Strand-specific attachment of avidin–spheres to double-stranded poliovirus RNA

(RNA–biotin–avidin interaction/electron microscopy/nucleic acid hybridization)

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ABSTRACT   Poliovirus-specific double-stranded RNA molecules containing covalently attached protein were coupled with a biotin ester through the protein moiety. Subsequent interaction of the RNA–biotin with avidin attached to electron-opaque plastic spheres led to the formation of complexes that were easily visualized in the electron microscope. Avidin-spheres were associated only with one end of the RNA–biotin molecules, as seen in the electron microscope. Avidin–sphere attachment to poliovirus double-stranded RNA is strand specific, as shown by molecular hybridization of strand-specific probes to the separated strands of denatured complexes. [³H]DNA complementary to polio virion RNA hybridized exclusively to the strands bearing associated spheres [(+) strands] whereas ¹²⁵I-labeled virion RNA hybridized predominantly with strands without spheres [(−) strands]. This biotin–avidin labeling technique provides a means for the isolation of full-length poliovirus (−) strands and may provide a general means for isolation of double-stranded polynucleotides containing tightly attached protein.

The RNA molecules encapsidated in the virions of poliovirus have been shown to be covalently attached to a small basic protein, designated VP$_g$ at the 5′ terminus of the genome RNA (1–3). The presence of a genome-linked protein is thus far unique among the single-stranded RNA viruses but appears to be a common feature of picornaviruses (4, 5). Although sequences coding for VP$_g$ have not been mapped on the viral genome, it is likely that the protein is virus-coded because it is synthesized after infection when virus-induced inhibition of host cell protein synthesis has occurred (1). In addition, the proteins isolated from poliovirus RNA and from encephalomyocarditis virus RNA, grown in the same host cell, display different mobilities in sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gels (6) and thus appear to be specific for the infecting virus.

The role that VP$_g$ plays in the replication of the picornaviruses has not been established. However, analysis of replicative intermediates and of double-stranded RNA isolated from the infected cell showed that the complementary (−) strands of these molecules were also linked to a protein (2, 7) and suggested that virtually all viral RNA molecules were attached to protein during their biosynthesis. With the development of the avidin–biotin technique utilized for gene enrichment and gene detection (8–10), a means was available for the selective labeling of poliovirus RNA molecules by attachment of electron-opaque avidin–spheres to the protein moiety of poliovirus RNA molecules that had been coupled to a biotin ester to form a RNA–biotin compound. Thereafter, these molecules can be specifically enriched by biochemical methods and can be examined in the electron microscope. In this report, we show that only one

strand of viral RNA in the double-stranded molecule is capable of reacting in the biotin–avidin–sphere reaction, and that this reaction is limited exclusively to the (+) strand. This labeling technique may prove useful for isolation of nucleic acids with an attached protein moiety.

MATERIALS AND METHODS

Cells, Virus, and Growth. Suspension cultures of HeLa S3 cells were grown as described (11). Cells were concentrated to 5×10^6/ml and were infected with the Mahoney strain of poliovirus type 1 at a multiplicity of 10–100 plaque-forming units per cell at 37°C. In some preparations, poliovirus RNA was labeled with [¹⁴C]uridine at 1 µCi/ml (1 Ci = 3.7×10^{10} becquerels) or with [³H]uridine and [³H]adenosine, each at 1 µCi/ml, in the presence of actinomycin D (5 µg/ml). Infected cells were collected by centrifugation 5–6 hr postinfection, poliovirus was purified from cytoplasmic extracts as described (11), and the virions were dialyzed against 0.15 M NaCl/0.01 M Tris·HCl/5 mM EDTA, pH 8.3 (TNE buffer).

