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Generation of a minigenome with non-coding sequences of infectious pancreatic necrosis virus

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Infectious pancreatic necrosis virus (IPNV) is an important aquatic pathogen causing a highly devastating disease in salmonids. The virus has been isolated from over 50 species across the world. For combating the disease, vaccines have been developed by different recombinant DNA technologies. Production of live virus vaccines with defined attenuations requires reverse genetics system and minigenome synthesis to study the attenuation and virus production *in vitro* systems. Towards this objective, the two open reading frames of the IPNV (West Buxton strain) were genetically engineered to replace them with bacterial chloramphenicol acetyl transferase (CAT) reporter gene while retaining the non-coding regions (NCR). The minigenome of IPNV without the coding regions was generated using a modified pUC 19 plasmid and was checked for the nucleotide correctness by dideoxy chain termination method. Expression of the reporter gene was verified after transfection studies in susceptible cell line. The synthesised minigenome is useful in carrying out a number of studies in the reverse genetics of IPNV.

Keywords: IPNV, minigenome, reverse genetics, virus vaccine

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Introduction

Infectious pancreatic necrosis virus (IPNV), the prototype virus of the family *Birnaviridae*, is perhaps the most well studied fish virus in the world today. Infectious pancreatic necrosis disease of salmonid fish is an acute, highly contagious and destructive disease, primarily of young salmonids caused by IPNV[1]. The virus has also been isolated from over 50 species of fish across the world[2]. The IPNV is a bisegmented double stranded RNA virus having 60 nm diam belonging to the *Aquabirnavirus* genus of the family *Birnaviridae*. There are 3 serogroups identified in *Aquabirnavirus* genus at present where Serogroup A containing 9 serotypes, while Serogroups B and C have one serotype each[2, 3].

IPNV genome consists of two segments of double stranded RNA with a molecular mass of 2.5×10^6 and 2.3×10^6 Da[4]. The larger genome segment (Segment A) of 3097 bp encodes a 106 kDa polyprotein in a single open reading frame (ORF) in the order 5'-pVP2 – NS – VP3 – 3'. The polyprotein is co-translationally cleaved by the non-structural viral protease to generate VP2 and VP3 structural proteins[5]. These two proteins are the major outer capsid proteins among which, VP2 is the major virion protein responsible for eliciting neutralisation antibodies in fish[6]. The Segment A also encodes for a Small 17 kDa protein by a partially overlapping ORF, which precedes the major ORF[7]. The second smaller genome segment (Segment B) of 2784 bp is monocistronic encoding a 94 kDa internal minor protein (VP1), which is the virion associated RNA-dependent RNA polymerase[4,8]. VP1 is present in the virions as a free polypeptide as well as a genome linked protein, VPg[9]. Both the segments A and B of IPNV have considerable length of non-coding sequences at 5' and 3' end, for which sequence data are now available[10]. There exists extensive homology between the non-coding regions of the 2 segments. While 32 of 50 nucleotides are conserved at the 5' terminal in segment A, 29 of 50 nucleotides are conserved at 3' terminus in segment B. These termini should contain sequences that are important due to its role in replication and packaging of virus particles as demonstrated in reoviruses and rotaviruses[11,12].

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Reverse genetics of different groups of viruses has already been established and many of the positive stranded RNA viruses, negative stranded and segmented RNA viruses and double stranded RNA viruses have already been rescued from cloned synthetic nucleic acids. A reverse genetics system for generating infectious IPNV from cloned cDNA has been developed by Yao and Vakharia[10]. Further studies with reference to the role of the 5' and 3' non-coding regions of the virus is yet to be carried out. In this context, the present work was carried out to generate a minigenome of the IPNV, which can be used for studying the *cis*-acting requirements for viral multiplication. The reverse genetics system in combination with the information on *cis*-acting requirements of IPNV would help in the generation of non-pathogenic vaccine strains of virus mutants with defined attenuations. Current vaccination methods of fish against IPNV included use of killed vaccines and sub-unit vaccines directed against the VP2 protein of the virus[13-15]. Although these vaccines offer certain amount of protection against the disease, there is still scope for improvement of the efficacy of the vaccination programme. Present work, therefore, was taken up to generate a minigenome of IPNV, replacing the coding region with the reporter gene, bacterial chloramphenicol acetyl transferase (CAT), with a view to understand the role of non-coding starter and trailer sequences in the replication and packaging of IPNV.

