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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

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The genome segment B sequence of infectious pancreatic necrosis virus was determined for both the Jasper and Sp serotypes. The sequences are 2784 and 2630 bp long, respectively, and contain a single large open reading frame encoding the VP1 protein, the putative RNA-dependent RNA polymerase (RdRp) of IPNV. The proteins exhibit an 88% homology with each other, but only 41% with infectious bursal disease virus (IBDV) VP1, another member of the Birnaviridae. Despite the low overall homology between the IPNV and IBDV VP1 proteins, homologous regions were detected within the central portion of the proteins. The carboxy-proximal regions of the VP1, which contain very low amino acid homology, displayed evidence of conservation in structural features such as a hydrophilic, highly basic domain. Consensus sequences associated with GTP-binding proteins and RdRps were also detected in VP1. However, unlike the RdRps associated with single-stranded plus RNA viruses, the birnavirus RdRp lacks the Gly–Asp–Asp motif characteristic of this enzyme family.

INTRODUCTION

Members of the family Birnaviridae possess a bisegmented double-stranded RNA (dsRNA) genome consisting of two segments (A and B) within an unenveloped, single shelled icosahedral capsid of 60 nm (Dobos et al., 1979). Members of the family include infectious pancreatic necrosis virus (IPNV), the causative agent of an acute, contagious disease of young salmonid fishes; infectious bursal disease virus (IBDV) which causes a highly contagious disease of chickens, characterized by the destruction of lymphoid cells in the bursa of Fabricius; and Drosophila X virus (DXV) of Drosophila melanogaster (Dobos et al., 1979; Brown, 1986).

Birnavirus gene expression involves transcription from the dsRNA parental genome segments by a virion-associated RNA-dependent RNA polymerase (RdRp) (Mertens et al., 1982; Spies et al., 1987). The enzyme directs the synthesis of nonpolyadenylated mRNA species corresponding in size to the A and B segments of the viral genome. No spliced messenger RNA species have been detected (Somogyi and Dobos, 1980; Bernard, 1980; Mertens and Dobos, 1982; Azad et al., 1985).

The sequence of the viral A segment (approximately 3.1 kbp) has been determined for two IPNV Jasper strains (Duncan and Dobos, 1986; Haverstein et al., 1990) and for three IBDV strains (Hudson et al., 1986; Spies et al., 1989; Kibenge et al., 1990). This genome segment A encodes an approximately 100K polyprotein which is cleaved to produce, in order from the amino terminal end, the major virion structural protein, VP2, and the minor structural proteins, VP4 and VP3 (Nagy and Dobos, 1984; Huang et al., 1986; Duncan et al., 1987; Jagadish et al., 1988). Autocatalytic protease activity has been associated with the VP4 protein (Duncan et al., 1987; Jagadish et al., 1988).

The viral B segment encodes VP1, the presumptive virion-associated RdRp. This protein is approximately 90 kDa in size as estimated by gel migration (MacDonald and Dobos, 1981; Nagy and Dobos, 1984; Azad et al., 1985). IPNV, IBDV, and DXV all possess genomically linked proteins (VPg) tightly associated with the ends of the genomic RNA (Persson and MacDonald, 1982; Revet and Delain, 1982; Muller and Nitschke, 1987). In the cases of IPNV and IBDV, this VPg has been shown to be VP1. The VPg of DXV is a 67K protein of undetermined origin (Revet and Delain, 1982). The RdRp may also contain guanylyl and methyl transferase activities (Spies and Muller, 1990).

