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Generation of Infectious Pancreatic Necrosis Virus from Cloned cDNA

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We developed a reverse genetics system for infectious pancreatic necrosis virus (IPNV), a prototype virus of the *Birnaviridae* family, with the use of plus-stranded RNA transcripts derived from cloned cDNA. Full-length cDNA clones of the IPNV genome that contained the entire coding and noncoding regions of RNA segments A and B were constructed. Segment A encodes a 106-kDa precursor protein which is cleaved to yield mature VP2, nonstructural protease, and VP3 proteins, whereas segment B encodes the RNA-dependent RNA polymerase VP1. Plus-sense RNA transcripts of both segments were prepared by in vitro transcription of linearized plasmids with T7 RNA polymerase. Transfection of chinook salmon embryo (CHSE) cells with combined transcripts of segments A and B generated infectious IPNV particles 10 days posttransfection. Furthermore, a transfectant virus containing a genetically tagged sequence was generated to confirm the feasibility of this system. The presence and specificity of the recovered virus were ascertained by immunofluorescence staining of infected CHSE cells with rabbit anti-IPNV serum and by nucleotide sequence analysis. In addition, 3′-terminal sequence analysis of RNA from the recovered virus showed that extraneous nucleotides synthesized at the 3′ end during in vitro transcription were precisely trimmed or excluded during replication, and hence these were not incorporated into the genome. An attempt was made to determine if RNA-dependent RNA polymerase of IPNV and infectious bursal disease virus (IBDV), another birnavirus, can support virus rescue in heterologous combinations. Thus, CHSE cells were transfected with transcripts derived from IPNV segment A and IBDV segment B and Vero cells were transfected with transcripts derived from IBDV segment A and IPNV segment B. In either case, no infectious IPNV or IBDV particles were generated even after a third passage in cell culture, suggesting that viral RNA-dependent RNA polymerase is species specific. However, the reverse genetics system for IPNV that we developed will greatly facilitate studies of viral replication and pathogenesis and the design of a new generation of live attenuated vaccines.

Infectious pancreatic necrosis virus (IPNV) is the causal agent of a highly contagious and destructive disease of juvenile rainbow and brook trout as well as Atlantic salmon (31). Highly virulent strains of IPNV can cause greater than 90% mortality in hatchery stocks less than 4 months old. Survivors of infection can remain lifelong asymptomatic carriers and serve as reservoirs of infection (21). Therefore, IPNV is a pathogen of major economic importance to the aquaculture industry. There are two distinct serogroups of IPNV, designated serogroups A and B. Serogroup A contains nine serotypes, whereas serogroup B contains a single serotype (19).

IPNV is the prototype virus of the *Birnaviridae* family and belongs to the *Aquabirnavirus* genus (10). The IPNV genome consists of two segments of double-stranded RNA that are surrounded by a single-shelled icosahedral capsid 60 nm in diameter (7). The larger of the two genomic segments, segment A (3,097 bp), encodes a 106-kDa precursor protein in a single large open reading frame (ORF) which is cotranslationally cleaved by the viral nonstructural (NS) protease to generate mature VP2 and VP3 structural proteins (8, 12). Segment A also encodes a 17-kDa arginine-rich NS protein from a small ORF, which precedes and partially overlaps the major polyprotein ORF. Although this protein is not present in the virion, it is detected in IPNV-infected cells (20). Similarly, in infectious bursal disease virus (IBDV), another member of the *Birnaviridae* family, segment A also encodes a 17-kDa NS protein (from a small ORF) which is found in IBDV-infected cells (22). Recently, it was shown that this NS protein of IBDV is not required for viral replication but plays an important role in pathogenesis (32). The genomic segment B (2,784 bp) encodes VP1, a 94-kDa minor internal protein, which is the virion-associated RNA-dependent RNA polymerase (9, 13). In virions, VP1 is present as a free polypeptide, as well as a genome-linked protein, VPg (2).

Although the nucleotide sequences for genome segments A and B of various IPNV strains have been published, the precise 5′- and 3′-noncoding sequences of these strains have not been determined or confirmed (11, 13, 18). Unlike IBDV, there is extensive homology between the noncoding sequences of IPNV segments A and B. For example, 32 of 50 nucleotides at the 5′-noncoding region and 29 of 50 nucleotides at the 3′-noncoding region between the two segments are conserved. These termini should contain sequences that are important in the packaging and replication of the IPNV genome, as demonstrated for other double-stranded RNA viruses, such as mammalian reoviruses and rotaviruses (17, 25, 30, 34).

In recent years, a number of animal RNA viruses have been recovered from cloned cDNA, such as poliovirus (a plus-stranded RNA virus), influenza virus (a segmented negative-stranded RNA virus), and rabies virus (a nonsegmented negative-stranded RNA virus) (14, 26, 29). Recently, we recovered IBDV (a double-stranded RNA virus affecting poultry) from clone-derived transcripts of its genome segments (24). How-

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TABLE 1. Oligonucleotides used for the construction of full-length cDNA clones of IPNV genomic segments A and B[a]

Nucleotide sequence	Orientation	Designation	Nucleotide no.
*TAATACGACTCACTATA*GGAAAGAGAGTTTCAACG	+	A-A5′NC	1–18
GTACCTCCTTGGTGCGATTG	−	A-ApaR	711–731
GGTCAACAACCAACTAGTGACC	+	A-ApaF	571–593
CTGAATCAGGTGTGATGC	−	A-SalR	2222–2240
GTAGACATCAAAGCCAT	+	A-SalF	2129–2146
GA**AGATCTCCCGGG**GGCCCCCTGGGGGGCC	−	A-A3′NC	3078–3097
CCAACCGACATGGACAAGATCA	+	A-A3′F	2753–2774
GTCCTGTGATGTTTCCAACCAC	−	A-A5′R	361–382
*TAATACGACTCACTATA*GGAAACAGTGGGTCAACG	+	B-B5′NC	1–18
GAAGGTAAGTTGCTTCAGAAGCG	−	B-HindR	477–500
GGACGATGGCAAGCTTAAGGACAC	+	B-HindF	352–375
GTGTTGTCCTGCAGTATGTAGATG	−	B-PstR	1267–1291
AGAGACAGTCTGGACAA	+	B-PstF	1217–1233
A**AGATCT**GGGGTCCCTGGCGGAAC	−	B-Bg13′NC	2766–2783
GA**AGATCTCCCGGG**GTCCCTGGCGGAACCGGATGTTAT	−	B-Sma3′NC	2756–2783
CAAGAGAGGCACTGGAGACCAT	+	B-B3′F	2454–2475
GTAGAATGCAGTGGTTCCTTCTG	−	B-B5′R	385–408
CGGAAACCCGGAGCCGAGATTG	+	B-SmaΔF	1898–1919
CAATCTCGGCTCCGGGTTTCCG	−	B-SmaΔR	1898–1919
CATGATGTACTACCTCCTGAC	+	B-SacF	1351–1371
GATGCCTGGAACGACATGTCA	+	B-BstF	2060–2080
GAGTTTGGTCCTTTGGTCTAG	−	B-BstR	2285–2305

[a] Composition and locations of the oligonucleotide primers used for cloning are shown. T7 promoter sequences are marked with italic type, the virus-specific sequences are underlined, and the restriction sites are marked in boldface type. Orientation of the virus-specific sequence of the primer is shown as sense (+) or antisense (−). The positions where the primers bind (nucleotide number) are in accordance with the published sequences of Jasper strain.

ever, to date, there is no report of a recovered infectious virus from an aquatic species.

