JOURNAL OF VIROLOGY, Dec. 1986, p. 1002–1011
0022-538X/86/121002-10$02.00/0

Vol. 60, No. 3

A Physical Map of the Viral Genome for Infectious Pancreatic Necrosis Virus Sp: Analysis of Cell-Free Translation Products Derived from Viral cDNA Clones†

MANLEY T. F. HUANG, D. SCOTT MANNING, MICHAEL WARNER, E. B. STEPHENS,‡ AND JoANN C. LEONG*

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804

Received 21 April 1986/Accepted 2 September 1986

The two segments of double-stranded RNA from infectious pancreatic necrosis virus Sp were cloned into the plasmid vector pUC8. Two sets of overlapping clones were identified by restriction enzyme and Southern blot analyses. Each of these sets was shown by Northern blot analysis to be exclusively related to either segment A or B of the genomic RNA. The entire lengths of the cloned segments were estimated to be 2.9 and 2.6 kilobases, respectively. Sequences from the two segments of viral cDNA were subcloned into the bacteriophage T7 RNA polymerase vectors pT71 and pT72. The activity of the single-stranded RNAs transcribed from these subclones in a rabbit reticulocyte lysate translation system provided information on the polarity of and the protein products coded for by each subclone. The four proteins encoded by the genome of infectious pancreatic necrosis virus were identified among the translation products of the individual cloned segments by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By constructing plasmids containing deletions in the sequences from either the 5′ or 3′ end of segment A, we were able to construct a physical map for the larger segment of double-stranded RNA. The proteins derived from these plasmids indicated that the linear gene order for viral proteins encoded in segment A is β, γ2, and γ1.

Infectious pancreatic necrosis virus (IPNV) of fish causes an acute, contagious disease in juvenile rainbow and brook trout (18). Highly virulent strains may cause greater than 90% mortality in hatchery stocks and are, therefore, a major concern within the aquaculture industry. No preventive or therapeutic treatments exist other than the destruction of infected stocks and disinfection of the hatchery facilities.

IPNV belongs to the family *Birnaviridae* (4, 6), whose members contain two segments of double-stranded RNA (dsRNA). The coding assignments for the two segments have been determined by genetic reassortment (13) and by analysis of the proteins synthesized in vitro from purified viral RNA (16). These studies have determined that segment A RNA encodes three proteins and segment B encodes the putative viral RNA polymerase.

The three major strains of IPNV are VR299, Sp, and Ab, although many strains have been isolated from shellfish and nonsalmonid species (18). VR299 is the type strain for North America, whereas Sp and Ab are the predominant strains found in Europe. A number of strains isolated from fish in Asia are closely related to the Ab strain (8, 9). These strains can be distinguished by variations in the sizes of their RNAs, as well as the sizes and relative proportions of the capsid proteins (8).

We cloned the viral genome of IPNV to establish a physical endonuclease map of the two RNA segments. We report here the molecular cloning of the Sp strain into the plasmid vector pUC8 and present a restriction endonuclease map of the segments of cDNA.

* Corresponding author.

† Oregon Agricultural Experiment Station Technical Paper no. 8018.

‡ Present address: Department of Microbiology, Medical Center, University of Alabama, Birmingham, AL 35294.

The cloned viral sequences of RNA segments A and B were subcloned into the plasmid vectors pT71 and pT72. These vectors are designed so that cloned DNA sequences may be transcribed into RNA from a bacteriophage T7 RNA polymerase promoter site. This technique yielded IPNV single-stranded RNA (ssRNA) that was active in a cell-free translation system. We describe here the proteins encoded by the cDNA clones and compare the in vitro translation products with those of purified virions and viral proteins synthesized within the first 8 h of infection in chinook salmon embryo cells (CHSE-214). In addition, we determined the physical map for the polycistronic segment A. The linear order of the three viral genes was determined from an analysis of the number and form of the proteins synthesized from a series of 5′- and 3′-end deletions of the segment A cDNA.

MATERIALS AND METHODS

Virus stock, growth, and purifications. IPNV Sp was obtained from R. P. Hedrick, University of California, Davis, and plaque purified in our laboratory before cDNA synthesis. The virus was propagated in CHSE-214 cells (7) at a multiplicity of infection of 0.05 to 0.1. Cell monolayers were grown at 18°C in glass bottles by using Eagle minimal essential medium with Earle salts (GIBCO Laboratories) supplemented with 0.11% bicarbonate, 10% fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin (GIBCO) per ml, and 10 μg of gentamicin sulfate (Sigma Chemical Co.) per ml. Virus particles were harvested between 3 and 5 days postinfection. After an initial low-speed centrifugation, the virus was pelleted in a Beckman T35 rotor at 30,000 rpm for 90 min and suspended in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 20 mM Tris hydrochloride [pH 7.5], 0.01% gelatin). The virus was initially purified by centrifugation through a step gradient consisting of 1.4 g of

CsCl per cm^3, 1.25 g of CsCl per cm^3, and 20% sucrose in SM buffer in either a Beckman SW41 or SW50.1 rotor at 35,000 rpm for 90 min. The virus banding between the 1.4- and 1.25-g/cm^3 CsCl layers was collected by side puncture, layered onto a 1.33-g/cm^3 CsCl equilibrium gradient, and centrifuged for 14 to 16 h at 35,000 rpm at 4°C. Intact virus was collected by side puncture or from above by using a Buchler Densi-flo IIC fraction collector. The fractions containing the virus were diluted with SM buffer, and the virus was pelleted at 35,000 rpm for 90 min in an SW41 rotor at 4°C.

Reagents. RNase H, terminal deoxynucleotide transferase, and oligo(dT)$_{12}$ primers were obtained from Pharmacia Molecular Biologicals. Poly(A) polymerase, *Escherichia coli* DNA polymerase I, all restriction endonucleases, and *E. coli* DNA ligase were purchased from Bethesda Research Laboratories, Inc., and avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences, Inc. Calf intestinal phosphatase was purchased from Boehringer Mannheim Biochemicals. The pT7 plasmids and T7 RNA polymerase were purchased from U.S. Biochemicals. [^{35}S]methionine and [^{32}P]ATP were acquired from New England Nuclear Corp., and [^{3}H]ATP was purchased from ICN Pharmaceuticals Inc. Promega Biotec was the source of RNasin and the rabbit reticulocyte lysate in vitro translation kit.

Preparation of viral RNA. The viral pellet was suspended in TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) containing 0.5% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K (Sigma) per ml and left at room temperature overnight or at 65°C for 2 h. The RNA was extracted twice with an equal volume of phenol–0.1% 8-hydroxyquinoline and then twice with chloroform-isoamyl alcohol followed by precipitation in ethanol.

Preparation of cDNA clones. Poly(A) tails with an average length between 40 and 80 bases were added to the 3′ ends of the dsRNA by using *E. coli* poly(A) polymerase (20). The reaction mixture was extracted with phenol and with chloroform-isoamyl alcohol followed by precipitation in ethanol in the presence of 2 M ammonium acetate.

Between 8 and 10 μg of poly(A)-tailed viral RNA was suspended in 20 μl of sterile distilled water (treated with 0.3% diethylpyrocarbonate), boiled for 3 min, and chilled in ice. The reaction volume was raised to 200 μl containing 50 μg of oligo(dT)$_{12}$ per ml, 80 μg of actinomycin D per ml, 2 mM each of dATP, dGTP, and TTP, 100 μM [α-^{32}P]dCTP (specific activity, 4 Ci/mol), 50 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 13 U of avian myeloblastosis virus reverse transcriptase per μg of RNA. After incubation at 42°C for 3 to 4 h, the reaction was stopped by the addition of EDTA and SDS. The RNA/cDNA hybrid was extracted with phenol and then chloroform-isoamyl alcohol, as described above. The nucleic acid was precipitated in ethanol twice in the presence of 2 M ammonium acetate before second-strand cDNA synthesis.

