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Evidence that Infectious Pancreatic Necrosis Virus Has a Genome-Linked Protein

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The double-stranded RNA segments of infectious pancreatic necrosis virus were extracted from virions by a method which avoids proteinase. In contrast to proteinase-treated RNA, such segments (i) exhibited a lower electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels and agarose gels, (ii) had a slightly lower buoyant density, and (iii) demonstrated a marked tendency toward aggregation as observed by electron microscopy. A small amount of protein tightly bound to the RNA could account for the above properties, and a 110,000-dalton protein was liberated from purified virion RNA by sequential digestion with RNase III and RNase A. The amount of radioactivity associated with RNA from virions labeled in vivo with [^{35}S]methionine suggested that an average of 1.4 molecules was bound per RNA segment. Interactions between RNA segments seen in electron micrographs appeared to occur only among the ends of the segments, suggesting these were the exclusive sites of protein attachment.

Infectious pancreatic necrosis virus (IPNV) is the prototype of a recently characterized family of viruses which encapsidate two segments of double-stranded RNA (dsRNA). The molecular weights of the segments vary somewhat among isolates, but 2.3×10^6 and 2.5×10^6 daltons are accepted values for the reference isolate VR299 (5). Only three structural proteins are known: VP1 (95,000 to 105,000 daltons), VP2 (50,000 to 54,000 daltons), and VP3 (31,000 to 32,000 daltons) (10). VP3 is an internal virion protein of unknown function (7) which may assist in the packaging of RNA. VP2 is the major capsid protein (7). VP1 may contain the RNA polymerase activity associated with virions (3, 12). Four properties of VP1 are consistent with a polymerase function: (i) it is similar in size to other known virion polymerases (1); (ii) only about 22 molecules are present per virion (6); (iii) it is an internal protein (7); and (iv) temperature-sensitive mutants are most readily isolated in the RNA segment encoding VP1, suggesting that it has an activity very sensitive to conformational changes (10).

Several viral families, including Adenoviridae and Picornaviridae, are known to have genome-linked proteins that are thought to become attached in the act of priming the synthesis of the viral nucleic acid (15). To date, no protein has been found attached to the genome of any dsRNA virus. We report here that a protein is tightly bound to IPNV dsRNA and suggest that this protein is a genome-linked primer.

The customary method of purification of IPNV RNA from virions has been proteinase digestion followed by phenol extraction. When virions were instead dissociated by 1 to 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol at 100°C, the RNA behaved rather differently from proteinase-treated RNA. This behavior suggested that some protein was tightly bound to virion RNA. We have characterized the presumed RNA-protein complex by comparison of its properties with those of naked IPNV RNA and have determined the molecular weight of a protein liberated from it by RNase digestion.

MATERIALS AND METHODS

Cells and virus. Chinook salmon embryo (CHSE-214) cells were grown in culture dishes in Eagle minimum essential medium (autoclavable) (GIBCO) plus 0.292 mg of glutamine per liter, 0.18% $NaHCO_3$, and 5% calf (bovine) serum (GIBCO) in CO_2 incubators at 20°C.

With one exception, an Ab isolate obtained from P. E. V. Jorgensen (State Serum Laboratory, Aarhus, Denmark) (see Fig. 5A), the virus used in all experiments was the Jasper, Alberta, Canada, isolate of IPNV. Virus purification was done by the method of Macdonald and Yamamoto (11). Purified virus was stored at 4°C in TNE buffer (0.01 M Tris-hydrochloride, 0.1 M NaCl, 0.001 M EDTA [pH 7.5]).

In vivo labeling of RNA and protein. After removal of the medium, 100-mm dishes with dense cell monolayers were each infected with roughly 1 PFU/cell in 0.3

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ml of Hanks balanced salt solution. After 30 to 60 min, the medium was replaced, and radioactive nucleosides or amino acids were added in small volumes of medium. For labeling of viral nucleic acid, the medium contained 2% fetal bovine serum (GIBCO) dialyzed against Hanks balanced salt solution. In separate experiments, the following compounds were included for labeling of virus RNA: [^{3}H]uridine (New England Nuclear Corp.; 40 to 50 Ci/mmol) at 12 μCi/ml and [^{14}C]uridine (New England Nuclear Corp., 0.52 Ci/ml) at 0.3 μCi/ml. For labeling of viral protein, the concentration of all amino acids in the normal cell growth medium was reduced 20-fold; it contained 2% dialyzed fetal bovine serum and [^{35}S]methionine (New England Nuclear Corp.; 1,400 Ci/mmol) at 16 μCi/ml.

