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STUDIES ON MOLECULAR MECHANISMS OF VIRULENCE OF INFECTIOUS BURSAL DISEASE VIRUS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the PhD Degree

in the Department of Pathology and Microbiology

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University of Prince Edward Island

Malliga M. Nagarajan

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ABSTRACT

Infectious bursal disease virus (IBDV) is the etiological agent of a highly contagious immunosuppressive disease in young chickens. Despite the use of vaccines, the incidence of IBDV infection and associated disease problems due to immunosuppression are still common. Spontaneous enhancement of virulence has occurred without significant changes in antigenicity of circulating classical virulent strains. The sites on the viral genome involved in such virulence variations and the underlying molecular mechanisms are poorly understood. Since the rate of viral replication influences virus virulence, attempts were made to map the regulatory (promoter/enhancer) sequences involved in IBDV genome expression and replication. A series of chimeric genes containing various lengths of the 5' noncoding sequences of IBDV segment A were constructed and analysed for promoter activity by measuring their capacity to direct the expression of the luciferase reporter gene. Progressive deletions of the 5' noncoding region of 131 bp of segment A of IBDV and transient transfection studies identified a minimal region of 32 bp that had maximal promoter activity without any enhancer activity. Attempts were also made to isolate virus variants able to escape neutralizing antibody. A partial neutralization resistant mutant of IBDV was isolated by serially passaging a QC2 strain of IBDV in Vero cells in the presence of polyclonal antibody, and its phenotypic and genotypic characteristics were studied. The selected mutant had similar growth characteristics as the parental virus but had a smaller plaque morphology. Its genome segment A had an additional *Pst* I restriction site in the major antigenic region. There were four and three nucleotide exchanges in the 3' noncoding regions of segments A and B respectively and none in the 5' noncoding regions. Moreover, less severe pathology in the bursa of Fabricius and higher virus neutralization titres (VN) were elicited by this mutant compared to the parental virus in specific-pathogen free chicken. Such a variant may be useful in the development of attenuated vaccines of IBDV. Furthermore, efforts were made to develop a reverse genetics system for IBDV by constructing two independent full-length cDNA

clones containing segments A and B of OH strain of IBDV. Infectious progeny viruses were recovered in Vero cells transfected with combined transcripts of segment A and segment B of these cDNA clones. Moreover, transfection of Vero cells with full-length segment A transcript and a partial length segment B transcript derived from a partial length cDNA construct of segment B without its 3' noncoding sequences also produced infectious virus that was almost indistinguishable from the wild-type IBDV. The 3' noncoding sequences that were absent in the partial length cDNA construct of segment B were regenerated in the genomic RNA of the progeny infectious virus indicating the functional significance of this region in establishing a productive infection. The cDNA clones constructed in this study offer great potential for further manipulation of the IBDV genome, to determine the nucleotide residue(s) involved in virulence in order to construct candidate vaccine strains of IBDV. They also provide a model system to study mechanisms underlying IBDV dsRNA synthesis and packaging of the IBDV genome.

Dedication

To my parents

ACKNOWLEDGEMENTS

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COMMONLY USED ABBREVIATIONS

Terms	Abbreviations
Standard units of measurement	
base pair	bp
centrifugal force	g
centigrade	C
hour	h
hydrogen ion activity	pH
kilodalton	kDa
kilobase	kb
millimolar	mM
revolutions per minute	rpm
microliter	µl
microgram	µg
millicurie	mCi
molar	M
second	s
single photon counts	SPC
volt	V
watt	W
Greek alphabet	
alpha	α
beta	β
gamma	γ
delta	Δ
phi	ϕ
Amino acid three letter code	
alanine	Ala
asparagine	Asn
aspartic acid	Asp
glycine	Gly
histidine	His
isoleucine	Ile
lysine	Lys

phenylalanine	Phe
serine	Ser
threonine	Thr
tryptophan	Trp

Other abbreviations

adenosine triphosphate	ATP
amino acid	aa
baby grivet monkey	BGM
B lymphocyte	B-cell
bacteriophage T7	T7
bacteriophage T4	T4
bovine serum albumin	BSA
5-bromo 4-chloro 3-indolyl β galactoside	X-gal
bursal disease antibody	BDA
carbon dioxide	CO_2
chicken embryo fibroblast	CEF
chicken embyro kidney	CEK
chloramphenicol acetyl transferase	CAT
complementary DNA	cDNA
cytopathic effect	CPE
deoxynucleotide triphosphate	dNTP
dideoxynucleotide triphosphate	ddNTP
diethyl pyrocarbonate	DEPC
dimethyl sulphoxide	DMSO
dithiothreitol	DTT
double-stranded	ds
drosophila X virus	DXV
eastern equine encephalitis virus	EEV
ethylene diamine tetraacetic acid	EDTA
fetal bovine serum	FBS
five prime	5'
fowlpox virus	FPV
geometric mean titre	GMT
genome linked viral protein	VPg
guanosine monophosphate	GMP
guanosine triphosphate	GTP
immune pressure	IP
infectious bursal disease virus	IBDV

infectious pancreatic necrosis virus	IPNV
infectious bronchitis virus	IBV
large plaque	LP
lithium chloride	LiCl
long terminal repeat	LTR
luciferase	LUC
magnesium sulphate	$MgSO_4$
magnesium chloride	$MgCl_2$
major histocompatibility complex	MHC
median tissue culture infective dose	$TCID_{50}$
messenger RNA	mRNA
middleburg virus	MBV
minimal essential medium	MEM
monoclonal antibodies	MAbs
mouse hepatitis virus	MHV
multiplicity of infection	MOI
natural killer cells	NK
noncoding	NC
nonimmune pressure	NIP
nucleotides	nts
open reading frame	ORF
ovine kidney	OK
oyster virus	OV
phosphate buffered saline	PBS
polyacrylamide gel electrophoresis	PAGE
polyclonal antiserum	APS
polyethylene glycol	PEG
polymerase chain reaction	PCR
potassium chloride	KCl
quail fibroblast cell line	QT35
restriction enzyme	RE
reverse transcriptase	RT
ribonucleic acid	RNA
ribonucleotide triphophate	rNTP
Ross River virus	RRV
simian immunodeficiency virus	SIV
sindbis virus	SIN
single photon counts	SPC
single-stranded	ss

small plaque	SP
sodium dodecyl sulphate	SDS
sodium chloride	NaCl
sodium hydroxide	NaOH
specific-pathogen free	SPF
standard challenge	STC
T-lymphocyte	T-cell
tellina virus	TV
N,N,N',N' tetramethyl 1,2-diaminoethane	TEMED
three prime	3'
transcription coupled with translation	TNT
turkey herpes virus	HVT
very virulent	VV
viral protein	VP
virus neutralization	VN
weight	wt
western equine encephalitis virus	WEE

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Infectious Bursal Disease Virus

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens (Nagarajan and Kibenge, 1997a; Kibenge et al., 1988a). The etiological agent, infectious bursal disease virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects pre B-lymphocytes (Burkhardt and Müller, 1987). Chickens are susceptible to infection at three weeks or more when maternal antibodies are on the wane, and display clinical disease. Economic losses in the poultry industry result from high mortality rates due to an acute clinical form of the disease in chickens above 3 weeks old or from a subclinical infection in chickens below three weeks old characterized by B-cell dependent immunodeficiency (Lukert et al., 1984). The latter enhances the susceptibility of chickens to other infections and depresses the response of infected chickens to vaccines against other diseases such as Newcastle disease, Marek's disease and infectious bronchitis (Lasher and Shane, 1994).

IBDV belongs to the family *Birnaviridae* of the genus *Avibirnavirus* (Dobos et al., 1995). Members of this family contain a double stranded (ds)RNA genome consisting of two segments, designated A and B, within a non-enveloped icosahedral capsid of 60 nm diameter (Nick et al., 1976). These include infectious pancreatic necrosis virus (IPNV) of young salmonid fishes, Tellina virus (TV), oyster virus (OV) and crab virus in the genus *Aquabirnavirus,* and Drosophila X virus (DXV)

in the genus *Entomobimavirus* (Dobos et al., 1995). Based on cryo-electron microscopic studies and computer image processing for IBDV, a model of the virion was proposed consisting of an outer layer of VP2, a major capsid polypeptide, and an inner layer of VP3, a minor capsid polypeptide with a very basic carboxy terminal region found in association with the genomic RNA molecules . VP4 is located at the inner 5-fold axes (Böttcher et al., 1997). Of the two recognized serotypes of IBDV, only serotype 1 strains are pathogenic and replicate in proliferating pre-B cells of the bursa of Fabricius (Müller, 1986). Individual serotype 1 strains differ markedly in their virulence (Winterfield and Thacker, 1978). The serotype 2 strains are nonpathogenic for chickens (Ismail et al., 1988) and do not replicate in lymphoid cells (Nieper and Müller, 1996).

The pathogenesis of IBDV is poorly understood. Since 1985, antigenic variant strains of IBDV causing marked immunosuppression without any clinical signs have been isolated from vaccinated flocks on the Delmarva peninsula (i.e. portions of Delaware, Maryland and Virginia) in the United States (Heine et al., 1991; Snyder, 1990). These strains are distinct from the classical virulent strains and cause low or no mortality but severe damage to the bursa of Fabricius. Since 1989, there have been outbreaks of disease in broiler and layer flocks caused by strains of IBDV referred to as very virulent (VV) strains. These strains induce severe clinical signs with more than 70% mortality and severe damage of the bursa of Fabricius, thymus, spleen and bone marrow (Tsukamoto et al., 1995). Outbreaks with VV

strains are a problem of growing importance in several countries including the Netherlands (Box, 1989), Great Britain (Chettle et al., 1989), Belgium (Van den Berg et al., 1991), Germany (Öppling et al., 1991), Poland (Minta and Daniel, 1994), France (Enterradossi et al., 1992), Japan (Lin et al., 1993), Taiwan (Tsai and Lu, 1992), Israel (Pitcovski et al., 1998), Middle East and Northern and Southern Africa (Lasher and Shane, 1994). Young birds with maternally derived antibody (MDA) are immune to infection while antibody levels are high but become susceptible when the titers drop. However, some of the newly emerged VV strains appear to be capable of breaking through MDA at an earlier age and of overcoming protective immunity induced by intermediate vaccine strains but their enhanced virulence was not accompanied by alterations in their antigenicity (Gaudry, 1993). Thus, there is a need to better understand the molecular basis of IBDV virulence so that better vaccines can be produced for effective control of IBDV.

1.1.1 Viral Genome Structure and Organization

IBDV genome consists of two segments of double-stranded RNA designated A and B. Genome segment A (approx. 3300 bp) contains two open reading frames (ORF); a small ORF preceding and partially overlapping the larger ORF encodes a 17 kDa protein (Kibenge et al., 1990). This protein, designated VP5, is of unknown function although it is present in IBDV infected cells (Mündt et al., 1995). The larger ORF encodes a 109 kDa precursor polyprotein (N-VPX-VP4-VP3-C) (Azad et al., 1987; Hudson et al., 1986) which is processed into two major structural proteins, VP2 (40-45 kDa) and VP3 (32-34 kDa) by the putative viral protease VP4 (28-30.5 kDa)

(Azad et al., 1988; Hudson et al., 1986). VP2 is the host-protective immunogen of IBDV and contains the major antigenic site responsible for eliciting neutralizing antibodies (Fahey et al., 1989). Most amino acid (aa) changes in VP2 between IBDV strains are located in a variable domain between aa positions 206 to 350 representing a major conformational domain (Azad et al., 1987).

VP3 contains the group-specific antigens (Becht et al., 1988) and a minor neutralizing epitope (Mahardika and Becht, 1995; Jagadish and Azad, 1991). The C-terminal region of VP3 has also been implicated in either packaging or stabilizing the RNA genome within the interior of the capsid (Fahey et al., 1989). Deletion expression studies of cDNA fragments of segment A of 002-73-IBDV suggest VP4 to be the viral protease involved in the processing of the precursor polyprotein to VPX, VP3 and VP4 (Jagadish et al., 1988; Azad et al., 1988). Even though the active site of the viral protease has not been established, polyprotein residues His at position 546, Asp at 589 and Ser at 652 are suggested to form the catalytic triad of a serine protease (Brown and Skinner, 1996). Recent work by Sànchez and Rodrieguez (1999) using a series of plasmids encoding polyproteins consisting of either deletions or single amino acid substitutions has demonstrated the existence of two major sites, Lys-Ala-Ala (aa residues 511-513) and Met-Ala-Ala (aa residues 754-756) that are involved in the processing of the VPX-VP4 and VP4-VP3 precursors respectively.

A precursor-product relationship has been suggested in the biosynthesis of VP2, VP3 and VP4 polypeptides. A two-step cleavage has been described in which a polypeptide of 50KDa could be pulse chased to form 49 KDa (VPX), the precursor of VP2 (40 KDa) (Müller and Becht 1982). Since VP2 does not accumulate intracellularly, post-translational modification of the 50 KDa polypeptide into the 40 KDa (VP2) may occur during virus maturation and assembly (Müller and Becht 1982). Similarly, a 55 KDa to 60 KDa polypeptide is suggested to be the precursor for VP3 and VP4 (Azad et al., 1987; Hudson et al., 1986). Recent studies on IBDV polyprotein processing using the expression of segment A cDNA in rabbit reticulocyte lysates *in vitro* and in Sindbis virus recombinant-infected cell cultures detected VPX, VP3 and VP4 but not VP2, indicating that VPX maturation to VP2 does not involve cellular proteases (Kibenge et al., 1997). Moreover, virus-like particles were formed when full-length segment A cDNA encoding the polyprotein precursor was expressed in recombinant baculovirus infected insect cells. No VP2 was detected in lysates of infected insect cells indicating that VP2 maturation is not necessary for capsid assembly (Kibenge et al., 1999).

The smaller genome segment B (approx. 2820 bp) encodes a single protein (VP1) of 90 kDa (Morgan et al., 1988). It is a minor component of the virion and has sequence similarity with known RNA-dependent RNA polymerase of other dsRNA and positive single-stranded (ss) RNA viruses (Duncan et al., 1991). VP1 of IBDV has no guanylyl-transferase activity. It cannot transfer the radiolabeled GMP to an acceptor molecule such as GTP (Kibenge and Dhama, 1997) indicating that it is not

a capping enzyme. VP1 also exists in the virions as a genome-linked protein (VPg) (Müller and Nitschke, 1987) and as a free form of 90 kDa polypeptide attached to short stretches of viral RNA sequences (Kibenge and Dhama, 1997; Magyar et al., 1998).

1.1.2 Coding Sequences of IBDV

The nucleotide sequences of segment A of several serotype 1 strains (Brown and Skinner; 1996; Vakharia et al., 1994; Kibenge et al., 1990; Bayliss et al., 1990; Hudson et al., 1986) and serotype 2 IBDV strains, OH (Kibenge et al., 1991) and 23/82 (Bernstein, 1993), and segment B of serotype 1 strain 002-73 (Morgan et al., 1988) and serotype 2 strains OH (Kibenge et al., 1996) and 23/82 (Bernstein, 1993) have been reported. Between the pathogenic serotype 1 and nonpathogenic serotype 2 strains that have been sequenced for the coding region of segment B, there exists high degrees of nucleotide (89%) and amino acid (93-98%) sequence identities (Brown and Skinner, 1996). The RNA dependent-RNA polymerase (RdRp) consensus sequence motifs 1-8 (Bruenn, 1991) are conserved in both IBDV serotypes. In segment A, lower nucleotide (83%-84%) and amino acid (90%) sequence identities are noted in the coding regions between serotype 1 and 2 strains (Kibenge et al.,1991). The lower sequence identities in segment A are mainly attributed to the hypervariable region corresponding to the serotype-specific epitope(s) in the structural protein VP2 (Kibenge et al., 1990; Bayliss et al., 1990; Kibenge et al., 1991).

1.1.3 Noncoding Sequences of IBDV

Complete 5' and 3' terminal noncoding sequences of segments A and B of North American (SK140a, IN, OH) and European strains (23/82, P2, Cu-1, Cu-1M) of IBDV have been reported (Kibenge et al., 1996; Mündt and Müller, 1995). The 5' terminal sequences in both genome segments consist of a 32-nucleotide consensus sequence GGA U(A/G)C GAU (C/G)GG UCU GAA CC(C/U) C(G/U)G(G/-)A GUC AC . There are short consensus sequence motifs at the 3' noncoding region which are homologous between the genome segments A and B of North American and European strains of IBDV. The last 5 nucleotides at the 3' end of the plus strands of genome segments A and B of North American strains of IBDV are also similar with a conserved pentamer -GCGGU (Kibenge et al., 1996).

At the 5' and 3' ends in both genome segments of IBDV, there are direct terminal and inverted repeats that are likely to contain important signals for replication, transcription and packaging (Kibenge et al., 1996) (Fig.1). In addition the inverted adjacent repeats at the 3' terminus on segment A and 5' terminus on segment B have the potential to form stem and loop secondary structures (Kibenge et al., 1996). In segment A of IBDV, there are differences between serotype 1 and 2 strains in the predicted secondary structures formed by the 5' noncoding region preceding the VP5 gene (Fig. 2); these structures have been predicted to have a role in viral replication, in determining host cell-type specificity and possibly virulence (Mundt and Müller, 1995). However, a direct demonstration of the

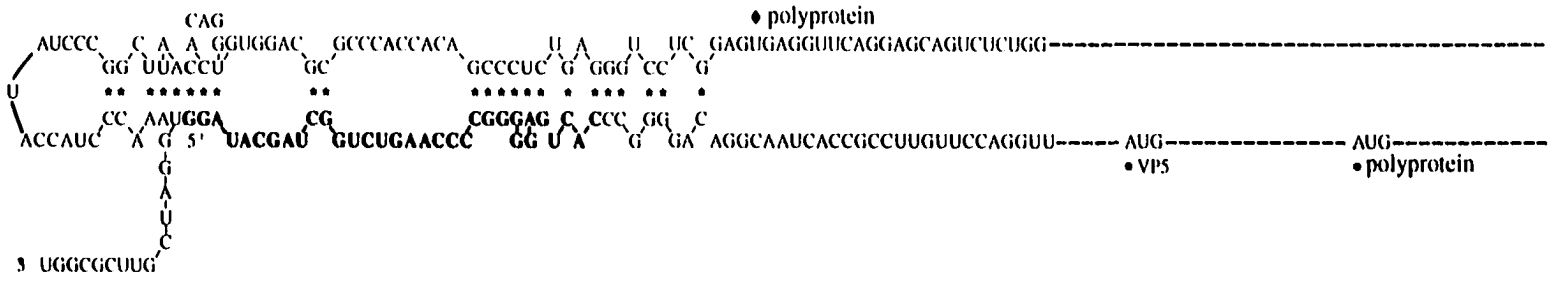
importance of these predicted secondary structures in viral replication has not been provided.

Segments A and B of IBDV also contain serotype-specific nucleotide changes in the noncoding regions. When serotype 2 strains of IBDV (OH, 23/82) were compared wiith serotype 1 strains, there were five nucleotide changes in the 5' noncoding region which were serotype-specific on segment A and three nucleotide change on segment B (Kibenge et al., 1996). No serotype-specific changes were found in the 3' terminal sequences of segment A whereas a single nucleotide change was identified in the 3' terminal sequences of segment B (Kibenge et al., 1996).

1.1.4. Sequence Analysis in Other Birnaviruses

There is a low overall amino acid sequence identity (41%) between VP1 of IBDV and IPNV, another member of the *Birnaviridae* family. Even though homologous regions were present within the central portions of these proteins, the regions near the carboxy-terminal of VP1 exhibited very low amino acid homology. Consensus sequences associated with GTP-binding proteins and RNA-dependent RNA polymerases (RdRps) were present in both IBDV and IPNV. The birnavirus RdRps lack the Gly-Asp-Asp motif associated with RdRps of single-stranded plus RNA viruses (Duncan et al., 1991).

Segment A (IBDV strain OH)



Segment B (IBDV strain OH)

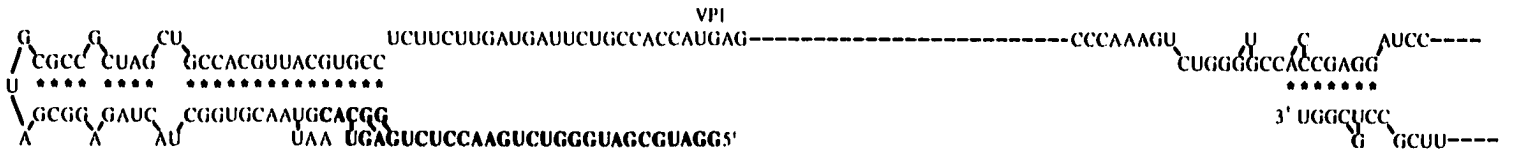


Fig. 1: Predicted RNA secondary structures for terminal sequences of segments A and B of IBDV strain OH. (*) denotes H-bonding. Hyphens indicate long stretches of sequence. The consensus 32-nucleotide 5' end sequence of the plus RNA strands in segments A and B is printed in bold and shaded; ♦denotes polyprotein stop codon and • the initiation codons of the VP5 and polyprotein ORFs on segment A and the VP1 ORF on segment B (Kibenge et al., 1996).

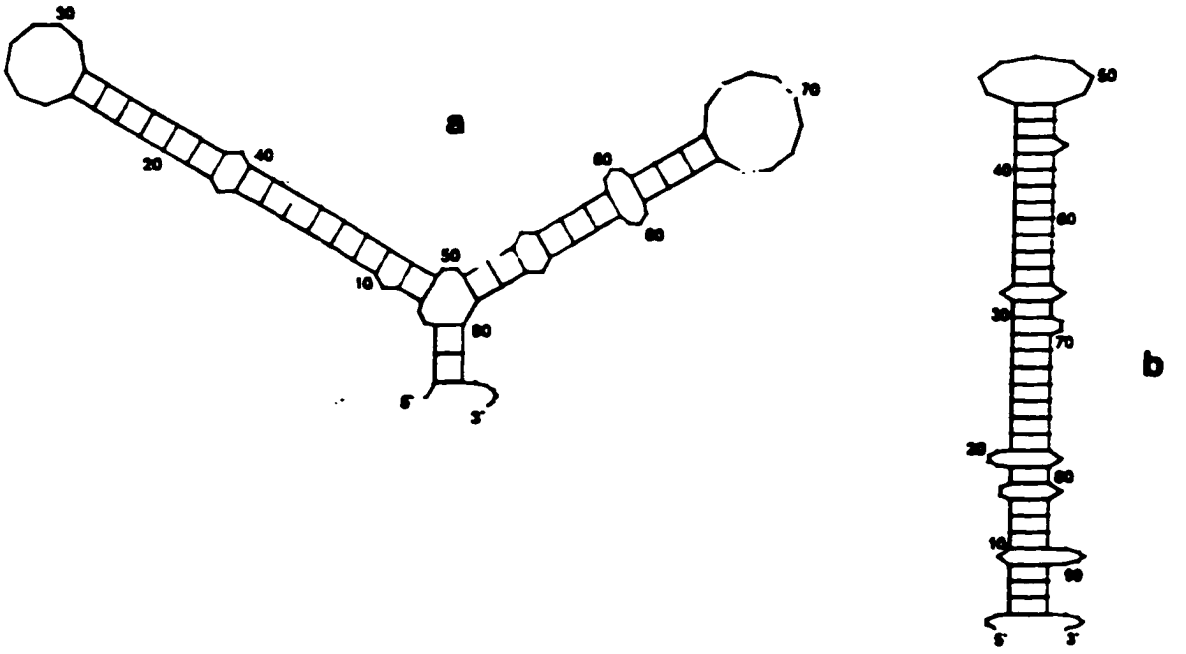


Fig. 2: Predicted RNA secondary structures for the 5'-noncoding regions (NCR) of segment A of (a) serotype 1 strain Cu-1 (nucleotides nt1-99) and (b) Serotype 2 strain 23/82 (nt 1-99) (Mundt and Müller, 1995) (Illustration used with permission).

The Drosophila X virus, another member of the *Birnaviridae* family has a relatively larger genome segment A (3360 base pairs) compared to other birnaviruses. It consists of a larger ORF encoding a 114 kDa protein and a smaller ORF unique to this member at the VP4/VP3 junction encoding a 27 kDa polypeptide. The amino and carboxy terminal regions of preVP2 and a particular 21 nt long residue near the carboxy terminal region of VP3 of the Drosophila X virus exhibited significant homology to IPNV and IBDV (Chung et al., 1996).

1.1.5 Viral Replication

A number of IBDV strains have been adapted to replicate and produce cytopathic effect (CPE) in primary cell cultures of chicken origin such as bursal lymphoid cells, chicken embryo kidney (CEK) and fibroblast (CEF) cells (Lukert and Davis, 1974). IBDV specific polypeptides were identified in chicken bursal lymphoid cells as early as 90 min after infection and in the culture medium of such cells 6 h after infection (Müller and Becht, 1982). Prolonged multiplication cycles of more than 48 h were noted in mammalian cell lines such as Vero and BGM-70 cells (Kibenge et al., 1988; Jackwood et al., 198, Lukert et al., 1975). Moreover, of all the known birnaviruses, only IBDV is able to replicate in mammalian cells, a unique cross-species biological property (Kibenge et al., 1988b). Viral particles assemble and accumulate in the cytoplasm. In the case of IBDV, VP4 is located in the nucleus as well as in the cytoplasm of infected cells (Granzow et al., 1997).

Incomplete IBDV particles with aberrant protein composition and which interfere with the replication of complete virus were formed by repeated passage of IBDV at high multiplicity of infection (MOI) in CEF cells (Müller et al., 1986). Such defective interference (DI) was also associated with viral persistence in the bursa and spleen at 28 days post-infection (PI) with an Arkansas variant strain IBDV-s977 serially passaged in CEF at a high MOI, suggesting that DI particles may be involved in virus attenuation (Bayyari et al., 1996).

Both pathogenic serotype 1 and nonpathogenic serotype 2 strains replicate efficiently in CEF, but only serotype 1 strains replicate in bursal lymphoid cells. Both CEF and bursal lymphoid cells have 46 kDa and 40 kDa common receptors for serotype 1 and 2 strains (Nieper and Müller, 1996). In addition, CEF has receptors specific for each serotype whereas lymphoid cells have receptors specific for serotype 2 strains only although the serotype 2 strains do not replicate in lymphoid cells (Nieper and Müller, 1996).