To study the function of RNA-dependent RNA polymerase of birnaviruses and to develop a reverse genetics system for IPNV, we constructed full-length cDNA clones of segments A and B of the West Buxton (WB) strain. Complete nucleotide sequences of these cDNA clones were determined, including those of the 5′- and 3′-noncoding regions. One of the cDNA clones was modified by site-directed mutagenesis to create a genetic tag in segment B. Synthetic plus-sense RNA transcripts of IPNV segments A and B were produced by in vitro transcription reactions on linearized plasmids with T7 RNA polymerase and used to transfect chinook salmon embryo (CHSE) cells. Furthermore, transcripts derived from IPNV segment A plus IBDV segment B and IBDV segment A plus IPNV segment B were used to transfect CHSE and Vero cells, respectively. In this report, we describe the recovery of IPNV from CHSE cells transfected with homologous RNA transcripts of IPNV segments A and B and evaluate the fate of IPNV or IBDV recovery in heterologous combinations. Furthermore, we demonstrate the need for the capping of the transcripts for virus rescue and the repair of the extraneous nucleotides synthesized at the 3′ end of these RNAs in the recovered virus.

MATERIALS AND METHODS

Cells and viruses. CHSE-214 cells (ATCC CRL-1681) were maintained at room temperature in minimal essential medium containing Hanks' salts and supplemented with 10% fetal bovine serum (FBS). Vero cells were grown in M199 medium supplemented with 5% FBS at 37°C in a humidified 5% CO_2 incubator. These cells were used for propagation of IPNV, transfection experiments, further propagation of the recovered virus, and immunofluorescence studies, essentially as described previously (24). The WB strain of IPNV (a reference serogroup A_1 strain) was kindly provided by Frank M. Hetrick (Maryland Department of Agriculture, College Park, Md.) and was purified as described previously with slight modifications (4). Briefly, CHSE cells were infected with IPNV, and after the cytopathic effect was visible, the cells were scraped into the medium and the crude virus was clarified by centrifugation at 5,000 × *g* for 30 min at 4°C. The pellet was resuspended in 10 ml of TNE buffer (0.1 M Tris-HCl [pH 7.4], 0.1 M NaCl, 1 mM EDTA), mixed with 1 volume of Freon, and homogenized for 5 min. After centrifugation at 8,000 × *g* for 20 min at 4°C, the aqueous layer was aspirated and mixed with the supernatant of the crude virus preparation. Polyethylene glycol (molecular weight, 20,000) was added to a final concentration of 10% (wt/vol), and the mixture was incubated overnight at 4°C. The solution was centrifuged at 8,000 × *g* for 30 min at 4°C to pellet the virus, which was resuspended in 10 ml of TNE buffer. After Freon extraction, the virus was pelleted at 100,000 × *g* for 1.5 h at 4°C and resuspended in 0.5 ml of TNE buffer. The virus was layered onto a cushion of 30% sucrose (wt/vol, in TNE buffer) and centrifuged at 120,000 × *g* for 1 h at 4°C. Finally, the virus pellet was resuspended in 100 μl of TNE buffer and stored at −20°C until use.

Determination of 5′ and 3′ termini of the IPNV genome. Complete nucleotide sequences of 5′- and 3′-noncoding regions from both genomic segments of IPNV were determined by two methods as described for IBDV (23). Briefly, viral RNA was isolated from purified virus by digestion with proteinase K (200-μg/ml final concentration) for 6 h at 37°C in the presence of sodium dodecyl sulfate (1%) followed by phenol-chloroform extraction and ethanol precipitation (27). To determine the 3′ termini of both strands of segments A and B, the viral RNA was polyadenylated and reverse transcribed with a poly(dT) primer (5′-GCGGCCG CCCTTTTTTTTTTTTTTTT-3′), and resulting cDNAs were amplified by PCR with either A-A3′F, A-A5′R, B-B3′F, or B-B5′R primer (Table 1) (3). The reverse transcription (RT)-PCR products were separated by agarose gel electrophoresis, purified by using a QIAquick gel extraction kit (Qiagen, Inc.), and directly sequenced by the dideoxy chain termination method, using the segment-specific primers described above (28). The 5′ termini of segments A and B were determined by the rapid amplification of cDNA ends method, using the 5′ RACE system (GIBCO/BRL) (16). Briefly, cDNA of segments A and B was synthesized by RT reaction with virus-specific primers A-ApaR and B-HindR (Table 1), respectively. The cDNA was purified by chromatography on GlassMAX columns and tailed with oligo(dC) by using terminal deoxynucleotidyltransferase. The tailed cDNA was amplified by PCR with nested virus-specific primer A-A5′R or B-B5′R (Table 1) and abridged anchor primer, in accordance with the manufacturer's protocol. The PCR products were gel purified and directly sequenced by using segment-specific primers as described above.

Construction of the full-length cDNA clones of IPNV. The cDNA clones containing the entire coding and noncoding regions of IPNV RNA segments A and B were prepared by standard cloning procedures and methods, as described previously for IBDV (24). Construction of full-length cDNA clones of IBDV genome segments A and B of strain D78 has been reported previously (24, 32). In addition, all manipulations of DNAs were performed in accordance with standard protocols (27). On the basis of published IPNV sequences of the Jasper strain and the determined 5′- and 3′-terminal sequences of the WB strain, several primer pairs were synthesized and employed in RT-PCR amplifications (Table 1).

To generate cDNA clones of segment A of the WB strain, three primer pairs (A-A5′NC plus A-ApaR, A-ApaF plus A-SalR, and A-SalF plus A-A3′NC) were used for RT-PCR amplification (Table 1). Using genomic RNA as a template, desired overlapping cDNA fragments of segment A were synthesized and amplified in accordance with the supplier's protocol (Perkin-Elmer). Amplified

