TERMINAL SEQUENCES OF GENOME SEGMENTS A AND B OF SIX INFECTIOUS BURSAL DISEASE VIRUS ISOLATES

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ABSTRACT

Highly virulent, pathotypic and antigenic variant strains of infectious bursal disease virus (IBDV) are emerging causing significant levels of mortality in young chickens. Better understanding of the genome organization of IBDV, its molecular diversity and pathogenicity is essential in developing strategies for effective control of infectious bursal disease in poultry. At present, considerable information is available on the coding sequences of IBDV. However, there is a lack of information on the noncoding terminal sequences of IBDV which harbour regulatory signals for viral replication, transcription, assembly and possibly for virulence. A novel method of PCR amplification across ligated 3' and 5' end junction of viral RNA strands was developed in order to determine the terminal sequences of segmented dsRNA genome of IBDV. Genome segments A and B of six IBDV strains OH, SK9, QC-2, IN, SK140a and GLS representing the two serotypes were studied. PCR amplification across 3'-5' ligated junctions was carried out using two primers, one from available known 5' sequence and the other from available known 3' sequence bracketing the unknown terminal sequences. For segment A, a PCR product of size approximately 155 bp for strains OH, IN and SK140a, 230 bp for strain GLS and approximately 265 bp for strains SK9 and QC-2 were obtained. For segment B, almost identical 260 bp PCR products were obtained for all the six IBDV strains. The PCR products were subsequently cloned and sequenced by the dideoxy-chain termination method using T7 DNA polymerase. Segment A of strains OH, SK140a and IN and segment B of all six strains of IBDV are proposed to begin and end with conserved pentanucleotide sequences GAACC- and -GGUCU, respectively. The genome segment A of IBDV strains GLS, SK9 and QC-2 have only GAA- and -CU of the consensus sequences. These common terminal sequences of both segments A and B of the IBDV genome may serve as recognition signals for viral RNA polymerase and for assembly of genome segments into progeny virus particles. 5' and 3' terminal sequences of segment B of all six IBDV strains are more than 97% identical indicating the significance of this region as this segment encodes the putative viral RNA polymerase. However, in segment A of strains SK9, QC-2 and GLS, large insertions in the 3' terminal regions were observed. No definite sequence pattern differentiating serotype 1 and 2 strains of IBDV was observed in the noncoding regions. However the significance of deletions and substitutions identified among the strains has to be further characterized. Regardless of the considerable terminal sequence divergence observed in segment A of strains GLS, SK9 and QC-2, consensus sequence motifs were identified between all six IBDV strains which may harbour regulatory elements for virus specific functions. The total lengths of segment A and B of IBDV strain OH were established to be 3,241 bp and 2,922 bp, respectively by determining the missing extreme terminal sequences in this virus strain. This would facilitate reverse genetics studies on IBDV.

Dedication

To my parents

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COMMONLY USED ABBREVIATIONS

Terms	Abbreviations
Standard units of measurement	
centrifugal force	g
hydrogen ion activity (negative logarithm)	pH
kilobase	kb
kilocalorie	kcal
kilodalton	kDa
microgram	μg
microliter	μl
millimolar	mM
revolutions per minute	rpm
volt	V
watt	W
Greek alphabet	
alpha	α
beta	β
gamma	γ
Amino acid one letter code	
alanine	A
glycine	G
serine	S
tryptophan	W
Other terms	
adenosine triphosphate	ATP
baby grivet monkey	BGM
bacteriophage T4	T4
bacteriophage T7	T7
B lymphocyte	B-cell
bovine serum albumin	BSA
bovine viral diarrhea virus	BVDV