Materials and Methods

Cells and Viruses

The West Buxton (WB) strain of IPNV was grown in the salmonid fish cell line CHSE-214. The cells were cultured using DMEM (Gibco-BRL, USA) supplemented with 10% FBS (Gibco-BRL, USA) penicillin/streptomycin/fungizone mix (Biowhittaker, USA). The cells were maintained at 15°C both before and after inoculation with IPNV.

Purification of Virus and Viral RNA Extraction

Once the cytopathic effect (CPE) is extensive, the cells were scrapped into the supernatant and spun at 1500 rpm for 15 min. The supernatant was collected and pellet was resuspended in 1.4 mL of 1% NP 40 in 10 m*M* Tris-HCl and kept on ice for 15 min with occasional stirring. The suspension was again spun at 500 rpm for 10 min and the supernatant collected was added to the supernatant collected earlier. The combined supernatant was overlaid on 30% sucrose cushion in a SW 28 rotor and again spun at 23,000 rpm for 90 min. The pellets were pooled and stored at 4°C in TNE [0.1 *M* Tris-HCl (*p*H 7.4), 0.1 *M* NaCl, 1 m*M* EDTA). Viral RNA was extracted by mixing 200 µl virus suspension in TNE, 190 µL PK buffer (0.01 *M* Tris, 0.005 *M* EDTA) and 7 µL proteinase K (20 µg/µL). The suspension was incubated at 37°C for 1 h and viral RNA was extracted by standard phenol-chloroform method. The pellet was washed in 70% ethanol (200 µl) and dried.

Construction of cDNA of 5' and 3' end non-coding sequences of IPNV Segments A and B

Primers were designed for amplifying the non-coding sequences both at the 5' and 3' end of both the segments. The sequences of the IPNV WB strain were obtained from the published information[10] at the GenBank database (Accession nos AF078668 and AFO78669). Each of the primers was designed with an overhang to incorporate at least one restriction enzyme site. Two primers for the 5' end of both the segments also had additional T7 promoter sequences in the overhang (Table 1). One *Sma* l site was also added at the 3' end of both the segments for linearisation of the DNA.

Reverse transcription of the viral RNA was carried out using all the 4 forward ('+') primers designed. This reaction was to generate 4 cDNA, two each for the Segment A and Segment B aimed at amplifying the regions of 5' and 3' non-coding sequences. The ds RNA was boiled in presence of the forward primer for 2 min and cooled on ice. The reaction was carried out using Thermoscript RT-PCR system as per the supplier's protocol (Life Technologies). The cDNA generated was used as the template appropriately for the subsequent PCR with the 4 forward and reverse primers for both the segments. PCR was carried out for 25 cycles with denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, following a single step of denaturation at 94°C for 4 min. The PCR products were purified using phenol/chloroform extraction method.

Generation of IPNV-CAT Minigenome

A modified pUC 19 plasmid that carried a chloramphenicol acetyl transferase (CAT) reporter gene was used for the generation of the minigenome. The plasmid (KSG) contained multiple cloning sites flanking the CAT reporter gene. The PCR amplified non-coding regions of both the segments (5' and 3' ends) were inserted into the cloning sites of the

Table 1—Details of non-coding sequences and designed primer sequences

A. Non-coding nucleotide sequences of 5' and 3' ends of IPNV-WB Segments A and B

Segment A	5'- 1 ggaaagagag tttcaacgtt agtggtaacc cacgagcgga gagctcttac ggaggagctc 61 tccatcgatg gcgaaagccc tttctaacaa
Leader	acaaccaaca attctattta catgaatc 119 **atg** (121)
Trailer	5'-3035 **taa**cggctac tctctttcct gactgatccc ctggccttaa ccccggcccc ccaggggggcc 3095 ccc (3097)
Segment B	5'- 1 ggaaacagtg ggtcaacgtt ggtggcaccc gacataccac gactgtttat gtatgcacgc 61 aagtgcccct taacaaatcc
Leader	ctatacacac aactcatgat **atg** (103)
Trailer	5'-2636 **taa**gaagacc aaaccgggaa gaatccgaaa tgacccagct ggactcatat gcaagctccg 2696 cgccgtaagg caagctgaac caaagtagtg acccgacaat gtgccaccaa catgacccca 2756 gataacatcc ggttccgcca gggacccc (2783)