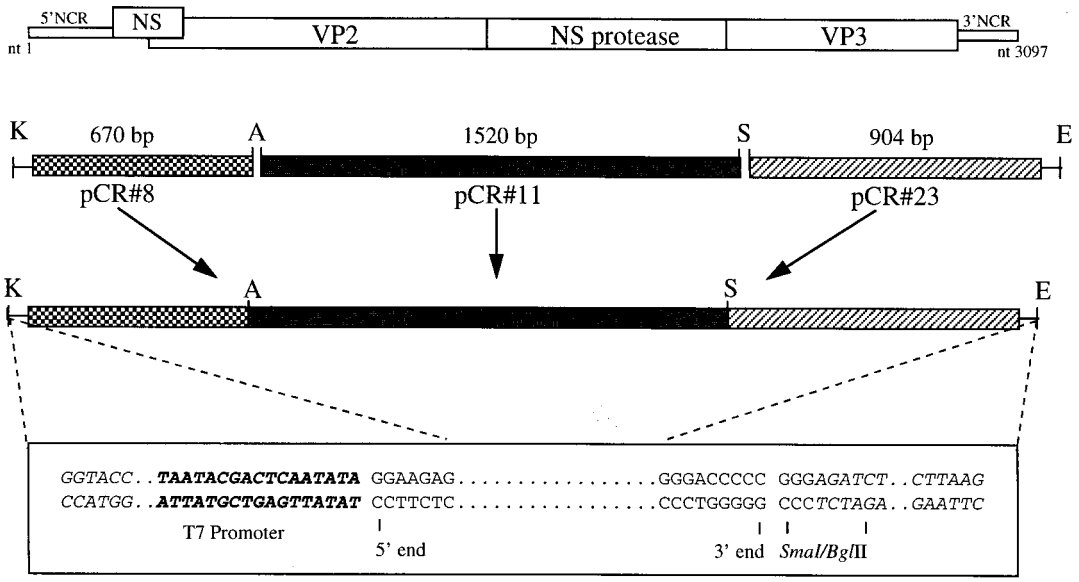

FIG. 1. Construction of the full-length cDNA clone of IPNV segment A for the generation of plus-sense RNA transcript with T7 RNA polymerase. The gene structure of IPNV segment A and its encoded proteins are shown at the top. Overlapping cDNA segments of IPNV were generated by RT-PCR and cloned into a pCR2.1 vector to obtain various pCR clones, as indicated. These plasmids were digested with appropriate restriction enzymes, and the resulting segments were cloned into a pUC19 vector to obtain plasmid pUC19WBA. This plasmid contains a T7 RNA polymerase promoter sequence at its 5′ end. Restriction enzymes used for the construction or linearization of the full-length clone are indicated. Abbreviations: A, *Apa*I; E, *Eco*RI; K, *Asp*718; S, *Sal*I.

fragments were cloned into the *Eco*RI site of the pCR2.1 vector (Invitrogen Corp.) to obtain plasmids pCR#8, pCR#11, and pCR#23 (Fig. 1). The insert DNA in all these plasmids was sequenced by the dideoxy chain termination method with an Applied Biosystem automated DNA sequencer, and the sequence data were analyzed by using PC/GENE (Intelligenetics) software. To construct a full-length cDNA clone of segment A, plasmids pCR#8, pCR#11, and pCR#23 were double digested with restriction enzyme pairs *Asp*718 plus *Apa*I, *Apa*I plus *Sal*I, and *Sal*I plus *Eco*RI to release 670-, 1,520-, and 904-bp fragments, respectively. These fragments were then cloned between the *Eco*RI and *Asp*718 sites of pUC19 vector to obtain plasmid pUC19WBA. This plasmid contains a full-length copy of segment A, which encodes all of the structural and NS proteins (Fig. 1). Similarly, to prepare cDNA clones of segment B, three primer pairs (B-B5′NC plus B-HindR, B-HindF plus B-PstR, and B-PstF plus B-Bgl3′NC) were used to generate overlapping cDNA fragments of segment B by RT-PCR amplification (Table 1). Amplified fragments were cloned into pCR2.1 vector as described above to obtain plasmids pCR#4.1, pCR#3, and pCR#29 (Fig. 2). DNA from these plasmids was sequenced and analyzed as described above. Since sequence analysis of plasmid pCR#3 revealed an internal *Pst*I site, it was necessary to make two additional plasmids. To construct these clones (pUC19B5′#2 and pUC19B3′#5), plasmids pCR#4.1, pCR#3, and pCR#29 were double digested with enzyme pairs *Eco*RI plus *Hin*dIII, *Hin*dIII plus *Asp*718 or *Asp*718 plus *Pst*I, and *Pst*I plus *Bam*HI. After these digestions, respective fragments of 361, 626 or 293, and 1,503 bp were released. The *Eco*RI-*Hin*dIII and *Hin*dIII-*Asp*718 fragments were first cloned between the *Eco*RI-*Asp*718 sites of pUC19 vector to obtain plasmid pUC19B5′#2.

The *Asp*718-*Pst*I and *Pst*I-*Bam*HI fragments were then cloned between the *Asp*718 and *Bam*HI sites of pUC19 vector to obtain plasmid pUC19B3′#5. Finally, to construct a full-length cDNA clone of segment B, plasmid pUC19B3′#5 was digested with *Asp*718 and *Bam*HI, and the resultant fragment was cloned into the *Asp*718-*Bam*HI-digested pUC19B5′#2 vector. A representative clone of segment B was selected and designated pUC19WBB, which encodes VP1 protein (Fig. 2).

To introduce a sequence tag into IPNV segment B, plasmid pUC19WBBmut was constructed by oligonucleotide-directed mutagenesis with specific primer pairs and PCR amplification of the parent plasmid pUC19WBB. To construct pUC19WBBmut, two primer pairs (B-SacF plus B-SmaΔR and B-SmaΔF plus B-BstR [Table 1]) were synthesized and used to amplify the DNA fragments of 568 and 407 bp, respectively. These fragments were combined and subsequently amplified by PCR with the flanking primers (B-SacF plus B-BstR) to produce a 954-bp fragment. This fragment was digested with *Sac*II and *Bst*EII enzymes, and the resulting fragment (798 bp) was cloned back into the *Sac*II-*Bst*EII-cleaved parent plasmid pUC19WBB. As a result of this mutation, the unique internal *Sma*I site in this plasmid was deleted. Another plasmid, pUC19WBB-Sma, was constructed which upon linearization with *Sma*I enzyme and transcription reaction would yield RNA transcript with precise 3′-end sequences as the genomic RNA. To construct this plasmid, primer pairs (B-BstF plus B-Sma3′NC [Table 1]) were synthesized and used to amplify by PCR a 723-bp fragment from pUC19WBmut template. The amplified fragment was digested with *Bst*EII and *Bgl*II enzymes, and the resulting fragment (584 bp) was cloned back into the same sites of this plasmid. Finally, a representative clone of segment B which lacked an internal *Sma*I site was selected and designated pUC19WBB-Sma. The integrity of the full-length constructs, pUC19WBA, pUC19WBB, and pUC19WBB-Sma, was tested by coupled in vitro transcription and translation in a reticulocyte lysate system with T7 RNA polymerase (Promega Corp.).

Transcription and transfection of synthetic RNAs. Plasmid pUC19WBA and plasmids pUC19WBB and pUC19WBB-Sma were digested with *Sma*I and with both *Bgl*II and *Sma*I, respectively (Fig. 1 and 2), and used as templates for in vitro transcription with T7 RNA polymerase (Promega). Similarly, plasmids pUC19FLAD78 and pUCD78B were linearized with *Bsr*GI and *Pst*I enzymes, respectively, as described earlier (24, 32). The linearized DNA templates (≈3 μg) recovered after phenol-chloroform extraction and ethanol precipitation were added separately to a transcription reaction mixture (50 μl) containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM (each) ATP, CTP and UTP, 0.1 mM GTP, 0.25 mM cap analogue [m7G(5′)ppp(5′)G], 120 U of RNasin, and 150 U of T7 RNA polymerase (Promega), and the mixture was incubated at 37°C for 1 h. Under these conditions, up to 70% of the transcripts were capped and purified by phenol-chloroform extraction and ethanol precipitation. As controls, the transcription products were treated with either DNase or RNase (Promega) before the purification step.