Morgan et al. (1988) reported the sequence of IBDV genome segment B (Australian strain 002-73). They
reported no homology between the predicted VP1 sequence and the sequences of putative ssRNA-dependent RNA polymerases (ssRdRps). Subsequently, Gorbalenya and Koonin (1988) reanalyzed the IBDV sequence and detected homology with consensus sequence elements found in ssRdRps (Kamer and Argos, 1984; Argos, 1988). We present in this report the genome segment B nucleotide sequences of the IPNV-Jasper and -Sp strains and the predicted sequences of their encoded VP1 proteins. Sequence analysis revealed the presence of extensive homology between IPNV and IBDV VP1 in the central regions of the proteins and the presence of several conserved domains associated with RdRps and GTP binding proteins. Contrary to other putative RdRps, however, IPNV VP1 lacks the conserved Gly–Asp–Asp motif, the proposed catalytic site of this enzyme family.

MATERIALS AND METHODS

Viral strains and cDNA production

The IPNV-Jasper and -Sp strains were grown in CHSE-214 cells and genomic viral RNA was isolated from purified virions as previously described (Huang et al., 1986; Duncan et al., 1987). The viral RNA was used as templates for cDNA production and recombinant plasmids were screened for viral inserts by specific hybridization to B segment RNA in Northern blots (Huang et al., 1986; Duncan et al., 1987). A recombinant plasmid with a 2.8-kbp cDNA insert of the B segment of IPNV-Jasper was isolated and subcloned into M13 mp18 and mp19 for DNA sequencing. A recombinant plasmid containing a 2.6-kbp insert of the B segment for IPNV-Sp was constructed in pT7 from two overlapping cDNA clones (Huang et al., 1986) and selected restriction fragments from this recombinant plasmid were recloned into M 13 mp18 and mp19 for sequence analysis. Subsequently, the large cDNA inserts were subcloned into Gemini vectors (Promega Biotec) for in vitro transcription and translation.

DNA sequencing and analysis

The sequence of the IPNV genome segment B for both virus strains was determined by the dideoxy chain termination procedure (Sanger et al., 1977) using standard 6% polyacrylamide, 8 M urea gels or buffer gradient gels (Biggin et al., 1983). The sequences were determined by sequencing overlapping subclones in both directions. The entire Jasper sequence was determined from both strands. Unambiguous sequence was determined in one direction for several small regions of the Sp sequence from several independently isolated cDNA clones. In addition, oligonucleotide primers complementary to Sp nucleotide positions 96–117, 681–697, 805–821, 1746–1763, 2079–2096, and 2371–2388 were used to confirm these sequences.

The cDNA sequence was analyzed on a VAX780 using the DNA sequence analysis package from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

RESULTS AND DISCUSSION

Nucleotide sequence comparison of IPNV segment B Jasper and Sp serotypes

Recombinant plasmids containing cDNA copies of the B segment of IPNV were detected by Northern blot analysis of cDNA libraries prepared from total genomic RNA of the Jasper and Sp serotypes of IPNV (Huang et al., 1986; Duncan et al., 1987). In the case of the IPNV-Jasper library, a recombinant plasmid containing an insert of approximately 2.8 kbp was identified and used for sequence analysis. The IPNV-Sp library contained no insert larger than 2 kbp. Thus, two of the IPNV-Sp recombinant plasmids were physically mapped using a series of restriction endonucleases and a large continuous insert was produced by ligation of insert fragments at overlapping restriction sites (Huang et al., 1986). The reconstruction of the complete coding regions for both R segment RNAs was verified by in vitro translation of RNA transcripts of segment B and radioimmune precipitation of the VP1 protein (Huang et al., 1986; R. Duncan, data not shown).

The nucleotide sequences of both serotypes of IPNV genome segment B were determined using the sequencing strategy outlined in Fig. 1 and the plus strand cDNA sequences are shown in Fig. 2. The sequences were aligned using the algorithm of Needleman and Wunsch (1970). The genome segment B sequence summary is outlined in Table 1.