5-bromo 4-chloro 3-indolyl β galactoside	X-gal
chicken embryo fibroblast	CEF
chicken embyro kidney	CEK
chicken embryo skin cells	CES
complementary DNA	cDNA
cytopathic effect	CPE
deoxynucleotide triphosphates	dNTP
diethyl pyrocarbonate	DEPC
dimethyl sulphoxide	DMSO
dideoxynucleotide triphosphate	ddNTP
dithiothreitol	DTT
double-stranded	ds
drosophila X virus	DXV
enzyme linked immunosorbent assay	ELISA
Escherichia coli	*E.coli*
ethylene diamine tetraacetic acid	EDTA
five prime	5'
fowlpox virus	FPV
genome linked viral protein	VPg
guanosine triphosphate	GTP
hepatitis A virus	HAV
hepatitis C virus	HCV
infectious bronchitis virus	IBV
infectious bursal disease virus	IBDV
infectious pancreatic necrosis virus	IPNV
International Committee on Taxomomy of Viruses	ICTV
leishmania RNA virus 1	LRV1
lithium chloride	LiCl
magnesium chloride	$MgCl_2$
magnesium sulphate	$MgSO_4$
median tissue culture infective dose	$TCID_{50}$
messenger RNA	mRNA
7-methyl guanosine pyrophosphate linkage to 2'-O-methyl nucleotide	$m^7GpppAmp$
minimal essential medium	MEM
monoclonal antibodies	MAbs
natural killer cells	NK

nucleotides	nts
open reading frame	ORF
ovine kidney	OK
oyster virus	OV
polyethylene glycol	PEG
polymerase chain reaction	PCR
potassium chloride	KCl
quail fibroblast cell line	QT35
ribonucleic acid	RNA
restriction endonuclease from *E.coli* RY 13	*Eco*RI
restriction endonuclease from *Providencia stuartii*	*Pst*I
single-stranded	ss
sodium dodecyl sulphate	SDS
sodium hydroxide	NaOH
specific pathogen free	SPF
standard challenge	STC
three prime	3'
tellina virus	TV
N,N,N',N' tetramethyl 1,2-diaminoethane	TEMED
T-lymphocyte	T-cell
viral protein	VP
virus neutralization	VN

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens (reviewed by Kibenge *et al.*, 1988a). The etiological agent, IBD virus (IBDV), has predilection for cells of the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B cell lineage (Burkhardt and Müller, 1987). The chickens are most susceptible at 3-6 weeks of age, and the clinical infection results in bursal atrophy, impaired growth, skeletal muscle hemorrhages and death. Economic losses to poultry industry result either from high mortality rates due to an acute course of the disease or from a chronic form of the disease due to B-cell dependent immunodeficiency resulting in immunosuppression. The latter exacerbates infections caused by other etiologic agents and interferes with effective vaccination of chickens against other diseases such as Newcastle disease, Marek's disease and infectious bronchitis. Therefore, effective control of infectious bursal disease (IBD) is of great economic significance to the poultry industry.

Infectious bursal disease virus belongs to the family *Birnaviridae.* Members of the family are characterized by a bisegmented double stranded (ds)RNA genome consisting of two segments A and B within a single-shelled icosahedral capsid of 60 nm (Dobos *et al.*, 1979). Other members of the family include infectious

pancreatic necrosis virus (IPNV) of young salmonid fishes, Drosophila X virus (DXV) of *Culicoides* sp, and tellina virus (TV), oyster virus (OV) and crab virus of bivalve molluscs (Bonami and Adams, 1991; Dobos, 1991).

There are two distinct serotypes of IBDV, 1 and 2 (Jackwood *et al.*, 1982); only serotype 1 strains cause disease. All known serotype 2 strains are naturally avirulent for chickens (Ismail *et al.*, 1988). In the past few years, highly virulent antigenic variants of serotype 1 strains of IBDV which are not neutralized by antibodies against standard challenge strains have emerged causing significant levels of mortality in young chickens (Heine *et al.*, 1991; Sharma *et al.*, 1989; Jackwood and Saif, 1987).

The nucleotide sequences of genome segment A of at least six serotype 1 IBDV strains, PBG98, Cu-1, 52/70 (Bayliss *et al.*, 1990), 002-73 (Hudson *et al.*, 1986), STC (Kibenge *et al.,* 1990), and GLS (Vakharia *et al.*, 1994) and one serotype 2 IBDV strain, OH (Kibenge *et al.*, 1991), and the nucleotide sequences of genome segment B of two IBDV strains, 002-73 (Morgan *et al.*, 1988) and OH (Dybing, 1992), have been determined. Sequence comparisons of the coding region of IBDV reveal amino acid differences in the VP2 region between serotype 1 and serotype 2 strains and between the highly virulent antigenic variants and the standard strains of IBDV. Not much information is available about the noncoding sequences of IBDV. In fact it is not known whether the

published sequences represent the complete genomic RNA sequence of IBDV since both 5' and 3' ends of genome segments A and B have not been determined.