The RNA/cDNA hybrid nucleic acid was converted into dsDNA by a modification of the method of Okayama and Berg (17). The nucleic acid was suspended in a reaction volume of 100 μl containing 30 U of *E. coli* DNA polymerase and 1 U of RNase H per μg of cDNA in a solution of 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.9), 4 mM $MgCl_2$, 15 mM beta-mercaptoethanol (BME), 70 mM KCl, 2 mM each of dCTP, dGTP, and dATP, 50 μM [α-^{32}P]TTP (final specific activity, 1 Ci/mmol), and 15 μM beta-NAD. The reaction mixture was incubated successively at 12°C and room temperature for 1 h each, and the reaction was terminated with EDTA and SDS. Ethanol precipitation in the presence of 2 M ammonium acetate was performed twice to eliminate free deoxynucleoside triphosphates from the double-stranded cDNA before poly(dC) tailing.

Transformation of cDNA. Complementary homopolymer tails of approximately 15 to 20 bases were added to the cDNA [poly(dC)] and to *Pst*I-cut plasmid vector pUC8 [poly(dG)] by using terminal deoxynucleotide transferase. Insert and vector DNAs were reannealed at 42°C for several hours and transformed into calcium chloride-treated *E. coli* host strain SC181 (2) by the method of Maniatis et al. (14). Transformants were isolated by plating onto LB agar supplemented with 120 μg of ampicillin per ml.

Preparation of cDNA probes. Viral genomic RNA (2 μg) was radiolabeled with avian myeloblastosis virus reverse transcriptase. The RNA, with added calf thymus primer DNA (22), was denatured by boiling in sterile distilled water for 2 min and then quickly cooled in an ice water bath. The reaction volume was raised to 30 μl containing 50 mM Tris hydrochloride (pH 8.3), 50 mM KCl; 10 mM $MgCl_2$, 5 mM dithiothreitol, and 50 μCi of [^{32}P]dCTP (specific activity, 3,200 Ci/mmol), and the mixture was incubated at 42°C for 2 h. EDTA was then added, and the labeled cDNA was purified by Sephadex G-50 column chromatography.

Colony blots. Transformants were examined for viral sequences by using the screening method of Maniatis et al. (14). Bacterial colonies were transferred onto nitrocellulose filter papers and hybridized with approximately 10^6 cpm of the viral cDNA probe per ml. Hybridization was performed at 42°C for 12 h in buffer containing 6× SSPE, 50% formamide, 0.5% SDS, 1% glycine, 5× Denhardt solution, and 200 μg of denatured salmon sperm DNA per ml. The filters were rinsed with 2× SSPE–0.5% SDS at 68°C until background radiation was minimized.

Plasmid DNA preparation. Two techniques were used for either the analysis of plasmid DNA by agarose gel electrophoresis or for the production of large quantities of DNA. Overnight bacterial colonies were treated with an alkaline SDS buffer for direct gel electrophoresis (11), and the boiling method of Holmes and Quigley (10) was used for large-scale DNA purification.

Restriction enzyme analysis. Restriction endonuclease maps were determined for those transformants positively identified by colony blot hybridized with viral cDNA probes. Enzyme reactions were performed as described by the manufacturer with 500 ng of plasmid DNA.

RNA denaturing gels and Northern blot analysis. RNA denaturing gels were run as described by Thomas (23) with slight modification for IPNV RNA. Both labeled and unlabeled genomic RNAs were denatured with glyoxal and electrophoresed in agarose gels followed by transfer to nitrocellulose paper by using 20× SSPE. Denaturation was performed for 1 h at 65°C in a solution containing 1.8 M deionized glyoxal, 50% dimethyl sulfoxide (DMSO), and 10 mM phosphate buffer (pH 7.0). The denatured RNA was loaded onto a 1.2% agarose gel in 10 mM phosphate buffer (pH 7.0) and electrophoresed at a constant 80 V for 4 to 6 hours with circulating buffer.

Viral RNA markers (Sp strain) were labeled before denaturation with cytidine 3′,5′-bis (phosphate) (pCp). Approximately 1 μg of dsRNA was incubated with 0.4 to 1.0 μg of phage T4 RNA ligase in 50 mM HEPES (pH 7.5), 18 mM $MgCl_2$, 3 mM dithiothreitol, 0.1 mM ATP, 10 μg of bovine serum albumin per ml, 10% DMSO, and 50 μCi of [^{32}P]pCp (specific activity, 2,900 Ci/mmol) for several hours at 4°C.

After transfer to nitrocellulose filters, lanes containing unlabeled RNA were separated from the [^{32}P]pCp-labeled sample, and the filter was prepared for hybridization as described above for colony blots. Nick-translated probes of individual cDNA plasmids were prepared and hybridized as described above.

[^{35}S]methionine labeling of viral proteins. Monolayers of CHSE-214 cells in 35-mm dishes were infected with IPNV Sp at a multiplicity of infection of between 0.05 and 0.1. At 40 to 42 h postinfection, the medium was removed and replaced with minimal essential medium without serum or methionine and supplemented with 70 μCi of [^{35}S]methionine. The infection was allowed to proceed to complete cytopathic effect, and the virus was harvested as described above.

Polyvalent antiserum to IPNV Sp. Intact virus was purified twice in equilibrium CsCl gradients as described above and dialyzed against 10 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–1 mM EDTA (TNE). Virus (0.5 ml; 1 mg/ml) in TNE was mixed thoroughly with an equal volume of Freund complete adjuvant and injected subcutaneously at four locations around the neck of a rabbit biweekly for 6 weeks. At the end of 8 weeks, the rabbit was sacrificed, and the blood was withdrawn by cardiac puncture. The whole blood was stored at 4°C overnight to permit clotting. Serum was collected after low-speed centrifugation and stored at −70°C. The serum was preadsorbed twice on monlayers of CHSE-214 cells.

Group-specific rabbit sera against the β and γ proteins of IPNV Sp. Purified IPNV Sp (1.5 mg) was suspended in 2 ml of 6 M guanidine hydrochloride–0.1 M BME. The solution was incubated at 25°C for 20 h and then 37°C for 1 h. Viral proteins were separated on a Sephadex G-200 column with 6 M guanidine hydrochloride–0.1 M BME as the elution buffer. Column fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12). The proteins were identified by silver staining (1), and proteins on a duplicate gel were transferred to nitrocellulose by the method of Towbin et al. (24). A Western blot was developed with polyvalent anti-IPNV Sp rabbit serum by the Immuno-Blot procedure of Bio-Rad Laboratories. The protein banding pattern of the purified IPNV Sp is shown in Fig. 1. The major viral proteins are divided into three size classes as described by Dobos and Rowe (5): α (90.5 kilodaltons [kDa]), β (51.5 to 63 kDa), and γ (28.5 to 32 kDa). Fractions containing viral proteins from the β and γ size classes were pooled separately, concentrated to a volume of 0.5 ml, and emulsified in an equal volume of Freund complete adjuvant. The emulsions were injected subcutaneously at three sites into the back of a New Zealand White rabbit. The fractionation procedure was repeated with 0.6 mg of viral protein. The resulting fraction pools were emulsified in Freund incomplete adjuvant and injected 4 weeks after the initial immunization. Serum samples were tested 2 weeks after the second immunization and found to react specifically with the immunizing protein by Western blot analysis (data not shown). The rabbits were bled shortly thereafter. The resulting group-specific sera were designated anti-β and anti-γ.