Preparation of samples of RNA-protein complex and naked RNA. Naked RNA samples were prepared by digesting purified virus suspensions overnight at 35°C with proteinase K (EM Biochemicals) at 0.2 mg/ml in TNE buffer plus 1% SDS. The RNA solution was extracted three times with equal volumes of distilled phenol (Fisher Scientific Co.), ethanol precipitated, and resuspended in TNE buffer.

RNA-protein complex samples were liberated from purified virions by heating in a boiling water bath in the presence of SDS. When a sample was to be loaded directly on an SDS-polyacrylamide gel, heating was for 2 min in Laemmli sample buffer, which contains 2% SDS and 5% 2-mercaptoethanol (9). When a sample was to be purified in a sucrose gradient, heating was for 3 min in TNE buffer plus 1% SDS. Aqueous samples of purified RNA-protein complex that lacked SDS customarily contained 0.1 to 1% Sarkosyl (Sigma Chemical Co.) to prevent aggregation.

In vitro radioactive labeling of RNA-protein complex. RNA-protein complex was purified twice in sucrose gradients. Labeling was by the method of Bolton and Hunter (2), with an ^{125}I-acylating agent (New England Nuclear Corp.; 2,000 Ci/mmol) to form amides with free amino groups. The RNA was suspended in 40 μl of borate buffer (0.1 M sodium borate, 0.001 M EDTA [pH 8.5]) plus 0.1% Sarkosyl (Sigma) and added to 1 mCi of dry reagent at 0°C. After 30 min with intermittent shaking, 0.25 ml of 0.2 M glycine in borate buffer was added. After an additional 15 min at 0°C, the mixture was layered on a 1- by 10-cm gel filtration column (Sephadex G-25 fine; Pharmacia Fine Chemicals, Inc.) and eluted with TNE buffer plus 0.1% Sarkosyl. Fractions (0.2 ml each) were collected, and 5-μl samples were spotted on glass fiber filters, which were then dried and counted in toluene–2,5-diphenyloxazole (PPO)–1,4-bis-(5-phenyloxazolyl)benzene (POPOP) with a Beckman LS 8000 scintillation counter. The three fractions at the leading edge of a peak of radioactivity near the start of the void volume were pooled for use in the experiments.

Sucrose gradient purification of RNA-protein complex. Sucrose (Fisher Scientific) solutions of 5 and 20% were prepared in TNE buffer. SDS was present in the solutions at a final concentration of 1%. Centrifugation was for 4 h at 45,000 rpm in a Beckman SW50.1 rotor at 22°C. Drops were collected from the bottom of the tube. Fractions containing labeled viral RNA were identified by radioactivity. When no labeled RNA was present, peak fractions were identified by agarose gel electrophoresis of samples.

Cesium sulfate gradients. Virus samples of 0.1 ml in TNE buffer plus 0.1% Sarkosyl were layered over 2 ml of a solution of cesium sulfate (Schwarz/Mann) in TNE buffer plus 0.2 M guanidine hydrochloride (Sigma) with a density of 1.5 g/ml. Mineral oil was used to fill the nitrocellulose tube (Beckman). Centrifugation was for 48 h at 35,000 rpm in a Beckman SW50.1 rotor at 4°C. Drops were collected from the bottom of the tube and kept on ice until the refractive index was taken with a Jena refractometer. The refractive indices were corrected for the contribution of the solutes in TNE buffer and that of the guanidine hydrochloride by subtraction of 0.0039 U from each reading, this being the difference between TNE buffer with 0.2 M guanidine hydrochloride and distilled water. The assumption in making this correction was that these solutes affect density proportionately less than they affect refractive index. Densities of the fractions were determined by using the equation: density = (refractive index−1.2646)/0.073. Yeast carrier RNA was added, and each fraction was precipitated with an excess of cold 10% trichloroacetic acid. The precipitates were collected on Whatman GF-A glass fiber filters. The filters were dried and counted in toluene-PPO-POPOP with a Beckman LS 8000 scintillation counter.