It is of prime importance to understand better the mechanism of viral replication and genome expression since the rate of replication directly influences the virus virulence. RNA-dependent RNA polymerase activity is associated with IBDV particles grown in CEF cells (Spies et al., 1987). *In vitro* studies indicate that RNA polymerase synthesizes viral ssRNA by a semi-conservative strand displacement mechanism, whereby the nascent strand displaces one of the parental strands (Spies et al., 1987). Two genome-length 24S mRNA hybridizing to the two IPNV

segments were detected both *in vivo* (Somogyi and Dobos, 1980) and *in vitro* (Mertens et al., 1982).

In the case of IPNV, more recent experiments indicate that virion-associated VP1 catalyzes a guanylylation reaction to form VP1pG complex that serves to prime viral RNA synthesis; apparently only the plus strands of the two genome segments are synthesized *in vitro* and remain base-paired to their respective templates (Dobos, 1995). It is suggested that the initiation of viral RNA synthesis may involve either two VP1 molecules, one serving as a primer and the other as polymerase for chain elongation or a single VP1 molecule being involved in both functions. Such protein primed RNA synthesis has been demonstrated *in vivo* as well (Magyar et al., 1998). Part of VP1-oligonucleotide complexes in which the RNA moiety consisted of 5' terminal sequences of genome segment A- and B-specific plus strands could be chased via replicative intermediates and genome length ssRNA to intracellular VP1-dsRNA and finally to virion VPg-dsRNA (Magyar et al., 1998). These observations with IPNV support an asymmetric and semi-conservative strand-displacement mechanism of replication for IBDV where the template minus strand remains annealed to the new plus strand. However reverse genetics studies with IBDV (Mundt and Vakharia, 1996) and IPNV (Yao et al., 1998) suggest a conservative mechanism similar to rotavirus replication where the template minus strand repairs with the parental plus strand (Patton et al., 1993) and the newly transcribed plus-strand RNAs serve as templates for minus strand synthesis. The ability to recover

infectious progeny IBDV and IPNV in cell culture by transfecting the *in vitro* generated plus sense transcripts of segment A and segment B support the above mechanism.

1.2 Gumboro Disease

Chickens less than three weeks of age infected with classical strains of IBDV usually do not show clinical disease but develop a subclinical infection characterized by immunosuppression (Saif, 1991). Chickens between three and six weeks of age develop clinical signs that include severe depression, ruffled feathers, incoordination, diarrhea and high mortality, a condition called Gumboro disease (Cosgrove,1962). Immunofluorescence studies detected IBDV four hours after infection in lymphoid cells and macrophages in the intestinal tract and five hours later in Kupffer cells of the liver. The virus disseminated through the blood thereafter to other organs including the bursa of Fabricius where intense replication occurred (Müller et al., 1979). The infected bursa increased markedly in size and weight due to edema and hyperemia 48 hours PI (Cheville, 1967) and appeared gelatinous with a necrotic core (Cosgrove; 1962; Cummings et al., 1986). There was complete lymphoid depletion by the eighth day of infection. However, lymphoid repopulation of spleen and thymus but not of the bursa of Fabricius occurred within 12 days after infection (Lukert et al., 1975, Cheville, 1967). Pronounced nonbursal lesions characterized by hemorrhages were present in the thigh and breast muscles in later stages of the disease (Parkhurst, 1964).

1.2.1 Immunosuppression

IBDV has a predilection for actively proliferating cells such as precursor B cells of the bursa of Fabricius (Dohms et al., 1988; Müller 1986; Sivanandan and Maheswaran,1980b) causing severe necrosis, lymphoid depletion, and subsequent immunosuppression. Other mechanisms of immunosuppression such as the development of suppressor cells in the spleen of infected chicks causing *in vitro* mitogenic hyporesponsiveness and impairment of helper T-cell function have also been suggested (Sharma et al., 1989a). Inhibition of the mitogenic response seemed to be mediated by inhibitory cytokines such as transforming growth factor-β and by nitric oxide produced by activated splenic macrophages (Sharma et al., 1994). There was also enhanced expression of several other cytokine genes in splenic macrophages such as interferon (IFN-α) and chicken myelomonocytic growth factor (cMGF) (Kim et al., 1998). The CD3+ cells comprising primarily TCR2 (αβ isotype) receptors persisted in the bursa of chickens infected with a virulent strain of IBDV even when IgM+ cells and IBDV antigen-positive cells disappeared (Tanimura and Sharma, 1997).

In vitro studies using IBDV infected chicken peripheral lymphocytes demonstrated features typical of apoptosis (Ojeda et al., 1997; Tham and Moon, 1996; Vasconcelos and Lam, 1994) suggesting that IBDV, in addition to causing necrosis, can induce apoptosis in avian lymphocytes. Indeed, there was depletion of cortical thymocytes due to apoptosis following infection with a highly virulent strain of IBDV

(HPS-2) (Inoue et al., 1994). VP2, the major antigenic protein of IBDV appeared to contribute to apoptosis (Fernandez-Arias et al., 1997). Programmed cell death ensued following transfection of a variety of mammalian cell cultures with vaccinia virus recombinants expressing VP2 of IBDV. VP2 expression resulted in strong inhibition of total protein synthesis in these cells and this effect was abrogated by coexpression of the proto-oncogene bcl-2. These results indicate the bona fide apoptotic inducing capabilities of VP2 and its involvement in apoptotic pathway in a step upstream of Bcl-2 (Fernandez-Arias et al., 1997).

The exact mechanisms of cellular apoptosis observed in IBDV infected cells have not been fully elucidated. There is a depletion of proliferating bursal lymphocyte population in chickens infected with IBDV and an increase in the proportion of apoptotic lymphocytes. It has been suggested that IBDV infection induces a change from lymphocyte proliferation to apoptosis (Ojeda et al., 1997). Recent work has shown that IBDV can also induce apoptosis in heterophils and macrophages with ensuing immunosuppression in the affected chickens (Lam, 1997; 1998). Moreover, infection of chicken CEF cells with Cu-1 strain of IBDV resulted in marked changes in potassium outward current properties that were independent of virus replication and presumably due to virus attachment and/or membrane penetration. It has been suggested that the reduction in potassium channel activity could be a mechanism utilized by IBDV in infected CEF cells to delay the onset of an apoptotic process and thereby prolonging the viability of the cell for production of maximum number of virions (Repp et al., 1998).

Chickens infected with some of the VV IBDV strains from Japan developed not only bursal lesions but also thymic and bone marrow lesions (Lin et al., 1993). High virus titres were detected not only in the bursa of Fabricius, but also in the thymus, spleen and bone marrow suggesting that these organs may also be involved in efficient replication of VV IBDV in susceptible chickens (Tsukamoto et al., 1995). Infection of three-week-old chickens with a mutant recombinant virus derived from a parental attenuated vaccine strain of IBDV-D78, in which the initiation codon of VP5 gene was changed to a stop codon failed to produce any pathological lesions or clinical signs of disease and demonstrated only a slight delay in replication *in vivo* suggesting that VP5 plays a role in viral pathogenesis (Yao et al., 1998).

1.2.2 Control of IBDV

In the past, a combination of live and inactivated vaccines used in the parent breeder flocks was sufficient to induce high levels of maternal antibody in the broiler progeny that prevented early infections and immunosuppression. However, most intermediate vaccine strains despite their high immunogenecity and reduced efficiency to cause bursal damage are inadequate in providing protection against VV IBDV. Some of the less attenuated vaccine strains or so called 'hot' strains with acceptable reduction of mortality have been evaluated by determining the optimum age for vaccination using a formula which predicts the decline in maternal antibody (Kowenhovan and van Den Bos,1993). A novel complex IBDV vaccine containing a mixture of IBDV with viral antibodies (bursal disease antibody; BDA) has been

shown to be safe and adequate for protection of chicks following subcutaneous administration (Whitfill et al., 1995).

With the increase in knowledge of the molecular structure and immunology of IBDV, better attenuated and genetically engineered vaccines are continually being developed. Structural protein genes of IBDV have been expressed in fowlpox, turkey herpesvirus and baculovirus-vector systems. VP2 from a virulent IBDV strain 52/70 expressed as a β-galactosidase fusion protein in a recombinant fowlpox virus (FPV), fpIBD 1, provided protection against mortality, but not against damage to the bursa of Fabricius (Bayliss et al., 1991). Recombinant FPV-VP2 containing the VP2 coding region under the control of the fowlpox early/late promoter provided a considerable level of protection when challenged with IBDV strain 002-73, although the level was lower than the protection provided by an oil adjuvanted inactivated whole IBDV vaccine (Heine and Boyle, 1993).

Baculovirus-expressed IBDV antigens are structurally similar to native virus particles and assemble to form virus like particles (VLPs) that are highly immunogenic and elicit virus-neutralizing antibodies that confer active and passive protection (Vakharia, 1997). A chimeric cDNA clone of the large segment A of an antigenic variant strain GLS, encoding VP3, VP4 and the VP2 with the incorporated classic B69 neutralization epitope was expressed in a recombinant baculovirus (Snyder et al., 1994). When used as a vaccine, the above recombinant clone conferred protection in SPF chicks against virulent challenge with the classical IM and STC

strains and the antigenic variant strains E/DEL and GLS (Vakharia et al., 1994). In another study, the VP2 and VP2-VP4-VP3 baculovirus recombinants provided partial protection (no clinical disease and death) in SPF chickens against challenge with STC IBDV but not against bursal damage. Similarly, when challenged with the variant MD IBDV, neither of these recombinants conferred protection against bursal damage (Dybing and Jackwood, 1998).

Recently, vaccination with turkey herpesvirus (HVT) vector with the VP2 gene inserted at the locus of gI gene under the control of the human cytomegalovirus immediate/early promoter provided good protection in one-day-old chickens against mortality and bursal damage after IBDV challenge (Darteil et al., 1995). Moreover, HVT is nonpathogenic to chickens and the persistent infection in chickens subsequent to vaccination enables presentation of the foreign antigens over a long period of time.

Genetically engineered recombinant IBDV vaccine vectors offer an alternative approach as candidate vaccines subsequent to the generation of infectious IBDV from cloned DNA (Mundt and Vakharia, 1996). It is now possible to determine the nucleotide residues or viral protein domains responsible for attenuation of virulence and to construct highly immunogenic attenuated vaccine strains of IBDV.

1.3 Antigenic and Virulence Characteristics

Since 1985, antigenic drift in field IBDV populations has been recognized in the United States with the isolation of several serotype 1 strains from the bursa of Fabricius of birds properly vaccinated with mild, live IBD vaccine (Rosenberger and Cloud, 1986). These virus isolates were named variant viruses since they infected broiler chickens with relatively high levels of maternal antibodies (Box, 1989). They were antigenically different from the classical strains isolated before 1985 and were highly immunosuppressive, causing rapid bursal atrophy without symptoms of clinical disease. However, the newly emerged very virulent strains (VV) of IBDV from different parts of Europe and Asia do not show any differences in antigenicity from the classical virulent strains (Brown et al., 1994) and the underlying molecular mechanisms for their virulence variations need clarification.

Since all known serotype 2 strains of IBDV are naturally avirulent for chickens (Ismail et al., 1988), considerable knowledge of the molecular basis for antigenicity and virulence has accumulated from identifying sequence differences between the naturally avirulent serotype 2 strains and the virulent serotype 1 strains (Kibenge et al., 1991). Previously, the majority of efforts focused on the VP2 coding region responsible for inducing virus neutralizing (VN) antibodies (Fahey et al., 1989). The greatest amount of amino acid sequence variations in VP2 among the various strains of IBDV is between amino acid residues 206 and 350 (Kibenge et al., 1990; Bayliss et al.,1990). This hypervariable region consisting of 151-152 amino acid residues encodes the conformational epitope recognized by VN monoclonal

antibody (mAb) 17/82 (Azad et al., 1987). Two symmetrically spaced hydrophilic regions (amino acid residues 212-224 and 314-324) are recognized in this hypervariable region (Schnitzler et al., 1993). These hydrophilic regions and the internal sequences of VP2 are not conserved between the pathogenic serotype 1 and nonpathogenic serotype 2 strains. The first hydrophilic region is speculated to be responsible for stabilizing the conformation epitope and the second hydrophilic region for recognition by VN mAb 17/82 (Heine et al., 1991).

In variant viruses such as Variant E, the amino acid substitutions in the second hydrophilic region appeared to enable variant viruses to escape VN by antibodies induced by vaccination with a classical type 1 vaccine (Heine et al., 1991). Six amino acid changes were identified within the variable domain of VP2 of Variant A virus when compared to the consensus sequence of five other IBDV isolates (Heine et al., 1991). In Variants DS326, E/DEL and GLS only one or two amino acid exchanges were noted in each of the two hydrophilic regions (Heine et al., 1991; Lana et al., 1992; Vakharia et al., 1994) and all these strains had a Gln $\rightarrow$ Lys substitution at position 249.

Comparison in the VP2 region of a classical serotype 1 virulent strain 52/70 (30%-50% mortality) and an attenuated vaccine strain PBG98 from the same geographic region revealed five amino acid changes which might be associated with virulence (Bayliss et al., 1990). Another region of interest in terms of virulence is a heptapeptide region (residues 326 to 332) adjacent to the second hydrophilic region

of VP2. Various VV IBDV strains isolated in Japan (Lin et al., 1993) and the antigenic and virulent variants isolated in the United States contain a conserved serine rich heptapeptide Ser-Trp-Ser-Ala-Ser-Gly-Ser in this region. The less virulent strains have fewer serine residues (Vakharia et al., 1994; Heine et al., 1991). Avirulent serotype 2 strain (OH) has three substitutions in this region (Kibenge et al.,1991). In serotype 2 strains (OH, 23/82), there is also an insertion of an amino acid residue at position 249 (serine) in the VP2 coding region and a deletion of residue at position 680 in the VP4 region which may be responsible for the loss of pathogenicity of the virus (Vakharia et al., 1994). Another region adjacent to the first hydrophilic area representing a 14 amino acid segment (residues 249-263) with 10 amino acid mismatches between the avirulent serotype 2 strain (OH) and the virulent serotype 1 strains (STC, 52/70, Cu-1) was also suggested to be associated with virulence (Dybing, 1992).

Recent work comparing the amino acid sequences of parental highly virulent Japanese strains OKYM and TKSM in the VP2 region with their attenuated progeny revealed 2 amino acid substitutions at residues 279 and 284 from Asp → Asn and Ala → Thr, respectively (Yamaguchi et al., 1996). Another attenuated Chinese strain GZ911, similar to cell culture adapted strains also had amino acid exchanges at position 279 (Asp → Asn) and at 284 (Ala → Thr) (Cao et al., 1998); but the Asp residue at position 279 is well conserved in avirulent serotype 2 strains OH and 23/82 (Nagarajan and Kibenge, 1997). However, it is speculated that the Ala residue at position 284 between the two hydrophilic regions may have a role in

virulence determination since it is not conserved in serotype 2 strains OH and 23/82. It is interesting to note that Thr is present at this position in serotype 1 virulent strains GLS and Cu-1 similar to the avirulent serotype 2 strains (Nagarajan and Kibenge, 1997a). Thus, the virulence determinants are not clearly established in spite of investigations of the VP2 region.

Sequence determination and analysis of segment A and segment B of a recent European VV isolate (UK661) of IBDV has revealed some other aspects of the virulence of IBDV. The coding sequence of segment B of serotype 1 (VV) UK661 isolate is closely related to segment B coding sequences of two nonpathogenic serotype 2 strains (OH and 23/82) (Brown and Skinner, 1996). Some amino acid substitutions identified in the (VV) UK661 strain in the VP4 viral protease, near the VP2-VP4 cleavage site, and in the antigenic sites of VP2 and VP3 were considered to affect its phenotype, possibly including the virulence (Brown and Skinner, 1996). Particularly, the substitutions in the polyprotein at 651 (Asn → Ser) adjacent to the predicted active site of the VP4 serine protease, and a substitution at 752 (His → Asp) near the alternative VP4-VP3 cleavage site Ala-X-Ala-Ala-Ser could modify the protease thereby affecting the polyprotein processing and the virus replication rate (Brown and Skinner, 1996). Furthermore, the presence of higher relative homology of VP1 coding sequences of (VV) UK661 with those of serotype 2 strains (23/82 and OH) suggested possible reassortment between segment A of virulent and segment B of avirulent strains (Brown and Skinner, 1996) implying that segment B is important in IBDV virulence.

By comparing the nucleotides in the variable region of VP2, the UK VV isolates (661, 74/89A, JY86, CS/89) were found to be closely related to each other (Brown et al., 1994). Among themselves, they differed by no more than two nucleotides and no amino acid changes whereas from the classical virulent strains such as STC and 52/70 they varied by at least 29 nucleotides and four amino acids. The Dutch VV isolate DV86 was also very closely related to the UK VV isolates differing by only three nucleotides and no amino acid changes in this region. Also, all the European isolates resembled the Japanese (VV) isolate 90-11 with no amino acid changes in the hypervariable region of VP2 but differing from it only by one or two nucleotides (Brown et al., 1994).

All the amino acid changes except one observed in the British and Japanese VV isolates were between the two hydrophilic regions of the VP2 gene. However, there was one amino acid exchange in the first hydrophilic region (Phe → Ala at 222) similar to that of antigenic variants A, E/Del and GLS. Mutations in the second rather than the first hydrophilic region are believed to be responsible for escape from antibody and for failure of neutralizing mAbs to recognize this site (Schnitzler et al., 1993; Heine et al., 1991; Lana et al., 1992). Hence some of the mutations observed in the VP2 coding region of Japanese and UK VV isolates were not considered to be related to their increased virulence.

In reviewing the investigations addressing the antigenic and virulence variations of serotype 1 strains of IBDV, it becomes apparent that some virulent IBDV strains

from the United States show antigenic drift whereas some European and Asian isolates are highly virulent without showing significant changes in antigenicity. The sites on the viral genome involved in such virulence variations and the underlying molecular mechanisms are not fully understood. Sequence comparisons between the virulent and attenuated strains and the antigenic variants of IBDV were unable to identify nucleotide or amino acids residues relevant to the virulence of IBDV (Bayliss et al., 1990; Dybing, 1992; Lin et al., 1993; Vakharia et al., 1994; Brown et al., 1994; Brown and Skinner, 1996; Yamaguchi et al., 1996; Nagarajan and Kibenge, 1997a).

It is speculated that nucleotide changes in other areas of the genome such as VP2-VP4, VP4-VP3 protease cleavage sites and the noncoding regions possibly contribute to a particular genotype necessary for the evolution of virulent strains of IBDV (Brown and Skinner, 1996; Nagarajan and Kibenge, 1997). Moreover, the replication strategy of IBDV is poorly understood. The rate of replication of IBDV in bursal and nonbursal lymphoblastoid tissues such as spleen, thymus and bone marrow may influence the pathogenicity of IBDV. Hence it is of interest to locate the regulatory sequences, particularly at the 5' end of segments A and B which may have an important role in viral genome expression and replication. Besides, noncoding sequence information on several IBDV strains revealed conserved regions which are likely to be recognition sites for initiation of transcription, translation or encapsidation (Kibenge et al., 1996; Mundt et al., 1995). There are

very few systematic studies on viral replication strategies and the regulatory mechanisms involved in IBDV genome expression. It is considered advantageous to identify and functionally characterize *cis*-acting elements such as promoters that are recognized by the viral polymerase and involved in regulating the transcription of IBDV genome. Such studies would enable better characterization of the mechanism of viral replication, the mode of IBDV evolution and virulence variations.

Another approach to better understand the mode of evolution and virulence of IBDV would be to isolate antibody resistant mutants from a parental wild-type strain by passaging the virus serially in cell culture under selective immune pressure. Such an attempt is likely to simulate field conditions and the behaviour of natural selection events exerted by antibodies of the protected host (Schiapacassi et al., 1995). Because the virulent variants emerge even in the presence of protective maternal antibodies against standard vaccine strains of IBDV, it is considered that the molecular analysis of the partial neutralization resistant mutants which may arise during serial passage in presence of a minimal limiting dilution of polyclonal antibody containing virus neutralizing antibodies is likely to provide information as to the location of genomic regions involved in virulence or attenuation of virulence. It is also hypothesized that nucleotide changes in genomic regions other than VP2 such as the VP2-VP4 and VP4-VP3 cleavage sites and the noncoding regions possibly contribute to the genotype necessary for the evolution of antigenic and virulent strains of IBDV. Hence an attempt would be made to isolate a partial

neutralization resistant mutant of IBDV during passage in cell culture under immune pressure. It is possible to identify regions involved in virulence by phenotypic and genotypic characterization of this mutant including its pathogenic and immunogenic profiles in specific-pathogen free chickens (SPF) in relation to the parental strain.

Furthermore, assignment of particular mutations to a specific phenotype such as virulence or attenuation of virulence is possible through construction of full-length cDNA clones from which infectious progeny virus can be produced (Rice et al., 1987; Polo et al., 1988). This approach will provide an improved experimental infection system to study the effects of specifically defined nucleotide changes introduced by *in vitro* mutagenesis within structural and nonstructural genes and *cis*-regulatory elements on biological functions such as viral replication and virulence. Therefore, it is considered advantageous to establish the conditions necessary for recovery of infectious IBDV from full-length cDNA clones of genome segments A and B by transfecting them into appropriate host cells to produce recombinant infectious progeny IBDV. Since the complete sequences of IBDV genome segments A and B have been determined in our laboratory (Kibenge et al., 1996), an attempt to obtain infectious clones will also facilitate the potential of investigations on viral replication and virulence. Moreover, it should be possible to determine which mutation or constellation of mutations best attenuates the virus, an essential step in the development of live modified candidate vaccine strains of IBDV.

1.4 Research Objectives

1. To map 5' regulatory sequences of the IBDV genome by

a) Constructing full-length and progressively deleted lengths of 5' terminal noncoding sequences of OH-IBDV which may contain potential regulatory sequences such as promoter and enhancer involved in IBDV genome expression and replication in a specially designed vector with a reporter gene encoding for luciferase.

(b) Transfecting the constructs prepared in (a) in uninfected and IBDV infected Vero cells and by monitoring the relative expression of luciferase directed by the potential regulatory sequences in the viral cDNA inserts.

2. To isolate a partial neutralization resistant mutant of IBDV during serial passage *in vitro* under selective immune pressure and to characterize the mutant with regard to the following:

(a) Phenotypic alterations such as (i) resistance to neutralization, (ii) plaque size, (iii) growth characteristics in cell culture (iv) virulence or attenuation of virulence in specific-pathogen free chickens.

(b) Genotypic changes in selected coding and noncoding regions of the IBDV genome.

3. To recover infectious recombinant IBDV progeny in cell culture by

(a) Constructing full-length cDNA copies of segments A and B of IBDV genome in a plasmid vector by PCR amplification and ligation of the cDNA fragments.

(b) Establishing parameters for transfection into Vero cells of various *in vitro* transcripts of linearized constructs made in (a) in order to recover recombinant infectious IBDV in cell culture.

2. MAPPING THE 5' REGULATORY SEQUENCES OF THE IBDV GENOME

2.1 INTRODUCTION

Infectious bursal disease virus is a member of the genus *Avibirnavirus* of the family *Birnaviridae*. Members of this family are characterized by a dsRNA genome consisting of two segments (A and B) within a single-shelled icosahedral capsid of 60 nm diameter (Dobos et al., 1995). The genome segments A (approx. 3300 bp) and B (approx. 2820 bp) have the putative viral RNA polymerase attached to their 5' ends through a phosphodiester bond between a guanine residue and the amino acid serine (Calvert et al., 1991) forming a genome linked protein, VPg (Muller and Nitschke, 1987; Dobos, 1993). This viral protein encoded by segment B (Morgan et al., 1988) also occurs in the virion as a 90 kDa polypeptide (VP1) linked to short stretches of viral RNA (Kibenge and Dhama, 1997). VP1 of IBDV has sequence similarity with known RNA-dependent RNA polymerases of other dsRNA and positive (ss)RNA viruses (Duncan et al., 1991).

In birnaviruses, replication and transcription are initiated when the virus penetrates the host cell without the need for uncoating or degradation of the capsid. The precursor molecules gain access to the polymerase-template complex by passing through the capsid (Spies et al., 1987). Recent studies with infectious pancreatic necrosis virus (IPNV), the prototype birnavirus, and IBDV indicate that virion-associated VP1 catalyzes a guanylylation reaction which serves to prime viral RNA

synthesis. Apparently only the plus strands of the two genome segments are synthesized *in vitro*, which remain base-paired to their templates (Dobos, 1995). Similar protein primed RNA synthesis occurs *in vivo* as well (Magyar et al., 1998). where VP1-oligonucleotide complexes are formed initially in which the RNA moiety represents the 5' terminal sequences of genome segment A- and B-specific plus strands. Such complexes are chased via replicative intermediates to genome length ssRNA, intracellular VP1-dsRNA and finally to virion VPg-dsRNA (Magyar et al., 1998). These observations suggest an asymmetric and semi-conservative strand-displacement mechanism of replication for IBDV.

Reverse genetics studies with IBDV (Mundt and Vakharia, 1996) and IPNV (Yao et al., 1998) suggest a conservative mechanism similar to rotavirus replication (Patton et al., 1993; 1996), where the plus-strand RNAs serve as templates for minus strand synthesis by VP1 or RNA-dependent RNA polymerase to form dsRNA. Aside from these studies, the regulatory mechanism involved in IBDV genomic RNA replication and expression are not well understood. In particular the *cis*-acting sequences involved in IBDV RNA synthesis are not known. It is of interest to map the sequences involved in genome expression and replication since the rate of virus replication may influence the pathogenicity of a virus.

Recently, the complete terminal sequences of genomic segments A and B of IBDV have been established (Mundt and Muller, 1995; Kibenge et al., 1996). The

precursor polyprotein ORF on segment A is flanked by 131 bp at the 5' end while the 3' end is variable; similarly the VP1 ORF on segment B is flanked by a variable 3' end and 111 bp at the 5' end. Both segments are 85.3% identical in the initial 32 nucleotides at the 5' end of the plus strands in the six IBDV isolates examined to date. This is the only significant region of homology between the two genomic segments although the noncoding sequences for each segment are highly conserved among the different IBDV strains.