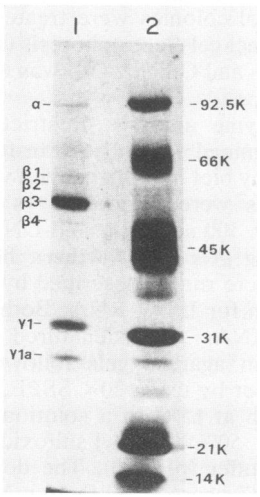

FIG. 1. Silver stain of purified IPNV Sp. IPNV Sp was prepared as described in Materials and Methods. The virus was electrophoresed in a 10% SDS-polyacrylamide gel and stained with silver by the method of Allen (1). Lane 1, IPNV Sp (viral proteins: α, viral RNA polymerase; β1 through β4, different forms of the major capsid protein; γ1 and γ1a, minor virion proteins); lane 2, Bio-Rad low-molecular-weight markers: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

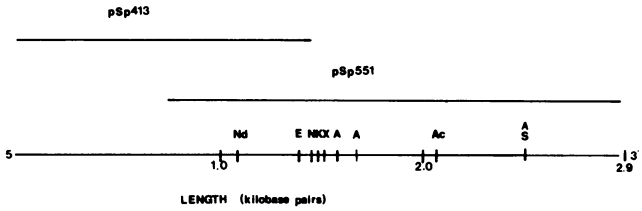

FIG. 2. Partial restriction map of cDNA clones from genomic RNA segment A. The two recombinant plasmids pSp413 and pSp551 contain overlapping sequences from segment A RNA. Each plasmid was digested with single or multiple enzymes and electrophoresed in 1.0% agarose or 4% NuSieve (FMC Corp.) agarose gels. The gels were stained with ethidium bromide, and the sizes of the individual DNA fragments were estimated from *Hin*dIII-cut lambda DNA markers. A, *Ava*I; Ac, *Acc*I; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; S, *Sma*I; Nd, *Nde*I; X, *Xho*I.

[^{3}H-labeled vesicular stomatitis virus mRNA. Baby hamster kidney cells (BHK-21) were infected with vesicular stomatitis virus in the presence of [^{3}H]uridine. Cell monolay-

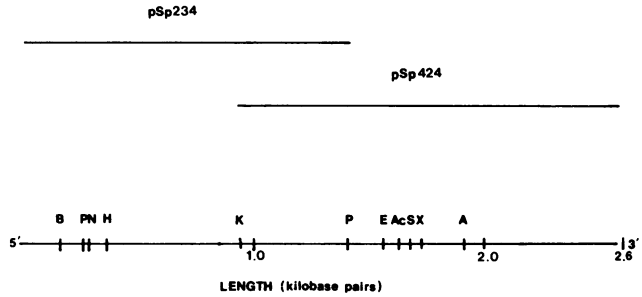

FIG. 3. Partial restriction map of cDNA clones from genomic RNA segment B. The two recombinant plasmids pSp234 and pSp424 contain overlapping inserts from segment B RNA. Each plasmid was digested with single or multiple enzymes and electrophoresed in 1.0% agarose or 4% NuSieve (FMC) agarose gels in Tris acetate-EDTA buffer. The gels were stained with ethidium bromide, and the sizes of the individual DNA fragments were estimated from *Hin*dIII-cut lambda DNA markers. A, *Ava*I; Ac, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sst*I; X, *Xho*I.

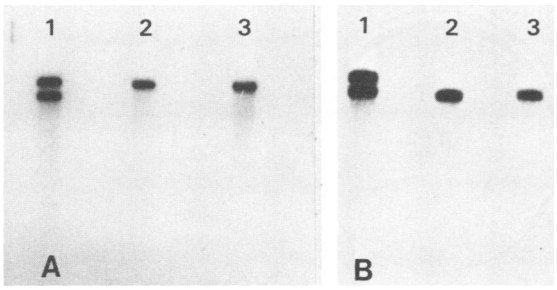

FIG. 4. Northern blot analysis of cDNA clones. Two sets of cDNA inserts were defined by Southern blot analysis as distinct from one another. The individual recombinant plasmids were labeled with ^{32}P-nucleotides by nick translation and hybridized to genomic RNA which had been denatured by glyoxal gel electrophoresis and transferred to nitrocellulose filters. (A) Northern blot analysis using ^{32}P-probes of pSp551 (lane 2) and pSp413 (lane 3). Genomic dsRNA was labeled with [^{32}P]pCp by using T4 RNA ligase (lane 1). Both probes hybridized to segment A RNA only. (B) Northern blot analysis using ^{32}P-probes of pSp234 (lane 2) and pSp424 (lane 3). RNA markers were the same as in panel A (lane 1). Both probes hybridized to segment B RNA only.

ers were infected with a 1:20 dilution of stock virus for 1 h after which the virus was replaced with minimal essential medium supplemented with 5% fetal bovine serum and 0.5 μg of actinomycin D per ml. At 2.5 h postinfection, [^{3}H]uridine was added to a final concentration of 15 μCi/ml. When approximately 25% of the cells exhibited cytopathic effect, the cells were harvested and total RNA was extracted by the guanidinium-cesium chloride method of Maniatis et al. (14). Poly(A)$^+$ RNA was purified by oligo(dT) column chromatography (14) and served as markers in agarose gel electrophoresis.

In vitro translation and SDS-PAGE. Individual reactions were performed in 25-μl volumes. Each reaction mixture contained 17.5 μl of rabbit reticulocyte lysate, 0.5 μl of a 5 mM stock solution containing 19 amino acids without methionine, 2.5 μl of [^{35}S]methionine (specific activity), 1,100 Ci/mmol), 37 U of RNasin, and approximately 2 μg of ssRNA in 3.5 μl of water previously treated with 0.3% diethylpyrocarbonate. Genomic dsRNA was denatured with methyl mercury (3) before addition to the translation mixture. The translation reaction mixtures were incubated at 30°C for 90 min.

Samples from each translation reaction mixture were added directly to an SDS-polyacrylamide gel or immunoprecipitated with anti-IPNV rabbit serum. Immunoprecipitated samples were prepared by the method of Rice et al. (19). Protein samples were electrophoresed in 10% SDS-polyacrylamide gels with a 3% stacking gel. The gel was fixed in 7.5% acetic acid for 1 h and dried onto filter paper before exposure to Kodak XAR-5 film.

Pulse-labeling of IPNV Sp-infected cells. CHSE-214 cell monolayers prepared in 35-mm-diameter dishes were infected with 0.5 ml of IPNV Sp at a multiplicity of infection 100 to 200 for 1 h at 18°C. The virus was then replaced with 2.0 ml of minimal essential medium plus 10% fetal bovine serum and incubated at 18°C. At various times postinfection, the medium was replaced with 2.0 ml of methionine-free medium for 1 h. This medium was then replaced with 0.5 ml of methionine-free medium supplemented with 50 μCi of [^{35}S]methionine (specific activity, 1,100 Ci/mmol). After 1 to 2 h, the medium was removed and the cells were rinsed twice with phosphate-buffered saline. The cells were harvested with 100 μl of lysing buffer (9.5 M urea, 5% BME, 2% Triton X-100). Samples were analyzed for acid-precipitable incorporation of [^{35}S]methionine and then electrophoresed in an SDS-polyacrylamide gel. Uninfected control cells were labeled and harvested in an identical manner.