SDS-polyacrylamide gel electrophoresis of RNA and RNA-protein complex. All samples were loaded in wells formed in 6% polyacrylamide (0.17% bisacrylamide) gels in a vertical slab gel apparatus. The buffer system of Laemmli (9) was used, but the stacking gel was omitted. Electrophoresis was for 6 h at 15 mA followed by 25 h at 100 V. Gels were stained with ethidium bromide (Sigma) at 2 μg/ml and photographed under UV light.

Agarose gel electrophoresis of RNA and RNA-protein complex. *Hin*dIII restriction endonuclease fragments of bacteriophage λ DNA were obtained from Boehringer Mannheim Corp. Purified reovirus type 3 was a gift from P. W. Lee (Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada). Reovirus RNA samples were prepared by proteinase K digestion and phenol extraction as for IPNV naked RNA. After sucrose gradient purification, RNA-protein complex was ethanol precipitated and suspended in TNE buffer plus 1% Sarkosyl. When loaded, all samples were suspended in Tris-acetate buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.1]) plus 20% glycerol plus 20% bromophenol blue tracking dye. The samples were loaded in wells formed in a horizontal submarine 1.5% agarose gel containing 0.1% Sarkosyl and 2 μg of ethidium bromide (Sigma) per ml. The gel was formed and run in Tris-acetate buffer at pH 8.1. Electrophoresis was for 6 h at 100 V. The gel was photographed under UV light.

Gradient polyacrylamide gel electrophoresis of proteins. ^{125}I-labeled RNA-protein complex was suspended in buffer containing 0.015 M Tris-hydrochloride, 0.05 M NaCl, 0.01 M $MgCl_2$, and 0.001 M EDTA at pH 7.5. RNase III in the manufacturer's storage buffer (Bethesda Research Laboratories; 0.8 U/μl) was added to 0.08 U/μl, and the mixture was incubated for 28 h at 35°C. RNase A (Sigma) in TNE buffer was added to 2 μg/ml, and the mixture was incubated for 30 min at 56°C. An equal volume of double-strength Laemmli sample buffer (9) was added, and the mixture was heated for 2 min in a boiling water bath. A control sample of ^{125}I-labeled RNA-protein complex was

treated similarly but without the addition of either RNase. Samples were run in 15-cm gradient polyacrylamide slab gels by using the discontinuous SDS-gel system of Laemmli (9). Electrophoresis of the 5 to 15% polyacrylamide gel (see Fig. 5A) was at 20 mA for 4 h followed by 75 V for 19 h. Electrophoresis of the 5 to 12% polyacrylamide gel (see Fig. 5B) was at 25 V for 2 h followed by 50 V for 18 h. Gels were fixed with a solution containing 30% methanol, 10% isopropanol, and 10% trichloroacetic acid. They were then treated with autoradiography enhancer (New England Nuclear Corp.), dried under vacuum, and fluorographed for 2 days at −90°C with Kodak AR-5 film.

Electron microscopy. RNA-protein complex was purified by sucrose gradient centrifugation and then ethanol precipitated and suspended in TNE buffer. Naked RNA in TNE buffer was used without additional purification. Samples were mixed with purified plasmid pBR322 from *Escherichia coli* and prepared for viewing by the formamide technique of Davis et al. (4) modified by the use of only the largest (N-terminal) cyanogen bromide cleavage fragment of cytochrome *c* rather than the whole molecule. Platinum-shadowed grids were photographed at magnifications of ×11,500 or ×18,000 (at the negative) with a Phillips 300 electron microscope. Measurements were made on prints enlarged three more times.

RESULTS

Behavior of SDS-released IPNV RNA. When RNA released from IPNV virions by SDS at 100°C was run in polyacrylamide gel electrophoresis, it migrated at a greatly reduced rate compared with naked (proteinase K-treated and phenol-extracted) RNA, as shown in Fig. 1. The only treatment that was effective in changing the mobility of the SDS-released RNA to that of naked RNA was digestion with proteinase. Neither RNase A nor 8 M urea affected the mobility of the complex. Because this evidence suggested the presence of protein, we refer to the SDS-released RNA as RNA-protein complex.