There are short consensus sequence motifs at the 3' noncoding region which are homologous between the genome segments of North American and European strains of IBDV. The last 5 nucleotides at the 3' end of the plus strands of genome segments A and B of North American strains of IBDV are also similar (Kibenge et al., 1996). Thus the termini of IBDV genome segments resemble those of other segmented RNA viruses such as reovirus (Antczak et al., 1982) and influenza virus (Stockle et al., 1987) where both termini are homologous between the genome segments. Further analysis of the terminal sequences of IBDV which are in regions of inverted repeats lend support that they may contain important signals for IBDV replication, expression, and packaging (Kibenge et al., 1996). Although the 5' noncoding region of IBDV genomic RNA is likely to contain elements necessary for RNA replication and expression, such elements have previously not been studied.

Preliminary studies were initiated to map the *cis*-acting regulatory sequences likely to be present at the 5' noncoding region of segment A of IBDV. Transient

transfection studies were conducted initially in uninfected Vero cells by employing a series of recombinant pGL3-reporter vector constructs with a luciferase (LUC) reporter gene (Promega). The level of luciferase expressed by these recombinant constructs was measured following transfection and was used as an indicator for the transcription of segment A (Alam and Cook,1990).

The Promega new pGL3 family of luciferase reporter vectors containing a cloning site for the introduction of cDNA sequences with potential regulatory sequences were employed along with appropriate pGL3-Control vector. These vectors offered other significant advantages because of a redesigned vector backbone allowing increased LUC expression, greater sensitivity, improved *in vitro* stability and greater flexibility in performing genetic manipulations. The constant light intensity generated in the LUC assays allowed for measurements up to three minutes. In addition, luciferase is a monomeric protein that does not require post-translational processing for enzyme activity. Hence it functions as a convenient genetic reporter immediately upon translation (De Wet et al., 1985).

The new pGL3 reporter vectors used in the study (Fig. 3A) were the following: pGL3-Enhancer vector which contained an SV40 enhancer downstream of LUC gene and the poly(A) signal. This was used in the verification of a functional promoter element because the presence of SV40 enhancer often results in transcription of LUC at higher levels. Another vector used was the pGL3-Promoter

vector which contained an SV40 promoter upstream of the luciferase gene. DNA fragments containing putative enhancer elements are inserted either downstream or upstream of the promoter-LUC transcriptional unit of this vector. The third vector used was the pGL3-Control vector which contained both SV40 promoter and SV40 enhancer to serve as a convenient internal standard for promoter and enhancer activities expressed by pGL3 recombinants. The regulatory sequences were mapped initially by making a set of unidirectional nested deletions in the insert cDNA of the recombinant vectors and then assaying for changes in biological activity.

The initial 32 bp stretch of sequence at the 5' end of segment A induced practically all the LUC activity when different constructs containing full-length and varying progressively deleted lengths of 5' NC region were examined for promoter strength *in vivo*. To further analyse the biological significance of this region, subsequent transfection studies were conducted in Vero cells complemented *in trans* by viral proteins and other factors by infection with IBDV. A different series of modified pGL3-Promoter recombinant plasmid constructs were made for this purpose where the terminal 32 bp fragment, full-length and 32 bp deleted fragment were inserted either in plus- or minus-sense form, flanked upstream by the SV40 promoter and immediately downstream by a LUC reporter gene. Observations of up-regulated stimulation of luciferase activity by transfected construct containing 32 bp in minus-sense form and abolition of such amplified expression in 32 bp deleted constructs

provided evidence that the 32 bp fragment at the terminal 5' end of segment A of IBDV harbours a promoter element for IBDV genome expression and replication.

2.2 MATERIALS AND METHODS

2.2.1 Virus and cells

IBDV strain OH of serotype 2 (Jackwood et al., 1982) was the source of all cDNA sequences used in this study. Vero cells (Salsbury Laboratories Inc., Charles City, USA) were used to propagate the virus and for transient plasmid DNA transfections. Vero cell monolayers were grown in Eagles Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) which was reduced to 2% for maintenance (Kibenge et al., 1988b).

2.2.2 RT-PCR and cDNA cloning

For first strand cDNA synthesis, IBDV dsRNA extracted from infected Vero cell cultures was used. Briefly, the virus culture was frozen and thawed 3 X and then clarified by low speed centrifugation (3200 X g for 20 min). Virus was pelleted by ultracentrifugation at 141,000 X g for 6 h at 4 °C. The viral dsRNA was released by digestion with Proteinase K (GIBCO-BRL, Burlington, Canada) at a final concentration of 2 mg/ml in the presence of 0.5 % sodium dodecyl sulfate (SDS) for 2 h at 37 °C followed by phenol-chloroform extraction and ethanol precipitation. Cellular DNA was removed by DNase I (Pharmacia, Baie d'Urfe, PQ) treatment at

a concentration of 100 u/mg of total nucleic acid for 1 h at 37 °C. The RNA pellet obtained subsequent to phenol-chloroform extraction and ethanol precipitation was further purified by chromatography on a CF-11 cellulose column (Mellor et al., 1985). Using this column, dsRNA was separated from contaminating ssRNA. The ssRNA was eluted with TSE buffer (50 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 6.9) containing 15 % ethanol, followed by elution of dsRNA in TSE buffer. The virus dsRNA obtained was denatured in 90% dimethyl sulfoxide (DMSO) at 65 °C for 90 min, snap chilled and the ssRNA was ethanol precipitated (Qian and Kibenge, 1994).

For first strand cDNA synthesis, purified denatured IBDV dsRNA and IBDV-specific (OH) primer SA06 (nt 1520-1539; 5'- TCTCGATTTGCATGGGCTAG-3'), were used to copy 5' end plus sense RNA of segment A with SuperScript II RT (GIBCO-BRL) (Kibenge et al., 1996). The cDNA clone containing the complete 5' noncoding region (corresponding to precursor polyprotein reading frame positions -131 to +12) was amplified by PCR with oligonuclotide primers SA11 (nt 1-18) 5'-gcgacgcgtGGATACGATCGGTCTGAA-3' and SA12 (nt 126-143) 5'-ctagatctCAGGTTTGTCATCGCTGC-3' from the IBDV genome segment A sequence (Kibenge et al., 1996), engineered to contain restriction sites *Mlu* I and *Bgl* II (underlined; the non-viral sequences are indicated in lowercase letters), respectively. The resulting PCR product was purified from low melting agarose gel (Qian and Kibenge, 1994). It was subsequently ligated to the pCR™II vector using the TA cloning kit, Version 2.1 (Invitrogen, San Diego, USA), according to the

manufacturer's protocol to give the clone (pPIIA) containing the insert of IBDV 5' terminal region (nt 1-143).

For ligation with pCRII vector, a 10 µl reaction was set up at 12 °C overnight in 1X ligation buffer containing 1 µl (4 units) of T4 DNA ligase (Invitrogen) and 1 µl of 10 mM ATP. Two microlitres of the ligation mixture were used to transform *E. coli* strain INV F' competent cells (Invitrogen, San Diego, USA). Uptake of plasmid was facilitated by heat-shock treatment at 42 °C for 90 sec followed by recovery of cells in SOC medium (2% bactotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose) and shaking at 225 rpm for 1 h at 37 °C. The transformants were grown on 2X YT agar (0.8% bactotryptone, 0.5% bacto yeast extract, 0.5% NaCl and 1.5% agar) plates containing 100 µg/ml ampicillin and 0.005% X-gal (Bio-Rad, Missisauga, Canada). The transformants were then screened for the desired inserts by digestion with *Mlu* I and *Bgl* II of the plasmid DNAs isolated from selected white colonies and susequent 2% agarose gel electrophoresis.

For plasmid DNA isolation for sequencing, the alkaline lysis method (Birnboim and Doly, 1979) as modified by Kibenge et al. (1990) was used. The harvested cells from overnight cultures in 50 ml of 2X YT broth (0.1% bactotryptone, 0.1% yeast extract, 0.05% NaCl) containing 100 µg/ml ampicillin were lysed by SDS-lysozyme-alkaline treatment. Contaminating cellular RNA was removed by RNase A (72 µl of 5 mg/ml) (Pharmacia, Baie d'Urfe, PQ) digestion followed by precipitation of

cellular proteins with 7.5 M ammonium acetate. Plasmid DNA was recovered from the supernatant by ethanol precipitation. The DNA pellet was resuspended in TE buffer (pH 8.0) and further purified by precipitation in 30% polyethylene glycol (PEG) (Fisher Scientific, Nepean, Canada) in 1.8 M NaCl. The PEG was removed by phenol-chloroform extraction and the plasmid DNA was precipitated with ethanol in the presence of 3 M sodium acetate (pH 5.2) and stored at -70 °C until used in sequencing reactions.

The nucleotide sequences of the DNA insert were determined by the standard dideoxy chain termination method (Sanger et al., 1977) using the Sequenase Version 2 kit (Amersham, Oakville, Canada). For this, purified plasmid DNA was denatured at 37 °C for 30 min with 0.1 vol of 2 M NaOH and 2 mM EDTA followed by ethanol precipitation. The DNA template was then annealed with forward (Universal primer M13/pUC) or reverse primer (Reverse sequencing primer M13/pUC) (Boehringer Manheim, Laval, Canada) or IBDV specific primer at a final concentration of 0.1 µM in a 10 µl Sequenase reaction mixture by heating the mixture at 65 °C for 2 min and cooling slowly to 37 °C. The annealed DNA-primer mixture was incubated for 5 min at room temperature with Sequenase enzyme, dithiothreitol (DTT) and all four dNTPs and radiolabeled dATP (Redivue™ [α^{35}S],10 mCi/ml, Amersham Canada Ltd, Oakville, Canada). Extension of the primer was terminated in four separate reactions, each containing a single dideoxynucleotide triphosphate ddNTP in addition to the four dNTPs. The termination reactions were carried out at 37 °C for 5 min and stopped by addition of 4 µl of stop solution (xylene

cyanol 0.1%, bromophenol blue 0.1%, 2 ml of 0.5 M EDTA in formamide) and stored at -70 °C until loaded in a sequencing gel of 6% acrylamide and 8 M urea. The sequencing reactions were denatured at 75-80°C for 2 min prior to loading into the gel wells and were electrophoresed at 55 W for 5 h. The gel was fixed in a solution containing 10% methanol and 10% acetic acid and dried under vacuum at 80 °C for 3 h. Autoradiography was performed at room temperature for approximately 36 h using Amersham Hyperfilm MP.

2.2.3 Subcloning of PCRII vector derived IBDV cDNA in pGL3-GeneLight expression vectors and nested deletions

The IBDV cDNA clone (pPIIA) was the source of the full-length 5' noncoding region of OH-IBDV genome segment A. The *Mlu* I-*Bgl* II insert DNA of clone pPIIA was purified from low melting agarose gel and inserted into both pGL3-Promoter GeneLight vector and the pGL3-Enhancer GeneLight vector (Promega, Madison, USA) digested with *Mlu* I and *Bgl* II. The insert was positioned immediately upstream of the luciferase (LUC) reporter gene sequences in these vectors, and transformed in *E.coli* DH5α competent cells (GIBCO-BRL, Burlington, Canada) to yield clones pPA131 and pEA131, respectively (Fig. 3B, 3C) . The pGL3-Promoter vector contains an SV40 promoter (enhancer-less pGL3-Promoter); the pGL3-Enhancer vector, an SV40 enhancer (promoter-less pGL3-Enhancer); the pGL3-Control vector contains both SV40 promoter and SV40 enhancer, all of them with firefly luciferase gene as the genetic reporter (Fig 3A).

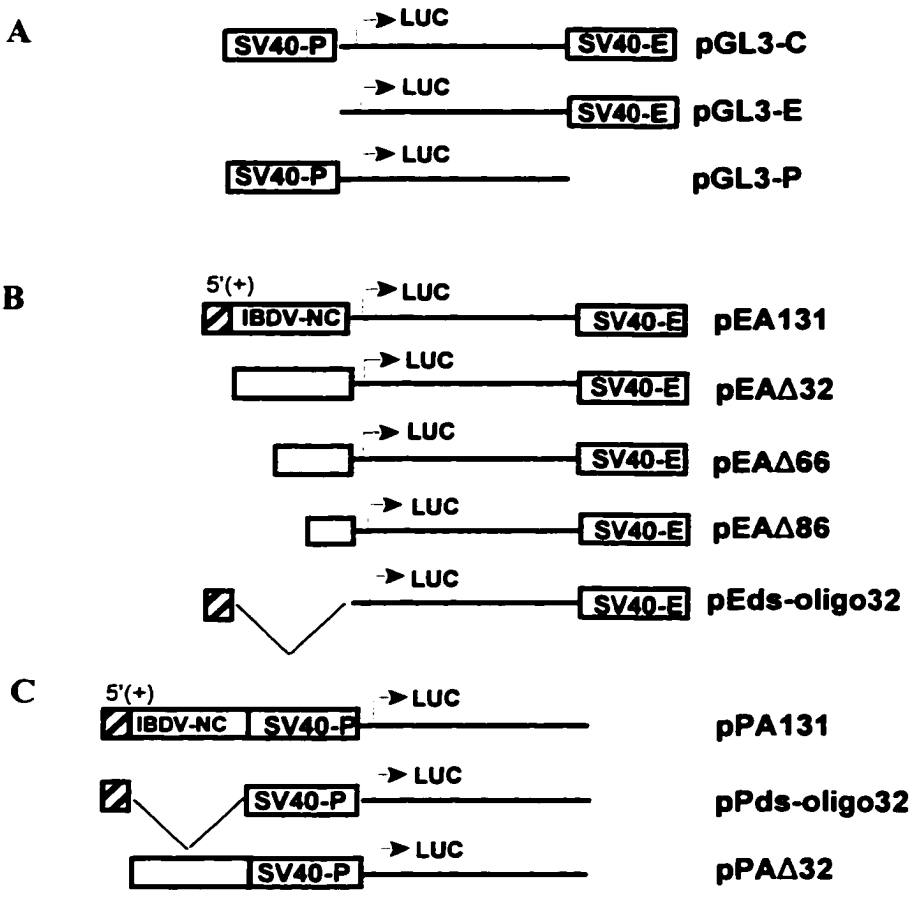


Fig. 3: pGL3 plasmid vector constructs. **A** PGL3-C denotes the pGL3-Control plasmid with S40 promoter (SV40-P), luciferase (LUC) reporter gene and SV40 enhancer (SV40-E); pGL3-E denotes pGL3-Enhancer plasmid without the SV40 promoter; pGL3-P denotes pGL3 promoter plasmid without SV40 enhancer. **B** Constructs of pGL3-Enhancer vector containing progressively deleted lengths of the 5' terminal region of segment A of IBDV (IBDV-5'NC); pEA131 contains the full-length noncoding region (precursor polyprotein ORF positions -131 to +12); pEAΔ32, pEAΔ66, and pEAΔ86 correspond to constructs containing -99 to +12, -65 to +12, - 45 to + 12 respectively of the 5' terminal region of segment A of IBDV genome. pEds-oligo32 contains the initial 32 nts of 5' terminal region of segment A of IBDV genome(-131 to -100 nt). **C** Constructs of pGL3-Promoter vector; pPA131 contains the full-length non-coding region (-131 to +12), pPAΔ32 contains noncoding region with the terminal 32-bp deleted; pPds-oligo32 contains the initial 32 nts of 5' terminal region of segment A of IBDV genome (-131 to -100 nt). Cross-hatched box represents IBDV 32bp consensus sequence in genome segments A and B. Δ denotes base deletion (Nagarajan and Kibenge, 1997b).

Progressive deletions on the recombinant plasmid containing the full-length 5' noncoding region of OH-IBDV genome segment A were created by digestion of pEA131 initially with *Kpn* I (nt 5 in the polylinker region of pGL3 vectors) to produce a 3' overhang resistant to exonuclease III. For this 15 µg of plasmid DNA (pEA131) was linearized by sequential digestion with 5 µl of *Mlu* I and *Kpn* I for 3 h at 37 °C followed by phenol-chloroform extraction and ethanol precipitation for 1 h at - 70 °C. The pellet was washed with 70% ethanol, vacuum dried and dissolved in 60 µl of exonuclease buffer. Exonuclease III (8.3 µl containing 65 units /µl) was added and the reaction was incubated at 10 °C. At 30 s intervals, for a total period of 4.5 min, 5 µl of the reaction mixture was removed, treated with 1 µl of S1 nuclease in 20 µl reaction mixture to remove the single-stranded (ss) regions and incubated at 30 °C for 30 min (Sambrook et al.,1989). At the end of incubation, 1 µl of S1 stop mixture was added to each tube and heated at 70 °C for 10 min. It was followed by Klenow fill-in by incubating each 20 µl sample with 2 µl of Klenow mixture at 37 °C for 5 min and with 2 µl of four dNTP mix (each at 0.5 mM) for another 15 min at room temperature with subsequent phenol chloroform extraction and ethanol precipitation. Three batches of pooled pellets from time points 30 s to 90 s, 90 s to 3 min, and 3 min to 4.5 min were used for ligation. They contained the linearized exonuclease treated plasmid vector with varying deleted lengths of IBDV 5' noncoding region.

The ligation reactions were in a total volume of 10 µl containing 1 µl (4 units) of T4 DNA ligase (Invitrogen, San Diego, USA) and 1 µl of 5 mM ATP, incubated overnight at 14 °C. Two microlitres of the ligation reaction were used for

transformation of *E. coli* strain DH5α competent cells (GIBCO-BRL, Burlington, Canada). The tranformants were screened for clones of desired deletion lengths by digestion of plasmid DNA isolated from randomly picked white colonies with *Bgl* II (a restriction site present at the 3' end of the various deleted lengths of the viral cDNA insert in the cloning site and *Dra* III (a restriction site in the vector backbone on the 5' end upstream of the various deleted lengths of the viral cDNA insert) and analysing the digestion products for the presence of different sized deleted fragments by electophoresis in 2% agarose gel. The nucleotide sequences of the progressively deleted noncoding regions in the selected plasmid constructs were determined to verify the number of nucleotides deleted and the sequences at the junction regions (Sanger et al., 1977). The constructs with respective 32, 66 and 86 nts deleted from the 5' end were selected (Fig. 3B) for use in subsequent studies.

To construct pPds-oligo32 and pEds-oligo32 clones containing 5' terminal 32-bp cDNA of OH-IBDV genome segment A, an *Mlu* I-*Bgl* II dsDNA fragment containing the initial 32 nucleotides of the 5' terminal sequence of segment A was commercially synthesized (Bio/Can) and subcloned into the *Mlu* I and *Bgl* II sites of the linearized pGL3-Promoter and pGL3-Enhancer vectors, respectively (Fig. 3B, 3C).

The pGL3-Promoter vector (Fig. 4A) was used to construct pUPS32, pUMS32, pUPS131, pUMS131, pUPS$_{\Delta}$32, pUMS$_{\Delta}$32 (Fig. 4B) for transfection of infected and

uninfected cells. They were engineered from a pGL3-Enhancer recombinant construct (pEA131) containing the full-length 5' noncoding (NC) region of IBDV by PCR using appropriate sets of primers containing an *Nco* I restriction site (Table 1) (SA14 and SA15 primers for full-length; SA16 and SA15 for terminal 32 bp deleted; and SA14 and SA17 for terminal 32 bp) in order to facilitate insertion of amplified products at the *Nco* I site immediately downstream of SV40 promoter. Such manipulations allow generation of recombinant viral-LUC RNAs endogenously in Vero cells by cellular RNA polymerase initiating transcription from the SV40 promoter in the modified pGL3-Promoter vector constructs (Fig. 8).

These amplified PCR products containing either the terminal 32 bp, the full-length or the 32 bp deleted fragment were initially subcloned into pCRII vector and subsequently into the pGL3-Promoter vector at the *Nco* I site immediately upstream of LUC reporter gene and downstream of SV40 promoter (Fig. 4B). The transformants were initially screened for appropriate size fragments of inserts by restriction of plasmid DNA isolated from the randomly picked white colonies with *Nco* I and analysing the digestion products by electrophoresis in 2.5% agarose gel. Subsequent screening for plus-sense and minus-sense orientation clones were carried out by PCR (Sandhu et al., 1989) using appropriate pGL3 vector specific (RV primer 3, Promega) and insert specific (SA15) primers for full-length and 32 bp deleted and pGL3 vector specific RV primer 3 and insert specific SA17 for clones containing terminal 32 bp only. The selected clones were confirmed by sequence analysis as described previously (see section 2.2).

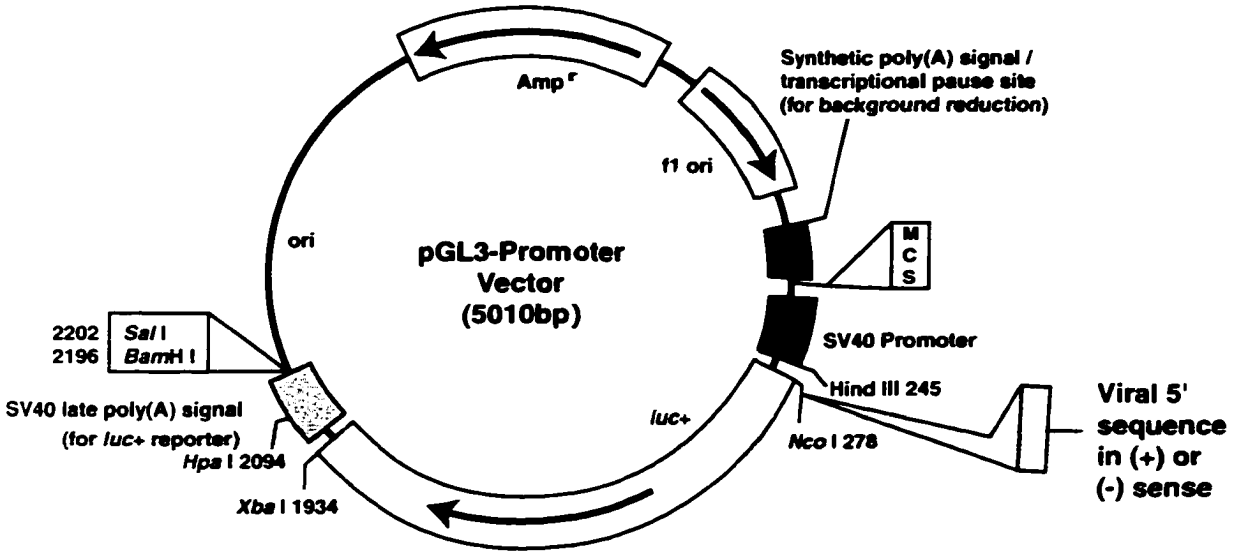


Fig. 4A: Engineered modifications of the pGL3-Promoter vector for *in vivo* assay of regulatory elements in genome segment A of IBDV. pGL3-Promoter vector with IBDV segment A 5' noncoding cDNA sequences inserted immediately upstream of LUC gene and downstream of SV40 promoter (the viral cDNA sequences are in plus-sense or minus-sense relative to the LUC gene). MCS represents the multiple cloning site (5-53 nt) of the pGL3-Promoter vector (Nagarajan and Kibenge, 1998).

Table 1: Oligonucleotide primers used for various constructs of modified recombinant pGL3-Promoter vector

Sequence	Orientation	Name	Map position[a]
CCATGGGGATACGATCGGTCTGAA	+	SA14	1-18
CCATGGCAGGTTTGTCATCGCTGC	-	SA15	126-143
CCATGGCCCGGGGACAGGCAATCA	+	SA16	33-50
CCATGGTGACTCCCCCGGGGTTC	-	SA17	16-32

[a]The map positions refer to published sequence of OH-IBDV (Kibenge et al., 1996). The restriction site for *Nco* I is in bold. (+) and (-) refer to virus specific sequences of sense and antisense primers respectively.

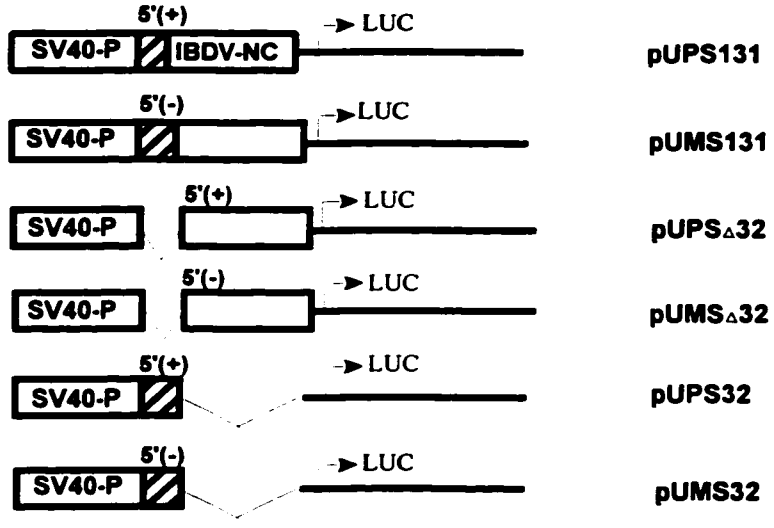


Fig. 4B: Individual recombinant plasmids used to transfect uninfected and IBDV infected Vero cells. pUPS131 and pUMS131 contain the full-length noncoding region of segment A of IBDV (precursor polyprotein ORF positions -131 to +12 nts) in plus- or minus-sense orientation, respectively, upstream of the LUC gene; pUPS32 and pUMS32 contain the terminal 32-bp fragment (-131 to - 100 nt) in plus- or minus-sense orientation, upstream of LUC gene; pUPSΔ32 and pUMSΔ32 with 5' terminal 32 bp fragment deleted (-99 to +12 nt), all inserted downstream of the SV40 promoter. Δ denotes deletion of bases in the constructs. Cross-hatched box represents IBDV segment A 5'-terminal 32 bp fragment (Nagarajan and Kibenge, 1997b).

2.2.4 Transient transfection of uninfected and IBDV-infected Vero cells

Both uninfected and infected Vero cells were transfected with 1.5 µg of respective plasmid DNAs by the Lipofectin method (Sumiyoshi et al., 1992; Khromykh et al., 1994) with some modifications. Briefly, plasmid DNA in 100 µl of OptiMEM I (GIBCO-BRL, Burlington, Canada) was gently mixed with 10 µl of Lipofectin reagent (GIBCO-BRL, Burlington, Canada) diluted in 90 µl of OptiMEM I and incubated at room temperature for 15 min. Vero cells grown to about 70% confluence in 35-mm tissue culture dishes infected with OH-IBDV at a multiplicity of infection of 1 (10^4 $TCID_{50}$) for 6 h or uninfected cells were washed two times with prewarmed OptiMEM I and incubated with the appropriate plasmid DNA-Lipofectin-OptiMEM I mixture in triplicates at 37 °C in a humidified incubator with 5% CO_2 atmosphere. After 6 h the transfection mixture was replaced with 2 ml of MEM growth medium.