The present study was undertaken to determine the complete terminal sequences of IBDV. A serotype 2 IBDV strain OH has been sequenced on genome segment A (Kibenge *et al.*, 1991) and B (Dybing, 1992) following random priming and reverse transcription and subsequent cDNA cloning. Almost the complete sequence of this virus is known except the extreme terminal sequences. In addition to OH-IBDV, another serotype 2 strain and four serotype 1 strains were selected for terminal sequence determination so as to compare and identify likely consensus sequences that may be involved in regulatory functions, and possible nucleotide residues that may play a part in virus virulence. It is considered that this would facilitate reverse genetics studies on IBDV to understand better the molecular basis of birnavirus diversity and the mechanism of viral pathogenicity.

1.2 Infectious Bursal Disease Virus

1.2.1 Classification

Infectious bursal disease virus (IBDV) is a member of the virus family *Birnaviridae* of the genus *Avibirnavirus* (Dr. Peter Dobos, personal communication, March 1995). Until now the family *Birnaviridae* contained only

one genus *Birnavirus*. The need for the possible division of the family *Birnaviridae* into three different genera was realized recently; one of the reasons being that there was no identity in the noncoding sequences between IBDV and infectious pancreatic necrosis virus (IPNV) (Kibenge *et al.*, 1990), another member of the same family. This was surprising and difficult to explain since both IBDV and IPNV belonged to the same genus and the coding regions of their genomes showed almost perfect identity in organization and function (Kibenge *et al.*, 1990). In fact, one would expect fewer similarities among coding sequences, but significant similarities among noncoding regions of the viral genome, as has been reported for RNA viruses belonging to the families *Bunyaviridae* (Clerx-Van Haaster *et al.*, 1982), *Reoviridae* (Antczak *et al.*, 1982; Li *et al.*, 1980; Omura *et al.*, 1988; Yan *et al.*, 1992) and *Picornaviridae* (Porter *et al.*, 1978). In these families, there is significant similarity in the noncoding regions for viruses belonging to the same genus but little terminal sequence similarity for viruses representing different genera. In 1993, the International Committee on Taxonomy of Viruses (ICTV), Birnavirus study group proposed the subdivision of the family *Birnaviridae* into three genera: *Avibirnavirus* for IBDV, *Aquabirnavirus* for IPNV, TV, OV, and *Entomobirnavirus* for Drosophila X virus and the proposal has been accepted (Dr. Peter Dobos, personal communication, March 1995).

1.2.2 Genome Organization

The larger genome segment A (approx. 3300 bp) consists of two open reading frames (ORF), a small ORF encoding VP5 (Kibenge *et al.*, 1990; 1991; Dybing, 1992; Mundt *et al.*, 1995a) and the larger ORF encoding a precursor polyprotein that is processed into mature VP2, VP4 and VP3 (Hudson *et al.*, 1986; Müller and Nitschke, 1987b). Mundt *et al.* (1995a) have recently expressed VP5 of strain P2-IBDV in infected chicken cells in tissue culture and in bursae of infected birds and identified a 21 kDa protein. The function of VP5 is unknown. VP4 is a viral protease involved in the processing of the precursor polyprotein (Azad *et al.*, 1987; Jagadish *et al.*, 1988). VP2 and VP3 are the major structural proteins of the viral capsid. VP2 contains the antigenic region responsible for eliciting neutralizing antibodies and serotype specificity, whereas VP3 contains the group-specific antigens. Genome segment B (approx. 2800 bp) has one ORF which encodes VP1, the dsRNA dependent RNA polymerase (Morgan *et al* ., 1988; Müller and Nitschke 1987a; Spies *et al.*, 1987). Some virulent IBDV strains from the United States show antigenic drift whereas some European and Japanese isolates are highly virulent without showing changes in antigenicity. The role of noncoding regions of the viral genome in genetic diversity and in virulence of IBDV is not presently known.