The Sp sequence contained 2630 bp while the Jasper sequence consisted of 2784 bp. Both sequences were colinear with no insertions or deletions outside of the terminal sequences and with the exception of an additional codon present in the Jasper sequence at the end of the VP1 coding region (nucleotides 2633–2635). There were a total of 521 nucleotide mismatches representing an overall homology of 80.7%. In view of the high degree of homology between the Jasper and Sp segment B sequences, it seems likely that the short 3′-nontranslated region of the Sp cDNA indicates that the clone is truncated. Each sequence contained a single large ORF encoding...
the 845-amino-acid Jasper or 844-amino-acid Sp VP1 proteins. The 2532-nucleotide Sp ORF begins at nucleotide 94 and ends with a single TAA termination codon at nucleotide 2626, while the 2535-nucleotide Jasper ORF begins at nucleotide 101 and terminates at a single TAA termination codon at nucleotide 2636 (Fig. 2 and Table 1).

There are no published birnavirus terminal RNA sequences available; therefore, it is not possible to state that the sequences presented represent the entire genome segment B sequence. However, the importance of the terminal sequoncs in transcription and translation initiation and possibly genome packaging prompted an analysis of these regions to identify consensus sequence elements. Figure 3 shows a comparison of the 5' and 3' terminal sequences of IPNV-Jasper segment B with the previously published Jasper and N1 serotype segment A sequences (Duncan and Dobos, 1986; Haverstein et al., 1990).

The Jasper segment A and B cDNA sequences both start with a conserved pentanucleotide sequence (GGAAA) and terminate with a conserved tetranucleotide sequence (CCCC). The N1 serotype segment A sequence contains an additional 11 nucleotides at the 5'-end and the first G of the GGAAA pentanucleotide was replaced by an A. In addition, the reported N1 sequence ended immediately prior to the conserved 3'-terminal CCCC sequence present in the Jasper segment A and B sequences. There was extensive homology between the three sequences near the 5'- and 3'-termini while there was little homology between the segment A and B coding regions. When the 5'-terminal sequences of the A and B segments of IPNV-Jasper were optimally aligned, 32 of the 50 nucleotides were conserved. Similarly, 29 of 50 nucleotides at the 3'-terminus of B were conserved in the segment A sequence (Fig. 3). These extensive nucleotide sequence homologies presumably reflect regions important for genome replication and expression. In addition, as previously reported for the N1 segment A sequence (Haverstein et al., 1990), the Jasper B sequence contained inverted terminal repeats of 14 nucleotides (Fig. 3). Unlike the segment A sequences, however, there was no indication of extensive adjacent inverted repeats in the segment B termini. The function of these small inverted repeats has not been determined. The consensus IPNV terminal sequences derived from the available IPNV sequence data are presented in Fig. 3. Refine-
FIG. 2. Nucleotide sequence of IPNV genome segment B. The cDNA nucleotide sequence of the plus-strand B segment of IPNV Jasper (Ja) and Sp strains. Nucleotide mismatches in the Sp sequence are shown. (--) Locations of insertions/deletions; (0) sequences not present in the Sp cDNA. The sequence is grouped in triplets beginning at the start of the VP1 coding region. These sequences have been deposited with the GenBank nucleotide sequence database and have been assigned Accession Nos. M58756 (Jasper) and M58757 (Sp).
The presence of perfectly conserved terminal nucleotides in the Jasper segment A and B cDNA sequences suggested that the cloned cDNAs represented complete genomic sequences. In fact, two large independent segment A cDNA clones were isolated and both contained identical terminal sequences (Duncan et al., 1987). In addition, an identical sequence was obtained by direct RNA sequencing of the 3'-end of the minus-strand genome segment A and B RNAs (E. Nagy and P. Dobos, unpublished data). While it is not possible to state unequivocally that the cDNA sequences represent complete genomic RNA sequences, it is reasonable to assume that extensive terminal sequences are not missing, and that a complete sequence determination of the viral genome of IPNV-Jasper has been made.