Preparation of RNA. RNA was prepared from dialyzed virions by making the suspension 1% in NaDodSO$_4$ and 10 µg/ml in polyvinyl sulfate and by extracting with TNE-saturated phenol two times at room temperature. The aqueous phase was collected and adjusted to 0.2 M sodium acetate (pH 5.5), and poliovirus RNA was precipitated with 2 vol of ethanol and kept at −20°C overnight. RNA was collected by centrifugation at 16,000 × g at 0°C for 30 min. The pellet was washed once with 70% ethanol/0.2 M sodium acetate, pH 5.5 and recentrifuged. The RNA was dissolved in TE (0.01 M Tris·HCl/0.01 M EDTA, pH 7.4 (TE buffer) and layered over a 15–30% sucrose gradient in 0.1 M NaCl/0.01 M Tris·HCl/2 mM EDTA/0.2% NaDodSO$_4$, pH 7.4 (NETS buffer) to obtain RNA sedimenting at 35 S (Fig. 1A). The bracketed fractions were combined, made 0.2 M in sodium acetate and 70% in ethanol, and kept at −20°C overnight. RNA was collected by centrifugation, as above.

Double-stranded poliovirus RNA (RF) was obtained from the crude cytoplasmic extract by adjusting it to 1% in NaDodSO$_4$, 10 µg/ml in polyvinyl sulfate, 0.1 M in NaCl, 20 mM in Tris·HCl, and pH 7.4, and 10 mM in EDTA, extracting two times with TNE-saturated phenol, and ethanol precipitating, as above. The cytoplasmic nucleic acids were fractionated in 2 M LiCl and in a 15–30% rate zonal sucrose in NETS gradient (12). Material sedimenting at 20 S was pooled and precipitated with ethanol. The RNA was dissolved in 0.4 ml of TE buffer and
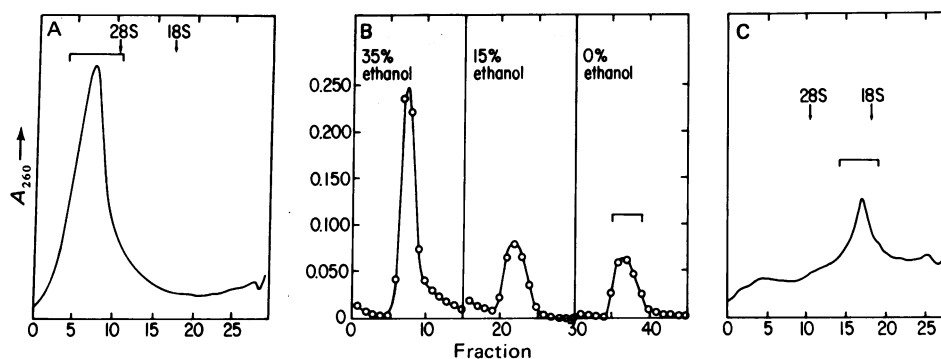
FIG. 1. Purification of polio virion RNA and double-stranded poliovirus RNA (RF). (*A*) Rate zonal (15–30% sucrose in NETS) sedimentation of polio virion [³H]RNA in SW 41 rotor at 40,000 rpm, 20°C, for 5.7 hr. (*B*) Cellulose CF-11 chromatography of ¹⁴C-labeled material sedimenting at 20 S in a rate zonal sucrose gradient; 2-ml fractions were collected in the appropriate eluting solvent. (*C*) Rate zonal (15–30% sucrose in NETS) sedimentation of bracketed fractions from *B* in SW 40 rotor at 25,000 rpm, 20°C, for 14.5 hr. Arrows in the rate zonal sedimentation profile denote positions of HeLa [³H]rRNA in external marker gradients. The brackets denote pooled fractions of purified 35S virion RNA (*A*) and 20S [¹⁴C]RF (*C*).