1.2.2.1 Segment A Coding Sequence

Sequence analysis of genome segment A has helped in identification of amino acid residues that are responsible for antigenic variation (Bayliss *et al.*, 1990; Heine *et al.*, 1991; Lin *et al.*, 1992; Schnitzler *et al.*, 1993, Vakharia *et al.*, 1994). Two prominent symmetrically spaced hydrophilic areas are recognized in a variable region in VP2 which harbour the exchanged amino acids (Schnitzler *et al.*, 1993). It has been suggested that the first hydrophilic region may be responsible for stabilizing the conformation epitope whereas the second hydrophilic region may be responsible for inducing virus neutralizing antibodies (Heine *et al.*, 1991). The hydrophilic regions and the internal sequences of VP2 are not conserved between serotype 2 and the serotype 1 strains. A serine rich heptapeptide S-W-S-A-S-G-S adjacent to the second hydrophilic region of VP2 is conserved only in the highly pathogenic serotype 1 strains of IBDV (Bayliss *et al.*, 1990). It has been hypothesized that the exchange of one amino acid in the hydrophilic areas 1 and 2 results in an antigenic drift (Schnitzler *et al.*, 1993). More exchanges may result in serotype 1 variant strains (Heine *et al.*, 1991). Replacement of four amino acids with the concomitant loss of hydrophilicity in hydrophilic region 1, and particularly in region 2 may result in antigenic shift and in emergence of a new serotype (Schnitzler *et al.*, 1993). Vakharia *et al.* (1994) compared the sequences of segment A of three variant strains of IBDV (GLS, DS326 and Delaware E) with the STC strain and found that the sequences were

very similar to STC in the VP4 and VP3 regions; most of the nucleotide and deduced amino acid changes occurred in the variable region of the VP2 gene.

1.2.2.2 Segment B Coding Sequence

Between the two genome segment B sequences determined so far of strains 002-73 of serotype 1 and OH of serotype 2, there is an overall nucleotide sequence identity of 90.7% and an amino acid sequence identity of 96% indicating very high conservation between the two serotypes (Qian and Kibenge, 1994). VP1, a 90-kDa protein (Azad *et al.*, 1985), has been suggested to circularize both segments A and B by tightly binding to their ends (Müller and Nitschke, 1987). Calvert *et al.* (1991) have demonstrated that VP1 as genome linked viral protein (VPg) is attached to the 5' ends of both genome segments of IPNV by a serine-5'-GMP phosphodiester bond. Since IBDV behaved similarly to IPNV during *in vitro* guanylylation reactions (Dobos 1993), VP1 of IBDV is presumed to be linked to the guanine residue at the 5' terminus. The exact amino acid involved in the linkage has not yet been identified for IBDV.

1.2.2.3 Noncoding Sequences of IBDV

It is not known whether the terminal sequence information of the IBDV genome is complete. The nucleotide sequences of the genome segments A and B obtained so far may not represent the complete genomic RNA sequence since both 5' and 3' terminal ends are not yet identified. The cDNAs used for

sequencing were synthesized with random hexanucleotides derived from calf thymus DNA as primers and it is possible that the terminal sequences were not present in these cDNA libraries. Even by direct RNA sequencing using specific primers which primed at terminal sites in the available DNA sequence, it was not possible to extend the sequence analysis to the terminal ends of genome segment B of IBDV strain 002-73 (Morgan *et al.*, 1988). This may have been due to inaccessibility of the termini of the viral RNA molecules because of VPg at the terminal ends.

Generally, the noncoding sequences of the genomes of many RNA viruses are of substantial interest as they contain several important regulatory elements. These include signals for replication (Kuge *et al.*, 1987; Niesters *et al.*, 1990; Rohll *et al.*, 1994), transcription (Schubert *et al.*, 1981; Shaad *et al.*, 1993), translation initiation (Jang *et al.*, 1988; Fukushi *et al.*, 1994; Levis *et al.*, 1993; Pelletier *et al.*, 1988, Perdue *et al.*,1982; Svitkin *et al.*, 1988), assembly and encapsidation (Blumberg *et al.*, 1983; 1991; Zou and Brown, 1992) and promoter sites for polymerase binding and initiation of transcription (Keene *et al.*, 1981; Fodor *et al.*, 1993; 1994).