The RNA-protein complex failed to enter gels unless SDS or Sarkosyl was present in the system. For example, the RNA-protein complex released from virions by SDS was unable to enter gels containing 0.2% Triton X-100 instead of SDS. Evidence from electron microscopy (see below) suggested that the RNA was aggregating at the top of the gel in the absence of an ionic detergent.

Size of RNA-protein complex. Estimates of the molecular weight of the RNA-protein complex were made by three methods: (i) polyacrylamide gel electrophoresis, (ii) agarose gel electrophoresis, and (iii) sucrose gradients. Figure 1 shows the migration of the RNA-protein complex in polyacrylamide compared with IPNV naked RNA and restriction fragments of DNA. The relative position of the RNA-protein complex indicated an apparent molecular weight twice that of naked IPNV RNA. The RNA-protein complex was not a subviral particle, since two

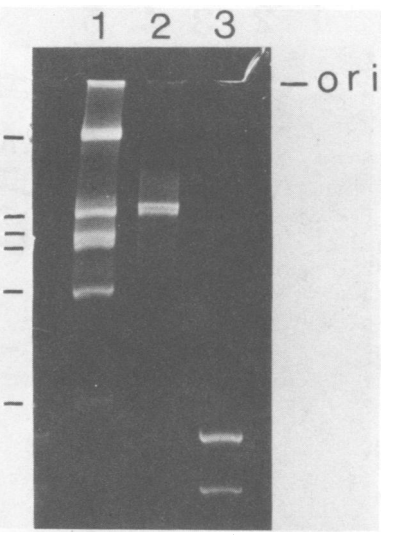

FIG. 1. SDS-polyacrylamide gel electrophoresis of IPNV RNA-protein complex and naked RNA. Lane 1, *Eco*RI restriction endonuclease fragments of bacteriophage λ DNA. The molecular weights of the six fragments indicated are ($\times 10^6$) 14.36, 4.98, 3.88, 3.66, 3.20, and 2.26 (Boehringer Mannheim product description). Lane 2, IPNV RNA-protein complex. Lane 3, naked RNA. ori, Origin of electrophoresis.

bands were distinguishable that represented the two different IPNV RNA segments. The content of the two bands was determined as follows. The upper and lower bands were individually cut from a gel, treated with proteinase K, and rerun in a second polyacrylamide gel (not shown). The upper band contained predominantly the large IPNV RNA, and the lower band contained predominantly the small IPNV RNA.

Analysis of the IPNV RNA-protein complex in agarose gels yielded an apparent molecular weight about 10% greater than that of naked IPNV RNA (Fig. 2). In relation to known dsDNA and dsRNA standards, the migration rate of naked IPNV RNA in agarose was the same as it was in polyacrylamide, but the migration rate of the RNA-protein complex was greatly increased.

Naked RNA and the RNA-protein complex sedimented to the same position in a sucrose gradient (Fig. 3). In some experiments, SDS-polyacrylamide gel electrophoresis of samples of the peak gradient fractions showed the presence of both naked RNA and the RNA-protein complex. Hence, conversion of one form to the other cannot explain their cosedimentation. These results suggest that there was at most only a slight molecular weight difference between naked RNA and RNA-protein complex, which seems consistent with their similar migration rates in agarose. We therefore consider the low migra-