adjusted to 0.1 M in NaCl, 0.01 M in Tris·HCl, 1 mM in EDTA, pH 7.0, and 30% in ethanol. This material was chromatographed through a cellulose CF-11 column (19 × 0.9 cm) according to Bishop and Koch (13). An elution pattern such as that in Fig. 1*B* was obtained. The material eluting in salt without ethanol (bracketed fractions) was pooled, adjusted to 0.2 M in sodium acetate and pH 5.5, and precipitated with 2 vol of ethanol. This precipitated material was further fractionated by rate zonal (15–30% sucrose in NETS) sedimentation to give an absorption profile as shown in Fig. 1*C*. Material sedimenting at 20 S was pooled (bracketed fractions) and collected by ethanol precipitation and centrifugation. The RF (20S) material was dissolved in 0.1 M NaHCO₃ for biotin labeling.

Coupling of Avidin–Spheres to RF. Either radiolabeled or nonradiolabeled RF (20S; 2.0–2.4 μg in 100 μl of 0.1 M NaHCO₃) was incubated with 10 μl of dimethylformamide containing 100 μg of the *N*-hydroxysuccinimidyl ester of biotin at 4°C for 2–3 hr. This mixture was dialyzed against 1 M NaCl/0.1 M Hepes, pH 7.5, with two changes of medium at 4°C overnight. The dialyzed solution was incubated with avidin-labeled polymethacrylate spheres (8) at a 5- to 6-fold excess of avidin–spheres to RF–protein, assuming one protein molecule per RNA strand, at 4°C for 2–20 hr. This mixture was layered onto a CsCl step gradient containing 2.45 ml of CsCl in 0.1 M Hepes (pH 7.5; ρ = 1.52) and 2.45 ml of CsCl in 0.1 M Hepes (pH 7.5; ρ = 1.23) and centrifuged in an SW 50.1 rotor at 45,000 rpm at 15°C for 22 hr. This step widely separates avidin–spheres coupled to biotin–RF molecules (sphere band in upper third of gradient) from uncoupled RF molecules (which pellet) (9). The sphere band, which is easily discernible by light scattering, was removed with a syringe and analyzed by three methods: (*i*) visualization of spheres coupled to RF molecules in the electron microscope, (*ii*) determination of acid-precipitable radioactivity, and (*iii*) denaturation and isopycnic centrifugation in CsCl to determine the strand specificity of the avidin coupling. The remainder of the CsCl solution was poured from the centrifuge tube and the pellet was dissolved in TE buffer containing 0.2% NaDodSO₄ for analysis of acid-precipitable material.

Electron Microscopy of RF Molecules Coupled to Spheres. RF molecules present in the sphere band were directly mounted for examination in the electron microscope by the formamide modification of the Kleinschmidt procedure (14). The RNA was spread from a hyperphase of 50% formamide in 0.1 M Tris·HCl/0.01 M EDTA, pH 7.5, onto a hypophase of 20% formamide in 0.01 M Tris·HCl/1 mM EDTA, pH 7.5. Electron micrographs were normally taken at a magnification of

×30,000. The relative lengths of the molecules were determined by using the Tektronix 4956 graphics tablet in conjunction with the Tektronix 4051 computer. The length of unit length RF was taken to be 7.60 kilobases (kb). G4 RF DNA was initially cospread with unit length RF. Using 5.5 kb for the length of G4 RF DNA (15), a relative base-pair separation (RF/RF DNA) of 0.91 was observed for these spreading conditions. This ratio was subsequently used for standardizing the lengths of fragmented RF molecules cospread with G4 RF DNA.