In some viruses the consensus sequences in the 5'- and 3'- noncoding regions are functionally important for the phenotype of the viruses. Of interest in this respect are nucleotide positions 10-34 in the 5' noncoding region of poliovirus

PV2. They have a potential to form a stable stem and loop structure and a deletion of base 10 in this region destabilizes this structure resulting in a mutant virus with the temperature sensitive phenotype (Sarnow *et al.*, 1986). The virulence or the attenuation of virulence has also been attributed to the conservation of certain nucleotide residues in the non-coding region of viral genomes (Almond, 1987; Pilipenko *et al.*, 1989; Lipton *et al.*, 1991; Macadam *et al.*, 1991, Kuhn *et al.*, 1992; Lawrenz-Smith *et al.*, 1994; Westrop *et al.*, 1989). The comparison of the nucleotide sequences of vaccine strains of poliovirus with those of the parental strains showed that there are several base substitutions scattered along the entire genome. Among the mutations identified as being important, those that are located in the middle of the 5'-untranslated region surrounding nucleotide positions 472-480 were reported to be involved in attenuation of neurovirulence (Evans *et al.*, 1985; Muzychenko *et al.*, 1991; Pilipenko *et al.*, 1989). Since the noncoding sequences are involved in the regulation of a number of virus specific functions, determining the complete genome sequence of IBDV is essential.

1.2.3 Replication

Replication of IBDV takes place within the cytoplasm. The virion associated dsRNA dependent RNA polymerase, VP1 (Spies *et al.*, 1987), acts as viral replicase as well as transcriptase. Based on the observation of a template-specific sequential addition of guanosine residues to VP1 to form VP1pGpG

complexes, it has been suggested that VP1 may also act as a primer during initiation of viral RNA synthesis (Dobos, 1993; 1995) similarly to VPgs in other viruses (Salas, 1991). *In vitro* single-stranded (ss)RNA synthesis studies using IPNV have shown that the viral RNA polymerase synthesizes virus ssRNA by a semi-conservative strand displacement mechanism, whereby the nascent strand displaces one of the parental strands (Spies *et al.*, 1987; Mertens *et al.*, 1982; Bernard, 1980). Two genome length mRNA transcripts are made that are translated into five different proteins. It has also been shown that the transcription of birnaviruses can be initiated when the virus has penetrated the host cell without any need for uncoating or degradation of the capsid (Spies *et al.*, 1987).

The reassortant IBDV isolates containing segment A from the virulent serotype 1 strain Cu-1 and segment B from the non-pathogenic serotype 2 strain 23/82 were shown to be non-lethal and exhibited only slight bursal lesions similar to those caused by some vaccine strains. This shows that both genome segments of a virulent strain are essential for optimal viral replication in chickens (Müller *et al.*, 1992).

IBDV is able to replicate and cause cytopathic effect (CPE) in primary cell cultures of chicken origin including chicken bursal lymphoid cells, chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) cells. The virus has

also been adapted to grow in several mammalian continuous cell lines such as Vero cells derived from African green monkey kidney (Lukert *et al.*, 1975), BGM-70 derived from baby grivet monkey kidney (Jackwood *et al.*, 1987) and QT35, a quail fibroblast cell line QT35 (Cowen and Braune, 1988). IBDV specific polypeptides have been identified in chicken bursal lymphoid cells as early as 90 min after infection and mature viral polypeptides have been demonstrated in the culture supernatant 6 h after infection. Because of a more extensive phase of maturation and less tendency to produce defective interfering particles (Kibenge *et al.*, 1988a), higher yields of virus for experimental studies were obtained when the virus strains were grown in Vero cells than in CEK cells (Kibenge *et al.*, 1988b).

1.2.4 Disease

IBDV infected chickens less than 3 weeks of age usually do not exhibit clinical signs but are immunosuppressed whereas those between 3 and 6 weeks of age show characteristic gross and microscopic bursal lesions. The lymphoid cells and macrophages in the gut carry the virus to the bursa of Fabricius, which increases in size due to inflammation followed by atrophy (Müller *et al.*, 1979). Some of the recent field isolates of IBDV, which are antigenic variants of serotype 1, do not induce inflammation but bursal atrophy due to destruction of lymphocytes (Snyder, 1990; Lukert & Saif, 1991). Vasconcelos and Lam (1994) studied this unusual phenomenon *in-vitro* using IBDV infected chicken peripheral

lymphocytes and found features typical of apoptosis (Cohen, 1991; Jeurissen *et al.*, 1992). These findings suggest that IBDV, in addition to causing necrosis, can also induce apoptosis in avian lymphocytes. Recently, chickens infected with some of the highly virulent Japanese strains have been shown to develop not only bursal lesions but also thymic and bone marrow lesions (Tsukamoto *et al.*, 1992). Inoue *et al.* (1994) found that the depletion of cortical thymocytes is due to apoptosis following infection with a highly virulent Japanese IBDV strain HPS-2.