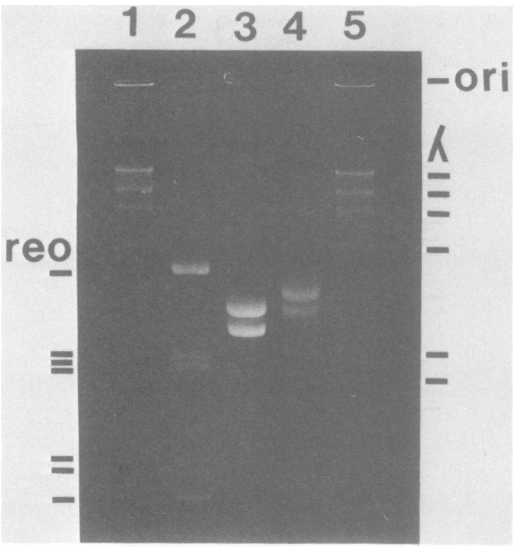

FIG. 2. 1.5% agarose gel electrophoresis of IPNV RNA-protein complex and naked IPNV RNA. Sarkosyl was present at 0.1% to prevent aggregation. Lanes 1 and 5, *Hin*dIII restriction endonuclease fragments of bacteriophage λ DNA. The molecular weights of the six largest fragments (indicated at right) are ($\times 10^6$) 15.6, 6.36, 4.38, 2.86, 1.49, and 1.31 (Boehringer Mannheim product description). Lane 2, reovirus type 3 RNA. The molecular weights of the segments indicated at the left were taken as ($\times 10^6$) 2.5, 1.6, 1.5, 1.4, 0.9, 0.8, and 0.7 (8). Lane 3, naked IPNV RNA. Lane 4, purified IPNV RNA-protein complex. ori, Origin of electrophoresis.

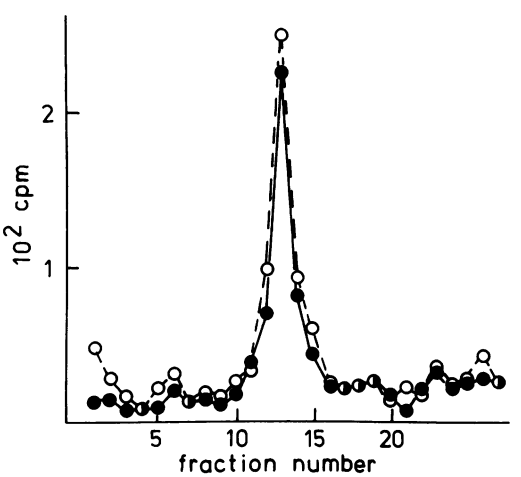

FIG. 3. Size of the RNA-protein complex by sucrose gradient centrifugation. Samples of [^{3}H]guanine-labeled naked IPNV RNA and [^{14}C]uridine-labeled RNA-protein complex were mixed and centrifuged in a 5 to 20% sucrose gradient containing 1% SDS in TNE buffer. Fractions were numbered in order of their collection from the tube bottom. Naked IPNV RNA has previously been shown to sediment as 14S (11). ○, ^{3}H counts; ●, ^{14}C counts.

tion rate of RNA-protein complex in polyacrylamide gels to be anomalous.

Buoyant density of RNA-protein complex. When the RNA-protein complex and naked RNA were centrifuged in the same cesium sulfate gradient to equilibrium, the RNA-protein complex banded at 1.56 g/ml and naked RNA banded at 1.58 g/ml (Fig. 4). Because we do not know the density of the protein in cesium sulfate, we cannot calculate the ratio of protein to RNA in the RNA-protein complex. However, the density shift on loss of the protein is so slight that there cannot be much protein relative to RNA.

Molecular weight of the bound protein. We attempted to label the RNA-protein complex in vivo with [^{35}S]methionine. Figure 5A shows the proteins from purified ^{35}S-labeled virions run on a 5 to 15% polyacrylamide gradient SDS gel and subjected to fluorography. The three unique IPNV virion proteins are indicated by their masses (in thousands of daltons). In addition, two minor bands were present that migrated at the position of RNA-protein complex (arrow). Densitometer tracings of similar fluorograms indicated that the intensity of the RNA-protein complex doublet was 0.15 times that of the VP1 band. Evidently, the bound protein contained methionine and could be labeled after infection.