2.2.5 LUC assays

The growth medium was removed from the cells after 40 h of incubation at 37 °C and the luciferase assay was carried out as follows. The cells were rinsed twice in phosphate buffered saline (PBS), being careful not to dislodge any of the cells. The cells were subsequently lysed by incubating with 175 µl of Cell Lysis Reagent containing 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diamino-cyclohexane N,N,N',N'-tetra-acetic acid, 10% glycerol and 1% Triton X-100 (Promega, Madison, USA) for 10 min at room temperature. The cells were scraped

from the culture dish and transferred to a microfuge tube and spun briefly (5 s) at 12,000 X g to pellet large debris.

LUC assays were carried out at room temperature by mixing 20 µl of clarified cell lysate with 100 µl of Luciferase Assay Reagent containing 470 µM luciferin, 270 µM coenzyme A and 530 µM ATP (Promega, Madison, USA) and immediately monitoring the reaction by placing the microfuge tube in a scintillation vial and measuring the amount of light produced as single photon counts (SPC) at 60 s in a scintillation counter (Packard) according to the manufacturer's protocol. For each experiment, serial dilutions of standard luciferase (Sigma, Oakville, Canada) diluted with bovine serum albumin solution (1 mg/ml in water) were carried out to confirm the linearity of the assay. Appropriate experimental controls were included wherever they were necessary. They were the pGL3-Control Genelight vector, pGL3-Promoter vector, pGL3-Enhancer vector and mock transfected Vero cell lysates. The plasmid construct yielding the maximum LUC activity measured as SPC in an experiment was considered to have 100% activity and all other constructs in the experimental group were represented relative to the construct with maximum activity.

2.3 RESULTS

2.3.1 Localization of promoter sequences in the 5' noncoding region of IBDV segment A

Transfection of Vero cells with a series of promoter-less pGL3-Enhancer vector constructs containing a LUC reporter gene encompassing progressively deleted lengths of the 5' noncoding sequences upstream of the precursor polyprotein ORF of genome segment A (Fig. 3B) were used to map promoter sequences in the IBDV genome. To determine whether the different constructs allowed expression of the LUC gene *in vivo*, plasmid DNA of these constructs was transfected into Vero cells in triplicate and assayed for LUC activity. Vero cells transfected with the recombinant pEA131 which contained the full-length 5' noncoding sequence had 7.5% LUC activity relative to that of the pGL3-Control GeneLight vector whereas constructs with the first 32, 66, and 86 nucleotides deleted (pEA$_{\Delta}$32, pEA$_{\Delta}$66, pEA$_{\Delta}$86), respectively (Fig. 3B) had only 0.09, 0.03, and 0.01% LUC activity (Fig. 5A) (Table 2). These results suggested that the terminal 32 nucleotides at the 5' end of segment A contained vital regulatory sequences.

To further confirm the regulatory activity of the 32-bp fragment of IBDV segment A, a construct containing only the first 32 bp (pEds-oligo32) was prepared in a promoter-less pGL3 vector (Fig. 3B) and transfected in Vero cells. The LUC activity of pEds-oligo32 was 54.8% relative to that of the pGL3-Control vector, a 7.8-fold

increase in the LUC activity over the pEA131 construct containing the full-length 5' noncoding region. Figure 5 B shows the relative LUC activity when pEds-oligo32 was transfected in Vero cells in parallel with pEA131, pEA$_{\Delta}$32, pEA $_{\Delta}$66 and pEA $_{\Delta}$86. The values for pGL3-Control vector and for pEds-oligo32 were obtained with 40-fold dilutions of the cell lysates and of pEA131 with 10-fold dilution of the cell lysate with bovine serum albumin (1 mg/ml) solution in water because of the high level of luciferase expression with these constructs whereas those of pEA$_{\Delta}$32, pEA$_{\Delta}$66, and pEA$_{\Delta}$86 were from undiluted cell lysates. These results clearly indicate the presence of a strong promoter element in the first 32 bp of the 5' terminal noncoding sequence of IBDV segment A.

2.3.2 Analysis of the 32-bp 5' terminal sequence of IBDV segment A for enhancer activity

To rule out enhancer activities in the identified promoter sequence, enhancer-less pGL3 constructs containing the full-length noncoding region (pPA131), the first 32 bp of the 5' end of segment A (pPds-oligo32) and the 5' noncoding region with the first 32 bp deleted (pPA$_{\Delta}$32) (Fig. 3C) were prepared in pGL3-Promoter vector.

These constructs were transfected into Vero cells in parallel with the pGL3-Control vector. As shown in Fig. 6, pPds-oligo32 had no LUC activity and therefore no enhancer activity whereas pPA131 and pPA$_{\Delta}$32 had 27% and 8.2 % LUC activity respectively, compared to the pGL3-Control vector. The background LUC activity

of the pGL3-Promoter vector which was 27.5% of the pGL3-Control vector (Table 3) was subtracted from the percent LUC activity exhibited by the different constructs made in this vector (Fig. 6).

2.3.3 Biological activity of the identified major promoter region for IBDV

To demonstrate the biological importance of the 32 bp 5' terminal sequence for IBDV genome replication and expression, transfection assays were carried out in IBDV infected and uninfected Vero cells with a different set of pGL3-promoter vector constructs that were engineered with either the plus-sense or the minus sense full-length sequences (pUPS131, pUMS131) respectively or with the first 32 bp deleted (pUPS$_{\Delta}$32, pUMS$_{\Delta}$32) or containing only the first 32 bp of the 5' NC region (pUPS32, pUMS32) downstream of the SV40 promoter (Fig 4B).

These constructs upon transfection into IBDV infected Vero cells allowed the generation of endogenous plus sense LUC RNAs by cellular RNA polymerase from the SV40 promoter. This RNA contained immediately upstream of the LUC initiation codon the minus-sense or the plus-sense respective viral RNAs. There was nearly 3 and 7-fold up-regulation of LUC expression levels by the minus sense full-length (pUMS131) and the terminal 32 bp (pUMS32) constructs, respectively, over those of the corresponding constructs with plus-sense orientation (pUPS131 and pUPS32) (Table 4, Fig. 7).

The amplified expression levels exhibited by the full-length (pUMS131) and terminal 32 bp (pUMS32) minus-sense constructs were in marked contrast to those of the other two constructs where the terminal 32-bp fragment was deleted (pUPS$_{\Delta}$32, pUMS$_{\Delta}$32) which showed no increase in LUC expression in IBDV infected cells. These data showed that IBDV replicative proteins in the IBDV-infected Vero cells recognized the regulatory regions in the 5' noncoding region resulting in modulation of the LUC expression. In uninfected cells, three-fold increased LUC activity was detected with constructs containing the full-length noncoding region pUPS131 and pUMS 131and the noncoding region with the 32 bp- fragment deleted (pUPS$_{\Delta}$32 and pUMS$_{\Delta}$32) (Table 4, Fig. 7). This was attributed to the presence of enhancer-like sequences in the noncoding region. Constructs containing only the terminal 32-bp fragment (pUPS32 and pUMS32) showed no such increase in LUC activity when transfected in uninfected Vero cells, in agreement with data from the transfection experiments with the pPds-oligo32 construct (Fig. 6).

The transfected pGL3-promoter (pGL3-P) vector in Fig. 7 showed slightly higher LUC activity over some of the other constructs used in infected and uninfected cells because the viral cDNA inserts were ligated at the *Nco* I site immediately upstream of the LUC initiation codon instead of the polylinker site as in pGL3-Promoter GeneLight vector (Promega, Madison, USA). This resulted in relatively lower translation efficiency for all the modified constructs (Fig. 4B), due to displacement of the Kozak consensus sequence (Kozak, 1989) 5' of the LUC gene of the pGL3-Promoter GeneLight vector.

Table 2: Luciferase expression levels in transfected Vero cells of full-length and varying deleted constructs of 5' NC regions of segment A of IBDV genome in pGL3-Enhancer vector

Construct transfected	Mean single photon counts (SPC) in millions	% Luciferase activity
pGL3-Control vector (pGL3-C)	532 (58)	100
5' NC full-length construct in pGL3-Enhancer vector (pEA131)	40 (0.7)	7.5
5' NC 32 bp deleted construct in pGL3-Enhancer vector (pEAΔ32)	0.46 (0.03)	0.09
5' NC 66 bp deleted construct in pGL3-Enhancer vector (pEAΔ66)	0.14 (0.01)	0.03
5' NC 86 bp deleted construct in pGL3-Enhancer vector (pEAΔ86)	0.07 (0.002)	0.01
5' end 32 bp ds-oligo construct in pGL3-Enhancer vector (pEds-oligo32)	292 (14)	54.8

Standard error of the mean (SEM) is indicated in the parenthesis.

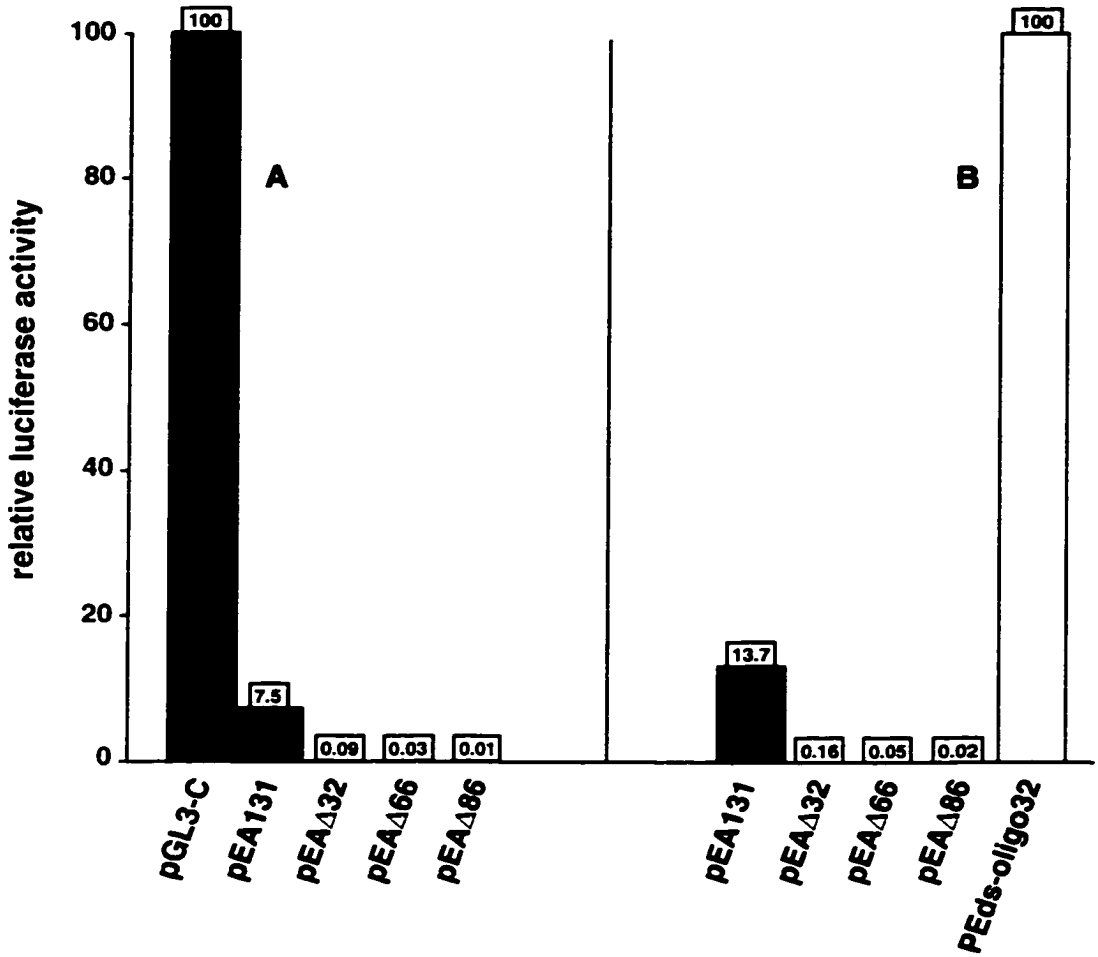


Fig. 5: Promoter strength of the 5' noncoding region of segment A of IBDV. The luciferase levels are arbitrarily relative to that of pGL3-Control vector (p-GL3-C). **A** LUC activity when pEA131, pEAΔ32, pEAΔ66, and pEAΔ86 were initially examined in parallel to that of pGL3-Control vector, and represent the results of at least three independent experiments where each construct was tested in triplicates. **B** Relative LUC levels when the same constructs were tested in parallel with pEds-oligo 32 and pGL3-Control vector (pGL3-C). The luciferase activity levels are represented relative to that of the pEds-oligo 32 construct. (Nagarajan and Kibenge, 1997b)

Table 3: Luciferase expression levels in transfected Vero cells of the full-length, 5' end 32 bp deleted and 32 bp ds-oligonucleotide constructs in pGL3-Promoter vector and localization of enhancer activity

Plasmid construct	Mean single photon counts (SPC) in millions	% Luciferase activity
pGL3-Control vector (pGL3-C)	1888 (19)	100
5' NC full-length construct in pGL3-Promoter vector (pPA131)	1024 (105)	54.2
5' end 32 bp ds-oligonucleotide construct in pGL3-Promoter vector (pPds-oligo32)	464 (32)	24.5
5' NC 32 bp deleted construct in pGL3-Promoter vector (pPAΔ32)	674 (70)	35.7
pGL3-Promoter vector (pGL3-P)	520 (17)	27.5

Standard error of the mean (SEM) indicated in the parenthesis

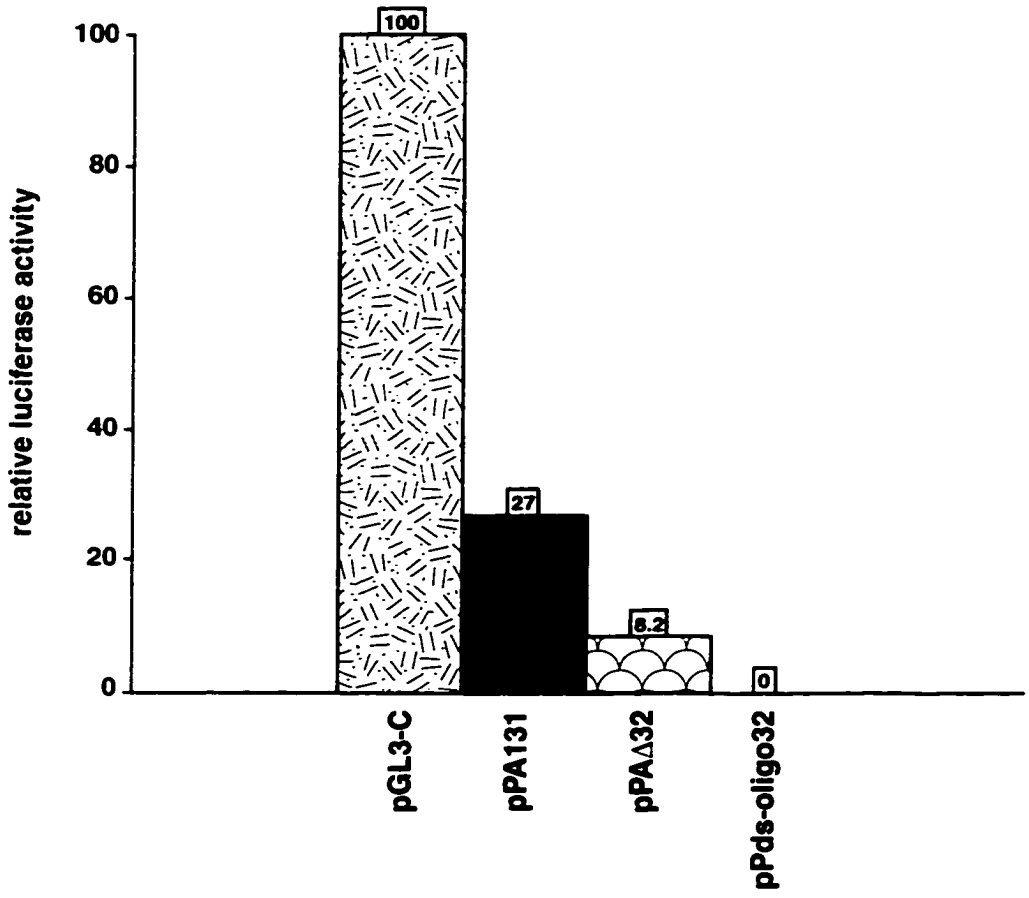


Fig. 6: Enhancer strength of the 5' noncoding region of segment A of IBDV. The luciferase activity levels of pGL3-Promoter constructs pPA131, pPAΔ32, and pPds-oligo32 are represented here arbitrarily relative to that of pGL3-Control vector (Nagarajan and Kibenge, 1997b).

Table 4: Luciferase expression levels in transfected Vero cells of plus-sense and minus-sense plasmid DNA constructs of 5' NC regions of segment A of IBDV in modified pGL3- promoter vector

Plasmid[a]	Uninfected cells		IBDV infected cells		Effect of IBDV replicative proteins on the IBDV promoter
	Mean SPC in millions	% LUC[b] activity	Mean SPC in millions	% LUC[b] activity	
pUMS32	11.3	13.3	52	100	Strong stimulation
pUPS32	11.8	14.5	7.1	13.9	Insignificant
pUMS131	17.8	21.0	32.6	63.9	Intermediate stimulation
pUPS131	19.1	23.0	12.3	23.9	Insignificant
pUMSΔ32	82.8	100.0	16.4	32.1	NA[c]
pUPSΔ32	76.7	92.7	18.4	35.9	NA
p-GL3-P[d]	25.1	30.1	10.2	19.8	NA

[a] Refer also to Fig 4B.
[b] Luciferase activity obtained from each set of reporter plasmids (pUMS and pUPS) in uninfected and IBDV infected Vero cells are expressed relative to the plasmid showing maximum activity taken as 100%.
[c] NA denotes not applicable (because these constructs did not contain the 5' terminal 32 bp fragment which is the putative IBDV promoter).
[d] pGL3-P is the pGL3-Promoter GeneLight vector without the IBDV segment A 5'-terminal noncoding sequences.

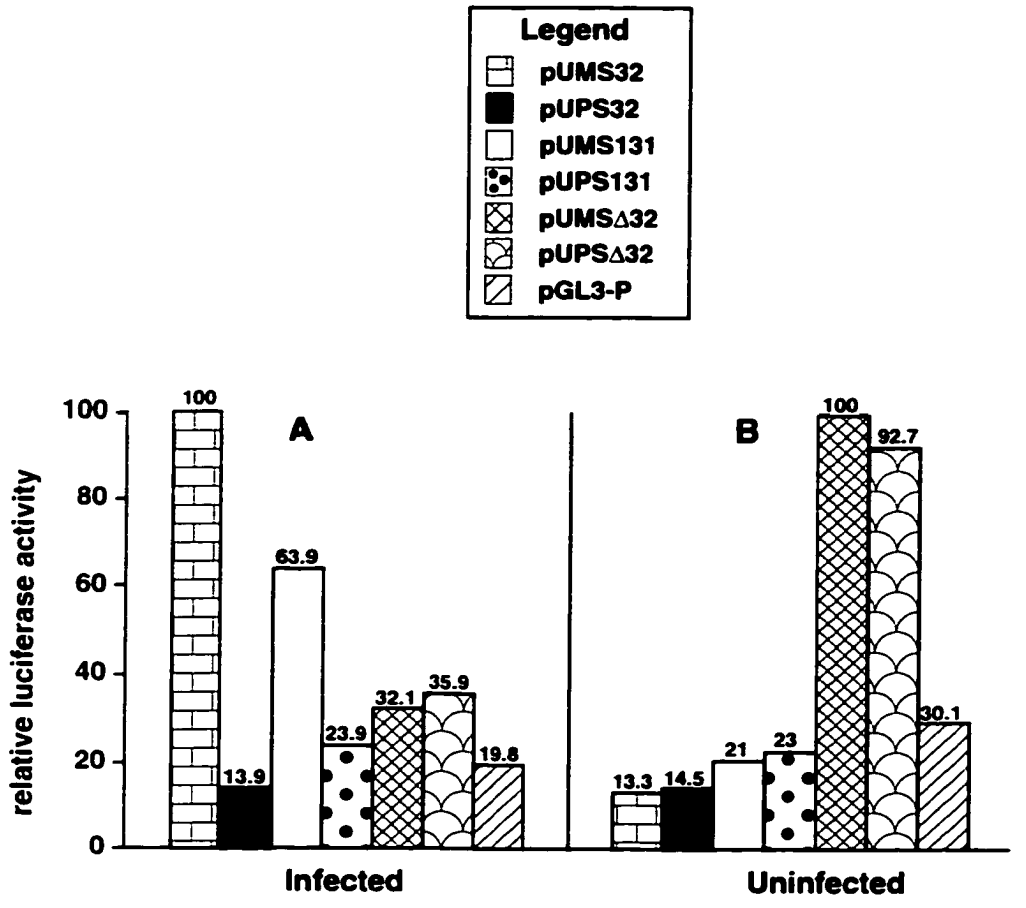


Fig. 7: Comparison of the promoter strength of the 5' noncoding region of segment A of IBDV in virus-infected and uninfected Vero cells. The values represent the results of at least three independent experiments in which the constructs pUMS32, pUPS32, pUMS131, pUPS131, pUMSΔ32 and pUPSΔ32 were tested in parallel with that of pGL3-Promoter vector. The values of the constructs in **A** are represented arbitrarily relative to that of pUMS32 and those in **B**, relative to that of pUMSΔ32 (Nagarajan and Kibenge, 1997b).

2.4 DISCUSSION

The 5' terminal sequence of the plus strand RNA in both genome segments consists of a 32 bp consensus sequence that is essentially similar in all six IBDV strains examined to date whereas the 3' end has the last 5 bases identical (Kibenge et al., 1996). The transcription of the bimavirus genome can occur by a semiconservative strand displacement mechanism with only the plus strand RNA of the two genome segments synthesized *in vitro* (Dobos, 1995) and *in vivo* (Magyar et al, 1998). It is highly probable that the plus-sense RNA transcripts are either sequestered by ribosomes for translation, or are packaged into procapsids, where they serve as templates for minus strand synthesis similar to what has been reported for other dsRNA viruses (Frilander et al., 1992; Patton, 1993; 1996). The data presented here demonstrate that the initial 32-bp stretch of sequence at the 5' end of genome segment A precursor polyprotein ORF positions -131 to -100 is a major promoter element of IBDV.

In the first set of transfection experiments in uninfected Vero cells with progressively deleted constructs of the 5' noncoding region of segment A, only the full-length construct (pEA131) and the construct with the first 32 bp fragment (pEds-oligo32) had significant LUC activity when examined for promoter strength. The full-length construct (pPA131) and the construct of the noncoding region with the terminal 32-bp deleted (pPA$_{\Delta}$32), but not the construct with the first 32-bp fragment (pPds-oligo32), also showed LUC activity when tested for enhancer activity (Fig. 6). The

promoter and enhancer activities of these constructs in uninfected Vero cells were unexpected as there is no report of sequence identity between IBDV VP1 which serves as the viral RNA dependent RNA polymerase and cellular DNA-dependent RNA polymerases. Since Vero cells are permissive for IBDV replication and IBDV replicates to very high titres in these cells (Lukert et al., 1975; Kibenge et al., 1988 b; Kibenge and McKenna, 1992) establishing a productive infection, the viral cDNA inserts in the constructs were presumably recognized by cellular transcription factors thereby modulating LUC expression in uninfected Vero cells. It is also possible that the putative promoter region of viral cDNA insert examined in the uninfected Vero cells has intrinsic DNA promoter activity thereby allowing recognition by the cellular DNA dependent RNA polymerase II and the same region could presumably serve as RNA promoter recognized *in trans* by IBDV RNA dependent RNA polymerase supplied by IBDV infected Vero cells.

To further determine whether the localized promoter region has functional significance for IBDV, selected regions of IBDV segment A terminal RNA sequences were generated endogenously *in vivo*. For this, transiently transfected modified pGL3-Promoter constructs were made in which *in vivo* transcription initiated by cellular RNA polymerase II recognizing the SV40 promoter positioned upstream of the viral terminal cDNA sequences (Fig. 4B) would generate recombinant viral-LUC RNAs. This strategy is analogous to that previously used in the mutational analysis of influenza virus promoter elements *in vivo* (Neumann and Hobom, 1995), where cellular RNA polymerase I was used for *in vivo* transcription of transfected

constructs containing recombinant viral terminal cDNA sequences with the chloramphenicol acetyl transferase (CAT) reporter gene. In this system, CAT RNAs were generated endogenously for the mutational analysis of influenza virus promoter where the viral coding sequence was replaced by the coding sequence for CAT. The viral terminal sequences were retained as cDNAs and were inserted between the mouse rDNA promoter and terminator sequences. Transfection of such recombinant DNA templates into mouse or MDCK cells followed by influenza virus infection and CAT assays demonstrated that the CAT vRNAs transcribed *in vivo* by cellular RNA polymerase I from mouse rDNA promoter were transcribed further *in vivo* by cellular RNA polymerase I into plus-sense mRNA and translated into CAT protein (Neumann and Hobom, 1995).

As shown in Fig. 7, virtually all the up-regulated LUC activity in infected cells could be attributed to the endogenous generation of viral minus-sense RNA of the terminal 32 bp upstream of the LUC AUG codon resulting from the transcription by cellular RNA polymerase II recognizing the SV40 promoter in constructs pUMS32 and pUMS131 (Fig. 4B).

The possible sequence of transcription events in the up-regulation of LUC expression in IBDV infected cells is depicted in Fig. 8 (Nagarajan and Kibenge, 1997b). Upon transfection, plasmid DNA constructs were transcribed by cellular RNA polymerase II recognizing the SV40 promoter. The resulting transcripts were

either translated into LUC or served as templates for transcription by the viral RNA polymerase provided *in trans* in IBDV-infected Vero cells.