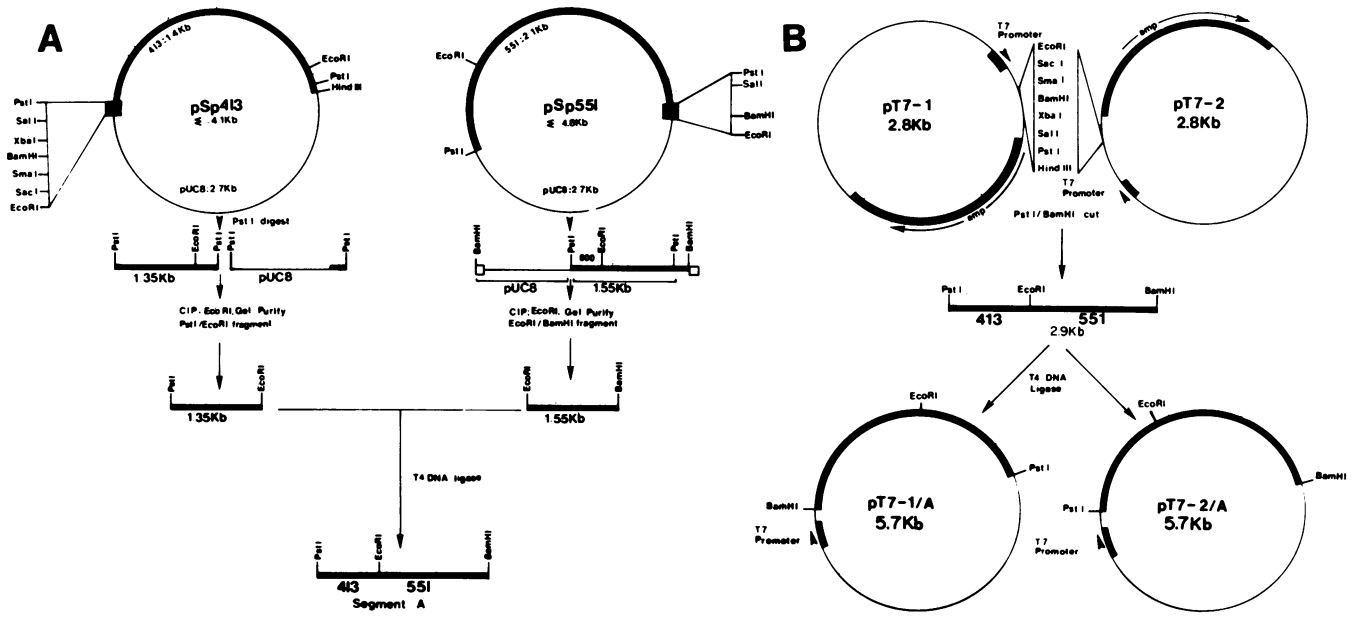

FIG. 5. Subcloning of segment A cDNA into T7 RNA polymerase vectors pT71 and pT72. (A) The two overlapping inserts from pSp413 and pSp551 were separated from sequences of pUC8 by restriction enzymes *Pst*I and *Bam*HI and ligated together at their common *Eco*RI site with T4 DNA ligase. (B) T7 RNA polymerase vectors pT71 and pT72 were both linearized by restriction enzymes *Pst*I and *Bam*HI. Segment A cDNA was ligated to each of the two plasmids with T4 DNA ligase and transformed into *E. coli* SC181. The resulting recombinants were designated pT71/A and pT72/A and contained the cDNA from segment A in opposite reading orientations with respect to the promoter site for T7 RNA polymerase.

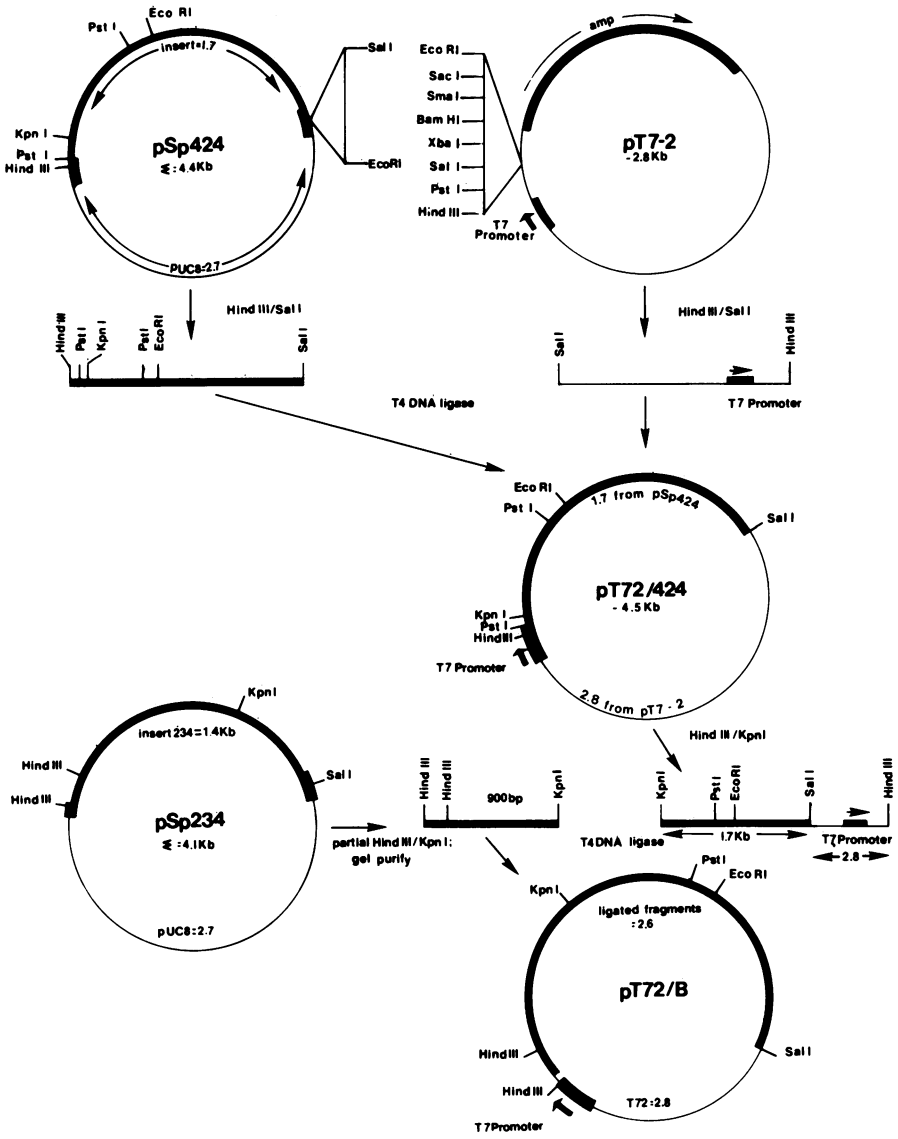

FIG. 6. Subcloning of segment B cDNA into T7 RNA polymerase vector pT72. The two inserts from recombinant plasmids pSp234 and pSp424 overlap at a common *Kpn*I site to form the continuous sequence of segment B. The *Hin*dIII-*Sal*I fragment of pSp424 was gel purified and ligated into the complementary sites of pT72. This recombinant was transformed into *E. coli* SC181 (the resulting recombinant was designated pT72/424) and used to subclone the adjacent sequence from pSp234 bordered by the *Hin*dIII and *Kpn*I sites. This portion of pSp234 was partially digested with *Hin*dIII, followed by complete digestion with *Kpn*I before subcloning into pT72/424. The final recombinant carrying segment B cDNA was designated pT72/B.