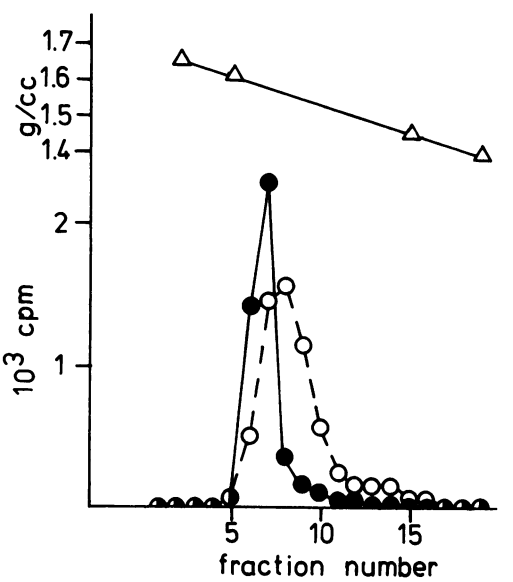

FIG. 4. Buoyant density of the RNA-protein complex. Samples of [^{14}C]uridine-labeled naked IPNV RNA and [^{3}H]uridine-labeled RNA-protein complex were mixed and characterized by centrifugation in cesium sulfate with 0.2 M guanidine hydrochloride. Fractions were numbered in order of their collection from the tube bottom. ●, ^{14}C counts; ○, ^{3}H counts after correction for ^{14}C spillover (about 10%); △, density.

However, we were unable to recover enough virion label in purified RNA-protein complex for further experimentation. Instead, we labeled the purified RNA-protein complex with ^{125}I by using the Bolton-Hunter reagent. Nearly all the label after iodination sedimented with the RNA-protein complex in a sucrose gradient, and all the label was sensitive to proteinase K treatment (not shown). The iodinated RNA-protein complex was treated with RNase III and RNase A and electrophoresed in a polyacrylamide gel for protein analysis (Fig. 5B). The most prominent band of liberated protein migrated with an apparent molecular weight of 110,000 (Fig. 5B, arrow). Some bands at positions indicating lower molecular weight were also present, but they were always less intense, and they could be detected in control samples not treated with RNase (Fig. 5B, lane 2). Perhaps the ^{125}I decay caused some degradation of the protein even while it was attached to the RNA. Because the 110,000-dalton protein was the largest species liberated from the RNA-protein complex, we infer that it constituted the entire molecule formerly attached to IPNV RNA.

Electron microscopy of naked RNA and RNA-protein complex. Purified RNA-protein complex and naked RNA were spread by the formamide technique of Davis et al. (4) and visualized in an electron microscope. Plasmid pBR322 was included as an internal length standard. IPNV naked RNA appeared as individual linear molecules with a median length equal to 61% of the length of pBR322 (Fig. 6A). IPNV RNA-protein complex appeared as similar linear molecules (Fig. 6B) with a median length equal to 62% of the length of pBR322. However, RNA-protein complex molecules were not present as individual molecules on the grid; rather, they tended to aggregate by end-to-end interaction. In a count of 166 molecules, 80% had at least one end very close to the end of another RNA (Fig. 6C) or were circularized (Fig. 6D). The size of the aggregates varied from a few molecules to hundreds of molecules.