Denaturation of RF Molecules Coupled to Spheres and Separation of RNA Strands. The sphere band from the initial CsCl isopycnic centrifugation was diluted with 0.5 M NaCl/0.1 M Hepes, pH 7.5, and the spheres were pelleted in an SW 50.1 rotor at 30,000 rpm at 4°C for 2 hr. The strands of RF molecules coupled to spheres were denatured either by suspension of the spheres in 10 μl of 10 mM Hepes (pH 7.5) plus 90 μl of dimethyl sulfoxide and heating at 40°C for 4 min or by suspension of the spheres in 30 μl of 10 mM Hepes (pH 7.5) and heating in a sealed capillary at 104°C for 3 min. These denatured samples were rapidly cooled to 15°C and centrifuged in an isopycnic CsCl gradient, as described above. After centrifugation the sphere band and pellet were collected for analysis of acid-precipitable radioactivity and/or hybridization with a poliovirus [¹²⁵I]RNA (+) strand probe or with a poliovirus [³H]cDNA (−) strand probe to determine the specificity of the strands present in the sphere band and in the pellet. The material in the sphere band was diluted and pelleted, as described above, and suspended in TE buffer containing 0.2% NaDodSO₄. The pellet was dissolved directly in TE containing 0.2% NaDodSO₄.

Preparation of Poliovirus ¹²⁵I-Labeled RNA (¹²⁵I-RNA). Virion RNA (35S; 5 μg) was iodinated in a polypropylene reaction tube with 1.5 mCi of carrier-free Na¹²⁵I (New England Nuclear) by the procedure of Prensky (16). The ¹²⁵I-RNA was purified by gel filtration through Sephadex G-50, and the excluded material was precipitated with ethanol and fractionated by isopycnic centrifugation in Cs(OAc)₃ as described by Burke *et al.* (17). Fractions containing ¹²⁵I-RNA were pooled, diluted 1:4 with water, adjusted to 0.2 M in sodium acetate, and ethanol precipitated. This RNA had a specific activity of about 3 × 10⁷ cpm/μg.