Since transfections with pUMS32 and pUMS131 constructs alone resulted in up-regulated LUC expression (Fig. 7), only the cellular RNA polymerase II transcripts containing the 5'-terminal viral RNA sequence in the minus-sense orientation were initially recognized by the viral RNA polymerase. It also follows that the viral RNA polymerase was able to utilize ssRNA as template for RNA synthesis, and that upon recognition of the promoter sequence in the terminal viral RNA, transcription could proceed in either direction. Probably 5'-3' interactions of recombinant viral RNA strands are important in determining the direction of transcription. Because of amplification in LUC expression in pUMS32 and pUMS131, the LUC transcripts used for translation must have been at least the second generation transcripts of the viral RNA polymerase. This implies that the viral RNA polymerase was also able to use its own plus-sense transcripts as templates for transcription. Since the plus-sense transcripts of cellular RNA polymerase II (in 5'-3' orientation) were apparently not used as templates for transcription initiating transcription, it is possible that the plus-sense transcripts of the viral RNA polymerase were somehow modified by viral RNA polymerase. It is possible that the IBDV RNA polymerase initiated the transcription at the 3' end of the viral transcript in the recombinant viral LUC RNA transcript. The transcription proceeded further to the other 3' end of recombinant LUC RNA by IBDV RNA polymerase probably circularizing and interacting with the 3' end of recombinant LUC RNA. Another second round of

transcription initiated at the minus sense transcript of recombinant LUC RNA would possibly result in the enhanced luciferase expression observed in IBDV infected cells transfected with pUMS32 and pUMS131. However, the results clearly indicate that while the viral terminal sequences of both negative and positive polarities served as templates for transcription by the viral RNA polymerase, the plus-sense templates were transcribed only if they were transcription products of the viral RNA polymerase (Fig. 8). This would account for the differences in LUC expression in IBDV-infected cells transfected with constructs in minus-sense and plus-sense orientations (Fig. 7).

It is possible that the regions analyzed for regulatory functions in the present study might have exerted differential translation efficiencies on the *in vivo* generated recombinant viral-LUC RNA transcripts. However, the absence of higher levels of expression in the constructs lacking the viral terminal 32-bp fragment (pUPS$_{\Delta}$32 and pUMS$_{\Delta}$32) in transfected infected cells in contrast to the strong stimulation by constructs with the 32 bp fragment (pUMS32 and pUMS131) (Fig. 7) indicates that such differential translation efficiencies were not a factor in the up-regulation of LUC expression, and that IBDV-specific amplification indeed took place rendering endogenously generated terminal viral RNA sequences from these constructs biologically active (Nagarajan and Kibenge, 1997b). Moreover, the LUC assays of the uninfected cells transfected with the same pGL3-Promoter recombinant constructs (Fig. 7) ran in close parallel with those of the initial transfections (Fig. 6) and ruled out any enhancer activity in the viral terminal 32-bp fragment.

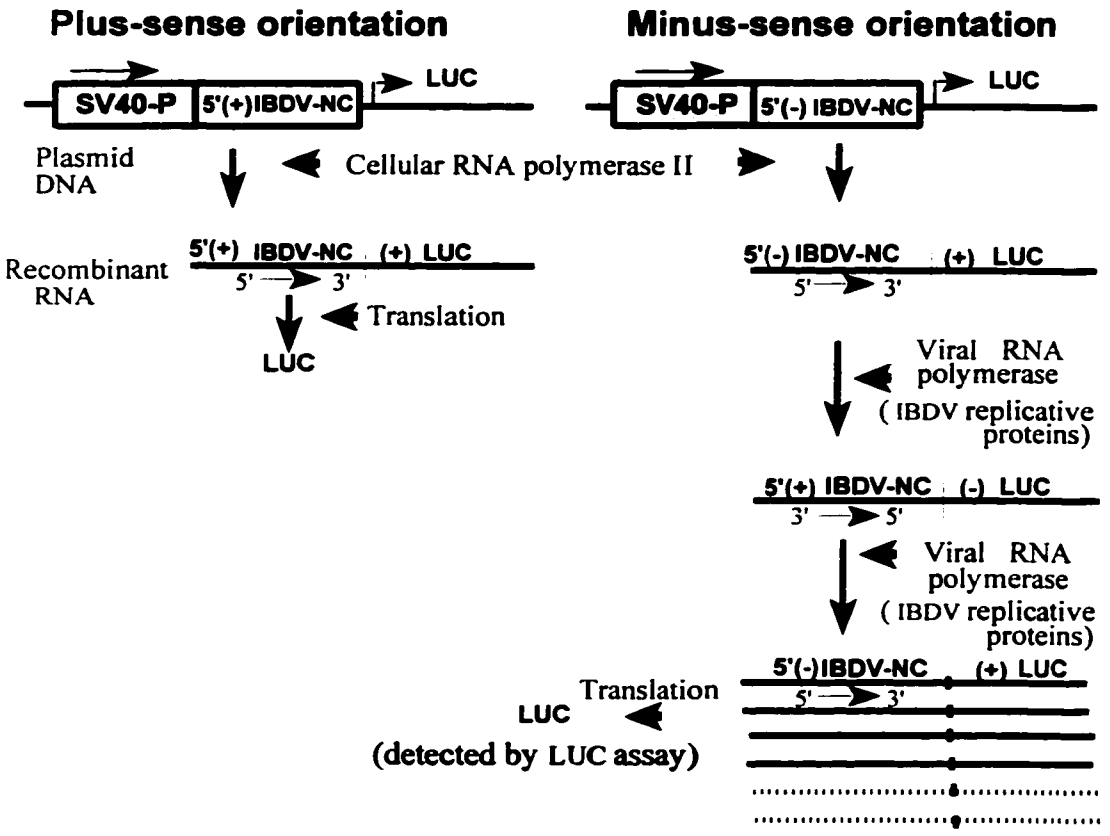


Fig. 8: Schematic diagram of the *in vivo* transcription of the recombinant viral-LUC DNA constructs. The plasmid DNA with 5'(+) IBDV-NC represents constructs pUPS131 and pUPS32 with the plus-sense orientation 5' → 3' of the noncoding region of segment A of IBDV downstream of the SV40 promoter. The plasmid DNA with 5'(−) IBDV-NC represents constructs pUMS131 and pUMS32 with the minus-sense orientation 5' → 3' of the noncoding region of segment A of IBDV downstream of the SV40 promoter. For the recombinant RNA, 5'(+) IBDV-NC and 5'(-) IBDV-NC denote the plus strand RNA and the minus strand RNA, respectively; the 5' → 3' or 3' → 5' direction is also indicated below each RNA strand.

In conclusion, the data taken together from infected and uninfected cells employing various combinations of recombinant pGL3-Genelight expression vectors strongly support the existence of a promoter element at the terminal 32 bp stretch of sequence at the 5' noncoding region of IBDV. Additional evidence consistent with the interpretation that the 5'-terminal 32-nucleotide sequence stretch of genome segment A contains a major promoter region of IBDV are: (i) this minimal region is highly conserved between the two genome segments (Kibenge et al., 1996), suggesting it has functional significance common to the two segments; (ii) this region had maximal promoter strength with no detectable enhancer activity, of the five constructs containing viral noncoding sequences transfected in uninfected cells; and (iii) this sequence at the 3' end of the minus strands for both genome segments A and B has considerable sequence identity with sequences at the alphavirus initiation site for 26S subgenomic RNA synthesis (Levis et al., 1990), the polymerase recognition core of rotavirus gene 8 mRNA (Patton et al., 1996) and the unique consensus intergenic or internal promoter sequences of mouse hepatitis virus (Shieh et al., 1987) (Fig. 9), which have been suggested to play an essential role in the generation of 3' co-terminal nested plus-sense transcripts from minus sense genomic RNA. Any nucleotide differences between IBDV segments A and B within the 32-bp conserved region (Fig. 9) may represent positions of lesser importance of recognition by the viral RNA polymerase. By the same token, sequence differences between viruses in the promoter sequences aligned in Fig. 9 may be a reflection of differences in specificity of the various viral RNA polymerases (Nagarajan and Kibenge, 1997b).

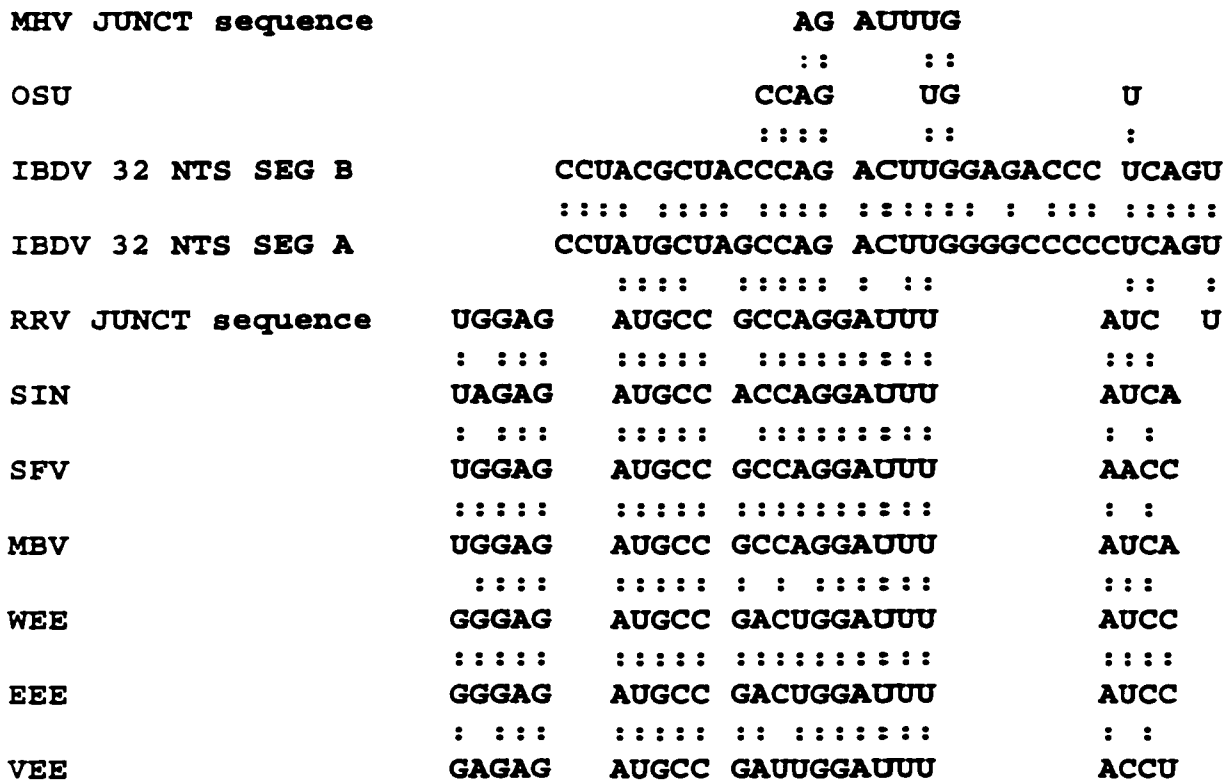


Fig. 9: Alignment of the 32-nucleotide stretch at the 3' end of the minus strands of genome segments A and B of IBDV with minus strand sequences at initiation sites of some alphaviruses (Levis *et al.*, 1990). *RRV* Ross river virus; *SIN* Sindbis virus; *SFV* Semliki forest virus; *MBV* Middleburg virus; *WEE* Western equine encephalitis virus; *EEE* Eastern equine encephalitis virus; *VEE* Venezuelan equine encephalitis virus; polymerase recognition core in rotavirus (OSU strain) gene 8 mRNA (Patton 1995); and consensus intergenic sequences of mouse hepatitis virus *(MHV)* (Shieh et al., 1987). Gaps are introduced to align the sequences. Colons denote sequence identity between viruses and between genomic segments A and B of IBDV (Nagarajan and Kibenge, 1997b).

The mode of IBDV genome replication may be similar to that of dsRNA bacteriophage ϕ6 (Emori et al., 1980; Usala et al., 1980; Van Etten et al., 1980) demonstrating a semiconservative strand displacement mechanism where the template minus strand remains annealed to the new plus strand. This is supported by studies on IBDV and IPNV by Dobos (1995) and Magyar et al. (1998). Birnaviruses are also unique among dsRNA viruses in having the VPg attached to the 5' end of both genome segments, circularizing them (Müller and Nitschke, 1987). Viral protein-primed RNA synthesis has been demonstrated *in vivo* (Magyar et al., 1998). Thus they seem to be different from other dsRNA viruses such as rotavirus (Gorziglia and Collins, 1992; Patton, 1993) and reovirus (Banerjee and Shatkin, 1970; Klett et al., 1971). Reverse genetics studies with IBDV (Mundt and Vakharia, 1996) and IPNV (Yao and Vakharia, 1998), however, suggest that a conservative mechanism may also occur where the plus-strand RNAs serve as templates for minus strand synthesis by VP1 or RNA-dependent RNA polymerase to make dsRNA. Nonetheless, the regulatory sequence for genome expression identified on the viral 5'-terminal noncoding region in the present study is likely to play a major role in the virus replication cycle. These observations do not rule out the possibility that other regulatory sequences may be located at the 3' terminus of the genome segments as well. The availability of an *in vivo* assay for viral regulatory regions provides a tool with which to further study these regulatory sequence interactions with specific viral and host factors thereby enhancing the potential of investigations on IBDV genome expression, replication and virulence (Nagarajan and Kibenge, 1997b).

Furthermore it is possible to introduce mutations in the identified promoter region of IBDV segment A coding for the polyprotein by using a reverse genetics system and to study the effect on the steady-state levels of polyprotein-specific mRNAs and any level of attenuation in recovered recombinant viruses. Such mutational studies of the vRNA promoter of an engineered influenza virus have resulted in the generation of mRNAs lacking poly A tails in most of them and thus having a differential effect on viral RNA transcription and level of viral replication efficiency (Fodor et al., 1998).

The model system used here comprising an *in vivo* assay with different recombinant constructs of LUC reporter gene ligated to various lengths of the 5' noncoding region of infectious bursal disease virus (IBDV), a dsRNA virus, can be extended to map the regulatory regions required for transcription of viral genes particularly of dsRNA, and negative sense RNA viruses which encode their own RNA-dependent RNA polymerases. It is a preferred way for characterizing the regulatory elements, since the target viral cDNA can be positioned in either minus- or plus-sense orientation immediately upstream of the LUC reporter gene and downstream of the SV40 promoter providing an *in vivo* system for generating authentic viral RNA sequences containing the initiation signals to be recognized by the viral RNA polymerase supplied by the respective virus-infected cells (Nagarajan and Kibenge, 1998).

3. ISOLATION AND CHARACTERIZATION OF A POLYCLONAL ANTIBODY ESCAPE MUTANT OF IBDV

3.1 INTRODUCTION

In recent years, virulence variations in IBDV have been associated with problems in properly vaccinated flocks (Snyder 1990). Antigenic and virulent variants of IBDV have emerged in vaccinated broiler flocks or in progeny of vaccinated parents (Müller et al., 1992). Rigorous vaccinations of commercial broiler layer flocks for prophylaxis are required with less attenuated ("intermediate") strains or more virulent strains that are capable of stimulating immunity in the presence of maternal antibody (Kowenhovan and van den Bose, 1993). The exact sites of the viral genome involved in virulence variations and the underlying molecular mechanisms are not yet defined. Comparisons between the sequences of the virulent and attenuated strains (Yamaguchi et al.,1996; Brown et al., 1994; Brown & Skinner, 1996) and antigenic variants of IBDV (Lin et al., 1993; Vakharia et al.,1994) have not adequately identified the specific nucleotide and amino acid residues relevant to the virulence of IBDV (Nagarajan and Kibenge, 1997a).

Over the years, a number of investigations have been carried out to characterize small and large plaque-forming mutants of IBDV. The classical IBDV strains decrease in virulence following passage in embryonated chicken eggs or in embryo

CEF cell cultures (Saijo et al., 1990; Izawa et al., 1978; Snedeker et al., 1967). Infection of chickens with attenuated large and small plaque clones derived by passaging the RF-1 strain of IBDV without immune pressure did not exert any immunosuppressive effect whereas the parental strain was immunosuppressive (Higashihara et al., 1991). However, there is no information about the effect of passage *in vitro* under immune pressure on the virulence, immunogenicity and other molecular properties of IBDV.

The present study was designed to compare the phenotypic and genotypic properties of a selected polyclonal antibody escape mutant with the parental virus in order to understand better the pathogenicity of IBDV. The selection exerted by antibody or other host immune mechanisms appears to play a major role in the rapid evolution of viruses in nature (Hyslop and Fagg, 1965; Schiapaccassi et al., 1995). For this study, an antibody resistant mutant of IBDV was isolated under immune pressure during serial passage *in vitro*. The antibody-resistant isolate was evaluated for mutations in selected parts of the genome and for pathogenicity in 3 week-old SPF chickens in comparison to the parental virus.

3.2 MATERIALS AND METHODS

3.2.1 Cells and virus

IBDV strain QC2 belonging to classical serotype 1 originally isolated from commercial chicken flocks in Quebec (Reddy and Silim, 1991b) was a kind gift from Dr. Amer Silim, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec. The virus was plaque-purified after a single passage in Vero cells in our laboratory and was used as the parental stock virus referred to as P0. P0 was serially propagated under immune pressure in Vero cells supplemented with 2% fetal bovine serum (FBS) and subsequent passaged viruses were designated as P1-IP, P2-IP, P3-IP etc., to denote the respective passage level under immune pressure.

3.2.2 Selection of polyclonal antibody escape mutants

In order to select IBDV variants partially resistant to neutralization by polyclonal antiserum, serial two-fold dilutions (1:20 to 1: 5120) of chicken polyclonal antiserum (APS) against serotype 1 strain QT2 (Reddy and Silim, 1991b) were made in MEM and mixed with 100 $TCID_{50}$ (median tissue culture infective dose) of parental IBDV strain QC2 in 96-well microtiter plates. The virus-antibody mixtures were incubated at 37 °C for one hour prior to addition of fresh Vero cells. Wells showing CPE after 5 days were harvested for the next passage. For this, cell lysates were frozen and thawed 3X and clarified by centrifugation at 3000X g for 20 min at 4 °C. The

supernatants were diluted, if necessary, and used for subsequent inoculations. The virus titre was determined every 5th passage so that appropriate dilutions could be made for passaging at low multiplicity of infection (0.1 - 0.01 moi). This selective cycle was repeated by mixing the progeny of each passage with the same and various gradually decreased two-fold dilutions of APS thereby increasing the immune pressure during each successive passage of QC2 in Vero cells. Duplicate series of infections were carried out for each passage. As a control, P0 was also passaged under the same conditions but in the presence of non-immune serum instead of IBDV specific polyclonal antiserum, and each successive passage under these conditions was designated as P1-NIP, P2-NIP etc.

3.2.3 Virus-neutralization (VN) test

Virus neutralization titres to the unpassaged virus (P0) and to the virus passaged in absence of antibody or nonimmune pressure designated P1-NIP, P5-NIP, P10-NIP etc. and virus passaged in presence of antibody and designated as P1-IP, P5-IP, P10-IP etc., were determined every 5th passage by using the constant-virus varying-antibody procedure in Vero cells (Skeeles et al., 1978). Virus neutralization (VN) titres were expressed as the reciprocal of the highest serum dilution that neutralized 100 $TCID_{50}$ of virus. They were performed in 96-well microtitre plates using duplicate two-fold serial dilutions of serum samples and 100 $TCID_{50}$ of the respective virus. For this, 50 µl of the serially diluted serum samples were mixed with 50 µl of the virus and incubated for an hour at 37 °C. A volume of 50 µl of

Vero cells at a density of 2×10^5 cells/ml in growth medium were added. A virus control and a cell control were also maintained on each row of the microtitre plate. The plates were incubated at 37 °C in a humidified 5% CO_2-in-air atmosphere and were examined for CPE at 72 h PI. The endpoints were determined as the reciprocal of the highest dilution at which there was no CPE. The geometric mean titres of the sera from each experimental group were then determined.

3.2.4 Growth Curve

Growth curves of P0 and P25-IP in Vero cells were studied as described previously (Kibenge et al.,1988). Monolayers of Vero cells in 25-cm^2 flasks were inoculated with 10^5 $TCID_{50}$ of virus in 0.1 ml of medium without FBS. They were washed once with phosphate buffered saline (PBS) and replaced with maintenance medium containing 2% FBS after adsorption of the virus for 1 h at 37 °C. The cultures were incubated further at 37 °C in a humidified CO_2 atmosphere. At specified intervals, two flasks were removed. The cell culture medium and the cell monolayer of one flask were harvested separately. The cell culture medium was clarified by low speed centrifugation to remove cellular debris and stored at -70 °C for the assay of extracellular virus. The harvested cell monolayer was pooled with the pellet from the centrifuged medium in 1.0 ml of fresh maintenance medium. This was frozen and thawed 3X, clarified, and the supernatant was assayed for cell-associated virus infectivity. The second flask was similarly frozen and thawed and the clarified supernatant was assayed for total virus infectivity. The viral titres were determined by serial dilutions of each virus sample in medium without FBS. Fifty microliters of

each dilution was transferred to four wells of a 96-well microtitre plate. An equal volume of growth medium was added to each dilution and the last column on each plate served as cell control. Fifty microliters of a fresh Vero cell suspension was added to each well and the plates were incubated at 37 °C in a humidified CO_2-in-air atmosphere for 4 days prior to reading the end points by CPE. The virus titre was calculated according to the method of Reed and Muench (1938).

3.2.5 Plaque phenotype

Plaque morphology was studied by inoculating 0.2 ml of serial 10-fold dilutions of the respective viruses onto a monolayer of Vero cells grown in 6-well tissue-culture plates. Overlay maintenance medium containing 0.8% agar was used as culture medium. After incubating at 37 °C for 4 days in an atmosphere containing 5% CO2-in-air, the cultures were stained with 0.02% neutral red and thirty discrete plaques selected at random from each plate of replicate plaque assays were analysed for their size and appearance. Plaques in replicate plaque assays were measured to obtain the average plaque size.

3.2.6 Nucleotide sequence analysis

In an effort to identify any molecular changes in the genomic regions of P25-IP relative to P0, total RNA was extracted from P0 and P25-IP by the TRIZOL method (GIBCO-BRL, Burlington, Canada). The RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) amplification using SuperScript™

One-Step™ RT-PCR system (GIBCO-BRL, Burlington, Canada) with appropriate primer pairs selected according to the published sequence of IBDV genome segments A and B (Kibenge et al., 1996). cDNA synthesis consisted of incubation at 50 °C for 30 min followed by pre-denaturation at 94 °C for 2 min. For PCR amplification, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 s and extension at 72 °C for 1 min followed by one cycle of final extension at 72 °C for 10 min were used. The following selected regions of segment A were amplified: the entire VP2 coding region (primer pairs SA06 and SA11 yielding a 1539 bp fragment), and a portion of the VP4 coding region spanning the presumed protease active cleavage sites (primer pairs SA03 and SA10 yielding a 918 bp product). Each PCR amplified cDNA of selected genomic regions of P0 and P25-IP was digested with a panel of restriction enzymes and the resulting restriction fragment length profile was determined. The PCR products containing the 5' noncoding regions of segments A and B amplified with primer pairs SA11 and SA12 (160 bp product) and SB14 and SB11 (593 bp product), respectively, and the 3' noncoding regions of A and B amplified with primer pairs SA09 and SA21 (111 bp product) and SB07 and SB15 (99 bp product), respectively, were cloned and at least three clones of each PCR product were sequenced by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase kit, Version 2.1 (Amersham, Oakville, Ontario).

3.2.7 Experimental infection of chickens

To compare the pathogenicity of P25-IP to P0, three-week old specific-pathogen free (SPF) white leghorn chickens of both sexes hatched from fertile eggs (SPAFAS Inc, Chicago, USA) were randomly divided into three groups of 15 chickens each. The chickens in the first two groups were inoculated orally with 0.2 ml of tissue culture media containing either P0 or P25-IP at a dose of 10^6 $TCID_{50}$ and the third group serving as a control was sham inoculated with tissue culture media containing no virus. The chickens were maintained in isolation in a facility designed for maintaining SPF status, and a broiler starter diet and water were provided *ad libitum*. Morbidity, mortality rates, clinical signs (prostration, diarrhea and ruffled feathers) and post mortem lesions such as edema of the bursa of Fabricius and hemorrhages were monitored. On day 5, 12 and 21 postinoculation, five chickens per group were randomly selected and bled by cardiac puncture. Blood samples were allowed to clot for about 2 h and the serum was collected for serological testing. The chickens were euthanised by halothane inhalation and examined for gross and microscopic lesions. The bursa (liver or spleen)-to-body-weight ratios were compared statistically using Fisher's F test. Differences were considered statistically significant at 5% probability.

3.2.8 Histopathology

The bursa of Fabricius, spleen, liver and thymus were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hemotoxylin and eosin

by standard procedures. Paraffin-embedded central cross sections from each bursa of Fabricius were examined for microscopic lesions. Attempts were made to determine the percentage of affected follicles per bursa on the basis of lymphoid necrosis and/or depletion as described elsewhere, with modifications (Rosales et al., 1989; Sharma et al., 1989a; Tanimura et al., 1995): 0 = no detectable lesions; 1 = less than 25% of lymphoid follicles affected; 2 = 25-50% of lymphoid follicles affected; 3 = 50-75% of lymphoid follicles affected; and 4 = greater than 75% of lymphoid follicles affected. Attempts were also made to score the splenic lesions (0 = no detectable lesions; 1 = mild decrease in lymphocyte numbers; 2= mild increase in macrophage numbers around sheathed capillaries and in red pulp; 3 = severe decrease in lymphocyte numbers, severe increase in macrophage numbers).

3.2.9 Virus re-isolation

The organs collected at various days PI were homogenized in PBS to obtain 1:4 wt:vol suspensions. They were frozen and thawed three times and clarified by low-speed centrifugation. The supernatant fluids were passed through 0.45-µm filter and the filtrates were used for inoculation of Vero cell monolayers as described (Kibenge and McKenna, 1992). Briefly, confluent monolayers of Vero cells in 24-well plates were inoculated with 100 µl of the filtrate samples using two wells per sample and incubated at 37 °C for 1 h for virus adsorption. The cultures were monitored daily for the appearance of CPE after addition of maintenance medium and incubation at 37 °C in humidified CO_2-in-air atmosphere. The samples were considered negative in virus isolation following 5 blind passages at 7 day intervals.

3.3 RESULTS

3.3.1 Selection of polyclonal antibody escape mutants under subneutralizing immune pressure

For the selection of antibody resistant mutants of IBDV strain QC2, 25 serial passages were carried out with gradually increasing amounts of antiserum used for partial neutralization thereby increasing the immune pressure imposed on the inocula. Increased neutralization resistance was noticed from P15-IP onwards as evidenced by consistent two-fold decrease in neutralization titre (1:160) when compared to P0 (1:320) and P25-NIP (1:320) (Fig. 10 and Table 5).