### Analysis of the segment B-encoded VP1 proteins

The predicted amino acid sequences of the IPNV-Jasper and -Sp serotype VP1 proteins are shown in Fig. 4 and were aligned with that of the IBDV VP1 (Morgan et al., 1988). Translation commencing at the first methionine codon of the B segment ORF produced a polypeptide of 845 amino acids for IPNV-Jasper and 844 amino acids for IPNV-Sp. The amino acid number corresponded to the estimated molecular weight of VP1, which has ranged from 95 kDa for the Jasper isolate (Duncan et al., 1987) to 89 kDa for the Sp and Ab isolates of IPNV (Hedrick et al., 1983). The amino acid...
TABLE 1
IPNV Genome Segment B Sequence Summary

| Serotype   | Jasper | Sp |
|------------|--------|----|
| Segment B  | 2784   | 2630 |
| 5'-noncoding | 100 | 93 |
| 3'-noncoding* | 149 | 6 |
| VP1 ORF (nucleotides*) | 101-2635 | 94-2625 |
| VP1 ORF (codons) | 845 | 844 |
| Predicted molecular weight | 94,441 | 94,064 |
| p1 | 6.60 | 6.60 |

* Including the termination codon.
* Excluding the termination codon.

compositions of the three VP1 proteins were similar to each other; the only unusual feature was their very low cysteine content (three cysteines in IPNV, VP1, eight cysteines in IBDV).

The Jasper sequence contained eight potential N-linked glycosylation sites, five of which were conserved in the Sp sequence and two in the IBDV sequence (Table 2). The Sp and IBDV sequences each contained one additional potential N-linked glycosylation site not found in the Jasper sequence. It is not known whether any of these sites are glycosylated in the birnavirus VP1 proteins.

Recent evidence indicates that Jasper VP1 in IPNV-infected cells exists in multiple phosphorylated forms (P. Dobos, unpublished observation). A similar situation has been recently reported for poliovirus RdRp, where multiple species of 3Dpol were detected which differed in their phosphorylation level of serine residues. (Ransone and Dasgupta, 1989).

While there is no recognition sequence for phosphorylation, preferred flanking sequences have been identified (Hunter, 1982; Bramson et al., 1984). The phosphorylation of Ser residues by cAMP-dependent protein kinases exhibits a marked preference for one or two basic amino acids located one or two residues upstream of the Ser or Thr target site. Similarly, Tyr-specific kinases like pp60src preferentially phosphorylate Tyr residues that are preceded by an acidic residue four amino acids upstream. The VP1 sequences were scanned for such motifs and the results are summarized in Table 2. Jasper VP1 contained six potential Ser phosphorylation sites, four of which were found at the same sites in Sp VP1, and one was conserved in IBDV VP1. In addition, Sp VP1 contained one, and IBDV VP1 contained five, unique potential Ser phosphorylation sites. There was one potential Tyr phosphorylation site in Jasper which was conserved in Sp, while IBDV VP1

![Fig. 3. IPNV terminal cDNA sequences. The 5'-terminal (top) and 3'-terminal (bottom) plus-strand cDNA sequences of IPNV Jasper segment B (Ja-B) and A (Ja-A) and IPNV N1 segment A (NI-A) are listed. Insertions (-) were introduced to optimize the alignment. (0) Sequences not present in the cDNA. The numbers refer to the last nucleotide listed of the 5'-terminal sequence or the first nucleotide listed of the 3'-terminal sequence. Arrows above the sequence indicate the location of the inverted terminal repeats which appear in the 5'- and 3'-terminal regions. A consensus sequence (con) for the IPNV terminal sequences is shown.](https://example.com/fig3.png)
Fig. 4. Amino acid sequence comparisons of birnavirus VP1 proteins. The predicted amino acid sequences of the IPNV Jasper (Ja) and Sp VP1 proteins were aligned with the sequence of IBDV (IB) VP1 using the algorithm of Needleman and Wunsch (1970) and insertions (--) were introduced to optimize the alignment. The amino acids are numbered on the right. Residues conserved in all three proteins are indicated (*). The conserved GTP-binding motif (GTP) and RdRp consensus sequence elements 1, 2, and 3 are overlined (see text).
TABLE 2

