Characterization of the small open reading frame on genome segment A of infectious pancreatic necrosis virus

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The genome of infectious pancreatic necrosis virus (IPNV) is composed of two segments of dsRNA. The larger segment contains a small ORF partly overlapping the 5' end of the polyprotein reading frame. Yet very little is known about this possible new gene, which presumably codes for a 17 kDa polypeptide (VP5). The region of the viral genome which encompasses the small ORF was reverse-transcribed and amplified by PCR before cloning and sequencing. Analysis of the sequences obtained from five different virus strains revealed that the small ORF is not found on one of them, and that it is truncated on two others. Moreover, the deduced amino acid sequences did not appear to be well conserved. Despite the large variations between IPNV strains at the genomic level, all predicted VP5 are arginine-rich basic polypeptides. To verify whether the small ORF is translated into protein in fish cells, the 17 kDa polypeptide of the VR-299 strain was expressed as a fusion protein in a prokaryotic expression vector and used to produce a specific antiserum. This antiserum reacted with concentrated virus in an immunodot assay, indicating that VP5 is synthesized in infected cells, but probably only in small quantities. When tested with 12 other IPNV strains, results were less conclusive than those obtained with strain VR-299. Nevertheless, three of the 12 viruses gave a clearly negative signal in the immunodot assay, suggesting that possibly more than one viral strain lacks the small ORF.

Infectious pancreatic necrosis virus (IPNV) belongs to the family Birnaviridae (Francki et al., 1991) and has been isolated from fresh-water and marine fish, molluscs and crustaceans. Its genetic information is contained within two segments of dsRNA (Dobos et al., 1979). The smaller, segment B, is approximately 2700 bp long and contains a single large open reading frame (ORF) encoding VP1 (Duncan et al., 1991), which is the putative RNA-dependent RNA polymerase (Cohen, 1975; MacDonald & Dobos, 1981; Dobos et al., 1991). Segment A (approximately 3100 bp) contains two partly overlapping ORFs (Duncan et al., 1987; Håvarstein et al., 1990). The larger one (2916 bases) encodes a polyprotein which is cleaved into three polypeptides in the order 5' VP2-NS-VP3 3' (Huang et al., 1986; Duncan et al., 1987; Nagy et al., 1987). The protease activity responsible for this cleavage has been associated with the viral protein NS (Duncan et al., 1987; Manning & Leong, 1990; Manning et al., 1990; Magyar & Dobos, 1994a). VP2 and VP3 are the major immunogenic and structural polypeptides of the virus and are associated with the capsid.

The small ORF (444 bases) on genome segment A starts 52 nucleotides upstream of the other ORF, but in a different frame. It could code for a 17 kDa arginine-rich protein, tentatively designated VP5 (Duncan et al., 1987; Håvarstein et al., 1990), but its function is still unknown.

Until now, only three strains of IPNV have been totally sequenced over segment A: Jasper (Duncan & Dobos, 1986), N1 (Håvarstein et al., 1990) and DRT (Chung et al., 1993). In each of these sequences, the location and length of the small ORF are identical. A few other experiments lend support to the presence of VP5. For example, a weak band at approximately 17 kDa was observed with [35S]methionine-labelled purified IPNV particles after SDS-PAGE (Håvarstein et al., 1990) and recently a polypeptide of similar molecular mass was immunoprecipitated from IPNV-infected cell lysates (Magyar & Dobos, 1994b).

We have compared sequences of the small ORF from various strains of IPNV which have already been assigned to different genogroups (Hepell et al., 1993). A specific antiserum against VP5 was also prepared in order to detect the protein in concentrated virus preparations.

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The nucleotide sequences reported in this paper have been submitted to the GenBank database and assigned the accession numbers L40580 to L40584.
IPNV strains used in this work were obtained from the ATCC (LWVRT 60-1 (VR-299), Ab, d'Honnincthun, Hecht, Canada 1, Canada 2, Canada 3, Tellina-2 and Jasper (ATCC), except for N1 (Dr K.E. Christie, University of Bergen, Norway), Jasper (Dr P. Dobos, University of Guelf, Canada), DK3315 (Dr N. J. Olesen, National Veterinary Laboratory, Denmark) and 122-88 (Dr W. Ahne, University of Munich, Germany). Note that the Jasper strain sequenced by Duncan & Dobos (1986) is different from the Jasper strain obtained from the ATCC (Berthiaume et al., 1992). Herein, we refer to the latter as Jasper (ATCC). All viruses were grown on Chinook salmon embryo CHSE-214 cells, except for DK3315 and 122-88 which required fat head minnow (FHM) cells, as described elsewhere (Heppell et al., 1992). Five strains (Ab, VR-299, d'Honnincthun, Canada 2 and Hecht) were selected for cloning and sequencing. The virus RNAs were extracted with phenol and chloroform as previously described (Heppell et al., 1992). One of the three clones obtained with the VR-299 strain was used for the amplification of the small ORF with primers p17F (5' GGAGGAGATCTCCGTCGATG 3') and p17R (5' CGCAAGCTTGACAGACTTCCTCGG 3'). The start codon and the complement of the stop codon of VP5 are shown in bold (the added restriction sites are underlined: BglII for p17F and HindIII for p17R). PCR amplification was performed with the following parameters: 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C for five cycles, then 30 more cycles of 1 min at 95 °C, 1.5 min at 65 °C and 1.5 min at 72 °C. The PCR product was inserted into the prokaryotic expression vector pMAL-c2 (New England Biolabs), then TB1 cells were transformed with the recombinant plasmid (p17pMAL). The insert was sequenced to ensure that no mismatches were introduced during the second PCR, and that the IPNV ORF was in the right reading frame for its expression. To verify the production of the fusion protein [maltose-binding protein (MBP)-p17], samples of bacterial cultures were taken before and after induction with IPTG and analysed by 12% SDS–PAGE.