3.3.2 Growth characteristics

P25-IP formed uniformly small smooth plaques (SP) of approximately 1-1.5 mm in diameter while P0 produced predominantly large irregular plaques (LP) of approximately 2-3 mm in diameter (Fig. 11). P25-NIP also produced predominantly large plaques of approximately 2-2.5 mm in diameter in Vero cells. In the present study the selected variant (P25-IP) with small plaque phenotype showed similar growth characteristics as the large plaque clone (P0) of the parental wild-type virus. No significant difference was noticed between the growth curves of P0 and P25-IP. Steady parallel increases in extracellular and cell-associated virus were noticed until approximately 30 h PI in Vero cells and similar maximum viral titres were reached by 48 h for both P0 and P25-IP (Fig. 12).

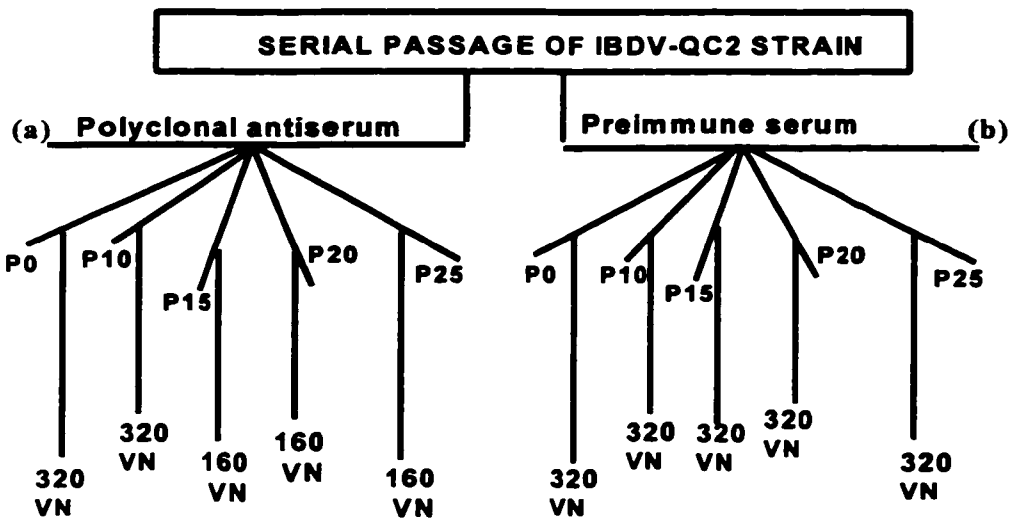


Fig. 10: Serial passage of IBDV-QC-2 strain (a) in the presence of serotype 1 IBDV- specific polyclonal antiserum and (b) in the presence of preimmune serum; P0-P25 represent different passage levels of the virus. Virus neutralization (VN) titer is expressed as the reciprocal of the highest dilution of antiserum to completely neutralize the virus and showing intact monolayer.

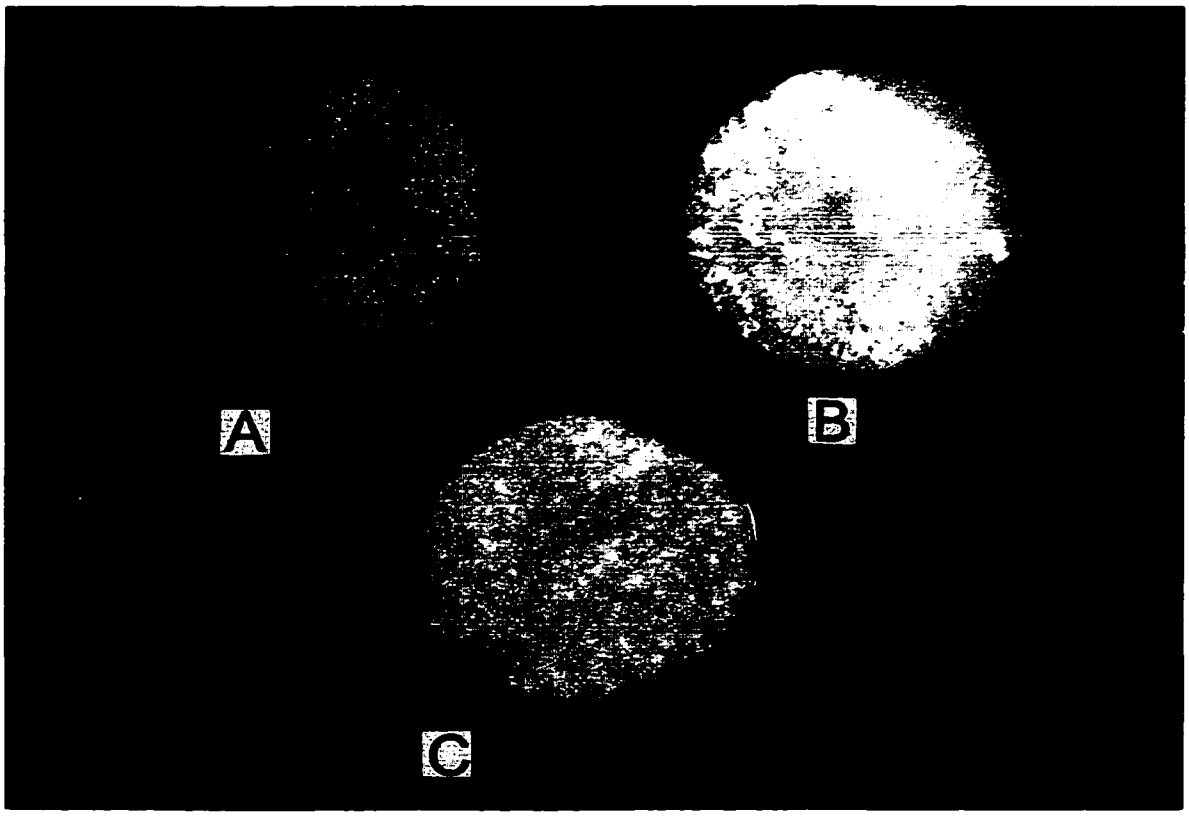


Fig. 11: Plaque assays of (B) IBDV-QC2 wild type isolate (P0) and (C) a variant (P25-IP) isolated after passaging IBDV-QC2 25X under immune pressure. (A) control Vero cells. Isolates were inoculated onto Vero cells as described in the text, incubated for 4 days at 37 °C and stained with crystal violet solution.

Table 5: Serum neutralization titres of QC2-IBDV

Passage level of QC2-IBDV with polyclonal antiserum[a]	$TCID_{50}$/50µl	SN titre[b] passaged under immune pressure	SN titre[b] passaged in absence of immune pressure
P0	$10^{7.0}$	320	320
P10	$10^{6.0}$	320	320
P15	$10^{4.25}$	160	320
P20	$10^{5.25}$	160	320
P25	$10^{5.5}$	160	320

[a] serotype 1 specific QT2 antiserum

[b]SN, serum neutralization titre is expressed as the reciprocal of the highest dilution showing no CPE ; P0-P25, denoting different passage levels.

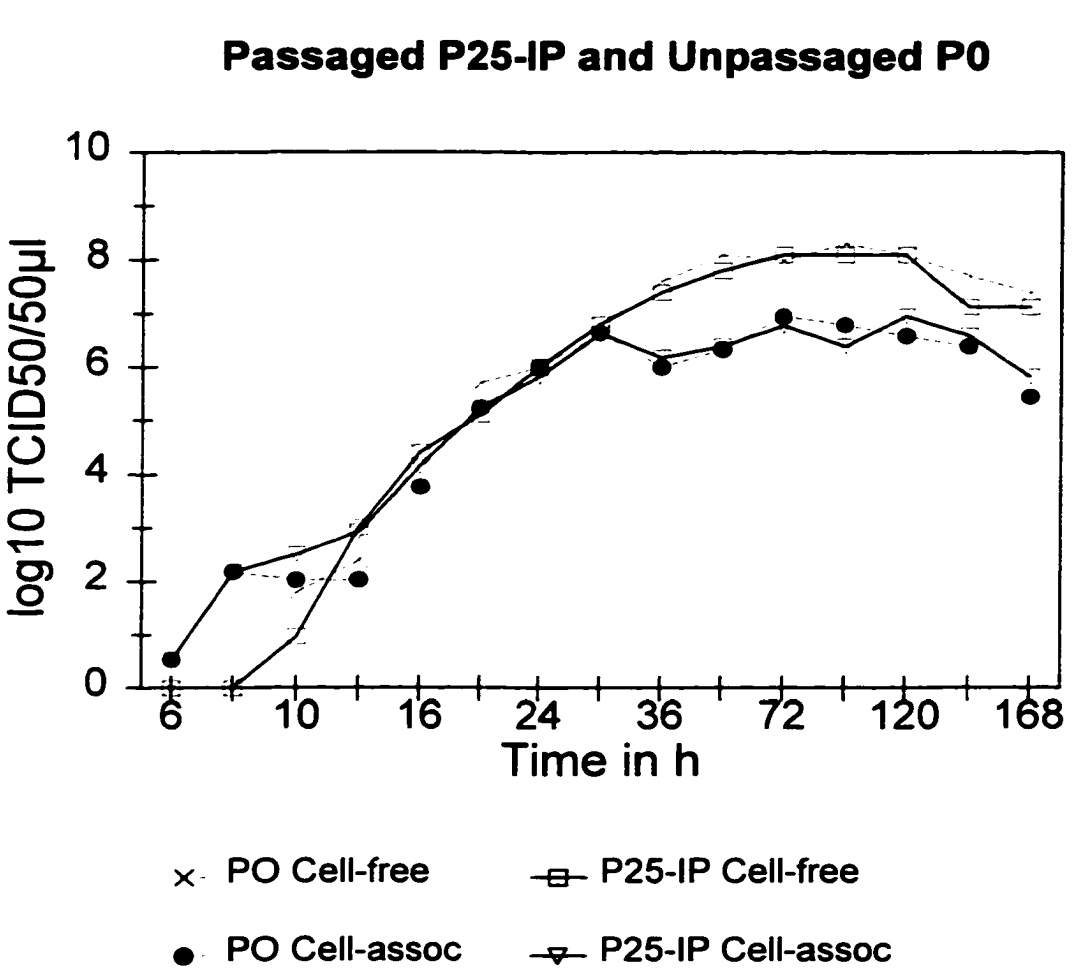


Fig. 12: Growth curve of IBDV strain QC-2 in Vero cells; P25-IP and P0 represent passaged virus in presence of antibody and unpassaged virus, respectively. Cell -free indicates extracellular virus in medium and Cell-assoc, denotes cell-associated virus.

3.3.3 Nucleotide sequence analysis

Restriction profiles of the viral cDNA were generated by restriction enzymes *Apa* I, *Bam*H I, *Bsu* 361, *Hind* III, *Kpn* I, *Mbo* II, *Pst* I, *Stu* I and *Xho* I (GIBCO-BRL, Burlington, USA) for the amplified product in the VP4 region including the putative cleavage sites between VP2 and VP4, and VP4 and VP3. No differences were observed between P0 and P25-IP. However one additional cleavage site difference was noticed in P25-IP when the product in the VP2 region was digested with *Pst* I. No other differences were detected with the other restriction enzymes (*Bam*H I, *Dra* I, *Sac* I, *Stu* I, *Mbo* I and *Taq* I) in the VP2 region. Sequence analysis of the 5' noncoding regions of cDNA copies of segment A and segment B revealed no differences for P0 and P25-IP (Figs. 13a and 14a). However, four and three nucleotide changes were identified in the 3' noncoding regions of segment A and segment B, respectively, which were not related to serotype specificity (Figs. 13b and 14b).

3.3.4 Pathogenicity study

Chickens in both infected groups appeared normal at day 1 postinfection (PI) but starting at day 2, some chickens in both experimental groups were recumbent and depressed. By 5 days PI, all the birds appeared normal. Birds in the control group remained normal throughout the experimental period. No mortality was recorded in any of the experimental groups.

(a) IBDV Segment A 5' noncoding region

```
QC2-P0       GGATACGATCGGTCTGAACCCCAGGGGAGTCACCCGGGGACAGGCCGTCAAGCGT

QC2-P25-IP   ------------------------------------------------------


QC2-P0       TGTTCCAGGATGGAACTCCTCCTTCTACAACGCTATCATTGATGGTTAGTAGAGA

QC2-P25-IP   ------------------------------------------------------


QC2-P0       TCAGACAACGATCGCAGCG

QC2-P25-IP   -------------------
```

(b) IBDV Segment A 3' noncoding region

```
QC2-P0       TGAGGCTCCTGGGAGTCTCCCGACACCACCCGCGCAGGTGTGGACACCAATTCGG

QC2-P25-IP   ----------------------------------------A----T-------


QC2-P0       CTTTACAACATCCCAAATGGATCGGTCGCGGT

QC2-P25-IP   ------------------T----T-------
```

Fig. 13: (a) Alignment of Segment A 5' noncoding sequences of unpassaged virus(QC2-P0) and passaged virus in presence of antibody (QC2-P25-IP). **(b)** Alignment of segment A 3' noncoding sequences of QC2-P0 and QC2-P25-IP. Hyphens denote identical sequences.

(a) IBDV Segment B 5' noncoding region

```
QC2-P0      GGATGCGATGGGTCTGAACCTCTGGGAGTCACGAATTAACATGGCTACTAGGGGC
QC2-P-25    ----------------------------------------------------

QC2-P0      GATGCGCCGCTAATTGCCACGTTAGTGGCTCCTCTTCTTGATGATTCTGCCACC
QC2-P25-IP  ----------------------------------------------------
```

(b) IBDV Segment B 3' noncoding region

```
QC2-P0      TAGCCATGATGGGAACCACTCAAGAAGAGGACACTAATCCCAGACCCCGTATCCC
QC2-P25-IP  ------------------T---------------------------------

QC2-P0      CGGCCTTCGCCTGCGGT
QC2-P25-IP  --------T---T----
```

Fig. 14: (a) Alignment of segment B 5' noncoding regions of unpassaged virus (QC2-P0), and passaged virus (QC2-P25-IP) in presence of antibody; **(b)** alignment of segment B 3' noncoding regions of QC-P0 and QC2-P25-IP. Hyphens denote identical sequences.

The average bursa:body weight ratios from chickens inoculated with P0 showed a significant difference ($P < 0.05$) from those of uninoculated controls at 12 days PI. In P25-IP inoculated birds, an initial increase in the ratio was noticed at 12 days followed by a decrease at 21 days PI (Table 6). Significant increases in spleen/body wt were observed at 12 days for chickens infected with either P0 or P25-IP (Table 7). There was a slight increase in liver/body wt for P25-IP at 12 days PI as compared to the control uninoculated group (Table 8). Microscopic lesions were not identified in thymus or spleen in any experimental group. Microscopic examination of sections of Bursa of Fabricius revealed depleted follicles and heterophilic infiltration in more chickens infected with P0 than with P25-IP (Table 9). Epithelial hyperplasia of follicular tissue and cystic degeneration were also marked in some of the P0 infected bursal tissues (Table 9). Thus, P25-IP demonstrated a slightly lowered pathogenic profile compared to P0.

3.3.5 Antibody response and virus re-isolation from infected chickens

Virus neutralization (VN) antibody titres were detectable from 5 days PI until the end of the experiment at 21 days PI. The VN antibody titres rose rapidly between 5 and 12 days for both P0 and P25-IP (Table 10). However, serum samples against P25-IP showed consistently higher geometric mean titres than those against P0. No antibodies were detected in chickens in the control group at any time. Viral CPE was demonstrated in Vero cells for both P0 and P25-IP isolated from the bursa of Fabricius, bone marrow and thymus of infected chickens at 5, 12 and 21 days PI following 3 blind passages.

Table 6: Bursa-to-body-weight ratios in chickens inoculated with unpassaged and passaged IBDV under immune pressure

Group	Bursa wt/body wt (×1000)		
	5 days PI	12 days PI	21 days PI
P0-Lp	5.75[b] (0.31)	5.75[a] (0.48)	5.44[a] (0.84)
P25-IP-Sp	5.55 (0.10)	6.29 (0.54)	4.98 (0.85)
Control	5.22 (0.14)	6.28 (0.14)	5.31 (0.17)

[a]Bursa-to-body-weight ratios differed statistically (F-test) from the control uninoculated group ($P < 0.05$).

[b]Bursa-to-body-weight ratios differed statistically (F-test) from P25-IP-Sp ($P < 0.05$).

P0-LP, unpassaged parent virus, large plaque phenotype; P25-IP-Sp, passaged virus (25x) with small plaque phenotype.

SEM (standard error of the mean) is indicated in the parentheses.

Table 7: Spleen-to-body-weight ratios in chickens inoculated with unpassaged and passaged IBDV under immune pressure

Group	Spleen wt/body wt (x1000)		
	5 days PI	12 days PI	21 days PI
P0-Lp	1.54[a] (0.12)	1.86[ab] (0.03)	1.82 (0.11)
P25-IP-Sp	1.58 (0.17)	2.01[a] (0.35)	1.88 (0.12)
Control	2.08 (0.42)	1.60 (0.08)	2.02 (0.12)

[a]Spleen-to-body-weight ratios differed statistically (F-test) from the control uninoculated group ($P < 0.05$).

[b]Spleen-to-body-weight ratios are significantly different from P25-IP-Sp ($P < 0.05$).

P0-Lp, unpassaged parent virus of large plaque phenotype; P25-IP-Sp, passaged virus (25X) in the presence of antibody and of small plaque phenotype.

SEM (standard error of the mean) is indicated in the parantheses.

Table 8: Liver-to-body-weight ratios in chickens inoculated with unpassaged and passaged IBDV under immune pressure

Group	Liver wt/body wt (×1000)		
	5 days PI	12 days PI	21 days PI
P0-Lp	32.48[ab] (0.59)	30.08[b] (0.92)	27.22 (0.86)
P25-IP-Sp	33.13 (1.65)	33.21[a] (0.96)	26.7 (0.92)
Control	33.42 (1.89)	28.61 (0.85)	28.16 (0.84)

[a]Liver-to-body-weight ratios differed statistically (F-test) from the control uninoculated group ($P < 0.05$).

[b]Liver-to-body-weight ratios are significantly different from P25-IP-Sp ($P < 0.05$).

P0-Lp denotes unpassaged virus with large plaque phenotype and P25-IP-Sp denotes 25 X passaged virus in the presence of antibody and of a small plaque phenotype.

SEM (standard error of the mean) is indicated in the parenthesis.

Table 9: Histopathologic characteristics of bursal lesions induced by IBDV (QC-2) in specific-pathogen-free chickens (N = 5)

Virus	Days PI	No. of chickens with bursal lesions characterized by			
		[a]Lymphoid depletion	Heterophil infiltration	Epithelial hyperplasia of follicular tissue	Cystic degeneration
P0	5	5	5	2	1
P25-IP	5	2	2	0	0
None	5	0	0	0	0
P0	12	3	2	0	2
P25-IP	12	2	0	0	0
None	12	0	0	0	0
P0	21	3	2	0	0
P25-IP	21	1	0	0	0
None	21	0	0	0	0

[a]less than 25% of lymphoid follicles were affected. P0 denotes unpassaged virus; P25-IP denotes virus passaged 25X under immune pressure.

Table 10: Virus-neutralizing (VN) antibody response in SPF chickens during IBDV infection

IBD virus[A]	VN antibody titres (GMT) at days post-infection[B]		
	5	12	21
P0-Lp[C]	70	139	139
P25-IP-Sp[D]	367	557	640
None[CD]	<20	<20	<20

[A] Each bird received $1x10^6$ $TCID_{50}$ virus in 0.2 ml via oral route at 3 weeks of age.

[B] Values are geometric mean titre (GMT) for five chickens.

[C] P0-Lp, the unpassaged virus with large plaque phenotype was used as homologous antigen in the virus neutralization (VN) test.

[D] P25-IP-Sp, the 25X passaged virus in presence of antibody and of a small plaque phenotype was used as homologous antigen in the virus neutralization (VN) test.

3.4 DISCUSSION

It is traditionally known that IBDV becomes attenuated following passage in cell culture. However, there is a lack of information about the effect of passage in cell culture in the presence of a limiting dilution of neutralizing antibody on the phenotype and other molecular properties of IBDV. Moreover, it has been hypothesized that antigenic and virulent strains emerge as a result of selection exerted by protective antibody in vaccinated flocks. Hence it is of interest to simulate such an antibody selection process *in vitro* in order to understand better the mechanism of pathogenicity. Thus, a partial neutralization resistant mutant of IBDV was selected by serial passage in the presence of polyclonal antiserum and its phenotypic and genotypic properties were compared with the parental virus.

A small plaque variant of QC2 strain of IBDV selected after 25 passages in the presence of polyclonal antiserum was analysed for its phenotypic and genotypic properties. This mutant was less efficiently neutralized than the parent strain by the serotype 1 antiserum against the QT2 strain used in this study. It was hypothesized that escape from imposed immune pressure during serial passage in Vero cells might favour the selection of antigenic and possibly pathogenic variants with accompanying phenotypic and genotypic changes.

Virus P25-IP was characterized for its growth characteristics in Vero cells. In general, serial passage of virus in cell culture favours the generation of attenuated strains that are better adapted for growth *in vitro* than the wild-type virus isolated from the natural host. It often results in simultaneous loss of pathogenicity of the passaged virus for the natural host (Cursiefen et al. 1979, Snedeker et al., 1967; Izawa et al., 1978). Since the selected variant under immune pressure in the present study demonstrated an altered small plaque phenotype and was less efficiently neutralized than the parental strain, it was considered that it may have altered pathogenicity for chickens compared to the parental virus.

In the present study precautions were taken to reduce the chances of generating defective interfering particles of IBDV during passage of the parental virus under selective immune pressure. Since such particles in some virus strains were reported to have small plaque morphology (Müller et al., 1986; Tsai and Saif, 1992), the moi between serial passages in Vero cells was kept low (0.1 - 0.01). The observations of no reduction in virus yield of the passaged virus and no significant differences in growth kinetics in Vero cells differed from those made earlier by Tsai and Saif (1992) with a variant IN strain of IBDV which exhibited poor replication efficiency during serial passage in a BGM-70 cell line. Moreover, the small plaque clone of P25-IP was not cell-associated, in contrast to another attenuated strain of IBDV (Cu1-M) which remained predominantly cell-associated with a slow replication rate (Cursiefen et al., 1979).

It was speculated that nucleotide changes occurred in the genome during serial passage of IBDV under immune pressure could modulate the pathogenicity of IBDV. Selected genomic regions spanning the VP2-VP4 and VP3-VP4 cleavage sites including the VP2 coding region were analysed by a panel of restriction enzymes (RE) and the noncoding regions by nucleotide sequencing. The RE profile revealed a cleavage site difference in the VP2 variable region. However, variation in RE profile is not necessarily an indication of variation in the pathogenic characteristics of a passaged virus. In the present study no mutations were identified in the 5' noncoding regions of either segment A or B of P25-IP which harbor many regulatory sequences. Moreover, P25-IP with a small plaque phenotype had similar growth characteristics as the parental strain with large plaque phenotype (P0) in Vero cells. It is possible that the determinants of small plaque morphology in passaged virus (P25-IP) map to some other regions of the genome other than the 5' NC regions. The significance of point mutations identified in the 3' noncoding regions of both segment A and B of P0 and P25-IP is not presently known. These 3' terminal regions demonstrating variability may be essential for replication, performing functions related to host range or virus assembly and packaging. It is also likely that these point mutations arose during serial passage in Vero cells during the adaptation of the parental virus to growth under immune pressure. However, it is not clear at the present time whether these mutations have any role in the slightly lowered pathogenic profile exhibited by the passaged virus in infected chickens.

From the pathogenicity studies, the spread of the IBDV-QC2 strain to other organs such as bone marrow was confirmed by virus re-isolation from these organs from chickens infected with both wild-type and passaged virus. Replication in bone marrow has been suggested to play a role in clinical IBD (Tsukamoto et al., 1995) because of the large number of bone marrow cells in the body. The virus was isolated from the thymus of chickens inoculated with P0 and P25-IP. Since no cortical lymphocyte condensation and depletion characteristic of thymic lesions were detected, it may be speculated that the viremia and/or circulation of monocytes infected with IBDV from the bursa contributed to the presence of virus in the thymus (Tsukamoto et al., 1995).

The seroconversion data indicated a rapid antibody response to IBDV which persisted throughout the duration of the experiment in both groups. However, higher VN titres were elicited by the SP clone of P25-IP than the wild-type virus suggesting a relatively stronger immune response. In addition, the parental virus induced significantly more bursal atrophy at 12 days post inoculation compared to the passaged virus (Table 6). The passaged virus induced slight liver and spleen enlargement at 12 days PI (Table 7 and 8) and slight inflammation of the bursa at 12 days PI followed by a slight reduction in bursal weight by 21 days PI. Thus, this isolated mutant acquired lowered pathogenicity and higher immunogenicity compared to the parent virus. Selecting such variant strains by passaging under immune pressure in cell culture may offer the possibility of these strains being developed into new and more effective live vaccines. The SP clone of P25-IP

appears to be a more suitable candidate vaccine strain than another IN strain of IBDV which lost its immunogenicity when used as a live vaccine (Hassan et al., 1996).

More pronounced changes in tissues may have been demonstrated by using a relatively larger dose of virus or a different route of administration. However evidence from previous pathogenicity studies with IBDV argues against these two ideas. Infections have been successfully initiated by using even lower titres of virus than what was used in the present study (Van den Berg et al., 1991; Okoye et al., 1992; Inou et al., 1994). Moreover the oral route of administration of IBDV resembling the natural route of infection of chickens has been used by several authors in previous pathogenicity studies with IBDV (Kaufer and Weiss, 1980; Tsukamoto et al., 1995; Yamaguchi et al., 1996). Indeed differences in susceptibility to IBDV among SPF chicken lines (Bumstead et al.,1993) and chickens with different histocompatibility complex (MHC) B haplotype have been previously reported (Nielsen et al., 1998). It is possible that the alleles inherited by the particular haplotype of the SPF chickens used in the present study may have determined the resistance of those chickens to viral infection and subsequently on the severity of the lesions observed.

Several conclusions can be drawn from the present study. The most compelling is that the overall clinical picture in infection studies using the virus passaged under immune pressure is similar to IBDV vaccination. The selected passaged virus

resembled the 'intermediate strains' of IBDV with its high immunogenicity and reduced ability to cause bursal damage. These observations are somewhat similar to previous findings (Haddad et al., 1997; Whitfill et al. 1995), where vaccination with IBDV-antibody complexes protected chickens from the effects of vaccination with challenge virus. However, the underlying mechanisms involved in such protection were not clarified in those studies.