Synthesis of [³H]cDNA from Poliovirus RNA. [³H]cDNA was synthesized from 35S poliovirus RNA (2.3 μg) by using avian myeloblastosis virus reverse transcriptase (gift of J. Beard, Life Sciences, Inc.) and 0.4 mCi of [*methyl*-³H]dTTP (New England Nuclear) by the procedures of Kacian and Myers (18) and Myers *et al.* (19). The [³H]cDNA was purified by described procedures (19) and fractionated in a 5–20% alkaline sucrose
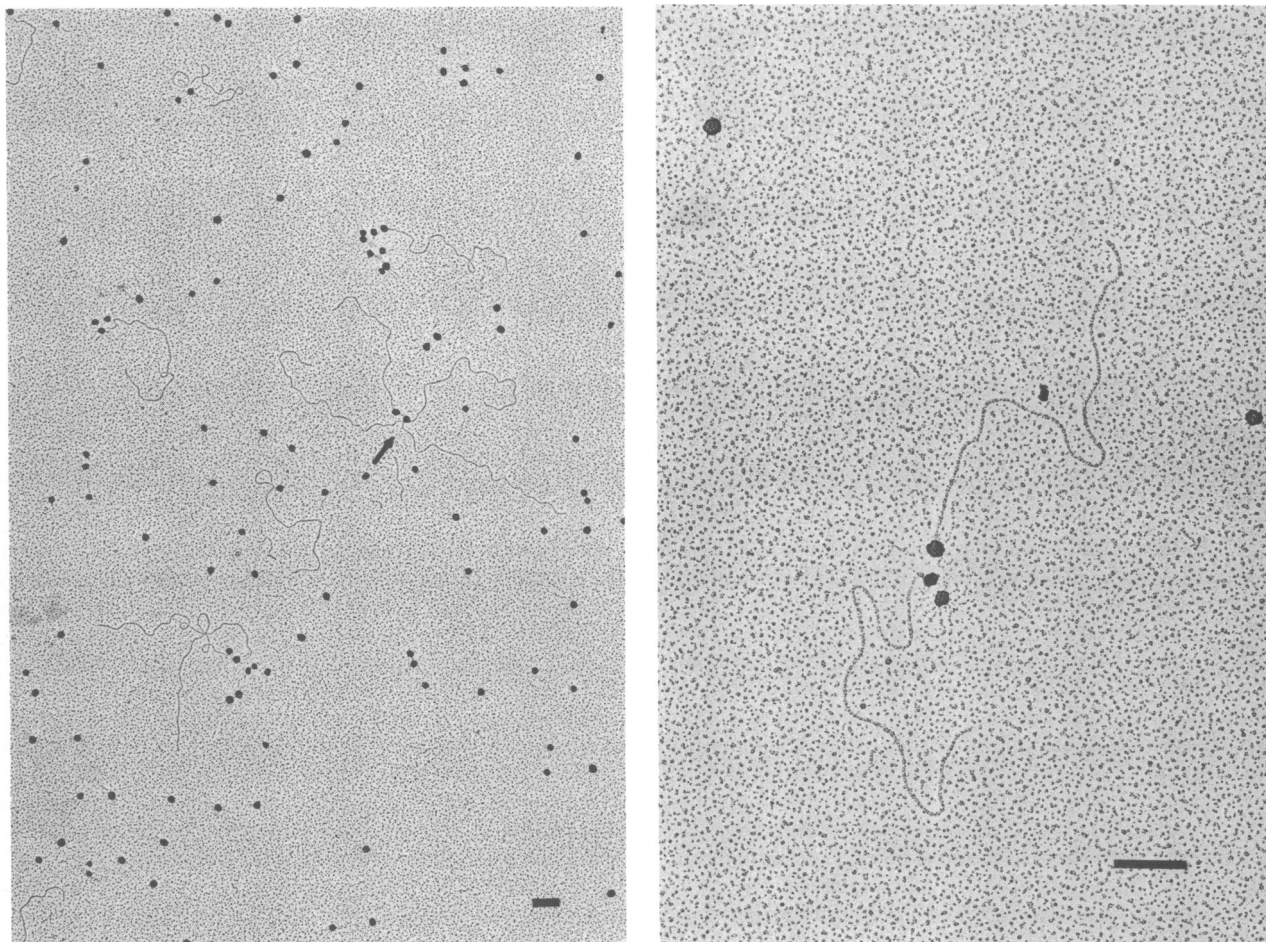
FIG. 2.   Electron micrographs of RF–biotin–avidin–sphere complexes. Isolated from a CsCl isopycnic gradient, dialyzed, and spread for examination. All RF molecules associated with spheres represent full-length RF RNA. Occasionally, multiple RF molecules were seen associated with a single sphere (arrow in *Left*). Scale = 1 kb of RNA.

gradient in 0.7 M NaCl/0.3 M NaOH/1 mM EDTA. Material sedimenting between 10 and 17 S was combined and neutralized, 100 μg of yeast RNA was added, and the mixture was adjusted to 0.2 M in sodium acetate and ethanol precipitated. The [³H]cDNA was fractionated on hydroxyapatite at 60°C and >95% of the probe eluted as single-stranded material; this material was dialyzed against TE buffer, adjusted to 0.2 M in sodium acetate, and ethanol precipitated. The [³H]cDNA saturated at least 65% of the ¹²⁵I-RNA probe and had a specific activity of about 8×10^6 cpm/μg.

Hybridization Reactions. RNA·RNA hybridization reactions with the ¹²⁵I-RNA probe were performed by denaturation of the samples plus 50 μg of yeast RNA in 1 mM EDTA at 104°C for 3 min followed by addition of the probe and salt mixture to 0.3 M in NaCl, 0.02 M in Tris·HCl, 2 mM in EDTA, and pH 7.5, to give a total volume of 30 μl. Incubations were in sealed capillaries at 75°C for 90 min followed by transfer to 1 ml of 0.30 M NaCl/0.03 M sodium citrate and incubation with a RNase mixture (50 μg of RNase A/ml and 5 units of RNase T1/ml) at 37°C for 30 min. Acid-precipitable material was collected and assayed for radioactivity.

