utilized in the attachment of the electron-opaque avidin-coupled spheres to the protein bound to viral RNA. Richards et al. (16) showed that avidin-coupled spheres were attached to the plus strand at only one end of poliovirus RF that had been reacted with biotin.

Our studies with mengovirus RF have shown that purified preparations of RF contain both circular and linear forms of viral double-stranded RNA (M. V. Marshall, R. B. Arling-

haus, and D. Robberson, *J. Cell Biol.* **70**:248a, 1976; Robberson et al., manuscript submitted for publication). The circular double-stranded viral RNAs are of genomic length, contain a short projection, and are noncovalent associations of the linear forms. Similar circular structures were first reported for RF RNA of encephalomyocarditis virus by Agol and his colleagues (1, 2) and subsequently shown to be noncovalent structures (18). In this study, the avidin-coupled sphere technique (11) (previously applied to poliovirus RNA) was used to demonstrate that both ends of biotin-linked mengovirus RF can bind avidin-coupled spheres, indicating the presence of VPg-like molecules at or near the termini, and to identify the location of the termini of linear RF within the circular structures.

MATERIALS AND METHODS

Cells and virus. BHK-21 clone 13 cells were grown and propagated in roller bottle cultures, as described (24). Mengovirus was plaque purified by Royce Z. Lockhart, Jr. (Du Pont Laboratories, Wilmington,

Del.); the initial stock of virus was obtained from his laboratory. In a typical experiment, two roller bottles, having attained 75 to 90% confluency of 5×10^6 cells, were infected with 10 to 50 PFU per cell. Fresh medium containing 5 μ g of actinomycin D per ml was added 2.5 h after infection, and the isotope (1 mCi of [3 H]uridine in 25 ml of medium) was added at 3 h after infection. At 5 to 6 h after infection, the virus-infected cells were harvested by scraping and collected by centrifugation.

Isolation and purification of mengovirus RF. Virus-infected cells were resuspended in a hypotonic buffer solution (0.02 M Tris [pH 7.5], 0.0036 M CaCl₂, and 0.0014 M MgCl₂) and allowed to swell for 5 min at 0°C. The cells were then mixed with an equal volume of hypertonic buffer (1.0% Triton X-100, 0.0036 M CaCl₂, 0.01 M MgCl₂, 0.5 M sucrose, 0.25 M KCl, 0.04 M Tris, pH 7.5) and homogenized with a loose-fitting pestle 10 times. The broken cell suspension was centrifuged at 5,000 rpm in a Sorvall SS-34 rotor for 10 min, and the supernatant fluid was decanted. Cytoplasmic RNA was extracted from the supernatant fluid by sodium dodecyl sulfate (SDS)-chloroform-phenol, as described (15). The RNA was made 0.2 M in lithium acetate (pH 5.1) and precipitated by the addition of 2 volumes of ethanol at -20°C overnight.

Mengovirus RF was separated from single-stranded virus-specific and other cellular RNAs based on its differential solubility in high-salt solutions (3, 4, 22). The cytoplasmic RNA was dissolved in 1 ml of TLE (10 mM Tris-hydrochloride [pH 7.5], 0.10 M LiCl, 1 mM EDTA), and an equal volume of 4 M LiCl was added to that solution. The resulting 2 M LiCl solution was stored overnight at 4°C. The precipitate, containing single-stranded RNAs, was removed by centrifugation at $3,000 \times g$ for 45 min. The 2 M LiCl-soluble supernatant fluid, containing the double-stranded RF, DNA, and small RNAs, was made 1 M in LiCl by the addition of an equal amount of sterile deionized water and ethanol precipitated overnight at -20°C. The ethanol precipitate was suspended in TLE-0.5% sodium dodecyl sulfate, made 2 M in LiCl, and kept at 4°C overnight. The precipitate, if any, was removed by centrifugation, and the soluble RNA was precipitated with ethanol and suspended once again in TLE that was then made 2 M in LiCl. After the third LiCl precipitation, the sample was ethanol precipitated and suspended in TLE-0.1% SDS and stored at -70°C.

The 2 M LiCl-soluble RNA (in 0.5 ml of TLE-0.1% SDS) was mixed with an equal volume of 5% sucrose-NLE-SDS (10 mM sodium acetate [pH 5.5], 100 mM LiCl, 1 mM EDTA, and 0.1% SDS) and layered onto a 5 to 25% sucrose-NLE-SDS gradient. Water (3.5 ml) was layered on top of the sample layer and the gradient was placed in a vertical rotor (TVA-865; Du Pont, Wilmington, Del.) and centrifuged for 75 min at 50,000 rpm at 4°C. The gradient was collected in 1-ml fractions and assayed for radioactivity, and the peak fractions, which sedimented at 20S, were collected and precipitated by ethanol.