Transfection assays were carried out in accordance with the protocol supplied by the manufacturer, using Lipofectin reagent (Life Technologies). Vero cells were transfected as described in detail previously (24). To transfect CHSE cells, the monolayers were grown to 80% confluence in 60-mm-diameter dishes and washed once with phosphate-buffered saline (PBS). Three milliliters of OPTI-MEM I (Life Technologies) was added to the monolayers, and the cells were incubated at room temperature for 1 h. Simultaneously, 0.15 ml of OPTI-MEM I was incubated with 12.5 μg of Lipofectin reagent for 45 min in a polystyrene tube at room temperature. Equimolar amounts of RNA transcripts of segments A and B (≈8 μg each) were resuspended in 0.15 ml of diethyl pyrocarbonate-treated water, added to the OPTI-MEM–Lipofectin mixture, mixed gently, and incubated on ice for 5 min. After removing the OPTI-MEM from the monolayers in 60-mm-diameter dishes and replacing it with a fresh 1.5 ml of OPTI-MEM, the nucleic acid-containing mixture was added dropwise to CHSE cells and swirled gently. After 3 h of incubation at room temperature, the mixture was replaced with minimal essential medium containing Hanks' salts and 10% FBS (without rinsing the cells). The cultures were incubated at room temperature for desired time intervals, and the cell supernatant was harvested and passaged (referred to as passage 2) onto fresh CHSE monolayers. These passage 2 cultures were incubated at room temperature for 4 to 6 days, and the virus present in the cell supernatant was harvested and passaged once more in CHSE cultures (referred to as passage 3). Cells transfected with combined plus-sense transcripts derived from plasmids pUC19WBA and pUC19WBB or pUC19WBA and pUC19WBB-

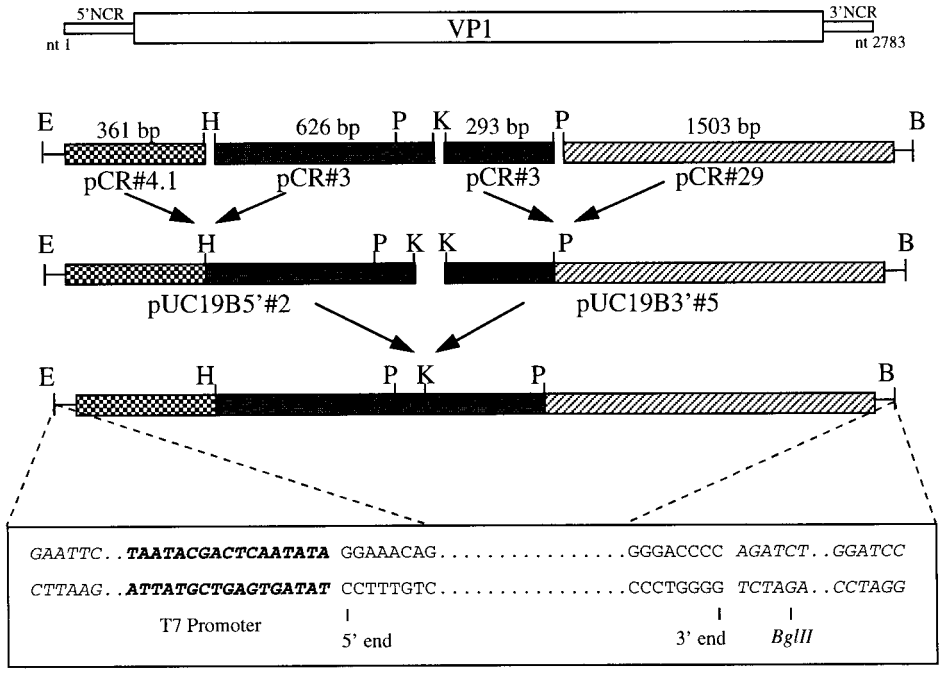

FIG. 2. Construction of the full-length cDNA clone of IPNV segment B for the synthesis of plus-sense RNA transcript with T7 RNA polymerase. The genome segment B of IPNV encodes the RNA-dependent RNA polymerase, VP1, which is shown at the top. Overlapping cDNA segments of IPNV were cloned into the pCR2.1 vector to obtain various pCR clones, as shown. These plasmids were digested with appropriate restriction enzymes, and the resulting segments were cloned into the pUC19 vector to obtain plasmids pUC19B5′#2 and pUC19B3′#5. Finally, a full-length plasmid, pUC19WBB, which contains a T7 RNA polymerase promoter sequence at its 5′ end, was obtained from these two clones. Restriction enzymes used for the construction of the above plasmids or for linearization of the full-length clone are indicated. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Asp*718; P, *Pst*I.

Sma gave rise to virus progeny which were designated recombinant WB (rWB) and rWB-Sma, respectively.

Characterization of recovered IPNV. To determine the specificity of the recovered viruses, CHSE cells were infected with the supernatants of rWB or rWB-Sma IPNV and the infected cells were analyzed by immunofluorescence assay (IFA) with rabbit anti-IPNV polyclonal serum. The anti-IPNV serum, prepared against the Jasper strain of serogroup A, was kindly provided by Ana Baya (Virginia-Maryland Regional College of Veterinary Medicine, College Park, Md.). CHSE cells, grown on coverslips to 80% confluence, were infected with the supernatants of rWB or rWB-Sma IPNV and incubated at room temperature for an appropriate time interval. The cells were then washed with PBS, fixed with ice-cold methanol-acetone (1:1), and treated with rabbit anti-IPNV serum. After being washed with PBS, the cells were treated with fluorescein-labeled goat anti-rabbit antibody (Kirkegaard & Perry Laboratories) and examined by fluorescence microscopy.

To identify the tagged sequence in recovered viruses, total nucleic acids of uninfected and IPNV-infected CHSE cells were isolated and analyzed by RT-PCR as described above. Segment B-specific primer B-BstR, binding to nucleotide positions 2285 to 2305 (Table 1), was used for RT of genomic RNA. Following RT, the reaction products were amplified by PCR with an upstream segment B-specific primer, B-SacF (binding to nucleotide positions 1351 to 1371 [Table 1]). The resulting PCR fragments (954 bp) were gel purified and either sequenced as described before or digested with *Sma*I enzyme to determine the tag sequence.

To determine the 3′-terminal sequence of segment B in recovered viruses, total RNA of rWB- or rWB-Sma-infected cells was polyadenylated, reverse transcribed with a poly(dT) primer, and amplified by PCR with the B-B3′F primer, as described above. The RT-PCR products (329 bp) were gel purified and directly sequenced with the B-B3′F primer (Table 1) and a Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Plaque assay. Monolayers of CHSE cells, grown in six-well plates (2×10^6 cells/well), were infected with serially diluted supernatants derived from transfected CHSE cells in duplicate (0.5 ml/well). One hour postinfection, the cells were rinsed with serum-free medium and overlaid with minimal essential medium containing Hanks' salts, 5% FBS, and 0.75% methylcellulose. The cells were incubated at 16°C for 7 to 10 days, fixed, and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing the cells with distilled water, the plaques were counted, and the titers of the virus were calculated as plaque-forming units per milliliter of supernatant.

Nucleotide sequence accession number. The complete nucleotide sequences of the IPNV genome segments A and B of the WB strain have been deposited in GenBank under accession no. AF078668 and AF078669, respectively.