cDNA·RNA hybridization reactions were performed by denaturation of the [³H]cDNA probe with the RNA samples and 50 μg of yeast RNA in 1 mM EDTA at 104°C for 3 min followed by addition of a salt mixture to give final concentrations of 0.6 M in NaCl, 10 mM in Tris·HCl, and 3 mM in EDTA and pH 7.4 in 30 μl and incubation in sealed capillaries at 67°C for 4 hr. After hybridization, samples were transferred to 1 ml

of 0.03 M sodium acetate, pH 4.5/0.3 M NaCl/3 mM ZnCl₂ containing 10 μg of alkali-denatured calf thymus DNA per ml for digestion with nuclease S1 (Sigma) at 50°C for 45 min. Acid-precipitable material was collected and assayed for radioactivity. Sufficient amounts of nuclease S1 were used such that 96% of single-stranded DNA was hydrolyzed in these incubation conditions with no detectable decrease in acid-precipitable material from duplex DNA.

RESULTS

Coupling of Avidin–Spheres to RF Molecules. Because poliovirus RNA has been shown to have a protein covalently attached to its 5′ end (1–3) and because the (−) strand of polio replicative intermediate has also been shown to possess a covalently linked protein (2, 7), these molecules appeared to be good candidates for coupling with the electron-opaque avidin–spheres (8) and subsequent analysis of the protein-bearing RNA strands. Initially, RF molecules were examined because they provide an exellent system for determining the efficiency with which a nucleic acid molecule may be physically isolated by virtue of possession of a tightly bound protein(s). Thus, RF molecules were treated with a biotin ester (8) under mild conditions such that only protein amino groups react. The vast excess of biotin ester molecules (about 300,000-fold excess) was removed by dialysis and the RF–biotin molecules were coupled to avidin–spheres (9). This reaction mixture was then fractionated directly in an isopycnic CsCl gradient to separate RF molecules coupled to avidin–spheres from those not coupled

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Table 1. RF molecules associated with spheres

RF	Molecules, no.	% of total
Sphere on one end	455	83
Spheres on both ends	7	1.3
No sphere on molecule	81	15
Sphere in middle	2	0.4

Table 2. Distribution of radioactivity in poliovirus RNA after reaction with avidin–spheres

Exp.	RNA	Sphere band,* cpm	Pellet,* cpm	% of total in sphere band
1	RF	8100	575	93
2	RF	2440	2820	46
	Denatured RF†	102	120	46
3	RF	1800	810	69

* Data refer to distribution of radioactivity in the sphere band and in the pellet from CsCl isopycnic gradients.
† Denatured RF refers to RF molecules coupled to avidin–spheres in an initial CsCl isopycnic gradient that were denatured in dimethyl sulfoxide and fractionated again in a CsCl isopycnic gradient.

to spheres and pelleted in the gradient. The spheres formed a sharp band in the top third of the gradient and were readily discerned by light scattering. Electron micrographs of RF molecules attached to avidin–spheres are shown in Fig. 2. Spheres were attached to RF molecules predominantly at one end of the structure only. Of a total of 545 full-length RF molecules picked at random on electron microscope grids, 83% (455) had spheres at one end only (Table 1); 15% of the molecules had no associated spheres, and only 1.3% had spheres at both ends. Clearly there is not a statistical distribution with molecules averaging one sphere per RF molecule, and we conclude that only one sphere couples to an RF molecule under our incubation conditions. In these experiments we started with full-length RF molecules (20 S; Fig. 1C).