DNase I treatment of RF. RF obtained by velocity sedimentation centrifugation as described above was suspended in 0.5 ml of NTM (100 mM NaCl, 5 mM MgCl₂, and 10 mM Tris [pH 7.8]). DNase I, obtained from Calbiochem-Hoescht, Behring, La Jolla, Calif.,

was added to a final concentration of 50 U/ml and incubated for 30 to 60 min at 25°C. The reaction was terminated by the addition of 50 μ l of 0.1 M EDTA (pH 7.0). An equal volume of 5% sucrose-NLE-SDS was added to the terminated reaction mixture, and the resulting solution was layered onto a 5 to 25% sucrose-NLE-SDS gradient and subjected to velocity sedimentation in a vertical rotor for 75 min at 50,000 rpm (4°C), as described above. The gradient was fractionated, and the peak fractions, which sedimented at 20S, were pooled and precipitated with ethanol.

Bonding of avidin-coupled spheres to RF. Avidin-coupled spheres were bound to RF molecules as described by Richards et al. (16). Briefly, RF prepared as described was suspended in 0.1 M sodium bicarbonate to yield a final concentration of 25 μ g/ml. A 100- μ l sample of the RF solution was mixed with 10 μ l of dimethyl formamide containing 100 μ g of the *N*-hydroxysuccinimidyl ester of biotin (kindly provided by Jerry Manning, University of California, Irvine) and incubated at 4°C for 3 h. This mixture was then dialyzed overnight against 1 M NaCl-0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5). To the dialysate was added 30 μ l of the avidin-coupled spheres (20 mg/ml), and the suspension was incubated at 4°C for 24 h. The preparation of avidin-coupled spheres bound to RF was then layered onto a CsCl step gradient (2.45 ml of CsCl in 0.1 M HEPES [pH 7.5], $\rho = 1.52$; 2.45 ml of CsCl in 0.1 M HEPES [pH 7.5], $\rho = 1.23$) and subjected to centrifugation in an SW50.1 rotor at 45,000 rpm for 22 h at 4°C. The avidin-coupled spheres and spheres bound to the RF molecules formed an opalescent band approximately one-third of the distance from the top of the gradient. The opalescent band contained 77% of the uridine-labeled RF RNA; the remaining 23% was found in the pellet. The band was removed with a syringe, dialyzed against TNE buffer (10 mM Tris [pH 7], 100 mM NaCl, and 1 mM EDTA), and examined by electron microscopy.

Electron microscopy. Samples from the CsCl gradient fractions containing RF reacted with polymethacrylate spheres were diluted in 0.1 M NaCl-0.1 M Tris [pH 8.0]-0.01 M EDTA to a final RNA concentration of 0.5 μ g/ml and prepared for electron microscopy by using the aqueous basic protein technique of Kleinschmidt as described previously by Davis et al. (5). Grids were stained with uranyl acetate as described (17) and rotary shadowed with Pt:Pd (80:20) after which they were examined in a Philips 300 electron microscope. Photographs were taken on 35-mm film, and lengths of projected images of the molecules were determined with a Numonics 1224 digitizer interfaced with a Hewlett-Packard 9825A computer.

RESULTS

Purification of mengovirus RF. Mengovirus double-stranded RNA, termed RF, was isolated from infected BHK-21 clone 13 cells and purified essentially as described previously by Spector and Baltimore (22). The isolation of the RF is based on its solubility in 2 M LiCl. Mengovirus RF, subjected to three LiCl precipita-

FIG. 1. Sucrose density gradient centrifugation of mengovirus-specific RF RNA. (A) Viral specific RNA was labeled with [³H]uridine in the presence of actinomycin D, and 2 M LiCl-soluble RNA was applied to a 5 to 25% linear sucrose gradient. The viral RNA tracing (●) was coplotted with a cellular RNA marker (○), and the latter was centrifuged in a parallel gradient. (B) The 20S peak (▲) was pooled, DNase I-treated, and centrifuged on a 5 to 25% linear sucrose gradient.

tions, sedimented as a homogenous peak at a rate of 20S (Fig. 1A). Treatment of RF, which had been isolated from a sucrose gradient, with DNase I had no effect on its sedimentation rate (Fig. 1B).