RESULTS

Sequence analysis of IPNV genome. We determined the complete nucleotide sequences of IPNV genome segments A and B, including the precise 5′- and 3′-terminal sequences. Segment A is 3,097 bp long and contains two overlapping ORFs. The major ORF encodes the structural VP2 and VP3 proteins and the NS protease, whereas the minor ORF codes for the NS protein (Fig. 1). Segment B is 2,783 bp long and encodes VP1, which is the RNA-dependent RNA polymerase (Fig. 2). Comparison of the 5′- and 3′-terminal sequences of segments A and B of WB strain with those of the Jasper strain (GenBank accession no. M18049 and M58756) showed some minor differences. For example, in segment A, a deletion of a T residue at nucleotide position 106 and an addition of a C residue at position 3097 were detected, whereas in segment B, a deletion of a C residue was found at position 2646 (Fig. 3). Comparison of the nucleotide and deduced amino acid sequences of WB strain segments A and B with those of the Jasper strain showed 91.64 and 90.37% identity at the nucleotide level and 97.22 and 97.16% identity at the amino acid level, respectively. This indicates that these two North American strains of IPNV are closely related. However, comparison of the nucleotide and deduced amino acid sequences of IPNV WB strain segments A and B with those of IBDV strain D78 showed 56.22 and 57.6% identity at the nucleotide level and 32.1 and 45.2% identity at the amino acid level, respectively.

Construction of full-length cDNA clones. To develop a reverse genetics system for IPNV, we constructed full-length

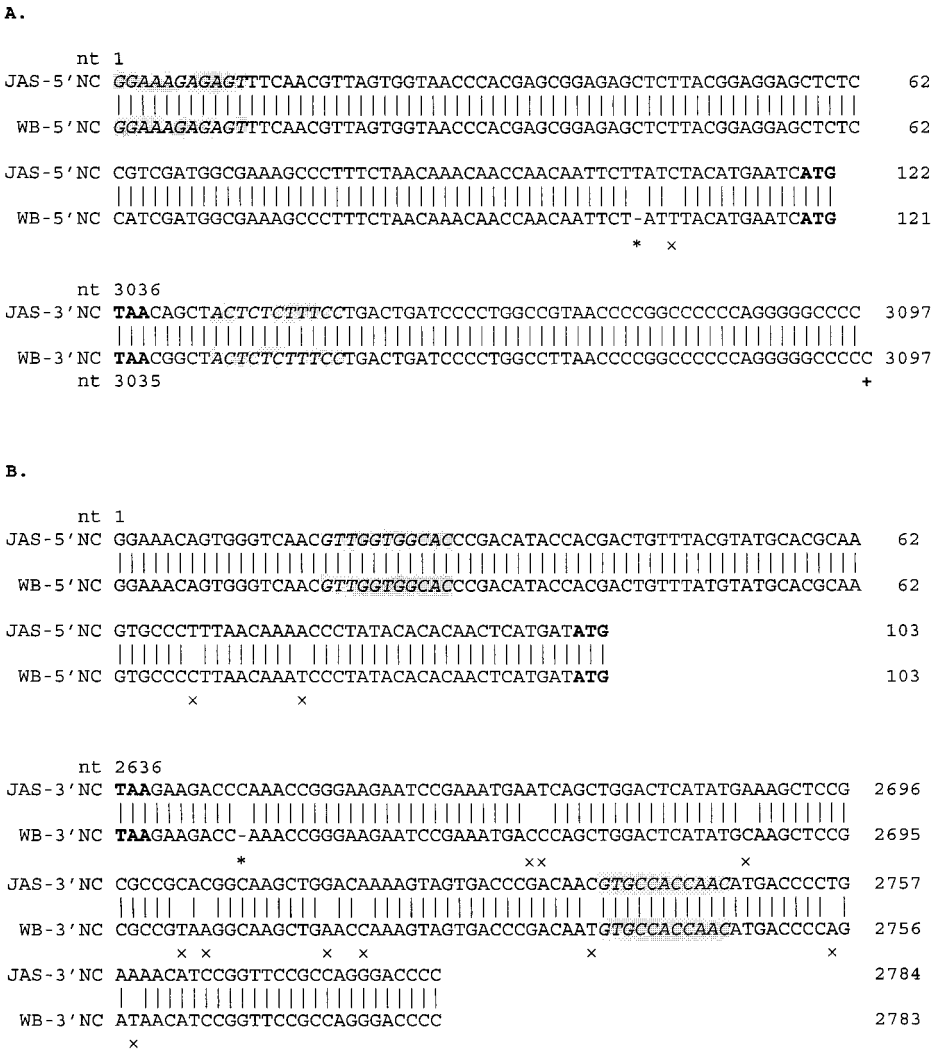

FIG. 3. Nucleotide sequence comparison of the 5′- and 3′-noncoding regions of segments A (A) and B (B) of IPNV strains Jasper (JAS) and West Buxton (WB). The start and stop codons of segments A and B major ORFs of both strains are in boldface type. Nucleotide differences between the two strains are marked by ×, and nucleotide identity is indicated by lines connecting identical residues. Nucleotide deletions in the WB strain are marked by ∗, and an additional C residue at the 3′ end of WB segment A is indicated by a +. Invert terminal repeats in segments A and B of both strains are shaded and italicized.

cDNA clones of segments A and B of IPNV strain WB. Plasmid pUC19WBA, upon digestion with *Sma*I and transcription in vitro by T7 RNA polymerase, yielded RNA with precise 5′ and 3′ ends, and it encoded all of the structural and NS proteins (Fig. 1). However, after linearization with *Bgl*II and transcription, plasmid pUC19WBB yielded RNA with the correct 5′ end but with an additional five nucleotides at the 3′ end, and it encoded VP1 protein (Fig. 2). We also constructed plasmid pUC19WBB-Sma with a genetic tag (the elimination of an internal *Sma*I site) to identify the virus as being of recombinant origin. Linearization of this plasmid with *Sma*I and transcription in vitro yielded RNA with precise 5′ and 3′ ends. Coupled transcription and translation of the above plasmids in a rabbit reticulocyte system yielded protein products which comigrated with the marker IPNV proteins after fractionation on a sodium dodecyl sulfate–12.5% polyacrylamide gel and autoradiography (data not shown).