The cDNA sequence of the small ORF and of the 5' non-coding region was determined for IPNV strains VR-299, Ab, d'Honnincthun, Hecht and Canada 2. Three previously published sequences, from strains Jasper (Duncan & Dobos, 1986), N1 (Hävarstein et al., 1990) and DRT (Chung et al., 1993), were also included in this study for the purpose of comparison. Except for additional nucleotides at the 5' end of the N1 strain and single mismatches for strains N1 (position 1), Ab (position 35), DRT (positions 50 and 64) and Hecht (position 76), the first 91 bases were identical on all sequences. However, the downstream region, located between bases 91 and 120 on the Jasper strain, appeared to be highly variable since only five bases were conserved among all strains.

Analysis of these sequences showed that the small ORF was not identical on all strains. Though its start codon was found on all eight viruses at the same position, insertion (in the case of Hecht) or deletion (in the case of d'Honnincthun and Canada 2) of one residue in the highly variable region was responsible for frame shifts in the small ORF. For this reason, the ATG of the small ORF moved into the same reading frame as the ORF of the polyprotein in the case of d'Honnincthun and Canada 2; these two strains had a truncated small
Comparison of the deduced amino acid sequences showed that there were more conserved residues in the N-terminal part of VP5 (43 out of 74) than there were in the second half of the protein (29 out of 74) (Fig. 1). However, no particular conserved motif or domain was found on either portion of the sequence, except for two potential phosphorylation sites which were found on all strains (tyrosine kinase and cAMP-dependent protein kinase phosphorylation sites at positions 25 and 144, respectively). Nevertheless, all VP5 had a similar hydrophobicity profile, as calculated by the method of Kyte & Doolittle (1982). In addition, they all had an estimated pI between 9.9 and 11.0, and they were all arginine-rich (11.4 to 12.8% of residues).

Percentage similarities between the nucleotide and the deduced amino acid sequences of the small ORF were calculated (Table 1). The Hecht strain appeared to be the most distant when compared to the others. However, there was no correlation between percentage similarity.
The 17 kDa polypeptide of VR-299 was expressed as a fusion protein in *Escherichia coli* (Fig. 2a). After purification with an amylose resin column (New England Biolabs) (lane 7), MBP–p17 was injected into a rabbit. The antiserum obtained was extensively adsorbed with MBP and tested by ELISA (data not shown) and Western immunoblot. For Western blotting, 12% SDS–PAGE was used and proteins were transferred to a PVDF membrane (BioRad). Detection was carried out with a chemiluminescence Western blotting kit (Boehringer Mannheim; Fig. 2b). Cleavage of the fusion protein with the Xa cleavage factor was performed in column elution buffer (10 mM-Tris–HCl pH 7.4, 200 mM-NaCl) at 20 °C for 1.25 h (Fig. 2a, lane 8). Digestion of MBP–p17 was only partial because of the rapid degradation of VP5 once separated from the MBP portion. For this reason, the whole fusion protein was injected into the rabbit. Fig. 2(b) shows that the antiserum recognized the fusion protein (lanes 6 and 7). After cleavage of MBP–p17, a weak band also appeared at approximately 17.5 kDa (lane 8). A better yield in the cleavage of the fusion protein would certainly improve these results. Despite the greater abundance of MBP compared to MBP–p17, as can be seen in Fig. 2(a), a stronger signal was obtained in chemiluminescence with the fusion protein, indicating that substantial amounts of anti-p17 antibodies were present.