DNase-treated mengovirus RF molecules were isolated as described above and examined by electron microscopy. Several forms were observed (Fig. 2): (i) linear RF molecules (Fig. 2A, B, and C), (ii) circular molecules possessing a short projection (Fig. 2A, arrow), and (iii) linear molecules with their ends opposed (Fig. 2B, arrow, and C, upper part). Although some molecules smaller than genomic length were present (Fig. 2C, arrows), the preparation of RF appeared to be rather homogeneous in length in each of the three forms. Before sphere attachment and CsCl gradient fractionation (see below), the RF preparation contained 7.1% circular duplexes with a projection, 6.0% linear duplexes with opposed ends, and only 1.0% circular duplexes without a detectable projection; a total of 298 molecules was examined by electron microscopy.

Binding of avidin-coupled spheres to RF molecules. The genome-linked protein, VPg, has been found to be covalently linked to the virus-specific RNAs of poliovirus (6, 7, 10, 16, 25), the genomic RNAs of foot-and-mouth disease virus (9, 20), encephalomyocarditis virus (8, 10), mengovirus (14), and calicivirus (21). In these studies, both biochemical and electron mi-

croscopic techniques have been employed. Using the avidin-coupled sphere method of Richards et al. (16), we initially sought to locate VPg on mengovirus linear RF molecules and circular RF molecules. Since VPg is expected to be found at or near the termini, its presence would then allow us to identify the termini of the molecules in the circular forms, a major objective in these studies.

An excess of the *N*-hydroxysuccinimidyl ester of biotin was reacted with mengovirus RF by means of the protein VPg. The mengovirus RF-VPg-biotin complex was then bound to avidin-coupled polymethacrylate spheres, as described above. Electron microscopic examination of the RF-bound spheres revealed several classes of linear molecules: (i) full length RF molecules possessing spheres on both termini (Fig. 3A and B), (ii) molecules possessing a single sphere on one end and multiple spheres on the other (Fig. 3C), (iii) molecules possessing multiple spheres on both ends (Fig. 3D), (iv) molecules of less than genomic length possessing a single sphere (Fig. 3E), and (v) infrequently occurring molecules possessing internally bound spheres (Fig. 3F). It should be emphasized that when multiple sphere attachment was involved, only one sphere of the aggregate was found to be in direct physical contact with the RF RNA molecule. Furthermore, in linear RF molecules, it was frequently observed that a sphere was attached at one terminus while a second sphere attached

FIG. 2. Electron micrographs of mengovirus RF RNA. Both linear and circular RF RNA molecules were detected. The circular molecules typically contained a short projection (indicated by the arrow in A). In addition, linear molecules with opposed ends were also detected (indicated by the arrow in B; upper part of C). Double-stranded molecules much shorter than genome length are indicated by the arrows in C. Bar, 1 μ m.

very near but not precisely at the other terminus, i.e., one terminus was seen to extend beyond the site of sphere attachment (Fig. 3A and F, arrows). This terminus extends approximately 1.0% of the genome length beyond the boundary of the sphere. Furthermore, a second site of sphere attachment appeared at a distance of 10 to 20% of the genome length from one of the termini (Fig. 3D at right and Fig. 3F) in approximately 6% of the linear duplexes and 13% of the circular duplexes with one or more attached spheres, which may reflect the presence of an additional VPg-like molecule or some other protein bound to the RF RNA.

Several unusual features require further comment. There were numerous unattached as well as attached spheres which possessed small protuberances that were distinguished from the projections on circular structures. A small protuberance is evident on the single sphere in the lower right corner of Fig. 3C. The source of these protuberances is unknown, but it should be

noted that very small RNAs have been observed in preparations of mengovirus and may account for these protuberances (manuscript submitted for publication). These protuberances were not observed in the poliovirus studies described by Richards et al. (J. Manning, personal communication). A second unusual phenomenon was the clumping of avidin-coupled spheres. The clumps of avidin-coupled spheres were rarely found except when bound to mengovirus RF. A random sampling of 212 spheres in the background of the CsCl gradient fraction containing RF revealed that 73% occurred as single nonaggregated spheres, 17% as aggregates of two spheres, 6% as aggregates of three spheres, 2% as aggregates of four spheres, and 2% as aggregates of five spheres. The significance of the clumps of spheres bound to one end of the RF remains to be determined. However, the clumping of spherical aggregates of antibodies was reported by Wu et al. (25) in antibody-treated 2,4-dinitrophenol derivatives of poliovirus RF

(see below).