Transfection and recovery of IPNV. Plus-sense transcripts of IPNV segments A and B were synthesized separately in vitro with T7 RNA polymerase with linearized plasmids pUC19WBA, pUC19WBB, and pUC19WBB-Sma as templates. Equimolar amounts of RNA transcript(s) of segments A and B (≈8 μg each) were then used to transfect CHSE cells. The efficiency of transfection was monitored by the β-gal staining assay, which measures the combined uptake of β-galactosidase and RNA-dependent RNA polymerase transcripts in transfected cells (33). Under these conditions, about 1.5% of the transfected CHSE cells would contain transcripts of both segments A and B. The cultures were incubated at room temperature for desired time intervals, and the cell supernatants were harvested and passaged (referred to as passage 2) onto fresh CHSE monolayers. Our results indicate that the transcripts derived from plasmids pUC19WBA and pUC19WBB were able to generate infectious virus (rWB) 12 days posttransfection, as evidenced by the appearance of cytopathic effect (CPE). Similarly, transcripts derived from plasmids pUC19WBA and pUC19WBB-Sma, either untreated or treated with DNase, gave rise to infectious virus (rWB-Sma) 10 days posttransfection. No CPE was detected when CHSE cells were transfected with either RNase-treated transcripts or uncapped RNAs of plasmids pUC19WBA and pUC19WBB-Sma, individual RNA of each plasmid, or Lipofectin reagent. These results indicate

TABLE 2. Recovery of IPNV at various times after transfection[a]

Days after transfection	No. of passages	IFA result	CPE	Titer of recovered virus (PFU/ml)
2	1	−	−	0
4	1	+	−	1.2×10^1
6	1	+	−	2.5×10^2
8	2	+	±	4.3×10^4
10	2	+	+++	6.7×10^7

[a] CHSE cells were transfected with synthetic RNAs derived from plasmids pUCWBB-Sma and pUCWBA as described in the text. The infectivity and specificity of the recovered virus were detected by cytopathic effect and indirect immunofluorescence antibody assays in CHSE cells. The titers of recovered virus present in the supernatants from the transfected cells were determined by plaque assay in CHSE cells.

that the capped and plus-sense RNA transcripts of segments A and B are required for the generation of IPNV, which is in agreement with the previous findings on IBDV reported from our laboratory (24).

To determine the time point for the recovery of infectious virus, CHSE cells were transfected with combined RNA transcripts of segments A and B. At 2, 4, 6, 8, and 10 days posttransfection, the cells were examined for the presence of viral antigens by IFA, and the cell supernatants were analyzed for the presence of transfectant virus by plaque assays, as shown in Table 2. Our results indicate that as early as 4 days posttransfection, infectious virus with a titer of 1.2×10^1 PFU/ml (which gradually increased) was produced. After several cycles of virus multiplication, the titer of the virus was high enough to cause overt CPE (about 80%) at day 10 posttransfection. After the third passage, the titer of these recovered viruses was comparable to that of the parental WB strain ($\approx 1 \times 10^8$ PFU/ml).

To determine the specificities of the recovered viruses, CHSE cells were infected with the supernatants derived from the third passage of either rWB or rWB-Sma viruses. At various time intervals, the cells were harvested and analyzed by IFA with anti-IPNV polyclonal serum. Figure 4 shows the results of immunofluorescence staining of IPNV-infected cells. CHSE cells infected with recovered IPNV gave a positive green immunofluorescence signal, indicating the expression of virus-specific proteins (Fig. 4b through e). However, no fluorescence was detected in the mock-infected cells at 24 h and 72 h (Fig. 4a and f).

Identification of tagged sequence. To demonstrate the utility of the reverse genetics system, two recombinant IPNVs were generated. To introduce a tagged sequence in segment B, plasmid pUC19WBB-Sma, in which a unique internal *Sma*I site in the VP1 gene was eliminated by site-directed mutagenesis, was constructed. Synthetic transcripts of this plasmid or of pUC19WBB and pUC19WBA were then used to transfect CHSE cells. To verify the presence or absence of mutation in recovered rWB and rWB-Sma viruses, genomic RNA was isolated and analyzed by RT-PCR with a primer pair specific for segment B. Figure 5 shows the analysis of RT-PCR products from recovered viruses before and after *Sma*I digestion. A 954-bp fragment was obtained from both rWB and rWB-Sma viruses (lanes 5 and 6), but not from the CHSE cells (lane 4). Moreover, no PCR product was detected in mock-infected or IPNV-infected cells if the RT was omitted from the reaction before PCR (lanes 1 to 3). This indicates that the PCR product was derived from RNA and not from contaminating DNA. *Sma*I digestion of the PCR product of rWB virus yielded the expected fragments of 403 and 551 bp. However, the PCR product of the mutant virus (lane 8), rWB-Sma, was not digested by *Sma*I (lane 7). Furthermore, sequence analysis of

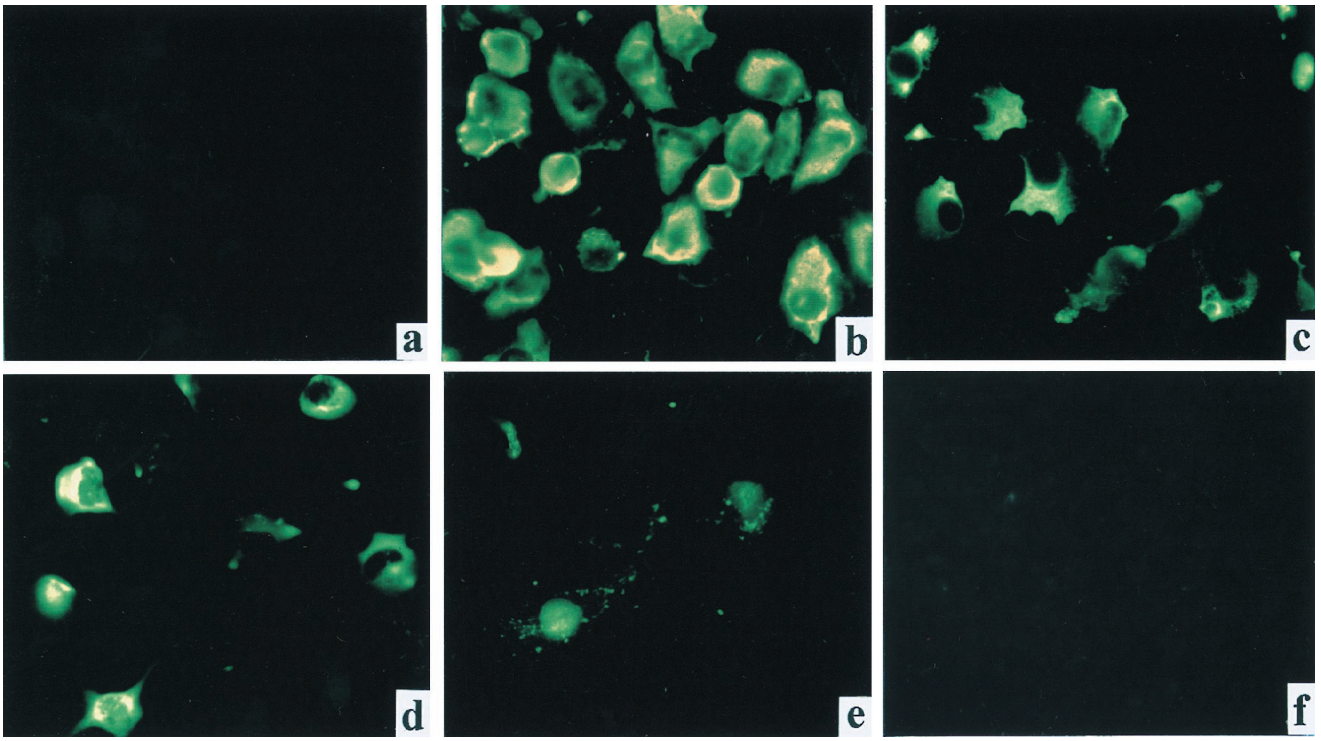

FIG. 4. Immunofluorescence staining of IPNV-infected cells for the detection of virus-specific proteins. CHSE cells were infected with the supernatant of rWB-Sma virus stock (after the third passage) and harvested at different time intervals. Cells were fixed at 24 h (b), 36 h (c), 48 h (d), and 72 h (e) postinfection and were analyzed by IFA with rabbit anti-IPNV polyclonal serum. Uninfected CHSE cells at 24 h (a) and 72 h (f) were used as negative controls. Magnification, ×184.

