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Nucleotide Sequence Analysis of the RNA-dependent RNA Polymerase Gene of Infectious Pancreatic Necrosis Virus DRT Strain

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To determine the nucleotide sequence of the ds RNA segment B containing the RNA dependent RNA polymerase (RdRp) gene of the DRT strain of infectious pancreatic necrosis virus (IPNV), the cDNA of the ds RNA segment B of the DRT strain of IPNV was synthesized using the reverse transcriptase (RT)-polymerase chain reaction (PCR) and its cDNA nucleotide sequence was determined. The DRT segment B was 2,783 bp long and contained only a single long open reading frame (ORF) of 2,535 bp in length. This ORF nucleotides encoded the VP1 protein, the putative RdRp of IPNV. The VP1 protein consisted of 845 amino acids. The molecular weight of the RdRp, as deduced from the nucleotide sequence, is 94,426. The nucleotide sequence of the ORF of the DRT showed 89.7% homology to the Jasper strain, but 80.8% to the Sp strain. The amino acid sequence of the ORF of the DRT showed 97.6% homology to the Jasper strain, but 88.7% to the Sp strain. The conserved GTP-binding motif was detected in VP1 protein.

Infectious Pancreatic Necrosis Virus (IPNV) is the prototype of the newly established Birnaviridae virus family and is an economically important fish pathogen (2). It mainly affects young salmonids, e.g. brook trout (*Salvelinus fontinalis*) and rainbow trout (*Salmo gairdneri*). IPNV infection causes an acute, contagious disease (11).

Birnaviruses possess a bisegmented, double-stranded RNA genome surrounded by a medium-sized (60 nm), unenveloped and icosahedral capsid (6). This virus family also includes infectious bursal disease virus (IBDV), which not only causes a highly contagious disease in chickens characterized by the destruction of B lymphocytes in the bursa of Fabricius, but also drosophila X virus (DXV) of the common fruit fly (2, 5).

IPNV genome contains two double-stranded RNA segments, A and B (6). Genome segment A, of approximately 3,100 bp in length, encodes a single polyprotein and an autocatalytic endoprotease which processes the polyprotein. It is cleaved from the amino-terminal end to produce, in order, VP2, a major structural and antigenic protein of the virion; NS, non-structural protein with which a protease activity has been associated; and VP3, an internal component of the capsid (8). Genome segment B, of approximately 2,800 bp in length, is monocistronic and encodes a single gene product, VP1 protein, which acts as the RNA-dependent RNA polymerase (RdRp) of the virus. This polypeptide is present in virion in two forms: as a free VP1 polypeptide and as a genome-linked protein or VPg (3, 17).

Birnavirus gene expression involves a transcription from the dsRNA parental genome segments by a virion-associated RNA dependent RNA polymerase (14). RdRp directs the synthesis of non-polyadenylated mRNA species corresponding in size to the A and B segments of the viral genome. The RdRp may also produce guanylyl and methyl transferase activities (23). No spliced messenger RNA species have been found (1, 22), and *in vitro* RNA synthesis revealed a semiconservative (strand displacement) mechanism in viral genome replication (14).

Several strains of IPNV have been isolated from salmonids and non-salmonids in North America, Europe and Asia (25). Recently, Park *et al.* (16) isolated a new strain of IPNV from rainbow trout (*Salmo gairdneri*) in Daechung Dam, Korea. The new strain, called DRT, has been identified to be serologically distinguishable from three reference strains of IPNV; Ab, Sp and VR-299 (16). However the base sequence of the segment A of IPNV was known in the Jasper (7) and N1 strains (10). In

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the case of segment B, the base sequences of the Jasper and Sp strains were known (9). There are not enough strains sequenced to better understand the molecular structure of IPNV. Thus, it is necessary to determine the cDNA construction and the nucleotide sequence of the IPNV. Also the cDNA of the RdRp gene is needed to study the mechanism of the replication of the viral genome of IPNV.

In this article, we report the cDNA cloning and sequencing of the RNA segment B of the DRT strain, also the nucleotide and deduced amino acid sequences of the segment B of the DRT strain were compared with other strains. Based on the results, the nucleotide sequence of the RdRp gene of the DRT strain of IPNV was found to be similar to that of the Jasper strain.