B. Primers designed for cloning of non-coding sequences

Designation		Nucleotide sequence*
SEGMENT –A (CAT LEADER)		
1.	T7 A FOR	5'- GCG CGC GGT ACC *TAA TAC GAC TCA CTA TAG* ***G*AA AGA GAG TTT CAA CGT TAG TGG T** -3' ('+' *Kpn*-I)
2.	T7 A REV	5'- TCT CCA TCT A**GA TTC ATG TAA ATA GAA TTG TTG GTT G** -3' ('-' *Xba* I)
SEGMENT –A (CAT TRAILER)		
3.	*PST* A FOR	5'- GCG TAA CTG CAG **CGG CTA CTC TCT TTC CTG ACT G** -3' ('+' *Pst*-I)
4.	*HIND* III A REV	5'- CCG CGC AAG CTT *CCC* ***GGG*** **GGC CCC CTG GGG GGC CGG GGT TAA** –3' ('-' *Hind* III, *Sma* I)
SEGMENT –B (CAT LEADER)		
5.	T7 B FOR	5'- GCG CGC GGT ACC *TAA TAC GAC TCA CTA TAG* ***G*AA ACA GTG GGT CAA CGT TGG TGG CAC** -3' ('+' *Kpn*-I)
6.	T7 B REV	5'- TCT CCA TCT AG**A TCA TGA GTT GTG TGT ATA GGG ATT TG** -3' ('-' *Xba* I)
SEGMENT –B (CAT TRAILER)		
7.	*PST* B FOR	5'- GCG TAA CTG CA**G AAG ACC AAA CCG GGA AGA ATC CGA** –3' ('+' *Pst*-I)
8.	*HIND* III B REV	5'- CCG CGC AAG CTT *CCC* ***GGG*** **GTC CCT GGC GGA ACC GGA TGT TAT CTG** –3' ('-' *Hind* III, *Sma* I)

* Orientation and name of the restriction enzymes are given in brackets. Restriction enzyme sites underlined, T7 promoter sequences italicised, IPNV sequences in boldface, *Sma* I site italics and underlined

plasmid as in Fig 1. For segment A minigenome, first the PCR product of primers 1 and 2 having T7 promoter and 5' non-coding sequence was double digested with *Kpn* I and *Xba* I and cloned into the appropriate double digested window of the KSG plasmid. Similarly, the PCR products of primers 3 and 4 were double digested with *Pst* I and *Hind* III and cloned into the plasmid downstream of CAT ORF by appropriate double digestion. Same procedure was carried out for the segment B minigenome. Two chimeric minigenomes were also constructed by switching the 5' and 3' end of segment A with that of segment B and vice versa. This resulted in the generation of 4 minigenomes, viz. A5'-CAT-A3' (C101), B5'-CAT-B3' (C104), A5'-CAT-B3' (C30) and B5'-CAT-A3' (C24). The sequence of the 4 minigenomes were confirmed with reference to the incorporation of the IPNV non-coding sequences by sequencing the nucleotides by the dideoxy chain termination method using Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit of USB Corporation, (USA) as per manufacturer's protocol. Two internal primers for the CAT reporter gene were used for checking the sequences. One of the primers, 3061 was a 19mer reverse primer at the

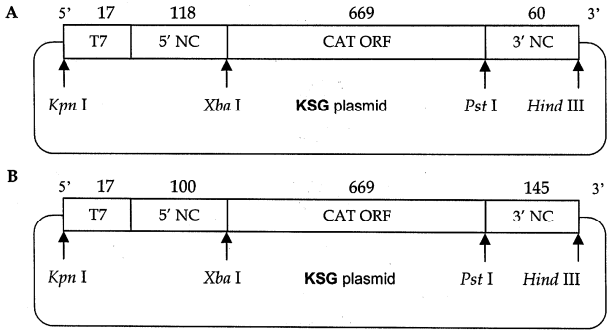

Fig. 1—Schematic diagram showing the construction of a minigenome of IPNV with CAT ORF (not to scale). CAT ORF is flanked by non-coding regions of segment A (A) and segment B (B) (open boxes) in separate constructs (A & B). T7 promoter for T7 polymerase is at the 5' end. Numbers of nucleotide are indicated. The restriction enzyme sites used for creation of the plasmid is also indicated.

5' end of the CAT gene and the second one, 3062 was a 22mer forward primer at the 3' end of the CAT gene; both on extension will run out to the flanking sequences cloned into the plasmid bearing the CAT gene. Following the correct incorporation of the non-coding sequences of both segments into the plasmids (verified by sequencing), all the plasmid DNA were amplified though transformation into competent

E. coli cells (MAX efficiency DH10B competent cells, Life Technologies, USA) as per the manufacturer's protocol and extracted using Qiagen midi prep kit. The DNA was linearised by restriction digestion with *Sma* I.