Having demonstrated that both termini, bearing VPg, could be labeled with the avidin-coupled spheres, it was of interest to determine the location of the termini in circular forms of mengovirus RF. As mentioned above, mengovirus circular RF molecules possessed a single short projection per circle. In 73% of the molecules scored (sphere-bound circles) the projection was obscured. The termini of the RF molecules, therefore, are at or in close proximity to the projection. Figure 4A presents a typical circular RF molecule with two spheres attached in close proximity but lying on the circular contour of

the RF molecule. Most circular forms had two spheres attached (Fig. 4A and B). There were also circular forms possessing a single attached sphere (Fig. 4D and E), multiple attached spheres (Fig. 4E), and internally bound spheres (Fig. 4F). In those circular structures possessing two or more spheres which indicated the positions of the termini of linear RF within the circular structures, it was found that the termini were physically separated by only a small unmeasurable distance on the circular contour (Fig. 4A, B, C, and F). This would indicate that in the circular forms of RF, the VPg molecules at the termini of the linear molecules are not

FIG. 3. *Electron micrographs of mengovirus linear RF RNA molecules with attached polymethacrylate spheres. (A and B) A single sphere is attached near each terminus of the linear molecules. The arrow (A) depicts one terminus which protrudes slightly beyond the boundary of the sphere. (C) An aggregate of four spheres is attached to one terminus and a single sphere is attached to the other terminus of the linear molecule. (D) Two spheres are attached at one terminus and an aggregate of six spheres is attached near but not at the other terminus, where a single sphere is attached. (E) A sphere is attached at only one terminus. (F) One sphere is attached near each terminus as well as a single site located on the linear molecule. The arrow (F) depicts the terminus of the molecule which protrudes beyond the boundary of the sphere. Bar, 1 μ m.*

FIG. 4. Electron micrographs of mengovirus circular RF RNA molecules with attached electron-opaque polymethacrylate spheres. Samples were prepared for electron microscopy by the aqueous technique of Kleinschmidt described previously by Davis *et al.* (5) and contrasted by rotary shadowing with Pt:Pd. (A and B) Two spheres are attached at apparently adjacent sites indicated by arrows on each of the circular molecules. (C) Two spheres joined in tandem appear attached at a single site on the circular molecules. (D and E [at left]) A single sphere is attached to the circular molecules. (E [at right]) An aggregate of seven spheres is attached at a single site of the circular molecule. (F) An aggregate of four spheres is attached at one site, and a single sphere is attached at a second site on the same circular molecule. Bar, 1 μ m.

juxtaposed but do lie in close proximity to one another.

Linear and circular dimers of mengovirus RF RNA have also been detected at a low frequency of occurrence (D. Robberson, M. Marshall, G. Thornton, and R. Arlinghaus, manuscript submitted for publication). These species also reacted with avidin-coupled spheres to give structures presented in Fig. 5. The circular dimers frequently contained two or more sites of sphere attachment (Fig. 5A), whereas linear dimers most frequently were seen to contain sites of sphere attachment at or near the termini (Fig. 5C) as well as at a site internally located on the molecule (Fig. 5D). A circular monomer was

occasionally seen attached to the terminus of a linear molecule through one or more avidin-coupled spheres (Fig. 5B). In such cases, the projection on the circular RF molecule was obscured by sphere attachment and, by inference, must be located near the site of sphere attachment.

Table 1 summarizes the frequencies of different structures observed in the avidin-coupled sphere bound RF preparation. Of significance is the fact that 63% of the linear RF molecules possessed spheres on both ends, indicating that at least one VPg molecule is at or near each terminus. However, 98% of the linear RF molecules had one or more spheres attached. Of the circular forms, 98% of the molecules contained

FIG. 5. Electron micrographs of mengovirus circular and linear oligomeric RF RNA molecules with attached polymethacrylate spheres. (A) The circular dimer form has two spheres attached near the same site (indicated by arrows) and a single sphere attached at a remote site. A linear monomer with a sphere at each terminus is shown at the top of the micrograph. (B) A circular monomer is joined to a linear monomer through an aggregate of four spheres in the region indicated by arrow. (C) A linear dimer has one sphere attached near one terminus and two spheres, close to each other, attached near the other terminus. (D) One sphere is attached at each terminus and one sphere is attached at an internal site indicated by the arrow on the linear dimer molecule. Bar, 1 μ m.

spheres, 43% contained one sphere, and 50% contained two spheres. It should be emphasized that sphere attachment nearly always occurred at a site that obscured the projection. Also, the frequency of circular forms in these preparations was significantly higher than in preparations not reacted with biotin-bound avidin-coupled spheres. One possible explanation for this occurrence is that the reaction with the avidin-coupled spheres may have induced a greater stability in circular forms, preventing their dissociation upon spreading for the electron microscopy.