RESULTS

Molecular cloning and cross-hybridization of IPNV Sp. The synthesis of the double-stranded cDNA, construction of the vector with the insert cDNA, and subsequent transformation of SC181 cells are described in Materials and Methods. Thirty-eight positive colonies were identified by colony blot analysis, and the plasmids containing the largest inserts were selected for further study. Repeated cross-hybridization analyses were performed using different radiolabeled probes prepared from recombinant plasmids containing IPNV cDNA. The resulting hybridization patterns led to the identification of two families of related inserts. The individual inserts were then digested with as many as 20 different restriction endonucleases to form a preliminary restriction map (Fig. 2 and 3). Each set was represented by two overlapping cloned DNA inserts. The largest set of recombinant clones, pSp413 and pSp551, has a combined length of approximately 2.9 kilobases (kb). The second set of clones, represented by pSp424 and pSp234, has a combined length of approximately 2.6 kb.

Northern blot analysis. Nick-translated probes were prepared from plasmids containing IPNV cDNA inserts and used to determine their relationship to genomic RNA segments A and B. The radiolabeled DNA from each set of overlapping clones hybridized to only one of the genomic segments (Fig. 4): clones pSp413 and pSp551 hybridized to segment A RNA, and pSp424 and pSp234 hybridized to segment B RNA.

Construction of pT7 subclones containing full-length cDNA

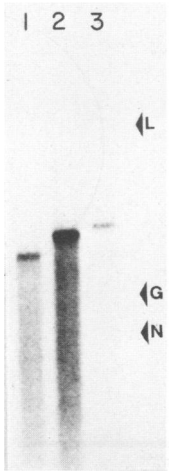

FIG. 7. Autoradiogram of the ssRNAs synthesized by T7 RNA polymerase. T7 RNA polymerase was used to synthesize ^{32}P-labeled ssRNA from pT71/A, pT72/A, and pT72/B. The RNAs were denatured in solutions containing 1.8 M glyoxal, 50% DMSO, and 10 mM phosphate buffer (pH 7.0) for 1 h at 65°C and electrophoresed in a 1.2% agarose gel for approximately 4 h at 90 mA. Lanes: 1, pT72/B; 2, pT72/A, 3, pT71/A. Vesicular stomatitis virus mRNA markers are indicated on the right; L = 6,380 bases, G = 1,672 bases, and N = 1,333 bases.

clones. Overlapping cDNA fragments, which are related to RNA segment A or B, as determined by Northern blot analysis, were ligated together at the common *Eco*RI restriction site and subcloned into the T7 RNA polymerase plasmids pT71 and pT72. The cDNA inserts from pSp413 and pSp551 (segment A) were joined at a single overlapping *Eco*RI site and subcloned into the *Pst*I and *Bam*HI sites of pT71 and pT72 (Fig. 5). Recombinant plasmids containing segment A cDNA in opposite orientations with respect to the promoter site for T7 polymerase were designated pT71/A and pT72/A.

The inserts from pSp234 and pSp424 (segment B) were ligated at a common *Kpn*I site and inserted into the *Hin*dIII and *Sal*I sites of pT72 to produce the recombinant plasmid pT72/B (Fig. 6). Each pT7 subclone contained a cDNA insert bordered by poly(dG-dC) tails and *Pst*I sites. The T7 RNA polymerase promoter was located just outside the polylinker region of the pT7 plasmids and added a number of nucleotides to the 5′ end of the ssRNA produced by T7 RNA polymerase. This represents 50, 53, and 20 bases for pT71/A, pT72/A, and PT72/B, respectively.

Synthesis of ssRNA by using T7 RNA polymerase. Each pT7 recombinant plasmid was cleaved at the 3′ end of the insert before ssRNA synthesis. The enzymes *Pst*I, *Bam*HI, and *Sal*I were used to linearize pT71/A, pT72/A, and pT72/B, respectively. The ssRNAs were labeled with [^{3}H]ATP or [^{32}P]ATP under reaction conditions provided by U.S. Biochemicals and analyzed by electrophoresis in glyoxal gels (Fig. 7). In each case, only one size class of RNA was produced, and it corresponded to the expected size for repeated runoff synthesis by T7 RNA polymerase at its promoter.

In vitro translation products of ss- and dsRNAs. The ssRNAs produced from pT72/A and pT72/B and the dsRNA from purified virus were active in rabbit reticulocyte lysate translation reactions. To establish the identities of the protein products synthesized in these reactions, a comparison of

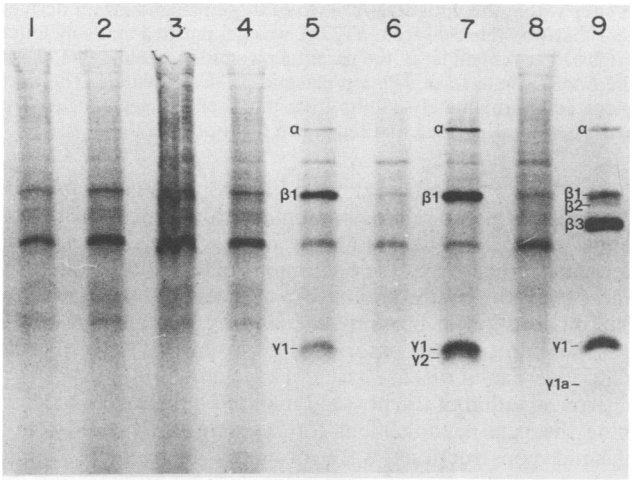

FIG. 8. Electrophoretic analysis of [^{35}S]methionine-labeled proteins from CHSE-214 cells infected with IPNV. Monolayers of CHSE-214 cells in 35-mm-diameter dishes were infected with IPNV at a multiplicity of infection of between 100 and 200. At various times postinfection, the cells were incubated in methionine-free medium supplemented with [^{35}S]methionine (100 μCi/ml) for 1 to 2 h. The cells were lysed and analyzed directly by SDS-PAGE. Shown are samples from uninfected cells (lanes 2, 4, 6, and 8) and from infected cells labeled at 2 to 3 h (lane 1), 3 to 4 h (lane 3), 5 to 7 h (lane 5), and 7 to 8 h (lane 7) postinfection. Lane 9 contained purified Sp virus. Viral proteins: α, RNA polymerase; β1, major capsid protein precursor; β2, a processed form of β1; β3, major capsid protein found in purified virus; γ1, minor virion protein; γ1a, 27-kDa minor virion protein processed from γ1 (Fig. 1); γ2, nonvirion protein seen only in infected cells.

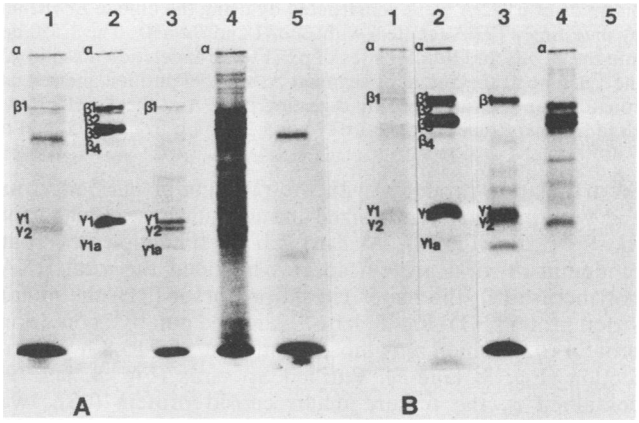

FIG. 9. Electrophoretic analysis of cell-free translation products of genomic dsRNA and ssRNA synthesized by T7 RNA polymerase. Samples of [^{35}S]methionine-labeled cell-free translation reactions were added directly to a 10% polyacrylamide gel with 3% stacking gel (A) or after immunoprecipitation with polyvalent rabbit antiserum prepared against whole virus (B). Lanes: 1, translation products from genomic dsRNA, 2, purified IPNV Sp; 3, translation products from pT72/A containing segment A cDNA; 4, translation products from pT72/B containing segment B cDNA; 5, endogenous proteins from the translation reaction. Viral proteins: α, RNA polymerase; β1, major capsid protein precursor; β2, first intermediate product, β3, major capsid protein found in purified virus; β4, third processed protein of β1; γ1, minor virion protein; γ1a, apparent breakdown product of γ 1, γ2, viral protein thought to occur only in infected cells. The major band below β1 in panel A, lane 1, is presumably a contaminating endogenous translation product present in the rabbit reticulocyte lysate, as shown in panel A, lane 5.