MATERIALS AND METHODS

Virus and Cell

The IPN virus, DRT strain (16) and Chinook salmon embryo (CHSE-214) cell line (12) used in this work were obtained from Dr. Y. C. Hah, Seoul National University, Korea. The virus was grown on a CHSE-214 cell at 18°C. The culture medium used for cell growth consisted of Eagle's minimum essential medium (EMEM) with Earle's salts, supplemented with 10% (v/v) fetal bovine serum (FBS), 2% sodium bicarbonate, 100 μg/ml streptomycin and 100 IU/ml penicillin. Once the cells were infected, only 5% FBS was used.

Viral RNA Extraction

Virus concentration was examined using the method of Yamamoto *et al.* (25) with slight modifications. Viral genomic RNA was extracted from the viral suspension by incubation with 1% SDS and 100 μg of proteinase K per ml at 65°C for 2 h followed by extensive phenol, phenol/chloroform and chloroform-isoamylalcohol extractions. Purified RNA was precipitated with 1/4 volume of 10 M ammonium acetate and 2.5 volume of 100% ethanol at −20°C overnight. The pellet was resuspended in 0.1% diethyl pyrocarbonate (DEPC, Sigma) H_2O. This solution was added to a 1×DNase buffer and 10 units/ml DNase I, and then was incubated at 37°C for 1 h, which was carried out phenol extraction and ethanol precipitation at −70°C for 2 h. The pellet was resuspended in TE buffer (10 mM Tris pH 7.6, 1 mM Na_2-EDTA).

The dsRNA was repurified from 1.0% low melting temperature agarose gel with EtBr. This RNA was precipitated with absolute ethanol containing 1/10 volume of 3 M sodium acetate at −20°C.

Oligonucleotides

Oligonucleotide sequences (Table 1) were deduced from the homology sequences between the Jasper and Sp reference strains (9). Oligonucleotides used for primers in RT-PCR were synthesized using an Applied Biosystem Model 380B DNA synthesizer in the Life Science Laboratory, Korea Basic Science Center (Seoul).

Table 1. List of primers for cDNA synthesis of the segment B of IPNV-DRT

Primer	Position*	Polarity	Sequence (5′→3′)
BU1	1 − 26	+	GGAAACAGTGGGTCAACGT-TGGTGGC
BL1001	977 − 1001	−	GCCCACAGCTCAGGTACCA-GAAGTC
BL1252**	1231 − 1252	−	CATCACTAGGTCCTCGTCG-TTG
BU982	982 − 1011	+	CTGGTACCTGAGCTGTGGG-CTGCTCTTCCC
BL1906	1880 − 1906	−	GGGTTTTCTGGCCAGGGCTT-TGTTCTC
BU1880	1880 − 1908	+	GAGAACAAAGCCCTGGCCA-GAAAACCCGG
BL2784	2759 − 2784	−	GGAGTCCCTGGCGGAACCG-GATGTTT

*Numbering is based on the sequences of the Jasper strain (7).
**Sequence of the primer is the sequence of the DRT strain.

cDNA Synthesis by RT-PCR

Purified genomic dsRNA was denatured by the treatment with dimethyl sulfoxide (DMSO), which was used as a template for first cDNA strand. The first cDNA strand was synthesized using genomic dsRNA segment B as a template, oligonucleotide primers (Table 1), reverse transcriptase and other reagents from an Amersham cDNA synthesis system kit under the conditions as described by the manufacturer. First strand cDNA was used as a template for the second strand cDNA synthesis and amplification of cDNA by PCR (19). The PCR reaction mixture (100 μl) contained 10×PCR buffer (Tris-HCl, 100 mM; $MgCl_2$, 15 mM; KCl, 500 mM; gelatine, 1 mg/ml; pH 8.3 (Boehringer Mannheim), 0.3 mM dNTPs, 100 pM of each primer and 2.5 units of Taq DNA polymerase. PCR reaction was subject to a 38 cycle program of 94°C for 1.5 min, 55°C for 2 min and 72°C for 3 min. The PCR products were extracted with an equal volume of chloroform-isoamylalcohol, precipitated with ethanol in the presence of 2.5 M ammonium acetate and resuspended in TE. The PCR products were eluted from the 1.0% low melting temperature agarose gel. These cDNAs were purified from the gel by melting the agarose in 5 volumes of TE (20 mM Tris pH 8.0, 1.0 mM EDTA) at 70°C for 10 min, followed by phenol/chloroform extraction and precipitation with ethanol.