| Potential Phosphorylation and Glycosylation Sites in VP1 |
|--------------------------------------------------------|
|                | Jasper | Sp | IBDV |
|----------------|--------|----|------|
| N-linked glycosylation sites | N-184<sup>a</sup> | N-184<sup>a</sup> | N-422<sup>b</sup> |
|                | N-226  | N-344<sup>a</sup> | N-449<sup>b</sup> |
|                | N-339  | N-409<sup>b</sup> | N-643  |
|                | N-344<sup>a</sup> | N-437<sup>b</sup> | N-658  |
|                | N-409<sup>b</sup> | N-595  | N-677<sup>a</sup> |
|                | N-437<sup>b</sup> | N-677<sup>a</sup> | N-677<sup>a</sup> |
| Ser phosphorylation sites | S-13<sup>a</sup> | S-13<sup>a</sup> | S-16<sup>b</sup> |
|                | S-245<sup>b</sup> | S-245<sup>b</sup> | S-256<sup>b</sup> |
|                | S-375  | S-292  | S-297  |
|                | S-738<sup>a</sup> | S-738<sup>a</sup> | S-304  |
|                | S-751  | S-781<sup>a</sup> | S-441  |
|                | S-781<sup>a</sup> | S-781<sup>a</sup> | S-716  |
| Tyr phosphorylation sites | Y-399<sup>a</sup> | Y-399<sup>a</sup> | Y-73<sup>b</sup> |
|                | Y-247  |        |        |

Note. N, Asn; S, Ser; Y, Tyr. Numbers refer to the amino acid.
<sup>a</sup>Conserved between Ja and Sp.
<sup>b</sup>Conserved between Ja, Sp, and IBD.

The comparison of the predicted secondary structures of the three VP1 proteins was made. The proteins were predicted to contain approximately 30% of their residues in an α helix conformation and in β sheet structures. The remaining 40% of the amino acids were predicted to exist in turns and random coil or loop structures characteristic of globular proteins. The low level of amino acid homology over the last quarter of VP1 and the large insertion in the IBDV sequence suggested that the function of this portion of the protein was dependent on the presence of similar, rather than identical, amino acids that impart a particular secondary or tertiary structure on the protein.

Interestingly, two of the most extensively conserved secondary structure motifs resided in the carboxy-terminal 30 amino acids were basic. The presumptive role of VP1 in RNA replication suggests a possible role for this region in RNA association.

The VP1 protein is the presumptive birnavirus RdRp. Comparative analysis of the amino acid sequences of nucleotide binding proteins and putative RdRps has revealed the presence of conserved sequence motifs...
which may be involved in the enzymatic functions of this group of proteins. The sequence GXXXXYGKS/T is a constant motif present in ras-type GTP binding proteins (Argos and Leberman, 1985; Moller and Amons, 1985) and is found in several viral proteins with a tentative role in RNA replication (Kaariainen et al., 1987). The same sequence is present in IPNV VP1 between residues 248 and 255 (GLPYIGKT). The corresponding region of IBDV VP1 is GLPYVYGR, a closely related sequence motif also found in the VSV N protein (Galione et al., 1981) and similar to the sequence GFIIKQRS found in the influenza A PA protein (Fields and Winter, 1982). As such, this region represents a potential GTP binding site in the VP1 of birnaviruses. This sequence may relate to the recently reported guanyl transferase activity associated with IBDV VP1 (Spies and Muller, 1990) and IPNV VP1 (P. Dobos, unpublished data).