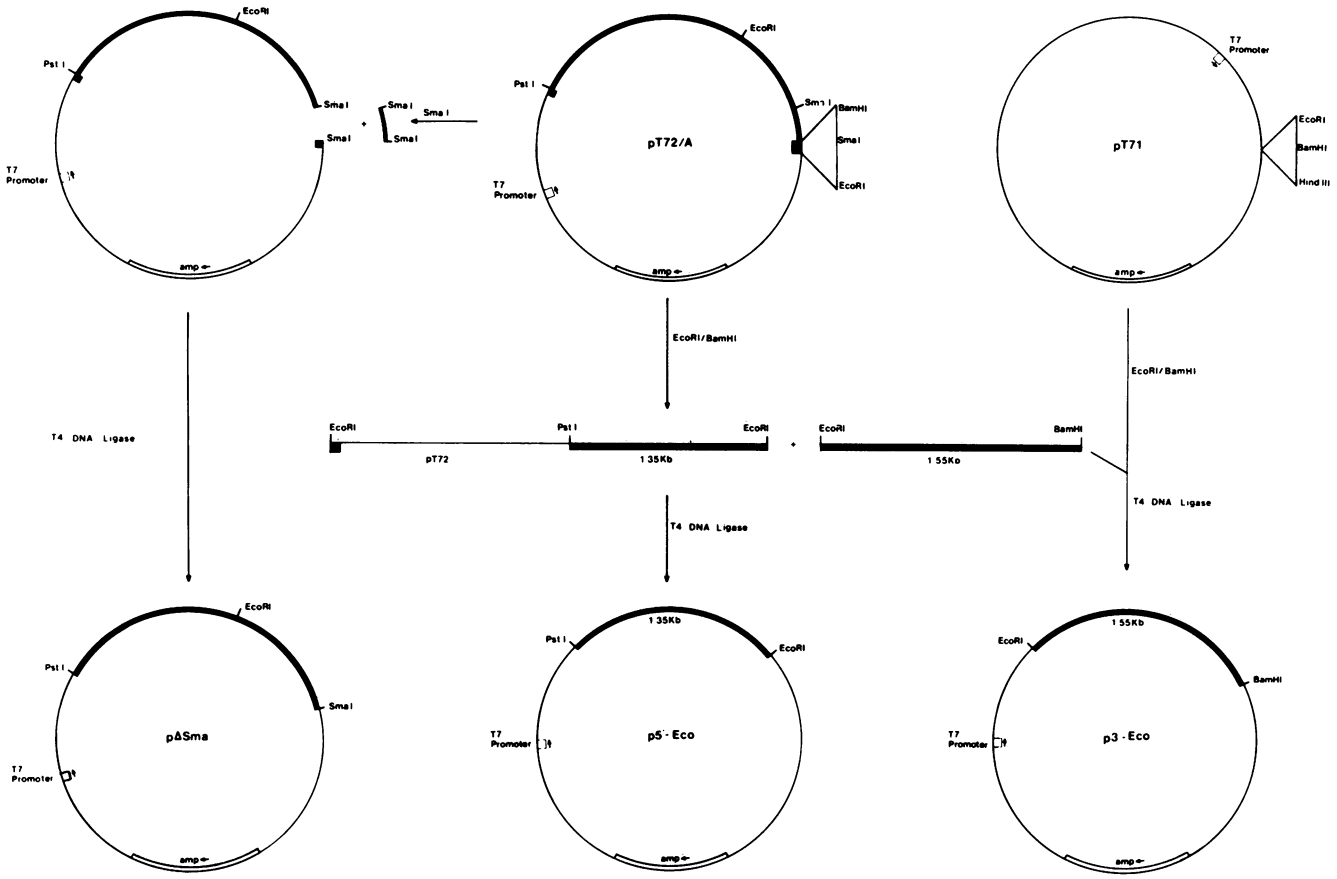

FIG. 10. Construction of three deletion plasmids of pT72/A used to determine the physical map of IPNV segment A. Three deletion plasmids of pT72/A were constructed by using the unique *Eco*RI and *Sma*I sites within the viral cDNA. Two of these plasmids were derived by digesting pT72/A (center) with *Eco*RI and *Bam*HI. The 1,550 bp fragment that was released from pT72/A was gel purified and subcloned into the *Eco*RI and *Bam*HI sites of pT71 to form deletion plasmid p3'-Eco (right). The remainder of the parental plasmid, containing pT72 and the 1,350 bp at the 5' end of segment A, was gel purified and religated at the *Eco*RI site to form deletion plasmid p5'-Eco (center). The third construction was prepared by digesting pT72/A at the *Sma*I site which is located approximately 430 bp from the 3' end of segment A. The large fragment that contained pT72 and 2,470 bp from the 5' end of segment A was gel purified and ligated to form pΔSma (left).

the translation products with the proteins of purified virus and with proteins synthesized intracellularly was made for the Sp strain (Fig. 8). At 5 to 7 h postinfection, the four predominant viral protein species included the viral RNA polymerase (α), the major capsid precursor (β1), the minor virion protein (γ1) (Fig. 8, lane 5), and the putative nonvirion protein (γ2), which was most prominent at 7 to 8 h postinfection (Fig. 8, lane 7). Purified Sp virus (Fig. 8, lane 9) contained α, the mature major capsid protein (β3), two precursor forms of the major capsid protein (β1 and β2), the minor virion protein (γ1), and a small protein (γ1a) which is thought to be a breakdown product of γ1 (5).

The major translation products from the dsRNA of purified IPNV Sp were α, β1, γ1, and γ2 (Fig. 9A, lane 1). After immunoprecipitation of the genomic RNA translation products with antisera made against purified IPNV, all four viral proteins were precipitated (Fig. 9B, lane 1). ssRNA from cloned segment A produced three major translation products (Fig. 9A and B, lanes 3). These bands corresponded in size to the major capsid protein precursor (β1), the minor virion protein (γ1), and the nonvirion protein (γ2), all of which were seen in virus-infected cells (Fig. 8). The ssRNA synthesized from pT71/A did not encode any virus-specific proteins and was presumed to be in the wrong orientation. ssRNA from cloned segment B (Fig. 9A and B, lanes 4) directed the synthesis of the viral RNA polymerase (α) plus a number of polypeptides presumed to be premature translation products. The results of these cell-free translation reactions indicate that the continuous cDNA clones of IPNV Sp contain the entire coding capacity of the viral genome. The distribution of proteins encoded by segments A and B was similar to that reported for strain VR299 (16) and infectious bursal disease virus of chickens (3).