The same rabbit antiserum was used in an immunodot assay for the detection of VP5 in virus preparations. Concentrated viruses (1 to 2 µg of total protein per µl), prepared as previously described (Tarrab *et al.*, 1993), and uninfected cell lysates (similarly processed) were spotted directly onto PVDF membrane along with purified MBP–p17. They were successively reacted with the rabbit anti-MBP–p17 serum and a peroxidase-labelled anti-rabbit IgG. A normal rabbit serum and a rabbit anti-VR-299 serum were employed as controls. Reactions were detected by chemiluminescence. As little as 8 pg of MBP–p17 could be detected with this test (Fig. 3, column 1). The reaction with concentrated VR-299 (Fig. 3a, column 2) was also clearly positive when compared to background levels (Fig. 3, column 3 and Fig. 3b). However, it was not possible to obtain an unambiguous positive reaction with some of the other viruses tested (Fig. 3a, columns 4 and 5), though Canada 2 and Jasper appeared positive while Hecht, Tellina-2 and Canada 1 were definitely negative. A rabbit anti-VR-299 serum, which cross-reacts with the other IPNV strains, showed that equivalent amounts of viral proteins were deposited onto the membrane for each virus (data not shown).

Overlapping reading frames in dsRNA genomes are not unique to birnaviruses. For example, human reovirus s1 mRNA, which codes for the viral haemagglutinin, also directs the synthesis of a 14 kDa polypeptide encoded by a different reading frame which overlaps the first (Ernst & Shatkin, 1985). Since the small ORF on genome segment A appeared to be well conserved among birnaviruses, it was surprising to find that at least one strain of IPNV did not contain such an ORF. The Hecht virus possesses neither the alternative ATG for the small ORF found on Canada 2 and d'Honnincthun strains, nor the stop codon (position 512 on Jasper) present in all other sequences. This is consistent with the negative result obtained by immunodot with this strain.

Canada 2 and d'Honnincthun are also special cases. Though the usual ATG of the 17 kDa protein is found in...
using the anti-MBP-pl7 serum, since it is a less sensitive protein. This could explain why it has not been possible expressed transiently. The configuration of the fusion conserved on any ORF of IPNV. Internal translation to identify the 17 kDa protein in Western immunoblots protein may also differ from that of the native viral of the anti-VR-299 antiserum (data not shown), the initiation of translation (Kozak, 1987), is not perfectly read as a start signal by ribosomes. In fact, Canada 2 that VP5 is produced in VR-299-infected cells. However, because the signal obtained was weak compared to that second ATG is not in a favourable context, since it lacks both the purine in the −3 position and the guanine at +4. It could, however, be read as a start signal by ribosomes. In fact, Canada 2 seemed to react positively with the anti-MBP-p17 serum. Furthermore, the Kozak consensus sequence for the initiation of translation (Kozak, 1987), is not perfectly conserved on any ORF of IPNV. Internal translation start sites have also been demonstrated in vitro (Huang et al., 1986; Duncan et al., 1987; Manning & Leong, 1990; Manning et al., 1990), showing that ribosomes do not have a strong preference for a particular ATG.

The results obtained in the immunodot assay suggest that VP5 is produced in VR-299-infected cells. However, because the signal obtained was weak compared to that of the anti-VR-299 antiserum (data not shown), the 17 kDa protein may be present in low amounts and/or expressed transiently. The configuration of the fusion protein may also differ from that of the native viral protein. This could explain why it has not been possible to identify the 17 kDa protein in Western immunoblots using the anti-MBP-p17 serum, since it is a less sensitive test. Negative results were also obtained by others using this technique (Magyar & Dobos, 1994b).

The presence of VP5 in some of the other IPNV strains tested was not clear. These results, however, are influenced by various factors, such as the affinity of the antisera toward heterologous strains and the level of expression of VP5 relative to the other viral proteins. Nevertheless, it appeared that when VP5 is encoded on the virus genome, it could be produced in infected cells. According to Håvarstein et al. (1990), this protein would be present on purified particles of the Ab, Sp, Jasper and N1 strains. This is consistent with our results since the small ORF is found in these viruses, and none of them were negative in the immunodot assay (strain Sp was not tested).

Apparently, there is no natural selection for a conserved primary structure of this small protein. The exact amino acid sequence is probably not critical for the fulfilment of whatever biological function it has. Despite the genomic variations all putative VP5 sequences are rich in arginine and basic. Such positively charged proteins are often found to be associated with nucleic acids and are common among negative-strand RNA viruses (Spiropoulou & Nichol, 1993). Thus, VP5 of IPNV is not a unique case.

Unlike IPNV, all strains so far sequenced of another birnavirus, infectious bursal disease virus (IBDV), contain the small ORF, though not all of them code for a basic VP5 (Kibenge et al., 1990). The fact that IPNV shows more variations than IBDV may result from an adaptation to a large variety of hosts. IPNV has been isolated from at least 20 different families of fish, as well as molluscs and crustaceans (Wolf, 1988). Multiple strains of the virus differ in virulence and serological response.

From the results presented in this paper, it is clear that the small ORF on genome segment A is not present in all IPNV strains. In cases where VP5 is encoded on the virus genome, the protein is probably synthesized in infected fish cells, as it is the case for VR-299. This, as well as the possible role of the protein or of the ORF itself in viral replication are currently under study.

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References


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