Northern Blot Hybridization

The dsRNA segment B of IPNV was transferred onto Hybond-N+nylon membrane and hybridized with the probe cDNAs by Northern blot analysis (13) using an

ECL gene detection system (18).

Restriction Map of cDNA

For the construction of a restriction map of the cDNA covering the entire genome segment B, the amplified PCR products were single or double digested with several restriction endonucleases.

Cloning and Sequencing of cDNA

PCR products of the expected size were eluted from the gel and treated with T4 polynucleotide kinase. Phosphorylated PCR products were cloned into the SmaI site of pBluescript SK(+) vectors (21). Both strands were sequenced by the dideoxynucleotide chain termination method (20) using Sequenase Version 2.0 system kit (United States biochemical). The plasmid DNA templates were sequenced using either T3 or T7 primer (Stratagene). The sequence analysis was carried out using the DNASIS and PROSIS software program (Hitachi Software, Pharmacia).

Nucleotide Sequence Accession Number

The nucleotide sequence data of segment B described in this paper have been acquired with the information deposited with the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases and have been assigned accession number D25627.

RESULTS AND DISCUSSION

cDNA Synthesis of the Segment B of IPNV-DRT

To determine the nucleotide sequence of the genome segment B of IPNV-DRT strain, five cDNA fragments were synthesized using a reverse transcriptase (RT)-polymerase chain reaction (PCR). First of all, I (NT 1-1001), II (NT 982-1906) and III (NT 1880-2784) cDNA fragments were synthesized as illustrated in Fig. 1 and 2. And to determine the nucleotide sequences of the primers (NT 977-1011, NT 1880-1908), IV (NT 1-1252) and V (NT 982-2784) cDNA fragments were synthesized (Fig. 1). To confirm the amplified cDNA fragments, the cDNAs were hybridized to the genome segment B in Northern blot hybridization (Fig. 3).

Nucleotide Sequence of the Segment B of IPNV-DRT

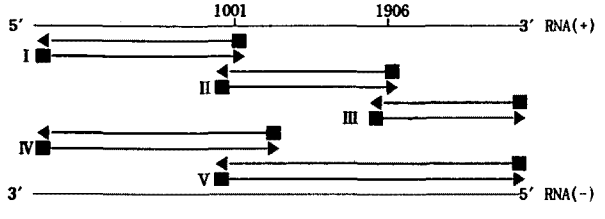

Fig. 1. Strategy of cDNA synthesis of genome segment B.
■: Primer for synthesis cDNA. Arrows indicate the direction of synthesis of cDNA.

We have determined the nucleotide sequence of IPNV-DRT genome segment B and deduced the amino acid sequence of an encoded VP1 protein from the nucleotide sequence. Furthermore, we have determined the nucleotide sequence of the RNA dependent RNA polymerase of the DRT strain of IPNV.

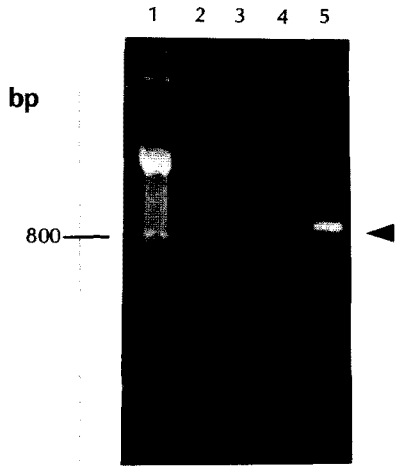

Fig. 2. Agarose gel electrophoresis of amplified cDNAs of the segment B of IPNV-DRT.
Eletrophoresis was carried out on 1% agarose gel. Lane 1, 100 bp ladder; lane 2, 1001 bp cDNA; lanes 3 to 4, 925 bp cDNA; lane 5, 905 bp cDNA.