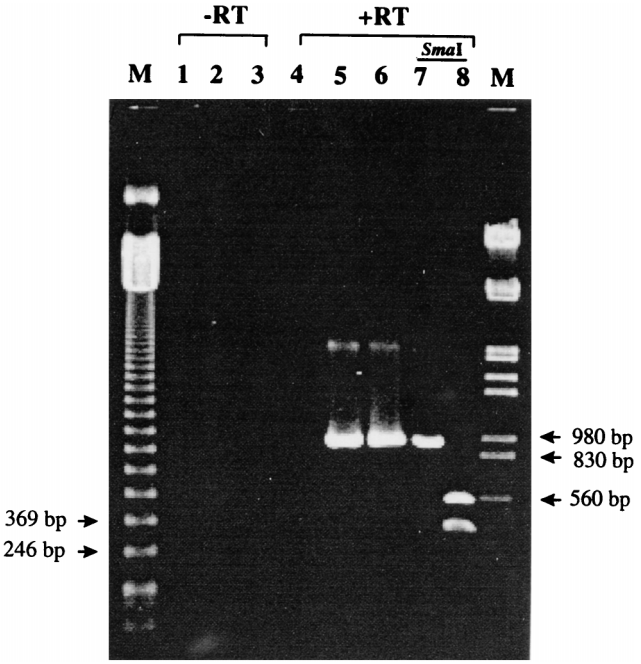

FIG. 5. Analysis of the RT-PCR products to identify the tagged sequence in segment B of recovered viruses. Genomic RNA isolated from recovered viruses was amplified by RT-PCR with segment B-specific primers B-BstR (binding to nucleotide positions 2285 to 2305) and B-SacF (binding to positions 1351 to 1371), and the products were analyzed on 1% agarose. A 954-bp fragment was obtained from rWB and rWB-Sma viruses (lanes 5 and 6), but not from the CHSE cells (lane 4) or the control(s) in which reverse transcriptase was omitted from the reaction (lanes 1 to 3). Gel-purified RT-PCR products were digested with *Sma*I as indicated (lanes 7 and 8). Only the DNA derived from parental virus (rWB) was digested, yielding fragments of 403 and 551 bp (lane 8), whereas the DNA of the rWB-Sma virus remained undigested because of an elimination of this *Sma*I site by site-directed mutagenesis (lane 7). A 123-bp ladder (left lane M) and lambda DNA digested with *Hin*dIII-*Eco*RI (right lane M) were used as markers.

this PCR product confirmed the expected nucleotide mutation (deletion of a *Sma*I site) in the VP1 gene (data not shown). These results show that the tagged sequence is present in the genomic RNA of the recovered virus.

Fate of extraneous nucleotides synthesized at 3′ end of RNA transcript. Since plus-sense RNA transcript derived from plasmid pUC19WBB contained five additional nucleotides at its 3′ end after linearization with *Bgl*II (Fig. 2), it was of interest to determine whether the double-stranded RNA present in the recovered virus also retained vector-derived nucleotides. Therefore, RNA from recovered viruses was polyadenylated, reverse transcribed with a poly(dT) primer, and amplified by PCR with segment B-specific primer, and the product was directly sequenced with the latter primer. Our results show that the extraneous nucleotides present in the synthesized RNA were not incorporated in the genome segment B of the recovered virus. This suggests that vector-derived nucleotides were precisely trimmed or excluded before being packaged into the virion.

RNA-dependent RNA polymerase of birnaviruses does not function in heterologous systems. To determine if RNA-dependent RNA polymerase of IPNV and IBDV can support virus rescue in heterologous combinations, CHSE cells were transfected with transcripts derived from IPNV segment A plus IBDV segment B, and Vero cells were transfected with transcripts derived from IBDV segment A plus IPNV segment B. In either case, no infectious IPNV or IBDV particles were generated even after the third passage in cell culture, indicating that viral RNA-dependent RNA polymerase is species specific.

DISCUSSION

In this communication, we describe the generation of IPNV from fish cells with the use of plus-stranded RNA transcripts derived from cloned cDNA of genomic segments A and B. This is the first report of an infectious RNA virus recovered from an aquatic species. The prerequisite for the construction of infectious clones of IPNV was the identification of the precise 5′- and 3′-terminal sequences, which are crucial for replication of viral RNA (25, 30). We determined the 5′- and 3′-noncoding sequences from both genomic segments of IPNV by poly(A) tailing and 5′ RACE methods. We found an additional C residue at the 3′ end of segment A of the WB strain, but the remaining terminal sequence was homologous to the published sequence of Jasper strain (11, 13). Based on these results, full-length cDNA clones of WB-IPNV genomic segments A and B were constructed. In these clones, a T7 RNA polymerase promoter sequence was also placed at its 5′ end to generate synthetic transcripts in vitro with T7 RNA polymerase. Upon transfection of CHSE cells with combined plus-sense RNA transcripts of segments A and B, recombinant IPNV (rWB) and recombinant IPNV bearing a tagged sequence (rWB-Sma) were efficiently recovered without the use of a helper virus or other viral proteins. The presence of the tagged sequence was verified by sequence analysis and restriction enzyme digestion of the RT-PCR products. These results unequivocally prove that the recovered virus was derived from the synthetic transcripts and that it was of recombinant origin.

The reverse genetics system for IPNV described here is very similar to the one developed for another birnavirus, IBDV, with which we demonstrated that synthetic transcripts of the IBDV genome are infectious and can give rise to a replicating virus (24). However, the significance of the cap analogue in generating infectious virus and the fate of extraneous nucleotides synthesized at the 3′ end during transcription were not determined. In the present study, we demonstrate that the "capping" of RNA transcripts was necessary to generate infectious virus, suggesting that the cap structure is essential for translation of synthetic mRNA. No significant difference in the stability of the synthesized RNAs, in the presence or absence of the cap, was observed in vitro. Moreover, 3′-terminal sequence analysis of RNA from recovered viruses shows that the extraneous nucleotides present in the synthetic RNA of segment B were not incorporated into the genome and were probably trimmed or excluded by the expressed RNA-dependent RNA polymerase during replication. Similar effects have been observed in several RNA viruses that have been recovered, including an unencapsidated hypovirus (1, 5). Nevertheless, this did not prevent the replication of the viral double-stranded RNA. It appears that recovery of this virus (rWB) was somewhat less efficient than that of the rWB-Sma virus, which was recovered with transcripts containing bona fide 5′ and 3′ ends. We did not examine whether extraneous nucleotides of segment A would also be excluded if plasmid pUC19WBA were linearized with *Bgl*II enzyme, but we would predict a similar fate. This is because when we analyzed the 3′-terminal sequence of RNA from recovered IBDV, all four vector-derived nucleotides were absent in segment A of the IBDV genome (33). However, the mechanism by which the omission of these nucleotides takes place is not known. One can speculate that since both segments of IPNV contain inverted terminal repeats (Fig. 3), these form a panhandle structure, similar to that reported for influenza virus, which the RNA polymerase could recognize to initiate transcription at a specific location (15). In addition, our results indicate that RNA-dependent RNA poly-

merase of birnavirus is species specific, since it does not support virus rescue in heterologous systems.