In general, it has been difficult to select the type of vaccine to use, and to determine the period at which it has to be administered in order to confer protection against pathogenic field strains of IBDV (Haddad et al., 1997). The present study forms the basis for an alternative approach where effective protection may ensue from the slightly altered pathogenicity and immunogenicity of the virus passaged under selective immune pressure with its associated phenotypic and genotypic changes. Hence it will be of great interest to evaluate other features of the SP clone of P25-IP such as the protection provided to the vaccinates challenged with a virulent IBDV strain. Since the parameters for recovery of an infectious progeny IBDV have also been established (Chapter 4, section 4.3), additional studies may be initiated where the recognized changes in the SP clone of P25-IP could be introduced into the respective cDNA clone by site-directed mutagenesis and the recombinant infectious virus could be evaluated for its protection efficiency in vaccinates. This may lead to the development of a new modified vaccine strain of IBDV for effective control of infectious bursal disease.

4. PARAMETERS FOR RECOVERY OF INFECTIOUS RECOMBINANT IBDV PROGENY IN CELL CULTURE

4.1 INTRODUCTION

Preliminary studies in our laboratory established the complete nucleotide sequences of segments A and B including the noncoding regions of the IBDV genome (Kibenge et al., 1996). In order to develop a model system to study mechanisms underlying the replication and virulence of IBDV and to facilitate the assignment of specific mutations to a particular phenotype such as virulence by a directed mutagenesis approach, attempts were made to recover infectious progeny IBDV in cell culture. Moreover, it would facilitate constructing IBDV genome chimeras for functional analysis. Such a chimeric IBDV was produced by exchanging genome segment A of a tissue culture adapted IBDV and a virulent strain of IBDV that grows only in CEC (Mundt, 1999). Use of site-directed mutagenesis of this construct allowed the identification of amino acids in the variable region of VP2 of IBDV responsible for tissue culture infectivity. Such knowledge of which mutation or constellation of mutations best attenuates the virus would greatly aid in the development of an efficacious and stable attenuated vaccine strain of IBDV.

Furthermore, a major promoter element involved in viral genome expression and replication has been identified in the initial 5'-terminal 32 bp conserved between

segments A and B (Nagarajan and Kibenge, 1997b). However, the specific mechanisms associated with complementary minus-strand RNA synthesis and the existence of other *cis*-elements at the 3'-end of the plus-strand thus involved in dsRNA synthesis or assembly are not clearly known. The 5' terminal sequence of the plus RNA strand in both segments contains a 32 nt consensus sequence which is essentially similar to that of IBDV strains from Europe (Mundt and Müller, 1995), whereas the 3' end appears to be variable among IBDV strains. However, there are a few short consensus sequence motifs at the 3' noncoding region which are homologous between the two genome segments of North American and European strains of IBDV. The last 5 nucleotides at the 3' end of the plus strands of genome segments A and B of North American strains of IBDV are also identical (Kibenge et al., 1996). In segment A, there are 87 nts in the 3' noncoding region of the polyprotein ORF whereas in segment B, there are 72 nts in the 3' noncoding region of the VP1 ORF. It is considered that the generation of an infectious virus clone would greatly enhance the potential of investigations on the biological functions of the IBDV genome. Moreover, development of such an infectious cDNA clone from a viral RNA genome overcomes the inherent heterogeneity in RNA viruses and problems associated with direct RNA manipulation. A cDNA form is thus easily obtained from which a wild-type virus and its mutants can be isolated, stabilized and then manipulated readily by recombinant DNA technology. Hence efforts were made to develop a reverse genetics system for IBDV by constructing two independent full-length cDNA clones that contain segment A and segment B of OH

strain of IBDV and to define the minimum required length for successful recovery of an infectious IBDV from cloned cDNA.

4.2 MATERIALS AND METHODS

4.2.1 Cells, virus, growth and purification

Vero cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Parental OH-IBDV and recovered progeny viruses were propagated in Vero cells supplemented with 2% and 5% FBS, respectively. Transfection studies were carried out using 80% confluent Vero cell monolayers in MEM with 5% FBS. Individual genomic RNAs of segments A and B of IBDV used as templates for the various constructs made in this study were recovered as described previously (see section 2.2.2).

4.2.2 Construction of full-length segment A cDNA clone

A full-length segment A construct, designated pRFLOA, was initially prepared in the pCRII vector (Invitrogen, San Diego, USA). To obtain the full-length cDNA fragment of segment A, RT-PCR amplification was carried out with a sense primer containing the *Sst* I recognition sequence, T7 polymerase promoter sequence and the 5'-terminal sequence of segment A and an antisense primer containing *Xba* I recognition sequence and the 3' terminal sequences of segment A on the complementary strand (Table 11). IBDV-specific sequences in the selected primers

were based on the published nucleotide sequences of IBDV strain OH (Kibenge et al., 1996). The SuperScript™ One-Step™RT-PCR system (GIBCO-BRL, Burlington, Canada) was used according to the manufacturer's protocol with some modifications. Approximately 0.5 µg of genomic RNA template was denatured with 90% dimethyl sulfoxide (DMSO) at 65 °C for 90 min, snap chilled, ssRNA was ethanol precipitated with 2.5 volume of ethanol and 0.1 volume of 3M sodium acetate, pH 5.2, and stored at -80 °C until used in RT-PCR. Prior to RT-PCR, approximately 50 ng of DMSO-denatured template RNA in a total 20 µl reaction in DEPC-water, containing 1 µl of each of the above mentioned primers (20 µM) was heated at 65 °C for 10 min and snap-chilled. This annealed mixture was used in a RT-PCR reaction containing 25 µl of 2X reaction mix, 1 µl of RT/*Taq* mix and 1 µl of elongase (GIBCO-BRL, Burlington, Canada). The thermocycler was programmed for cDNA synthesis at 50 °C for 30 min followed immediately by pre-denaturation at 94 °C for 2 min and PCR amplification for 35 cycles, each cycle with denaturation at 94 °C for 1 min, annealing at 55 °C for 30 sec and extension at 72 °C for 2 min and 45 s with a final extension at 72 °C for 10 min. The RT-PCR products were analyzed by electrophoresis using 0.9% agarose gels. The PCR products were purified from a low melting temperature agarose gel and cloned into pCRII vector (Invitrogen, San Diego, USA) (see section 2.2.2). The pRFLOA clone thus obtained in the pCRII vector was retrieved by restriction with *Sst* I and *Xba* I and subcloned into the pUC19 vector double-digested with the same enzymes to obtain the clone pUFLOA. This clone contained the full-length cDNA template of

IBDV segment A under the control of synthetic T7 RNA polymerase promoter (Fig. 15a).

4.2.3 Construction of segment B full-length and partial-length cDNA clones

Several attempts to clone the full-length RT-PCR amplified cDNA fragment of segment B into pCRII vector were unsuccessful. Hence a different strategy was followed in the construction of IBDV segment B clones. The full-length clone and a partial length clone of segment B were constructed in pUC19 vector from ligations of subgenomic length clones using appropriate restriction sites as depicted in Fig. 15b. Initially, the entire ORF of IBDV segment B from clone pSB27 was subcloned into the *Eco*R I site of pUC19 vector. The new construct was designated pUORFB (Fig. 15b).

The plasmid containing the 5'-terminus of segment B (nt 1 to 567) designated as pR5B was obtained by RT-PCR amplification from genomic RNA of segment B of OH-IBDV strain with a sense primer (SB14) containing an *Sst* I site, T7 promoter sequence and the first 21 bases of the segment B 5' end sequence. The SB14 primer was designed in such a way that the first nucleotide of the IBDV segment B cDNA sequence would be immediately downstream of the T7 promoter sequence (Table 11). The antisense primer (SB11) used contained the *Bst*E II site to facilitate the ligation of 5'-terminal fragment (567 bp) with the fragment obtained from the plasmid pUORFB by digestion with *Bst*E II and *Xba* I. The 5'- end fragment

between the *Eco*R I sites in the plasmid pR5B was transferred to the pUC19 vector linearized with *Eco*R I to obtain clone pU5B (Fig. 15b).

For construction of a partial-length cDNA clone pUPLOB of segment B without the 3' terminal sequences, the plasmid pU5B was digested with *Bst*E II and *Xba* I to facilitate introduction of a 2100 bp fragment digested from the plasmid pUORFB with *Bst*E II and *Xba* I. The partial-length clone pUPLOB (Fig. 15b) thus obtained had the entire 5' terminal sequences and the ORF of segment B without the 3' terminal sequences of segment B.

To construct the full-length clone of IBDV segment B, the 3' end of genomic RNA of segment B was amplified by RT-PCR by using a sense primer (SB12) containing *Bsm* I site (position 1568 nt) and an antisense primer (SB15) containing *Xba* I site immediately downstream of the 3'-terminal sequence of segment B of IBDV (Fig. 15b). The PCR product (1259 bp) obtained was cloned in the *Eco*R I sites of the pCRII vector to obtain plasmid pR3B. The *Eco*R I fragment containing the 3'-termini of segment B in the plasmid pR3B was transferred to the pUC19 vector linearized with *Eco*R I and the sense clone pU3B was obtained by screening with *Bsm* I and *Xba* I.

Table 11: Oligonucleotide primers used for the construction of full-length cDNA clones of OH-IBDV genome segments A and partial-length cDNA clone of segment B

Sequence[a]	Orientation[b]	Name	Map position[c]
GAG**AGCTCT***AATACGACTCACTATAGG* ATACGATCGGTCTGAACC	+	SA20	1-20
GCG**TCTAGA**ACCGCGAACGATCCATTT GGG	-	SA21	3221-3241
GAG**AGCTCT***AATACGACTCACTATAGG* ATGCGATGGGTCTGAACCT	+	SB14	1-21
GGTAACCTCATCCTTTAAGCCCTCGTT GGCTTCTGGAACCTGGA	-	SB11	523-566
GAATGCAGCCACGTTCATCAACAACCA	+	SB12	1562-1588
GCG**TCTAGA**CCGCAGGCGAAGGCCGG GGA	-	SB15	2799-2820

[a]T7 promoter sequences are italicized; the virus specific sequences are underlined and the restriction sites are printed in bold. [b](+) and (-) refer to virus specific sequences of sense and antisense primers, respectively. [c]The map positions refer to published sequence of OH-IBDV, Gene Bank Accession numbers U30818, U30819 (Kibenge et al., 1996).

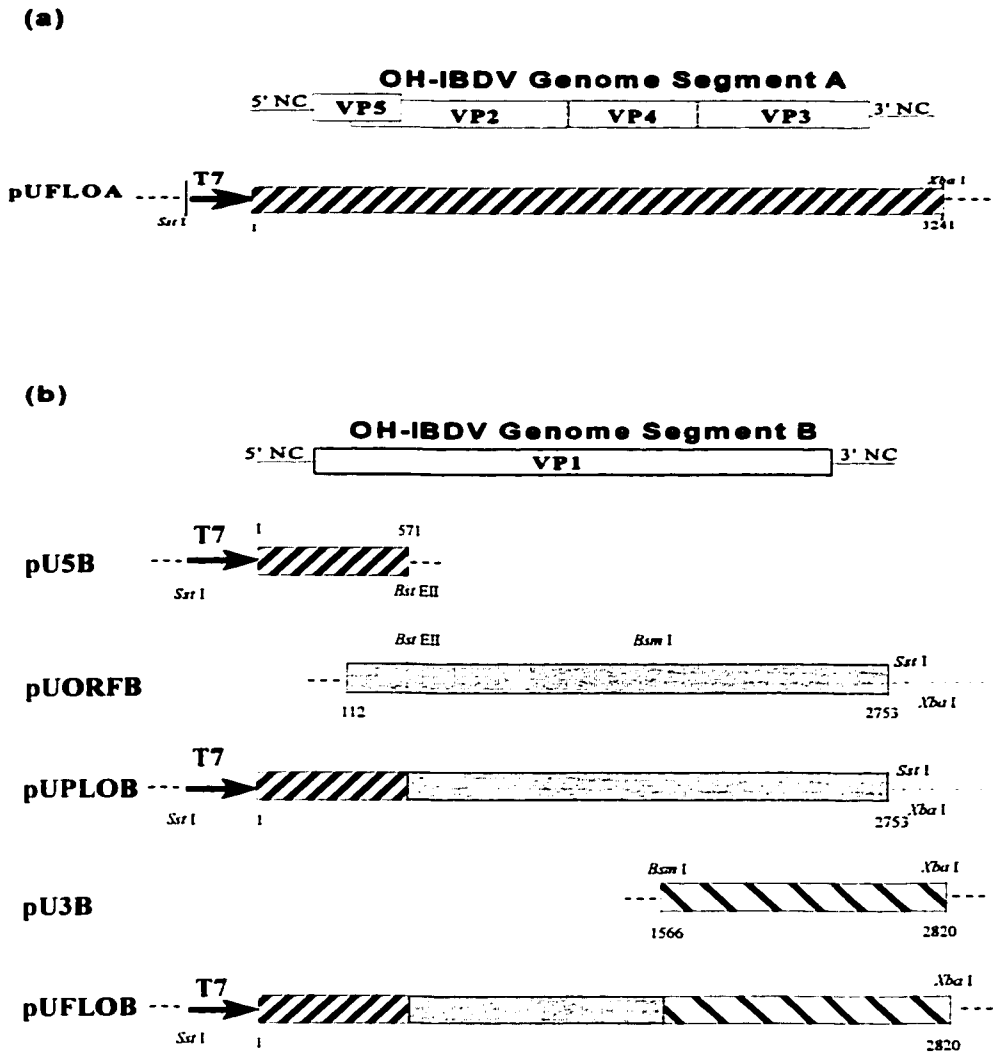


Fig. 15: Construction of full-length cDNA clones of segments A **(a)** and B and of partial-length cDNA clone of segment B **(b)** of OH-IBDV. Hatched boxes represent PCR amplified cDNA fragments transferred from pCRII vector to pUC19 vector; shaded box represents cDNA fragment derived from clone pSB27 in our laboratory containing the entire VP1 ORF of segment B. The numbers represent nucleotide positions within the IBDV genomic sequences. T7 arrow, denotes the RNA polymerase promoter sequence; *Sst* I, *Bst*E II, *Bsm* I and *Xba* I, restriction enzymes used for ligation of cDNA fragments at appropriate sites are shown. *Xba* I was used for linearization of the respective plasmid clones for *in vitro* transcription with T7 RNA polymerase. The *Xba* I site in the plasmids, pUORF and pUPLOB lies in the multiple cloning site of pUC19 vector.

To construct a full-length clone of IBDV segment B, the plasmid pUPLOB was digested with *Bsm* I and *Xba* I to facilitate the ligation of a 1259 bp fragment obtained by digestion of the plasmid pU3B with *Bsm* I and *Xba* I. The clone obtained was designated pUFLOB and contained the final version of the full-length cDNA copy of segment B of IBDV in pUC19 vector (Fig. 15b).

4.2.4 *In vitro* transcriptions

The plasmid constructs pUFLOA and pUFLOB containing full-length cDNA of segments A and B, respectively were verified for their translational activity by the TNT Quick Coupled Transcription/Translation System using rabbit reticulocyte lysate. For this, 2 µg of plasmid DNA was added to a reaction mixture in 50 µl containing 40 µl of the TNT Quick Master Mix, 2 µl of Redivue™ [^{35}S]methionine (Amersham, Oakville, Canada) and DEPC-water and incubated at 30 °C for 90 min. The translation products were analysed by SDS-PAGE. A positive control reaction was carried out using 2 µl of Luciferase control plasmid DNA instead of respective plasmid template DNA. When the translation reaction was complete, 12.5 µl of the reaction mixture was added to 12.5 µl of SDS 2X sample buffer, heated at 100 °C for 2 min to denature the proteins and loaded onto 12% SDS-polyacrylamide gel and electrophoresed at a constant current of 15 mA in the stacking gel and 30mA in the separating gel. Following electrophoresis, the gel was fixed in 7% acetic acid, 7% methanol, 1% glycerol for 5 min, placed on a sheet of Whatman 3M filter paper, covered with plastic wrap and dried at 80 °C for 3 h using a slab dryer (Hoeffer

Scientific SE1160). It was then subjected to direct autoradiography by exposing to Kodak X-Omat film at room temperature for approximately 36 h.

For generating *in vitro* transcripts for transfections, the respective plasmid DNAs were linearized with *Xba* I and purified by phenol-chloroform extraction and ethanol precipitation. The linearized cDNA templates (~1.5 µg each) were added separately to a 40 µl reaction mixture containing 8 µl of T7 Transcription 5X buffer, 12 µl of rNTP (25 mM ATP, CTP, GTP, UTP), and 4 µl of T7 enzyme mix (T7 Ribomax system, Promega, Wisconsin, USA). *In vitro* transcription reactions were performed in the absence of any cap analog, according to the manufacturer's instructions. The reaction mixture was incubated at 30 °C for 4 h, the transcription products were treated with RNase-free RQ1 RNase-free DNase (1u/µg of template DNA) for 20 min at 37 °C and the transcripts were purified by phenol-chloroform extraction and ethanol precipitation and stored at -80 °C until used.

4.2.5 RNA transfections

Transfections were performed essentially as described (Mundt and Vakharia, 1996) with minor modifications. Confluent monolayers of Vero cells in 35 mm dishes were washed with phosphate-buffered saline (PBS) and incubated with 2 ml of opti-MEM I (GIBCO-BRL, Burlington, Canada) for 1 h at 37 °C in a humidified CO_2-in-air atmosphere. Briefly, equimolar amounts of RNA transcripts obtained by *in vitro* transcription with T7 RNA polymerase from either the linearized templates of

pUFLOA and pUFLOB or those of pUFLOA and pUPLOB were resuspended in100 µl of DEPC-treated water. They were then added to previously equilibrated lipofectin (GIBCO-BRL, Burlington, Canada) -Opti-MEM I mixture (10 µg of lipofectin in 100 µl Opti-MEM) and incubated on ice for 15 min. The RNA-lipofectin-Opti-MEM I mixture was added to 0.8 ml of Opti-MEM I and used for transfection of Vero cells as described (GIBCO-BRL, Burlington, Canada). The transfected cell cultures were incubated at 37 °C for 2 h, the transfected mixture was then removed and replaced with MEM containing 5% FBS. Incubation was continued for another 48 h at 37 °C. The cell culture medium was harvested, clarified and subsequently transferred to a fresh monolayer of Vero cells and was monitored daily for CPE.

4.2.6 Characterization of infectious progeny virus

The inoculated cell cultures showing CPE were frozen and thawed three times. These harvests were clarified and RNA was extracted from 300 µl volumes using TRIZOL reagent (GIBCO-BRL, Burlington, Canada) according to manufacturer's instructions. The total RNA was analysed along with appropriate controls by RT-PCR. For this, Superscript II reverse transcriptase (GIBCO-BRL, Burlington, Canada) was used at 50 °C for 30 min with appropriate primers. To confirm the presence of virus specific sequences of segment A and B in the progeny viruses in the harvests, the following primers were used; SA03 (sense) and SA10 (antisense) spanning the VP4 coding region (position nt 1419-2337), SB03 (sense) and SB04 (antisense) spanning the VP1 coding region (position nt 1465-2115) and SB07

(sense) and SB15 (antisense) spanning the entire 3' noncoding region of segment B (position nt 2730-2820) of OH-IBDV strain. In all cases, a preparation of the OH-IBDV parental virus was used as a positive control.

4.3 RESULTS

Transfection of Vero cells with RNA transcripts of full-length cDNA clones of segments A and B generated infectious progeny virus designated r-OH. Transfection with combined transcripts of the full-length cDNA clone of segment A and the partial-length cDNA clone of segment B generated r-OH3NCB. The respective full-length plasmid constructs used for producing *in vitro* RNA transcripts for the above transfections were found to be active by coupled *in vitro* transcription-translation in a rabbit reticulocyte lysate system (T7-TNT, Promega, Wisconsin, USA) and analysis by 12% SDS-PAGE (Fig. 16). Some anomalous small molecular weight bands observed in the gel were attributed to internal initiation of translation as they were evident in the *in vitro* translation products of the control Luciferase plasmid as well.

The generation of infectious progeny virus was evident in both transfections using either combined transcripts of full-length segment A and segment B cDNA clones or transcripts of full-length segment A and partial-length segment B clones. It was characterized by the appearance of CPE (Fig. 17b) within 72 h of transfer of clarified

supernatants to fresh monolayers of Vero cells; the CPE was complete and covered the entire monolayer by seven days post-inoculation. No CPE was observed in Vero cells sham-infected with clarified supernatant of transfected lipofectin mixture without the RNA transcripts (Fig. 17a).

In order to characterize the recovered infectious progeny viruses, total RNA was isolated from culture supernatants of Vero cells infected with the viruses r-OH, r-OH3NCB and the parental virus OH-IBDV. The presence of segment A specific coding sequence in the recombinant viruses was verified by obtaining a PCR product of expected size using a primer pair specific for the coding region of segment A. RT-PCR analysis with a primer pair specific for the coding region of segment B also yielded the expected product from all the viruses but not from the uninfected Vero cell culture (Fig. 18). The expected PCR product (Fig. 19, lanes 1 and 3) was obtained not only from the recombinant virus r-OH and the parental OH-IBDV respectively but surprisingly also from the recombinant virus r-OH3NCB (Fig 19, lane 2) when the primer pair specific for the 3'-terminal sequences of segment B were used. If RT was omitted in the reaction mixtures, no PCR product was detected from the respective viruses (Fig 19, lanes 5, 6 and 7) indicating that the PCR product originated from template RNA and not from contaminating DNA.

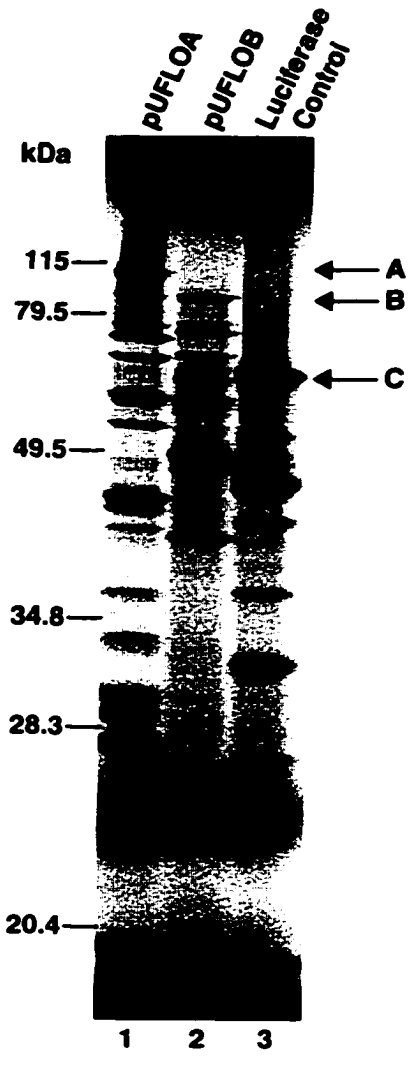


Fig. 16: Autoradiograph of radiolabeled translation products synthesized by using the TNT Quick Coupled Transcription /Translation Reticulocyte Lysate system (Promega, Wisconsin, USA). Lanes1 and 2 represent the translation products of unlinearized or circular plasmid constructs pUFLOA and pUFLOB containing full-length cDNA of segments A and B, respectively, and lane 3, the control TNT luciferase plasmid containing the luciferase gene. The prestained molecular weight marker proteins (Bio-Rad) are drawn in on the left while the corresponding expected IBDV proteins from segments A (polyprotein) and B (VP1) and the luciferase protein (control, C) are indicated on the right.

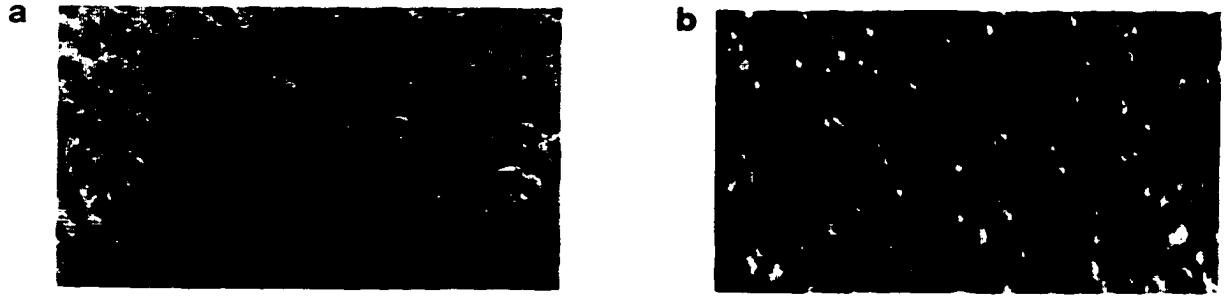


Fig. 17: Morphology of Vero cells infected with recombinant viruses. (a) The morphology of uninfected control Vero cells 6 days after the previous passage. (b) cytopathic effect of Vero cells 6 days after inoculation with recombinant viruses rOH or rOH3NCB. Both magnifications are x160.

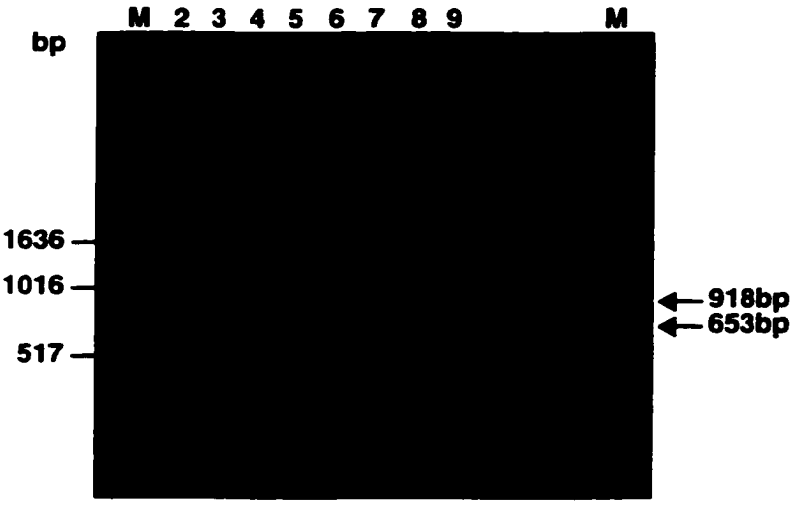


Fig. 18: Location of virus specific sequences of segment A and B in recovered recombinant progeny viruses. RT-PCR products of total RNA of r-OH, r-OH3NCB and OH-IBDV amplified with primer pairs SA03 (sense) and SA10 (antisense) spanning the VP3 gene and yielding a predicted product (arrowed) around 918 bp (lanes 2, 3 and 5 respectively); with primer pairs SB03 (sense) and SB04 (antisense) on VP1 yielding a predicted size product (arrowed) around 653 bp (6, 7 and 9 respectively); lanes 4 and 8 representing control uninfected Vero cells. RT-PCR products were analyzed on 1% agarose gel. M, 1 Kb ladder.