It was essential to show that the molecules observed in the electron microscope remained full-length. A histogram of the lengths of 117 RF molecules bearing avidin–spheres is shown in Fig. 3A. Of the 117 molecules measured, 98 were unit length with a mean (±SEM) length of 7.59 ± 0.39 kb, and all had spheres on one end. For molecules bearing spheres but of less than unit length, the mean was 4.17 ± 1.49 kb. For molecules without spheres (Fig. 3B), the mean length was 3.7 ± 2.4 kb. The sum of the mean lengths of the latter molecules and those containing spheres but less than unit was 7.87 kb or full length. This excludes the possibility that the spheres were predominantly attached to molecules of less than full length, possessing only one of the two termini of the parent RF molecule. Additionally, rate zonal sedimentation analysis of RF molecules that were not coupled to avidin–spheres in the same reaction mixture and were obtained from the pellet in the CsCl gradient still sedimented at 20 S, indicative of full-length RF molecules.

Frequently the RF molecules were radiolabeled so that their distribution in the CsCl gradients could be ascertained. The
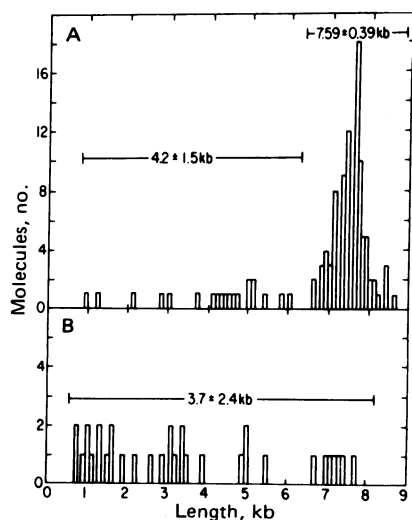
extent of coupling of RF molecules to avidin–spheres was variable (46–93%) (Table 2). This variability may be attributed to the incubation conditions either for the biotin reaction or for the coupling with avidin–spheres.

Specificity of Sphere Binding to Strands in RF Molecules. It was important to establish whether avidin–sphere coupling to RF molecules occurred at a specific end or was a random event. The sphere band from the initial CsCl isopycnic centrifugation, containing RF-biotin-avidin–sphere complexes, was collected and the RF molecules were denatured either by heat or by dimethyl sulfoxide and then fractionated again in an isopycnic CsCl gradient. There was an equal distribution of ^{3}H in both fractions (Table 2, Exp. 1), indicating that the strands had been separated under these conditions of denaturation.

Next, the sphere band and the pellet from CsCl gradients fractionating denatured RF–biotin–avidin–sphere complexes were analyzed for strand specificity by hybridization with strand-specific probes. Hybridization with an ^{125}I-labeled poliovirus RNA probe was utilized to detect the presence of (−) strands. Only the pellet contained significant levels of (−) strands (Table 3). Therefore, the avidin–spheres were not attached to (−) strands under our incubation conditions. Furthermore, the strands of RF remained essentially intact because (−) strands were not distributed between both fractions. This finding was supported by hybridization of both the sphere band and the pellet with a [^{3}H]cDNA probe that detects the presence of (+) strands. Only the sphere band had detectable (+) strands (Table 4), clearly showing that the presence of the sphere does not prevent hybridization. It must be concluded, therefore, that coupling of avidin–spheres to RF–biotin molecules is strand

FIG. 3. Histograms of RF with (*A*) and without (*B*) avidin–spheres. Means ± SEM are shown.

Table 3. Hybridization of strands of RF with ^{125}I-labeled poliovirus RNA

Sample*	No RNase	With RNase	% RNase resistance†
Sphere band, 2 μl	6270	149	0.1
Sphere band, 6 μl	6114	151	0.2
Sphere band, 18 μl	5990	194	0.9
Pellet, 2 μl	6940	735	8.3
Pellet, 5 μl	6350	2545	38
Pellet, 15 μl	6570	4268	63

* The sphere band from the CsCl gradient was concentrated to 50 μl total volume in which the concentration of RNA strands was approximately 0.3 to 0.6 ng/μl. The pellet from the CsCl gradient was resuspended in 50 μl of TE/0.2% NaDodSO₄ at a concentration of 0.3 to 0.6 ng/μl.
† The RNase resistance of the probe was 2.3% and has been subtracted in determinations of % RNase resistance.