DISCUSSION

We have been able to demonstrate the presence of protein, presumably VPg, on both termini of mengovirus RF by the avidin-coupled sphere technique of Manning et al. (11). Previous studies with this method of poliovirus RF (16) revealed a preferential labeling of only one end of the RF molecule with the avidin-coupled spheres, the end possessing the 5' terminus of the positive strand. Wu et al. (25), however, by using a different method (that of reacting anti-

2,4-dinitrophenol antibodies with the 2,4-dinitrophenol-derivatized VPg of RF molecules), were able to label both ends of poliovirus RF. It was suggested by Richards et al. (16) that the inability to label the 5' end of the negative strand of the poliovirus RF might be due to one of several factors, the most probable of which were (i) that the polyadenylic acid sequence on the 3' end of the positive strand is longer than the polyuridylic acid sequence on the 5' end of the negative strand, which might mask VPg at the 5' end of the negative strand, preventing esterification with biotin, and (ii) that there are two different VPg molecules, one capable of being esterified to biotin more readily. Both of these possibilities may, in fact, be responsible for attachment of only a single sphere to poliovirus RF. In fact, the studies described in this paper were initially undertaken, in part, because mengovirus has been shown to possess a short sequence of polyadenylic acid on its 3' terminus (12, 23). With this in mind, we felt that we could label both termini of the mengovirus RF without having to contend with a long polyadenylic acid

TABLE 1. *Frequencies of mengovirus RF RNAs with attached avidin-coupled polymethacrylate spheres^a*

RF RNA	No. of molecules	% of total
Genomic-length molecules		
Linear forms	43	45
Circular forms	44	46
Opposed ends	8	9
Genomic-length linears		
No spheres	1	2
Spheres on one end only	15	35
Spheres on both ends	27	63
Genomic-length circles		
One sphere, projection absent	19	43
Two adjacent spheres, projection absent	13	30
Two adjacent spheres, projection absent (loops)	9	20
Spheres present on projection	2	5
No spheres	1	2

^a The frequencies of internal sphere attachment mentioned in the text have been omitted from this tabulation.

sequence similar to that found in poliovirus RNA. As reported here, 65% of the mengovirus RF molecules were labeled at both termini, in contrast to the labeling of 13% of the RF molecules of poliovirus (16). Although we were able to label both ends of a picornaviral RF possessing a short polyadenylic sequence, it cannot be ruled out that there are chemically different VPg molecules possessing different labeling affinities (see below). In fact, King et al. (9) have recently demonstrated that there are two chemically different VPg's in foot-and-mouth disease virus RNA preparations.

Wu et al. (25) have described a class of RF molecules possessing a protein aggregate on one end of poliovirus RF and have termed these complex molecules. Most of these protein aggregates could be removed by banding the RF in CsCl-guanidine hydrochloride gradients. These additional protein molecules were not, therefore, covalently bound.

We report here the occurrence of complex RF molecules in preparations of mengovirus RF and note that there appears to be a preferential labeling of one or the other end of these molecules with two or more spheres; however, the observation that only one sphere of an aggregate lies in contact with the RF molecule suggests that only one VPg molecule has reacted with a sphere or aggregate of spheres at this site. Other VPg molecules may in fact be nearby but are not detected as a result of physical exclusion by

the sizes of spheres that were utilized. Whether or not the VPg or protein molecules on both ends of the RF are the same or different remains to be determined. It is also possible that one type of VPg molecule, or protein, has a greater affinity for one end of the RF molecule than the other.

A second major objective of this work was locating the termini of linear RF molecules within the mengovirus circular structures previously described (Marshall et al., *J. Cell Biol.* 70:284a, 1976) (Fig. 2). We have presented evidence that the spheres which are bound to the circular structures obscure a small projection we have previously observed to occur on circular molecules (Marshall et al., *J. Cell Biol.* 70:284a, 1976). The observation that the spheres are joined to the circular RF molecules at two distinct but closely separated points and that most circles possess two or more such spheres lying side by side suggests the close relative position of the VPg's but excludes the possibility that the VPg molecules are juxtaposed within most circular structures.

Another observation which must be accounted for is the increased frequency of circular RF RNA in the preparations reacted with avidin-coupled spheres. It appeared that the reaction increased the stability of the circles, possibly by cross-linking the VPg molecules or effecting a more stable structure which is able to withstand the spreading forces during preparation for electron microscopy.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants CA-25465, CA-16672 and CA-16527 from the National Institutes of Health and grants G-429 and G-841 from The Robert A. Welch Foundation. G.B.T. was supported by funds from the Research Council of Abilene Christian University.

We thank Susan Berkley, Carolyn Peterson, and James Syrewicz for excellent technical assistance and Rebecca Bazer for assistance in manuscript preparation. We especially thank Jerry Manning for providing reagents with which to do the biotin cross-linking and the avidin-coupled sphere binding.

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