Kamer and Argos (1984) were the first to report the presence of conserved sequence motifs in the aligned sequences of the picornavirus polymerase, 3Dpol and putative RdRps from other plus-strand RNA viruses of plants and animals. Three regions of similarity which were grouped in the central region of the proteins were detected. The most distinctive pattern detected was a GDD motif flanked by predominantly hydrophobic residues. This motif is highly conserved in putative RdRps and has been proposed to represent the functional site of RdRps (Kamer and Argos, 1984; Gorbalenya et al., 1989).

We scanned the IPNV VP1 sequence for the conserved RdRp motifs and aligned these regions with the corresponding regions of 38 putative RdRps from several plus-strand RNA and dsRNA virus families of animals and plants. Representative sequences from each of the virus families examined are listed in Fig. 5. The first two conserved regions, appropriately spaced, were clearly present in IPNV VP1 in the highly homologous central portion of the protein. The generalized consensus sequence for these regions is DXXXXD/E(X)_{45-88}S/TGXXXTXXXN, where X represents any amino acid. In fact, the amino acids flanking the absolutely conserved residues show a decidedly nonrandom distribution leading to a more extensive consensus sequence as outlined at the top of Fig. 5.

The absence of the GDD motif in IPNV VP1 should serve as a caution for anyone analyzing the sequence of a putative RdRp. The GDD sequence in region 3 is a highly conserved motif that is present in almost all putative RdRps. The IPNV VP1 proteins represent the only putative RdRps that deviate from the conserved GDD motif so typical of RdRps. While IBDV has conserved the Asp–Asp sequence, IPNV does not contain this motif in the corresponding region of its VP1. The Sp sequence contained a Lys–Asp while the Jasper sequence showed even less conservation with a Lys–Asn sequence at the corresponding region of VP1 (Fig. 5). This represents a significant change in the proposed catalytic site with acidic residues being replaced by a basic residue of very different size and a polar residue of similar size.

Although IBDV contained the Asp–Asp sequence in region 3, the flanking residues show a high degree of variation from the conserved sequence. As evident in Fig. 5, the flanking residues are hydrophobic or neutral, while the birnavirus flanking residues are for the most part charged or polar hydrophilic residues. Argos (1988) has recently analyzed the GDD motif and found that the Asp–Asp or Asp–Thr–Asp sequence is present in numerous RNA-dependent and DNA-dependent polymerases, as well as several reverse transcriptases. An analysis of these sequences led to the development of a series of rules governing the composition of the residues flanking the Asp–Asp sequence present in this 15-amino-acid consensus sequence (Argos, 1988).

Interestingly, none of the putative RdRps from dsRNA viruses in Fig. 5 fulfill the Argos rules for a core polymerase sequence, even those that contain the GDD motif (reovirus matches the consensus sequence at the top of Fig. 5, but the Gln in position 6 does not agree with the Argos core polymerase sequence). All of the plus-strand RNA viruses fulfill all of the rules except for infectious bronchitis virus, which has a Ser at position 7. It is possible that there is something intrinsically different about RNA polymerases from dsRNA viruses, such that the regions flanking the "core" are structurally distinct from RdRps of plus-strand RNA viruses. Alternatively, the Asp–Asp sequence may represent a highly conserved structural motif, and not the active site, which is more diverged in the case of RdRps from the dsRNA virus families. The fact that IPNV VP1 lacks the GDD sequence motif would seem to argue in favor of the latter possibility. Regions 1 and 2 do not appear in DNA-dependent RNA polymerases or reverse transcriptases (Argos, 1988) and, as such, represent conserved sequence motifs of RdRps.
merely scan their sequence for the presence of this motif. Indeed, outside of IPNV the only exceptions to the GDD motif are IBDV (IDD) and IBV (JDD) which were not identified as RdRps until more recently analyzed (Gorbalenya and Koonin, 1988; Gorbalenya et al., 1989). Our data indicate that even the Asp–Asp sequence may not be present in some putative RdRps. Proof of the role of birnavirus VP1 as a RdRp and the regions involved in polymerase function awaits direct experimental analysis and the development of a functional in vitro polymerase assay.

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