In vitro Transcription of Viral RNA from Cloned Minigenome DNA

The linearised DNA obtained by digestion with *Sma* I restriction enzyme was extracted by phenol/chloroform method to purify the DNA for transcription. All the 4 plasmid DNA generated by cloning of the non-coding sequences of the 5' and 3' regions of both the segments of IPNV were used for *in vitro* transcription using T7 RNA polymerase by standard procedures. The four RNAs generated were analysed in a denaturing agarose gel electrophoresis system.

Transfection of CHSE-214 Cells with Viral RNA

CHSE-214 cells were seeded into 6 well plates and after attaining 80% confluence, washed with PBS and Opti-MEM (Life Technologies, USA). After adsorption of 0.5 mL of virus (IPNV-WB) in Opti-MEM into cells for 90 min at 25°C, transfection was carried out with the *in vitro* transcribed RNA. The reactions were done in duplicate, while one set of four wells served as control for the 4 mRNAs. Transfection mix was prepared by incubating 1 mL Opti-MEM, 12 µL of RNA (containing approximately 1 µg RNA) and 12 µL Dimre-C (Life Technologies) for 15 min. The cells were washed with Opti-MEM before adding transfection mix. The transfection mix was then added dropwise and the plates were swirled gently. After 3 h incubation at 23°C, the mixture was replaced with MEM containing 10% FBS. The transfected cells were incubated at 23°C for observing the growth of the virus and noting the expression of CAT gene.

Determination of CAT Activity in Transfected Cells

The cells from the 6 well plates were harvested 48 h after infection and the cell lysates were checked for CAT activity by CAT assay protocol using ^{14}C chloramphenicol and autoradiography (Fig. 2) by standard procedures[16].

Results and Discussion

Minigenomes were created for both the A and B segments of IPNV, in which all the protein coding sequences that lie between the 5' and 3' end non-

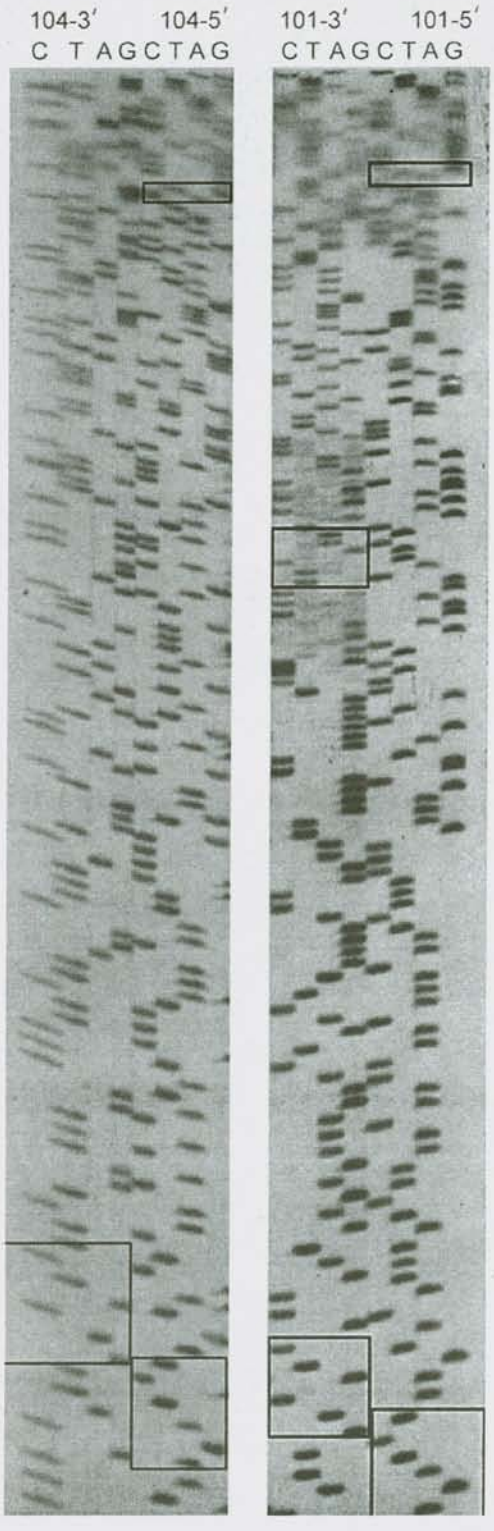

Fig. 2—Autoradiograms showing the nucleotide sequences of the IPNV minigenomes 104 and 101 clones. The restriction sites are boxed; *Hind* III (top) and *Pst* I (below) in 3' clones, and *Kpn* I (top) and *Xba* I (below) in 5' clones. (Sequence of 104-3' clone in the gel is till the *Hind* III site)

coding sequences were removed and replaced by a positive sense copy of the bacterial CAT gene. Two chimeric forms of the minigenomes were also constructed by switching the 5' and 3' end of segment A with that of segment B and vice versa. The sequence analysis has shown that the 4 minigenomes had correct predicted sequences including the positive sense CAT ORF (Fig. 2). Functionality of the plasmids was also verified by the successful transformation of the *E. coli* cells and amplification of the minigenomes.