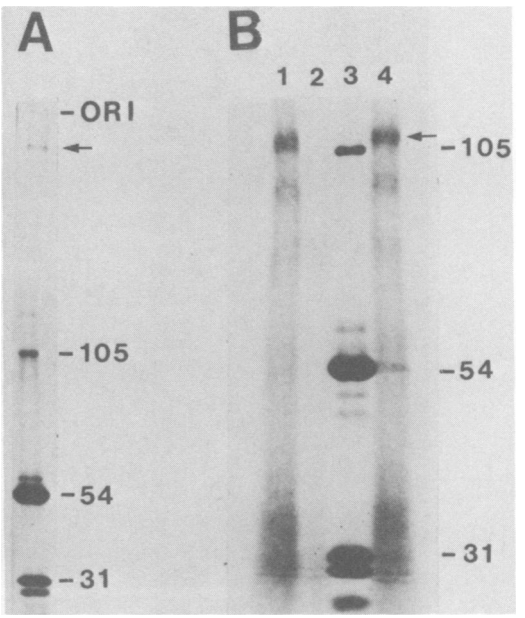

FIG. 5. Gradient SDS-polyacrylamide gel electrophoresis of virus proteins. (A) [^{35}S]methionine-labeled virion proteins. Arrow indicates a doublet with the mobility of RNA-protein complex. ORI, origin of electrophoresis. (B) The lower portion of a similar gel showing a protein liberated from RNA-protein complex labeled in vitro with ^{125}I-labeled Bolton-Hunter reagent. Lane 2, undigested complex; lanes 1 and 4, RNase-digested complex; lane 3, marker virion proteins labeled in vivo with [^{35}S]methionine. Lanes 1, 2, and 4 each contained roughly 3,000 precipitable cpm. The arrow shows the position of the major species of protein (110,000 daltons). The numbers to the right of each part of the figure are the molecular weights (in thousands) of the indicated virion proteins (10).

DISCUSSION

Our results provide evidence that a protein remained strongly attached to IPNV genomic dsRNA after virions were disrupted with SDS. This attached protein affected the mobility of the RNA in polyacrylamide and agarose gels and lowered its density in cesium sulfate. We have not yet characterized the bond between the protein and the RNA, but the strength of the union suggests that it may be covalent. The end-to-end interaction of molecules observed in an electron microscope suggests that the protein was located at the ends of the RNA segments.

The enormous retardation by protein of RNA electrophoresed in polyacrylamide has not been fully explained. Evidently, the protein had a much greater influence on RNA mobility in gels of small pore size. Perhaps, in addition to increasing RNA size, the protein affected the orientation in which the RNA passed through gel pores. This secondary effect might somehow have been critical only when the RNA was drawn through small pores.

If the aggregation of RNA segments seen in electron micrographs was due to interactions among bound proteins, the proteins must have been restricted to the ends of the segments, since these were the exclusive sites of close contact. Both ends of some RNA segments participated (Fig. 6C and D), so there would have to have been protein at both ends of some segments at least. If the magnitude of the forces tending to separate segments during spreading is considered, it would seem reasonable that all