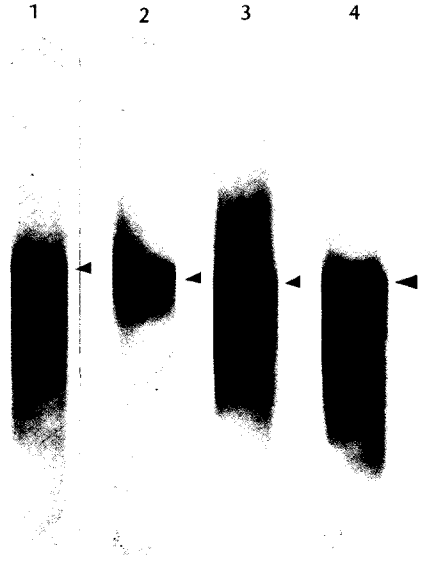

Fig. 3. Northern blot analysis of PCR amplified cDNAs of the segment B of IPNV-DRT.
Purified B segment of DRT genome RNA was electrophoreses on formaldehyde gel and transferred onto Hybond (N+) papers. The Hybond (N+) papers were incubated with labeled PCR products. The northern blot was incubated with detection solution and autoradiographed. Lane 1, standard, segment A hybridized with cloned cDNA; lane 2, 1001 bp cDNA; lane 3,925 bp cDNA; lane 4, 905 bp cDNA.

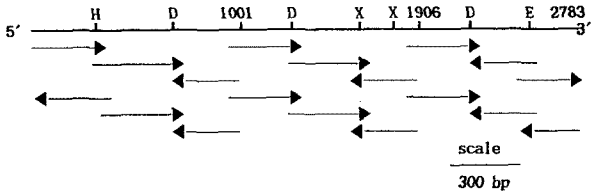

Fig. 4. Physical map and nucleotide sequencing strategy of the IPNV-DRT segment B.
The partial physical map of segment B is diagrammed. Arrows indicate the extent and direction of nucleotide sequence. Restriction enzyme sites: D, *Dra*II; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

Subcloning was performed according to the restriction map (Fig. 4) of the cDNA of the genome segment B in the DRT strain. Ten cDNA clones from the dsRNA segment B were selected and sequenced. Each bases was confirmed from both directions using two different cDNA clones. The direction and extent of determination of the sequences are shown Fig. 4.

The cDNA sequence of the genome segment B of IPNV-DRT was 2,783 bp long and contained only a single ORF of 2,535 bp (Fig. 5). The ORF encodes the VP1 protein that was composed of 845 amino acids. The 2,535-nucleotide DRT ORF began at nucleotide 101 and terminated at a single TGA termination codon at nucleotide 2,636. Duncan *et al.* (9) have previously reported the nucleotide sequence of the genome segment B of the IPNV-Jasper and -Sp strains.

The Jasper sequence contained 2,784 bp and the Sp sequence consisted of 2,630 bp. Each sequence contained a single large ORF encoding the 845-amino-acid Jasper or 844-amino-acid Sp VP1 proteins. The result of the comparison between the genome segment B of three IPNV strains is summarized in Table 2. A unique feature of the genome segment B sequence of the DRT strain was the deletion of the 2,645th nucleotide in the Jasper strain.

We found 89.7% homology of the ORF nucleotide sequence of the B segments between the DRT and Jasper strains, whereas a comparatively lower 80.8% homology exsisted between those of the DRT and Sp strains. The ORF amino acid sequence of the DRT strain showed 97.6% homology with that of the Jasper strain, but only 88.7% with the Sp strain. The result shows that the DRT strain is more closely related to the Jasper strain than to the geographically isolated European strain Sp.

Amino Acid Sequence Analysis of the Segment B Encoding VP1 Protein

The VP1 protein is the presumptive birnavirus RdRp. The predicted amino acid sequences of the IPNV-DRT, -Jasper and -Sp strains VP1 proteins, shown in Fig. 6, were aligned with that of the IBDV VP1 (15). The comparison of the IPNV VP1 sequences with the 878 amino acid IBDV sequence revealed the conserved 372 amino acids located in all four proteins. Duncan *et al.* (9) have reported potential N-glycosylation sites of Jasper and Sp strains. The DRT sequence contained eight potential N-glycosylation sites (Fig. 5). We found the GTP-binding sequence in VP1 protein of the DRT strain (Fig. 6). Comparative analysis of the amino acid sequences of the

Fig. 5. cDNA plus-strand nucleotide and deduced amino acid (in one-letter code) sequences of the segment B of the IPNV-DRT.
The start and stop codons are indicated by open bars. The GTP-binding motif is underlined. Potential N-linked glycosylation are dot lines.