In vitro transcription indicated the functionality of T7 promoter. This was documented by the successful generation of mRNA of the expected size, verified by running the RNA in the denaturing agarose gel electrophoresis system (Fig. 3). CAT activity albeit faint was observed in the entire 4 virus RNA (vRNA) infected wells of CHSE-214 cells. The requirement of capping the 5' end of the RNA transcripts by cap analogue 7-methyl guanosine (5') triphospho (5')-guanosine [m7G(5')ppp(5')G] for efficient translation of synthetic mRNA has been shown by various workers[10,11]. However, this was not done during the *in vitro* transcription of cDNA, which could have reduced the efficiency of translation of CAT gene.

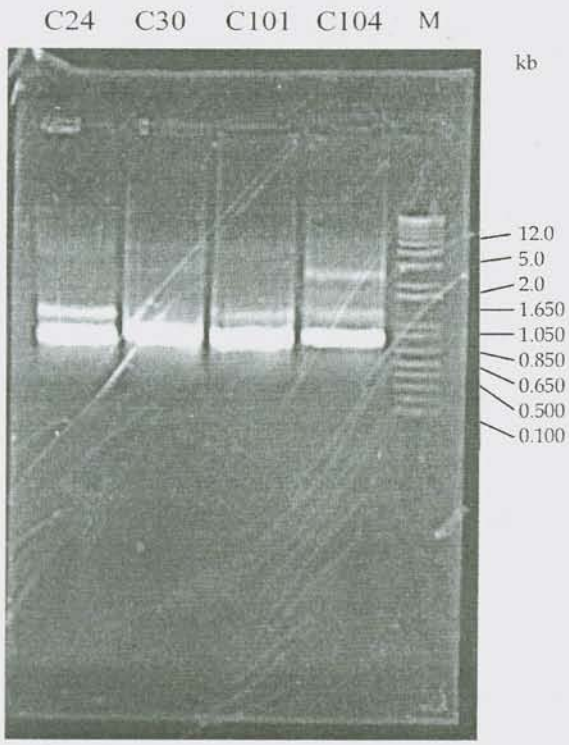

Fig. 3—The *in vitro* transcribed RNA from 4 plasmid DNA run in 1% agarose gel along with markers; C24 (5'B – CAT – 3'A), C30 (5'A – CAT – 3'B), C101 (5'A – CAT – 3'A), C104 (5'B – CAT – 3'B) and M (Marker)

Minigenomes of rotaviruses carrying CAT gene with non-coding sequences have shown to render the synthetic RNA biologically active when super infecting helper viruses supplied viral proteins[11]. They have observed trace amount of CAT activity even in the absence of superinfection by homologous viruses arising from direct translation when the RNA was capped. Similarly, in the present study, the cells transfected by CAT RNA alone did not show CAT activity presumably because of the absence of RNA capping[10]. Functional minigenomes provide an ideal model for the study of virus replication and packaging of many groups of RNA viruses. They also help in analysing the minimal *trans*-acting requirements of the virus genome[17]. Any possible attenuating effect that may be noticed in the minigenome replication system may have potential application in the production of live attenuated vaccines for viral diseases. An extension of minigenome system has resulted in the generation of infectious viruses from cloned DNA and this has great potential in designing vaccine strains of viruses with reduced pathogenicity aimed at controlling devastating viral infections[18].

The present study indicated that the synthetic RNA was recognised by the viral proteins to direct CAT expression in the infected cells. Since the CAT activity was noticed in the 4 different RNA transfected cells, it could be assumed that the viral proteins could recognise synthetic CAT RNA with the 5'and 3' NCR of both the IPNV segments interchangeably. This also indicates that NCR of both segments of IPNV carry *cis*-acting signals, which play an active role in the translation of RNA. Generation of the functional minigenomes provides ample scope for further research in studying the mutational analysis of the IPNV, and further studies incorporating NCR mutations would be required to delineate the exact *cis*-acting role of these regions of both A and B segments of IPNV. An understanding of this would facilitate the generation of recombinant IPNV strains with defined mutations to reduce pathogenicity and improved vaccine potential.

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