Table 4. Hybridization of strands of RF with [³H]cDNA

Sample*	cpm		% S1 resistance†
	No S1	With S1	
Sphere band, 2 µl	972	126	8.3
Sphere band, 5 µl	1159	235	15.5
Sphere band, 15 µl	1132	435	34
Pellet, 2 µl	1184	38	0
Pellet, 5 µl	1154	39	0
Pellet, 15 µl	1232	47	0

* The sphere band and the pellet from CsCl gradients were concentrated and suspended in 50 µl total volume, as described in the Table 3 legend, at a concentration of 0.3–0.6 ng/µl.
† The nuclease S1 resistance of the probe was 4.7% and has been subtracted in determinations of % S1 resistance.

specific and clearly specific for the protein at the 5′ terminus of the (+) strand.

DISCUSSION

This study has shown that avidin–spheres are attached to one end of RF and that attachment is exclusively to the (+) strand. It is assumed that this coupling is specific for the protein found covalently attached to the 5′ terminus of this strand (1–3). Nomoto *et al.* (2) and Pettersson *et al.* (7) have presented evidence for a protein also being covalently attached to the 5′ terminus of the (−) strand in replicative intermediate and RF structures, but we did not find sphere attachment to (−) strands. The reason for this is uncertain but there are several possible explanations: (*i*) the absence of protein on the 5′ terminus of the (−) strand in our RF molecules, (*ii*) the presence of different proteins on the (+) and (−) strands having different reactivities with biotin ester, and (*iii*) the presence of a conformational restriction that protects the protein on the 5′ terminus of the (−) strand from reaction with biotin or avidin spheres.

With respect to first possibility, electron microscope (20) and biochemical (2, 7) data indicate that a protein is present at the 5′ terminus of the (−) strand. Therefore, this possibility does not seem likely. With respect to the second possibility, Pettersson *et al.* (7) and Nomoto *et al.* (2) showed that the protein associated with the 5′ terminus of the (−) strand of replicative intermediate RNA and RI has the same electrophoretic mobility as the virion RNA VP_g. However, it should be noted that identical electrophoretic mobilities of small proteins does not necessarily indicate that they have identical molecular weights (21). Therefore, the second possibility cannot be completely eliminated. It would appear that the third possibility is the most likely explanation for the appearance of avidin–spheres on only one end of the RF molecules. If, as is likely, the third possibility is correct, some proteins may be isolated by this biotin–avidin–sphere technique and some may be sterically shielded. With reference to the present study, the data of Yogo *et al.* (22) suggests that the poly(U) segment at the 5′ terminus of the (−) strand in RF is 60–90 nucleotides long. The data of Yogo and Wimmer (23) and of Spector and Baltimore (12) indicate that the poly(A) segment on the 3′ terminus of the (+) strand of RF is 140–200 nucleotides long. Thus, the possibility exists that a nonpaired segment of a longer poly(A) stretch in the (+) strand may protect a protein on the 5′ terminus of the (−) strand, preventing reaction with either biotin or avidin–spheres.

The specific coupling of avidin–spheres to the (+) strand of RF presents a novel approach for the preparation of a pure population of poliovirus (−) strands, perhaps in a much better yield than possible heretofore by other techniques (24). RF–biotin–avidin–sphere complexes isolated in an initial CsCl isopycnic gradient may be collected, pelleted, denatured, and rebanded in a CsCl isopycnic gradient. The pellet from this gradient contains an essentially pure population of (−) strands, as shown by the hybridization analyses in this study. These strands may then be utilized in various ways for studies of the replication process of poliovirus. Furthermore, this procedure offers a means for the physical purification in high yield of double-strand polynucleotides containing a tightly bound protein moiety that is reactive with avidin–spheres.

After initiation of this work we learned that Wu *et al.* (20) have independently examined the position of protein moieties on poliovirus RF by use of electron-opaque labeling techniques.

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