Determination of the physical map of segment A. To determine the gene arrangement for segment A, three deletion plasmid constructions were prepared from pT72/A (described above). Two of these were derived by digesting the plasmid with *Eco*RI and *Bam*HI (Fig. 10). Religation at the *Eco*RI site of the plasmid molecule containing pT72 and the first 1,350 base pairs (bp) of the 5' end of segment A yielded the construction designated p5'-Eco (Fig. 10). Subcloning of the 1,550-bp *Eco*RI-*Bam*HI fragment of segment A into pT71 resulted in the plasmid designated p3'-Eco (Fig. 10). The third construction was prepared by *Sma*I digestion of pT72/A and religation of the resulting molecule with the 430-bp *Sma*I fragment missing. This construction contained approximately 2,470 bp of the 5' end of segment A and was designated pΔSma (Fig. 10).

In vitro translation products of the deletion constructs from pT72/A are shown in Fig. 11 and 12. Translation

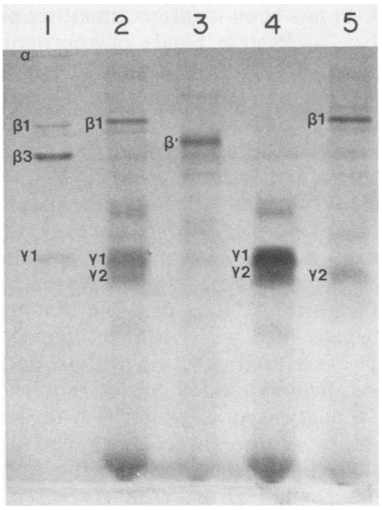

FIG. 11. Electrophoretic analysis of cell-free translation products from p5′-Eco, p3′-Eco, and pΔSma plasmids containing deletions of pT72/A. T7 RNA polymerase was used to prepare ssRNA from four plasmids containing sequences from IPNV segment A. ssRNA was added to rabbit reticulocyte lysate reactions, and samples were removed for electrophoresis in a 10% SDS-polyacrylamide gel with 3% stacking gel. Lanes: 1, ^{35}S-labeled purified IPNV Sp; 2, translation products from pT72/A; 3, translation products from p5′-Eco; 4, translation products from p3′-Eco; 5, translation products from pΔSma. Viral proteins: β1, major capsid protein precursor; β′, truncated major capsid protein precursor; β3, major virion protein found in purified virus; γ1, minor capsid protein found in purified virus; γ2, a viral protein associated with infected cells.

products from each of the three constructs were characterized by (i) a size comparison with both purified virus and proteins synthesized from a full-length transcript of segment A cDNA (Fig. 11) and (ii) immunoprecipitation with group-specific antisera prepared against either the β or γ size class viral proteins (Fig. 12).

The p5′-Eco transcript, which contains the first 1,350 bp of segment A, directed the synthesis of a 57-kDa polypeptide designated β′ (Fig. 11, lane 3). This protein is approximately 5 kDa smaller than the full-length precursor synthesized from the complete transcript of segment A (Fig. 11, lane 2). Both the full-length and partial translation products encoded at the 5′ end of segment A were recognized by the anti-β rabbit serum but not by the anti-γ serum (Fig. 12). Thus, the β protein is encoded at the 5′ end of segment A.

The p3′-Eco transcript encoded two proteins of 31 and 29 kDa (Fig. 11, lane 4). These two polypeptides are similar in size and electrophoretic mobility to proteins obtained by translation of the IPNV genomic dsRNA (γ1 and γ2) or from purified IPNV SP (γ1) and were recognized only by the anti-γ rabbit serum. Therefore, the 3′-terminal 1,500 bp of segment A contain the genes for γ1 and γ2 and are translated independently of the β protein initiation signal.

The immunoprecipitation of a protein band migrating as γ2 with antiserum to purified virion γ1 protein was unexpected. It was reported previously that γ2 is a nonvirion protein (5). A silver-stained gel of the purified γ1 proteins used to generate the antiserum revealed no γ2 protein band. However, the γ2 protein may have been a small, highly immunogenic contaminant of the preparation.

By the same criterion, we determined that the third deletion transcript, pΔSma, encoded the full-length β precursor and that the γ2 protein was recognized only by its group-specific antiserum (Fig. 12, lanes 5 and 9). Since this construction removed 430 bp at the 3′ terminus of segment A, we expected to see a truncated form of γ1 at 17 to 18 kDa. However, immunoprecipitation of the proteins from this reaction identified only the full-length β precursor and a protein migrating at the position of a γ2-size molecule. Proteins from the nonimmunoprecipitated gel sample also lacked a truncated γ1 molecule (Fig. 11, lane 5). Analysis of the protein patterns seen for the three translations suggested that (i) the gene order for segment A is linear and (ii) the arrangement of the genes is β, γ2, and γ1.

DISCUSSION

The cloning of the genomic RNA from IPNV Sp led to the construction of two sets of overlapping cDNA clones. All inserts within each set were shown to be related to each other by cross-hybridization analysis. In addition, each set of inserts was related to only one of the two genomic RNA species, as determined by Northern blot analysis. These genomic species have been designated segments A and B in accordance with the terminology first established by MacDonald and Dobos (13).

Restriction endonuclease maps of the two cDNA segments were constructed by comparing both the restriction and cross-hybridization patterns of individual cDNA inserts. Measurements of the resulting overlapping fragments (Fig. 2

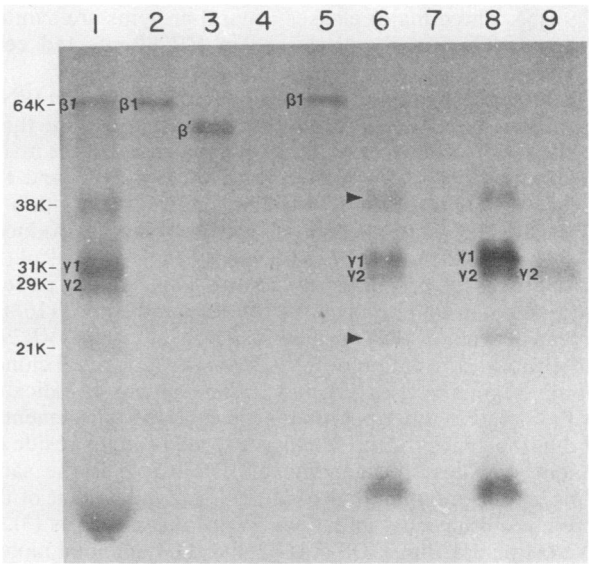

FIG. 12. Immunoprecipitation and SDS-PAGE of cell-free translation products from p5′-Eco, p3′-Eco, and pΔSma deletion plasmids (Fig. 8). Samples of the translation reactions shown in Fig. 8 (lanes 2 through 5) were immunoprecipitated with either anti-β or anti-γ group-specific rabbit sera. Lanes: 1, nonimmunoprecipitated translation products from pT72/A; 2 through 5, translation products, immunoprecipitated with anti-β group-specific rabbit serum, from pT72/A (lane 2), p5′-Eco (lane 3), p3′-Eco (lane 4), or pΔSma (lane 5); 6 through 9, translation products, immunoprecipitated with anti-γ group-specific rabbit serum, from pT72/A (lane 6), p5′-Eco (lane 7), p3′-Eco (lane 8), or pΔSma (lane 9). Viral proteins: β1, major capsid protein precursor; β′ truncated major capsid precursor; γ1, minor virion protein; γ2, nonvirion protein. The arrows indicate two proteins recognized by anti-γ rabbit serum whose sizes do not correspond to any viral proteins reportedly associated with IPNV.

and 3) gave an estimated size of 2.9 kb for segment A and 2.6 kb for segment B. These values are smaller than those predicted by Hedrick et al. (8). In their report, the dsRNAs from IPNV strains VR299, Ab, and Sp were electrophoresed in polyacrylamide gels and compared to reovirus type 3 RNA. The estimated molecular masses of segments A and B for strain Sp were 2.2 and 2.0 megadaltons, respectively. An average molecular mass of 640 daltons per RNA bp would convert these estimated molecular masses to 3.75 kb for segment A and 3.44 kb for segment B. These sizes are approximately 850 bp larger than those of the cDNA clones constructed in this study.