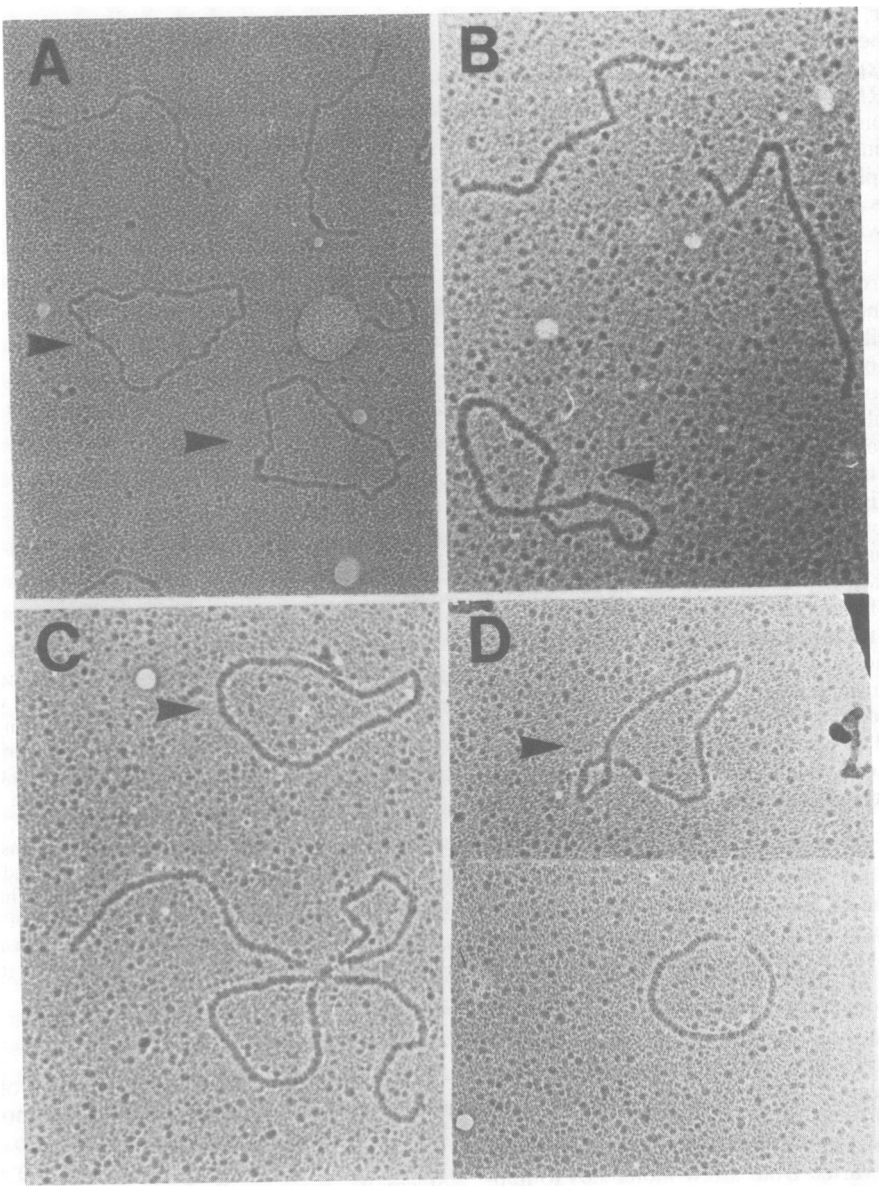

FIG. 6. Electron microscopy of IPNV naked RNA and RNA-protein complex. (A) Naked RNA. (B, C, and D) Various molecular conformations observed in samples of RNA-protein complex. Individual linear molecules (B) and circular molecules (D) were minor constituents. Most linear molecules observed were members of aggregates of three to eight molecules, such as the one in (C). Arrowheads indicate molecules of pBR322 DNA, which has a molecular weight of 2.88×10^6 (14).

RNAs had protein at both ends but that some interactions were disrupted.

The relative intensities of the bands representing free VP1 and RNA-protein complex after electrophoresis of dissociated virions previously labeled in vivo with [^{35}S]methionine (Fig. 5A) provide a rough estimate of the number of molecules of genome-linked protein in those virions. Since VP1 comprises 4% of virion protein (6) and protein bound to RNA produced only 0.15 times the band intensity of VP1, the linked protein represented 0.6% of virion protein. Taking 50×10^6 as the mass of protein in the IPNV virion (6), this corresponds to 2.7 molecules of the 110,000-dalton protein per virion, or 1.4 per RNA segment. Of course, this entire calculation assumes uniform incorporation of methionine and linear fluorographic response to radioactivity, neither of which has been established in this instance.

The gene for the IPNV 110,000-dalton genome-linked protein has not been mapped to an RNA segment, but certain genetic considerations are nevertheless worth noting at this time. There is no room for an additional large independent gene on the RNA segment encoding VP2, VP3, and NS1 (combined molecular weight, 113,000); similarly, there is no room on the RNA segment encoding VP1 (molecular weight, 105,000) (10). This leaves three possibilities: the genome-linked protein is the product of an overlapping gene; it is a cellular protein; or it is identical to a known viral protein. If the last possibility is correct, VP1 would be the logical candidate protein, since it is similar in size to the attached protein.

The two best-studied examples of viral genome-linked proteins are in poliovirus and adenovirus (15). Here precursor proteins are used as primers in viral replication, and these precursors are ultimately cleaved into an encapsidated terminal protein. Because of these precedents, we suggest that the IPNV genome-linked protein as well may be a primer for replication. Preliminary results have shown that the protein is present on all detectable IPNV intracellular single-stranded RNA (R. G. Williams and R. D. Macdonald, unpublished data). These single-stranded molecules act both as intermediates in viral genome replication and as mRNA (13). Recent evidence favors a mechanism of strand displacement for the synthesis of these single-stranded RNAs in purified virions (12). This mechanism distinguishes IPNV from reovirus (the other dsRNA virus prototype), since reovirus employs fully conservative single-stranded RNA synthesis (8). Moreover, strand displacement is a feature that IPNV has in common with poliovirus and adenovirus, which both have terminal proteins. Wimmer (15) has speculated that all viruses that replicate their nucleic acid by strand displacement may have a common ancestral replication system which utilizes a protein primer. If the genome-linked protein of IPNV is truly a primer, and if IPNV RNA synthesis is indeed by strand displacement, that speculation is further supported.

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