From our study, it is evident that transfected RNAs from both segments had to be translated in the host cells to produce functional proteins VP2, NS protease, VP3, and VP1 (RNA-dependent RNA polymerase). Since only the plus-strand RNAs of both segments were introduced into the cell, the minus-strand RNAs must have been synthesized from the plus-strand RNA templates by VP1 or RNA-dependent RNA polymerase to form double-stranded RNA. These results accord with the general features of rotavirus replication, where the plus-strand RNAs serve as a template for the synthesis of negative strands, which then yield double-stranded RNA (6, 25, 30). However, Dobos reported that in vitro transcription of IPNV is primed by genome-linked VP1 and that only the plus-strand RNAs, which remain base paired to their respective negative-strand templates, are synthesized by RNA-dependent RNA polymerase (9). These results indicate an asymmetric and semiconservative strand-displacement mechanism of replication, a possibility that should not be excluded.

The development of a reverse genetics system for IPNV presents new opportunities for studies directed at viral replication, persistence, and pathogenesis. In addition, it will greatly facilitate the construction of novel, live attenuated, marked vaccines for IPNV that are nonpathogenic to fish.

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REFERENCES

1. **Boyer, J. C., and A. L. Haenni.** 1994. Infectious transcripts and cDNA clones of RNA viruses. Virology **198:**415–426.
2. **Calvert, J. G., E. Nagy, M. Soler, and P. Dobos.** 1991. Characterization of the VPg-dsRNA linkage of infectious pancreatic necrosis virus. J. Gen. Virol. **72:** 2563–2567.
3. **Cashdollar, L. W., J. Esparza, J. R. Hudson, R. Chmelo, P. W. K. Lee, and W. K. Joklik.** 1982. Cloning of double-stranded RNA genes of reovirus: sequences of the cloned S2 gene. Proc. Natl. Acad. Sci. USA **79:**7644–7648.
4. **Chang, N., R. D. MacDonald, and T. Yamamoto.** 1978. Purification of infectious pancreatic necrosis (IPN) virus and comparison of peptide composition of different isolates. Can. J. Microbiol. **24:**19–27.
5. **Chen, B., M. G. Craven, G. H. Choi, and D. L. Nuss.** 1994. cDNA-derived hypovirus RNA in transformed chestnut blight fungus is spliced and trimmed of vector nucleotides. Virology **202:**441–448.
6. **Chen, D., C. Q.-Y. Zeng, M. J. Wentz, M. Gorziglia, M. K. Estes, and R. F. Ramig.** 1994. Template-dependent, in vitro replication of rotavirus RNA. J. Virol. **68:**7030–7039.
7. **Dobos, P.** 1976. Size and structure of the genome of infectious pancreatic necrosis virus. Nucleic Acids Res. **3:**1903–1919.
8. **Dobos, P.** 1977. Virus-specific protein synthesis in cells infected by infectious pancreatic necrosis virus. J. Virol. **21:**242–258.
9. **Dobos, P.** 1995. Protein-primed RNA synthesis *in vitro* by the virion associated RNA polymerase of infectious pancreatic necrosis virus. Virology **208:** 19–25.
10. **Dobos, P.** 1995. The molecular biology of infectious pancreatic necrosis virus (IPNV). Annu. Rev. Fish Dis. **5:**24–54.
11. **Duncan, R., and P. Dobos.** 1986. The nucleotide sequence of infectious pancreas necrosis virus (IPNV) dsRNA segment A reveals one large ORF encoding a precursor polyprotein. Nucleic Acids Res. **14:**5934.
12. **Duncan, R., E. Nagy, P. J. Krell, and P. Dobos.** 1987. Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. J. Virol. **61:**3655–3664.
13. **Duncan, R., C. L. Mason, E. Nagy, J. A. Leong, and P. Dobos.** 1991. Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp-Asp motif. Virology **181:**541–552.
14. **Enami, M., W. Luytjes, M. Krystal, and P. Palese.** 1990. Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA **87:**3802–3807.
15. **Fodor, E., D. C. Pritlove, and G. G. Brownlee.** 1994. The influenza virus panhandle is involved in the initiation of transcription. J. Virol. **68:**4092–4096.
16. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full-length cDNA rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85:**8998–9002.
17. **Gorziglia, M. I., and P. L. Collins.** 1992. Intracellular amplification and expression of a synthetic analog of rotavirus genomic RNA bearing a foreign marker gene: mapping cis-acting nucleotides in the 3′-noncoding region. Proc. Natl. Acad. Sci. USA **89:**5784–5788.
18. **Hävarstein, L. S., K. H. Kalland, K. E. Christie, and C. Endresen.** 1990. Sequence of the large double-stranded RNA segment of the N1 strain of infectious pancreatic necrosis virus: a comparison with other *Birnaviridae*. J. Gen. Virol. **71:**299–308.
19. **Hill, B. J., and K. Way.** 1995. Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. Annu. Rev. Fish Dis. **5:**55–77.
20. **Magyar, G., and P. Dobos.** 1994. Evidence for the detection of the infectious pancreatic necrosis virus polyprotein and the 17-kDa polypeptide in infected cells and of the NS protease in purified virus. Virology **204:**580–589.
21. **McAllister, P. E., W. J. Owens, and T. M. Ruppenthal.** 1987. Detection of infectious pancreatic necrosis virus in pelleted cell and particulate components from ovarian fluid of brook trout (*Salvilimus fontindis*). Dis. Aquat. Org. **2:**235–237.
22. **Mundt, E., J. Beyer, and H. Müller.** 1995. Identification of a novel viral protein in infectious bursal disease virus-infected cells. J. Gen. Virol. **76:**437–443.
23. **Mundt, E., and H. Müller.** 1995. Complete nucleotide sequences of 5′- and 3′-noncoding regions of both genome segments of different strains of infectious bursal disease virus. Virology **209:**10–18.
24. **Mundt, E., and V. N. Vakharia.** 1996. Synthetic transcripts of double-stranded birnavirus genome are infectious. Proc. Natl. Acad. Sci. USA **93:**11131–11136.
25. **Patton, J. T., M. Wentz, J. Xiaobo, and R. F. Ramig.** 1996. *cis*-acting signals that promote genome replication in rotavirus mRNA. J. Virol. **70:**3961–3971.
26. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214:**916–919.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schnell, M. J., T. Mebatsion, and K. K. Conzelmann.** 1994. Infectious rabies viruses from cloned cDNA. EMBO J. **13:**4195–4205.
30. **Wentz, M. J., J. T. Patton, and R. F. Ramig.** 1996. The 3′-terminal consensus sequence of rotavirus mRNA is the minimal promoter of negative-strand RNA synthesis. J. Virol. **70:**7833–7841.
31. **Wolf, K.** 1988. Fish viruses and fish viral diseases. Canstock Publishing Associates, Cornell University Press, Ithaca, N.Y.
32. **Yao, K., M. A. Goodwin, and V. N. Vakharia.** 1998. Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. J. Virol. **72:** 2647–2654.
33. **Yao, K., and V. N. Vakharia.** Unpublished data.
34. **Zou, S., and E. G. Brown.** 1992. Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. Virology **186:**377–388.