By subcloning the cDNA from each segment of IPNV Sp into the T7 RNA polymerase plasmids pT71 and pT72, we were able to synthesize full-length ssRNAs of each cloned segment. Full-length synthesis was extremely efficient when an excess of nucleotide precursors was used. Further modification of the RNAs, either by 5′ capping or 3′ polyadenylation, was not required to obtain translatable RNA. However, the translation efficiency of these RNAs (counts per minute incorporated per microgram of RNA) was approximately 10-fold less than that of control mRNA from brome mosaic virus (data not shown).

The cell-free translation products from the genomic dsRNA and the ssRNAs of cloned DNA were analyzed by immunoprecipitation and SDS-PAGE. Four major viral proteins were shown to be encoded by both the genomic RNA and cDNA sequences of IPNV Sp (Fig. 9). The relative banding pattern and coding assignments of these proteins were similar to those characterized previously for strain VR299 (5). Three major classes of viral proteins are synthesized and differentially processed in VR299-infected cells (5).

We demonstrated that CHSE-214 cells infected with IPNV Sp synthesize four viral proteins that correspond to the three size classes of viral proteins: RNA polymerase (α), the major capsid protein (β), the minor virion protein (γ1), and the nonvirion protein (γ2) (Fig. 8). The cell-free translation of both the ssRNAs and the genomic dsRNA (Fig. 9) produced protein bands that would be expected for the synthesis of the four unprocessed precursor molecules. This banding pattern was identical to that reported by Mertens and Dobos (16) for the translation of the purified dsRNA of strain VR299. Furthermore, translation of RNA from the individual cloned genomic segments (Fig. 9A and B, lanes 3 and 4) indicated that the putative RNA polymerase is encoded by segment B and that the genes for the remaining three proteins reside on segment A. Thus, the genome of IPNV Sp has the same coding arrangement as that of VR299, as well as that of the nonrelated birnavirus, infectious bursal disease virus (3).

We estimated from SDS-PAGE that the combined molecular mass for the unprocessed β protein and two γ proteins is approximately 120 kDa. A nonoverlapping arrangement of these proteins would require approximately 3,000 bp, a value very close to the estimated size of Sp segment A (2,900 bp). We showed that the selective deletion of 5′- and 3′-end regions of segment A cDNA results in the synthesis of partial or full-length viral proteins (Fig. 11). The simplest explanation of these results is based on a nonoverlaying linear gene arrangement along segment A in the order 5′–β-γ2-γ1–3′.

The ssRNAs from pT72/A and p3′-Eco also directed the synthesis of two polypeptides of approximately 38 and 21 kDa (Fig. 11, lanes 2 and 4). Although these proteins were recognized by the γ-specific rabbit serum (Fig. 12, lanes 6 and 8), their origins are not clear. The 38-kDa protein is too small to be a full-length precursor of γ1 and γ2, and no viral protein of 21 kDa has been identified in either purified virus or in infected cells. Protein bands of approximately these sizes are visible in the cell-free translation products from strain VR299 genomic RNA which were labeled with either [^{35}S]methionine or [^{35}S]formylmethionine (16). It is possible that the translation pattern seen here for the Sp strain of IPNV is not unique but is common to IPNV in general.

The γ-specific serum was expected to be specific for the γ1 and γ1a proteins of purified virus. However, the γ-specific serum recognized not only γ1 and γ1a but also a protein migrating at the position of γ2 in a gel of proteins translated in vitro from segment A. It is possible that the γ-specific antiserum was generated with virion protein contaminated with γ2 or that γ2 is actually a virion protein, detectable only in silver-stained, heavily loaded gels of the virion proteins. DNA sequence analysis of segment A will determine the precise boundaries of the three genes and enable us to examine possible similarities between the nucleotide and amino acid sequences of γ1 and γ2.

ACKNOWLEDGMENTS

This work was funded by grants R/FSD-9 and R/FSD-11 from the Sea Grant Office, Oregon State University, Corvallis.

We gratefully acknowledge the critical review of the manuscript by C. Passavant and the typing of the manuscript by C. Pelroy.

LITERATURE CITED

1. **Allen, R. C.** 1980. Rapid isoelectric focusing and detection of nanogram amounts of protein from body tissues and fluids. Electrohoresis **1**:32–37.
2. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a double lysogenic strain derived from *Escherichia coli* K12. Genetics **39**:440–452.
3. **Azad, A. A., S. A. Barrett, and K. J. Fahey.** 1985. The characterization and molecular cloning of the double-stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology **143**:35–44.
4. **Dobos, P., B. J. Hill, R. Hallett, D. T. C. Kells, H. Becht, and D. Teninges.** 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J. Virol. **32**:593–605.
5. **Dobos, P., and D. Rowe.** 1977. Peptide map comparison of infectious pancreatic necrosis virus-specific polypeptides. J. Virol. **24**:805–820.
6. **Fields, B. N.** 1985. Virology. Raven Press, New York.
7. **Fryer, J. L., A. Yusha, and K. S. Pilcher.** 1965. The *in vitro* cultivation of tissue and cells of Pacific salmon and steelhead trout. Ann. N.Y. Acad. Sci. **126**:566–586.
8. **Hedrick, R. P., J. L. Fryer, S. N. Chen, and G. H. Kou.** 1983. Characteristics of four birnaviruses isolated from fish in Taiwan. Fish Pathol. **18**:91–97.
9. **Hedrick, R. P., N. Okamoto, T. Sano, and J. L. Fryer.** 1983. Biochemical characterization of eel virus european. J. Gen. Virol. **64**:1421–1426.
10. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
11. **Kurath, G., K. G. Ahern, G. D. Pearson, and J. C. Leong.** 1985. Molecular cloning of the six mRNA species of infectious hematopoietic necrosis virus, a fish rhabdovirus, and gene order determination by R-loop mapping. J. Virol. **53**:469–476.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **MacDonald, R. D., and P. Dobos.** 1981. Identification of the proteins encoded by each genome segment of infectious pancreatic necrosis virus. Virology **114**:414–422.
14. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
16. **Mertens, P. P. C., and P. Dobos.** 1982. Messenger RNA of infectious pancreatic necrosis virus is polycistronic. Nature (London) **297**:243–246.
17. **Okayama, K., and P. Berg.** 1982. High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. **2**:161–170.
18. **Pilcher, K. S., and J. L. Fryer.** 1980. The viral diseases of fish: a review through 1978. Crit. Rev. Microbiol. **7**:287–364.
19. **Rice, C. M., C. A. Franke, J. H. Strauss, and D. E. Hruby.** 1985. Expression of Sindbis virus structural proteins via recombinant vaccinia virus: synthesis, processing, and incorporation into mature Sindbis virions. J. Virol. **56**:227–239.
20. **Sipple, A. E.** 1973. Purification and characterization of adenosine triphosphate:ribonucleic acid adenyltransferase from *Escherichia coli*. Eur. J. Biochem. **37**:31–40.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Taylor, J. M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**:324–330.
23. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
24. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.