Table 2. Comparison of genome segment B of the IPNV strains

	DRT	Jasper*	Sp*
Segment B (nucleotides)	2,783	2,784	2,630
VP1 ORF (nucleotides)	101~2,635	101~2,635	94~2,625
VP1 ORF (codons)	845	845	844
Predicted molecular weight	94,426	94,441	94,064
Termination codon	TGA	TAA	TAA

*Sequence data from Duncan *et al.* (9).

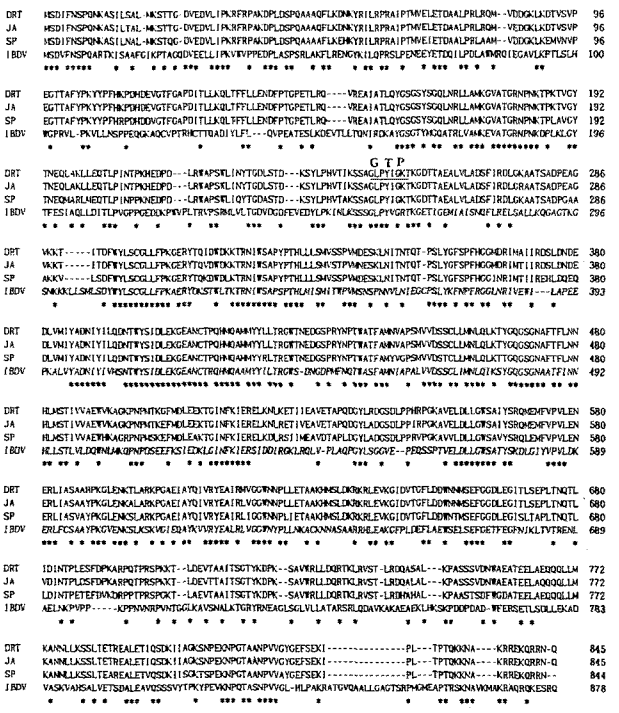

Fig. 6. Alignment of the Amino acid sequence of the VP1 protein of three IPNV strains and IBDV.
Residues conserved in allfour proteins were indicated by asterisk (*). The conserved GTP-binding motif were underlined. The predicted amino acid sequences of IPNV-Jasper Sp and IBDV were from Duncan et al. (1991) and Morgan et al. (1988), respectively.

nucleotide binding protein and putative RdRps has revealed the presence of the conserved GTP-binding motif in four VP1 proteins.

Dever *et al.* (4) reported the GTP-binding consensus sequence present in nucleotide binding proteins (e.g. elongation factors, ras p21 protein, phosphoenolpyruvate carboxykinase). The consensus sequence is composed of three elements GXXXXGK, DXXG and NKXD with spacings of either 40~80 or 130~170 amino acid residues between the first and second eliments and 40~80 amino acid residues between the second and third sequence elements (4). The same sequence is present in IPNV VP1 between residues 248 and 255 (GLPYIGKT). The corresponding region of IBDV VP1 is GLPYVGRT. As such, this region represents a potential GTP binding site in the VP1 of birnaviruses.

In this study a cDNA of the segment B of the DRT strain of IPNV was constructed and will be useful in the future in manipulating the RdRp gene. A direct experiment evidence is still required to establish a RNA dependent RNA polymerase activity for IPNV replication. So a cDNA which contains the complete coding region for VP1 should be constructed in order to express the protein in both Eucaryotic and *Escherichia coli* cells. The future studies on the expression and activity of the RNA dependent RNA polymerase of IPNV-DRT would give some insight into the IPNV replication mechanism. The determined nucleotide sequence of the segment B of the DRT strain of IPNV will also be useful for studying the function of the RdRp gene.

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