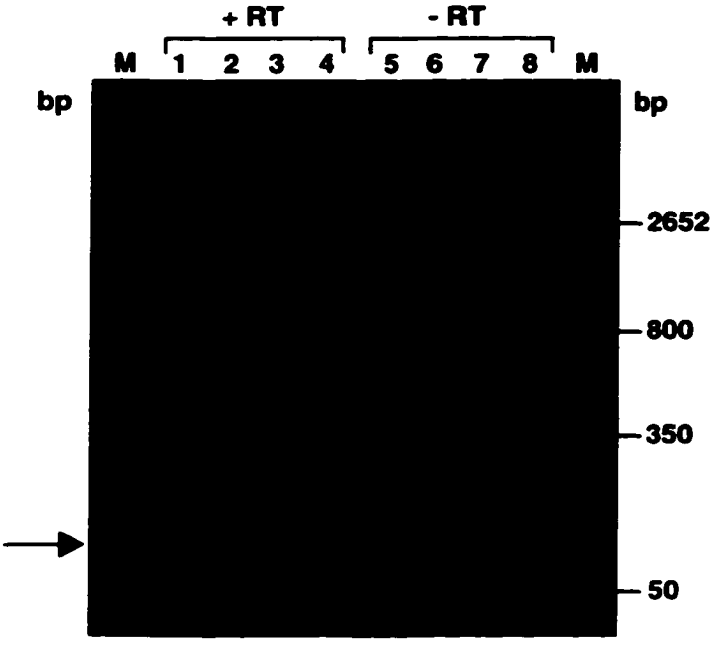


Fig. 19: Characterization of the recovered recombinant progeny viruses. RT-PCR of total RNA purified from Vero cells infected with r-OH, r-OH3NCB and parental OH-IBDV with primer pairs SB07 (sense) and SB15 (antisense) spanning the entire 3'-noncoding region of segment B yielded an expected size product around 99 bp (arrowed) (lane 1, 2 and 3) but not from Vero cells (lane 4) or the respective controls in which RT was omitted from the reaction (lanes 5, 6, 7and 8). RT-PCR products were analyzed on 2.2% agarose gel. M, 50 bp ladder.

The sequence analysis of the RT-PCR products confirmed the presence of almost identical segment B specific 3' NC sequences (5'-CCA TGA TGG GAA TCA CTC AAG AAG AGG ACA CtA ACC CCA GAC CCC gTA TCC CCG GCC TTC GCC TGC GGT-3') in the progeny virus r-OH3NCB except for two base substitutions (lower-case letters) (Fig. 20). The results also demonstrate that the 3'-NC sequences of segment B had been restored to essentially identical wild-type sequences in the recombinant virus r-OH3NCB, derived from partial length cDNA clone of segment B and full-lengh cDNA clone of segment A. The recombinant virus designated r-OH, derived from full-length cDNA clones of segments A and B had also identical 3' NC sequences as that of r-OH3NCB (5'-CCA TGA TGG GAA TCA CTC AAG AAG AGG ACA CTA ACC CCA GAC CCC GTA TCC CCG tCC TTC GCC TGC GGT-3') with an additional base substitution (lower-case letter) in the primer sequence region at the 3' end. The 3' NC sequences of segment B of both recombinant viruses were identical if the primer sequence region at the 3' end was excluded (Fig. 20). The observed mutations in the recombinant viruses divergent from the wild-type 3' NC sequences of segment B were predominantly nucleotide transitions between G and T.

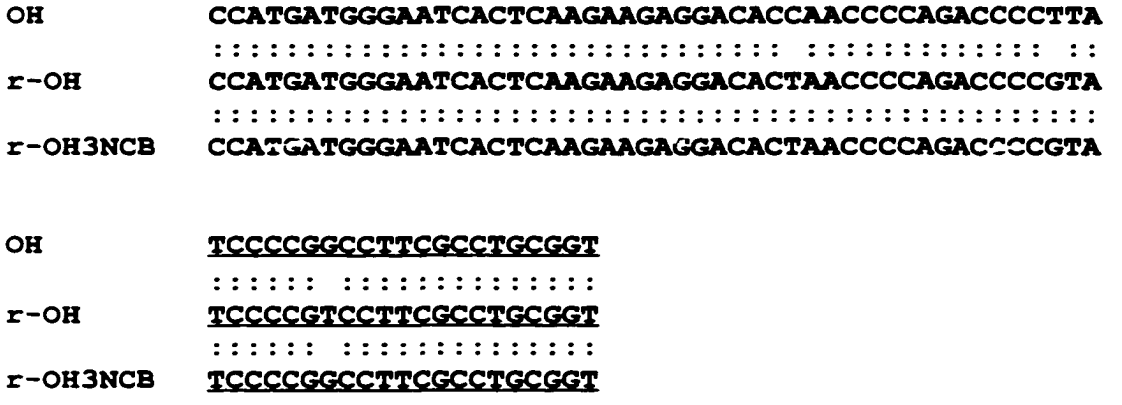


Fig. 20: Alignment of 3' NC sequences of recombinant viruses r-OH and r-OH3NCB with 3' NC sequences of segment B of parental strain OH. Gaps are introduced to align the sequences. : denotes identical nucleotides between the viruses OH, the recombinant viruses r-OH and r-OH3NCB. Primer sequence region at the 3' end is underlined.

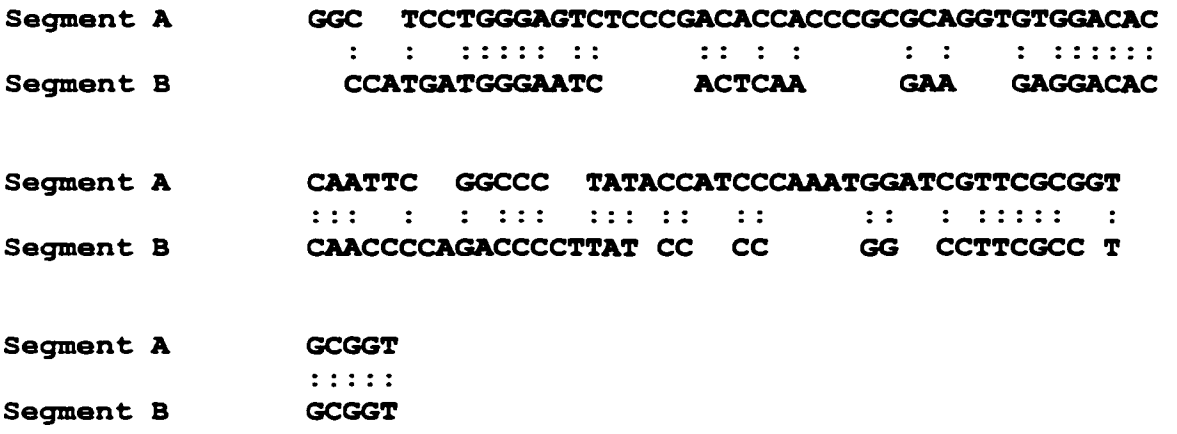


Fig. 21: Alignment of 3' NC sequences of segment A and segment B of OH-IBDV. Gaps are introduced to maximally align the sequences. : denotes identical sequences between segments A and B.

T7 RNA polymerase was expected to initiate transcription at the bona-fide 5'-terminal G-residue of cDNA clones of segments A and B of OH-IBDV (Table 11). Although the RNA transcripts derived from full-length constructs of segments A and B contained two extra non-viral residues at the 3'-end upon linearization with *Xba* I and at least 33 non-viral vector sequences at the 3' end upon similar linearization of the partial-length construct of segment B with *Xba* I, these extraneous non-viral nucleotides did not affect the recovery of infectious viruses.

4.4 DISCUSSION

Full-length cDNA of segments A and B and a partial-length cDNA of segment B without the 3'-terminal sequences of IBDV were cloned into a pUC19 vector under the control of a synthetic T7 promoter. Plus-stranded RNA transcripts were prepared by *in vitro* transcription of linearized plasmids with T7 RNA polymerase. Transfection of Vero cells with full-length segments A and B or full-length segment A and partial length segment B transcripts generated infectious virus that was indistinguishable from the wild-type IBDV. Overt cytopathic effects were observed in Vero cells within seven days upon passage of the supernatant harvested 48 h post-transfection of Vero cells. The 3' noncoding sequences (67 nts) that were not present in the partial length cDNA construct of segment B were restored in the genomic RNA of the progeny infectious virus. These sequences were of essentially

same length and similar to the wild-type segment B 3' NC sequence except for a few base substitutions.

The sequencing of cloned RT-PCR product obtained from recombinant virus r-OH3NCB with primer pair specific for the 3'-terminal sequences of segment B suggest that the 3'-terminal sequences of segment B have been restored to wild-type sequences in the recovered recombinant virus r-OH3NCB (Fig. 20). r-OH3NCB was generated from transfection of Vero cells with combined *in vitro* transcripts derived from a full-length segment A clone and a partial length segment B clone without its 3' NC sequences. The restoration of these 3' NC sequence primarily to the wild-type sequence also suggests the functional significance of the 3'-terminal sequences of segment B perhaps in assembly and packaging and in establishing a productive infection in transfected Vero cells.

Although the exact mechanism of regeneration of segment B 3' NC sequences in the recombinant virus r-OH3NCB is not known, several possibilities may be considered. A defective progeny virus may have been produced in the initial stage unable to establish a productive infection. A strong selection pressure may have been imposed to adapt and to regenerate the missing 3' NC sequences of segment B perhaps to restore some secondary structure motifs necessary for packaging in this region. Such restoration would have been facilitated at ease, taking into consideration the number of homologous sequence motifs present in the 3'

noncoding region between the two genome segments A and B (Fig. 21). Repair of deletion may have been accomplished due to polymerase jumping to homologous regions of segment A and copying accompanied by RNA editing by deletions, insetions and base substitutions by RNA dependent RNA polymerase to regenerate the segment B 3' noncoding sequences in the progeny recombinant virus r-OH3NCB essentially similar to the wild-type.

It is conceivable that the transfected *in vitro* plus-sense transcripts of full-length segment A and partial-length segment B served initially as templates for the synthesis of their respective minus-strands to direct the replication of the viral genome. This implies that a *cis*-element involved in minus-strand synthesis of segment B encoding putative RNA-dependent RNA polymerase must reside within the VP1 ORF, where presumably viral RNA polymerase had initiated the minus strand synthesis. However, the nascent segment B minus-strand being defective for generating infectious progeny virions might have undergone another round of transcription by viral RNA polymerase restoring the missing 3'-terminal sequences by possible mechanisms described earlier in the newly synthesized plus-sense strand of segment B.

The observation that infectious IBDV rescue in transfected tissue culture cells is not constrained by a strict requirement of the 3'-terminal sequences of segment B appears similar to observations reported earlier for non-segmented positive-sense

RNA virus, Venezuelan equine encephalitis virus (VEE), where a transcript from a cDNA construct containing more than two-thirds of the VEE genomic sequences but lacking the 102 nts at the 3' end of the nsp gene initiated a productive infection (Davis et al., 1989). However in case of IBDV, unlike VEE, there was regeneration of missing 3' terminal nucleotides in the recovered recombinant virus r-OH3NCB. Nonetheless, this is the first report to our knowledge on a segmented dsRNA virus where one of the transcripts derived from a partial length segment B cDNA construct of IBDV without its 3' terminal sequences had yet been able to ensure a productive infection upon transfection of Vero cells in conjunction with a transcript derived from a full-length cDNA construct of segment A. Meanwhile, it is also a remarkable observation where the missing terminal sequences in the cDNA construct of segment B of IBDV have been restored in the genomic RNA of recovered recombinant virus. It denotes the immense capacity for repair of deletions in segmented RNA viruses and highlights the significance of the 3' terminal sequences of segment B of IBDV in the viral replication cycle. It is possible that these sequences harbor cognate recognition motifs for viral or cellular factors and have an essential role in the assembly and packaging of the bisegmented viral genome in the progeny virion.

Sequence evolution and repair mechanisms have been reported for some other viruses (Olsthoorn and van Duin, 1996; Whatmore et al., 1995). In RNA phage MS2, an evolutionary reconstruction of a 19-nt deletion introduced in the

intercistronic region between the maturation and the major coat protein genes containing important regulatory and structural information has been reported (Olsthoorn and van Duin, 1996). In lentiviruses, a four amino acid deletion in the overlapping *nef* 3' long terminal repeat (LTR) region in simian immunodeficiency virus was repaired by sequence duplication and sequence evolution mechanisms *in vivo* resulting in restoration of the size of this region to the wild-type and production of a virus indistinguishable from that of the virulent wild-type (Whatmore et al., 1995). However, little is known about sequence evolution and repair mechanisms of dsRNA viruses harboring specific deletions.

In this study, the uncapped transcripts of IBDV cDNA proved to be efficient in generation of infectious progeny virus. This is not surprising, and for some viruses such as tomato bushy stunt virus (Hearne et al., 1990) uncapped transcripts have been shown to be more infectious. It is likely that a cap structure is not essential for translation of *in vitro* transcripts of IBDV since wild-type IBDV transcripts generated *in vivo* are not capped. Moreover, VP1 translated initially from the full-length and partial length transcripts of segment B may have exerted some stabilizing effect on the newly synthesized viral RNA strands with its suggested role as a primer for *in vivo* RNA synthesis (Magyar et al., 1998) and as VPg attached to the 5'-ends of the genome segments (Müller and Nitschke, 1987).

The fate of additional non-viral nucleotides that were present at the 3'-termini of transfected RNA transcripts of segments A and B of IBDV was not determined. Obviously, this did not interfere with the recovery of infectious progeny virus. In all other cases of recovered infectious clones, where this has been confirmed by either sequencing of progeny virus or molecular hybridization, extraviral 3' sequences are eliminated in cells upon viral replication and the wild-type sequence length is restored (Muelenberg et al., 1998; Yao and Vakharia, 1998; Boyer and Haenni, 1994). This has been attributed partly to host cell nucleases presumably removing the nonviral extensions and permitting the transcript to enter replication cycles. Viral RNA-dependent RNA polymerase might also play a role by initiating replication of elongated transcripts by internal recognition of their cognate binding site and removing the extra nonviral sequences in the progeny virus.

The present study demonstrates that the 3' terminal sequences of genome segment B are not required for the generation of infectious progeny virus from cloned DNA. Moreover, it was possible to recover infectious progeny viruses from transfected Vero cells and propagate them in Vero cells without resorting to CEF cells thereby demonstrating that the reverse genetics system for IBDV is highly reproducible and efficient.

The cDNA clones constructed in this study offer great potential for further manipulation of the IBDV genome. Specific IBDV mutants can be created *in vitro*

and their effect on expression, RNA replication and virulence can be tested *in vivo*. Particular attention can be focussed on suspected nucleotide differences which might influence the virulence or attenuation of IBDV. It is then possible to differentiate between silent incidental mutations versus those responsible for phenotypes such as virulence. Identifying such candidate virulence determinants might ultimately facilitate vaccine design strategies that depend on generating modified infectious IBDV strains from molecularly cloned infectious IBDV. Furthermore attenuated variants could be selected by construction of intratypic and heterotypic chimeras from infectious cDNA clones.

5. GENERAL DISCUSSION

Infectious bursal disease virus (IBDV) is an important poultry pathogen which belongs to the genus *Avibimavirus* of the family *Bimaviridae*. Despite the wide use of vaccines, the incidence of IBDV infection and associated disease problems due to immunosuppression is still common. It is considered that continual shift in antigenic components within field IBDV populations leads to the emergence of new variants and strains with enhanced virulence (Rosales et al., 1989). Even though the antigenic variation may represent an adaptive strategy of the virus, it does not explain the recent virulence variations in IBDV.

Spontaneous enhancement of virulence has occurred without any differences in antigenicity (Gaudry 1993) from the classical virulent strains. Some virulent IBDV strains from the United States show antigenic drift whereas some European and Asian isolates are highly virulent without showing significant changes in antigenicity. The sites on the viral genome involved in such virulence variations and the underlying molecular mechanisms are not fully understood (Nagarajan and Kibenge, 1997a). Considerable economic losses have been sustained due to the emergence of virulent variants of IBDV. It is speculated that nucleotide changes in genomic regions other than VP2 such as VP2-VP4,VP4-VP3 protease cleavage sites, and noncoding regions possibly contribute to the gene constellation necessary for the evolution of recent virulent strains of IBDV.

Moreover, the virulent variants are arising even in presence of maternal antibodies against standard vaccine strains of IBDV. Sequence analysis of naturally occurring avirulent, virulent and vaccine strains of IBDV have implicated several coding nucleotide differences and specific genomic regions as harboring determinants of virulence (Nagarajan and Kibenge, 1997a; Pitcovski et al., 1998; Cao et al., 1998). However, the assignment of specific mutations to a particular phenotype such as virulence has not been established.

A combination of studies were therefore initiated to understand better the mechanism of virulence of IBDV at the molecular level because it is crucial in the development of safer and more effective vaccines for control of IBD.

Because important features that modulate replication efficiency at the level of transcription may have an influence on the virulence of IBDV, crucial promoter/enhancer sequences of 5' flanking region of IBDV were mapped. A series of chimeric genes containing variable lengths of the 5' flanking sequence of segment A encoding the polyprotein and the coding region for luciferase were constructed and the activity of the promoter was measured by its capacity to direct the expression of a luciferase reporter gene. Progressive deletions of the 5' noncoding region of 131 bp of segment A of IBDV and transient transfection studies localized a minimal region of 32 bp that had maximal promoter activity without any enhancer activity (Nagarajan and Kibenge, 1997b).

The available assay systems for mapping regulatory sequences of RNA viruses until recently involved mainly reporter gene constructs encoding chloramphenicol acetyl transferase (CAT). Such systems are cumbersome and insensitive. Therefore, luciferase reporter gene constructs (pGL3-GeneLight vector constructs) were modified in the present study and used for localizing IBDV regulatory sequences. Moreover, the technique developed for localizing biologically functional promoter sequence of IBDV is much more sensitive and simple to adapt and to extend to further *in vivo* studies of putative regulatory elements of RNA viruses, particularly the dsRNA and negative-sense RNA viruses which encode their own RNA-dependent RNA polymerase (Nagarajan and Kibenge, 1998). The advantage remains where the target viral cDNA derived from any animal RNA viral genome can be positioned in either minus- or plus-sense orientation immediately upstream of the LUC reporter gene and downstream of SV40 promoter providing an *in vivo* system for generating authentic viral RNA sequences containing the initiation signals to be recognized by the viral RNA polymerase supplied by the respective virus-infected cells (Nagarajan and Kibenge, 1998).

An alternative approach for mapping the virulence determinants has been the isolation of variants which are closely related to the parental virulent strain and of their ability to escape neutralizing antibody. Hence efforts were made to isolate partial neutralization resistant mutants of IBDV under selective immune pressure *in vitro* so that phenotypic and genotypic changes can be characterized and their pathogenicity and immunogenicity can be studied in specific-pathogen free

chickens. It was reasoned that escape from immune pressure may result in the acquisition of functions that are critical determinants of pathogenicity. From the data gathered, the polyclonal antibody escape variant of QC2-IBDV selected following serial passage under immune pressure appears to have undergone changes not only in the major antigenic region but also in other parts of the genome as well such as the 3' terminal sequences of segments A and B of the viral genome during selection and growth procedures. The significance of these mutations with regard to the slightly lowered virulence exhibited by the selected variant is not presently known. Moreover, many of the recent virulent isolates have not been completely sequenced in the non-coding regions. Therefore, it is difficult at present to assess the full impact of some of the observed nucleotide changes in the 3' non-coding regions of the selected variant in the present study. However, compared to the unpassaged virus, high VN titres and a stronger immune response were elicited by live preparations of this polyclonal antibody escape variant in SPF chickens which also demonstrated slightly lowered pathology in the bursa of Fabricius. Selection of such a variant may offer promise for the development of an attenuated vaccine strain of IBDV.

Furthermore, recombinant technology now allows specific insertions or deletions in viral genomes and facilitates construction of attenuated viruses with defined mutations. Identifying the part of the virion responsible for virulence is necessary. It is then possible to create live modified vaccine strains of IBDV that are immunogenic as well as safe to use.

The assignment of particular mutations to specific phenotype has been greatly facilitated by the construction of full-length cDNA clones (Rice et al., 1987; Polo et al., 1988). Hence it was considered advantageous to develop a reverse genetics system for IBDV by constructing two independent full-length cDNA clones that contain segment A and segment B of OH strain of IBDV. It was of additional interest to define the minimum required length for successful recovery of an infectious IBDV from cloned cDNA. Hence parallel efforts were made to recover infectious IBDV progeny from a full-length clone of segment A, and a partial length clone of segment B containing its respective 5' terminal sequences and the entire coding regions without its 3' terminal sequences. Infectious progeny virus was successfully recovered in Vero cells transfected with combined transcripts of these cDNA clones. Moreover, the 3' noncoding sequences that were absent in the partial length cDNA construct of segment B were regenerated in the genomic RNA of the progeny infectious virus and the nucleotide sequence of the progeny virus in this region was almost indistinguishable from that of the wild-type virus. Such a phenomenon observed in the progeny infectious IBDV is unusual and highlights the significance of the 3' terminal sequences of segment B in the viral replication cycle. Moreover, efficient transfection of *in vitro* RNA un-capped transcripts of IBDV and subsequent recovery of infectious virus were accomplished in the Vero cell line, without the use of secondary chicken embryo cell cultures suggesting that the reverse genetics system for IBDV is highly efficient.

Infectious molecular clones of IBDV that have become available now will facilitate the study of a number of interesting phenotypes of this virus including mutants which affect morphogenesis, host range, virulence, lymphocidal and apoptotic effects of IBDV. Furthermore, site-specific genetic changes introduced into the genome of IBDV with subsequent recovery of infectious mutant viruses will enable studies related to the molecular basis for replication and pathogenicity of IBDV. Engineered deletions in the non-coding regions could be carried out for functional analysis of RNA structure and for isolation of deletion mutants to be evaluated as live attenuated vaccines (Cahour et al., 1995). Important features that modulate replication efficiency may be present in the promoter primary sequences. They may be evaluated by introducing changes in this region. Since the mutation is placed upon a known genetic background, it is possible to study the subtle effects of mutation and such studies would conceivably allow the skillful construction of IBDV with defined multiple attenuating mutations. Moreover, the ability to generate specific mutations within IBDV makes all aspects of IBDV replication amenable to study. Any mutation including those studied previously in other contexts can now be examined in the most relevant and important context, that of the virus life cycle. Through identifying multiple deleterious mutations, it should be possible to engineer several mutations affecting various steps in the virus life cycle. Such a virus would likely be highly attenuated and not able to revert to virulence.

5.1 Future Directions

The present study has added new information on the replication strategy of IBDV by mapping the promoter sequence of IBDV. The finding that infectious progeny IBDV could be successfully recovered by transfection of Vero cells with combined transcripts derived from a full-length segment A cDNA construct and a partial length segment B cDNA construct without its respective 3' noncoding sequences is also relatively new. Moreover, as an outcome of the present investigations on the passaged virus under immune pressure, some point mutations have been identified in the 3' noncoding regions of both segments A and B of the passaged virus in relation to the unpassaged virus. These sequences could be targeted for site-directed mutagenesis on cDNA to determine whether they are responsible for the slightly lowered pathogenic profile exhibited by the above passaged virus in infected chickens. The specific requirements in the VP2 region responsible for the ability of IBDV strains to infect cultured cells has been investigated extensively by using IBDV segment A chimeras and a reverse genetics system for IBDV (Mundt, 1999). However there is lack of information on any specific structural requirements in the 3' noncoding regions of IBDV genome which may be involved in tissue culture infectivity and in lowered pathogenic profile in the infected chickens. The reverse genetics systems established for IBDV could be exploited for such an endeavor.

A great deal remains to be resolved about the virulence mechanisms of IBDV and they are especially important in the context of widespread outbreaks of IBD in

recent years in vaccinated flocks. A comprehensive picture of the virulence and replication strategy of IBDV still remains elusive. However, the data gathered in the present study provide new opportunities for studies directed at the pathogenesis and replication of IBDV. The nature of the primary promoter sequence can be determined and evaluated for its effect on the expression of IBDV by introducing mutations in the identified promoter region of IBDV genome segment A encoding the polyprotein by using the reverse genetics system developed in the present study. The steady-state levels of polyprotein-specific mRNAs can be estimated at the level of transcription. By introducing desired mutations in the promoter region the level of attenuation in the recovered recombinant viruses can be evaluated by using the reverse genetics system. Such efforts may facilitate the construction of attenuated vaccine strains of IBDV.

The finding that passage of IBDV under selective immune pressure *in vitro* allows the selection of an attenuated virus is perhaps not surprising. However, the enhanced immunogenicity demonstrated by live preparations of this virus in SPF chickens is worthy of further exploration to improve upon vaccine strains that are currently available. Further experiments are necessary to evaluate other features of this selected virus under immune pressure such as its capacity to provide protection to vaccinates challenged with a virulent IBDV strain.

An appropriate framework seems to have been established by constructing molecular clones of infectious IBDV and for testing in infected chickens the

significance of various mutations identified by site-directed mutagenesis. Another approach worth pursuing would be the evaluation of the virus variant selected by passaging under limiting dilution of neutralizing antibody in the present study. Suspected nucleotide changes possibly associated with virulence or attenuation of virulence could be introduced into the respective cDNA clones of the parental virus. The modified recombinant infectious IBDV thus recovered from these cDNA clones could be evaluated for its protection efficiency in vaccinates. A superior strategy may then be adapted by engineering several mutations that can modulate the rate of replication and level of attenuation of IBDV. Moreover, such efforts would facilitate construction of better recombinant and attenuated vaccine strains of IBDV for control of infectious bursal disease.

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