1.2.4.1 Immunosuppression

Infection with IBDV at an early age significantly affects both humoral and local immune responses of chickens (reviewed by Saif, 1991). The virus seems to have a predilection for actively proliferating cells such as the immature or precursor B cells (Sivanandan and Maheswaran, 1980b). Infection causes severe necrosis, lymphoid depletion, and subsequent immunosuppression. Adverse effects on the local immune system of chicks 1 to 5 days of age, following IBDV infection have also been reported with significant reduction in plasma cells in the harderian gland of chicks (Dohms *et al.*, 1988). Nusbaum *et al.* (1988) observed a less severe effect on cell-mediated immune responses such as suppression of T-cell response. No consistent effect on natural killer cells (NK) cell activity has been detected (Sharma and Lee, 1984). Other mechanisms of immunosuppression such as the development of suppressor cells

in the spleen of infected chicks have also been suggested (Sharma *et al.*, 1989b).

1.2.4.2 Antigenic and Virulence Characteristics

IBDV capsid proteins VP2 and VP3 contain epitopes that determine group antigenicity (Becht *et al.*, 1988) which can be detected by the agar gel precipitation test and the fluorescent antibody test. In addition, VP2 contains the serotype-specific antigens responsible for inducing virus neutralizing antibodies. Among IBDV strains, sequence analysis of the VP2 region of genome segment A has revealed considerable variation between amino acid residues 206 to 350 (Bayliss *et al.*, 1990; Håvarstein *et al.*, 1990; Kibenge *et al.*, 1990). This hypervariable region, about 151-152 amino acid residues long (Kibenge *et al.*, 1991) encodes the conformation dependent epitope recognized by VN MAb 17/82 (Azad *et al.*, 1987) and the serotype-specific epitope of IBDV (Becht *et al.*, 1988).

Since 1985, antigenic shift in IBDV field populations has been recognized with the isolation of variant strains Delaware E (E/Del) (Rosenberger *et al.*, 1985), GLS and DS326 variant strains (Snyder *et al.*, 1988c). It has been found that these strains are not only antigenic variants, but are also pathotypic variants and differ from standard strains in causing an extremely rapid bursal atrophy without severe inflammation, haemorrhage or edema of the bursa of Fabricius. Infection

with these strains rarely cause mortality. Splenomegaly is a common feature of infection with these virus variants (Snyder, 1990). The pathogenic viruses isolated in the United States which cause classical Gumboro disease lesions (i.e. mortality, haemorrhagic bursas, edematous and yellowish discolouration of bursa of Fabricius) and all commercial vaccines derived from these standard virus strains carry the neutralization epitope defined by MAb B69. But the "virulent antigenic and pathotypic variants" (GLS, E/Del and DS326) do not carry the epitope defined by MAb B69. Vakharia *et al.* (1994) have shown that most of the amino acid substitutions in the US variants (GLS, DS326 and E/Del) occur in the central region between residues 212 to 332, especially in the two hydrophilic regions between residues 212-223 and residues 314-324 of VP2.

Recently "very virulent" (VV) strains of IBDV have been reported in Netherlands (Box, 1989), Great Britain (Chettle *et al.*, 1989), Belgium (Van Den Berg *et al.*, 1991), Germany (Öppling *et al.*, 1991), and France (Enterradossi *et al.*, 1992). In France, mortality rates close to 70% have been reported in broiler and pullet flocks. These strains cause bursal lesions and inflammation typical of standard strains of IBDV, do not show changes in antigenicity and carry the B69 epitope found on all standard strains (Snyder, 1990), but differ from the standard strains in causing high mortality rates. These variant strains could be referred to as "very virulent" pathotypic and nonantigenic variants to differentiate them from the "very virulent" pathotypic and antigenic variants of the United States (strains

GLS, E/Del and DS326). However both types are alike in causing infection even in presence of protective maternal antibody against the standard strains (Chettle *et al.*, 1989). Since the latter part of 1990, acute cases of IBD with more than 50% mortality in layer flocks have been reported in Japan (Nunoya *et al.*, 1992; Tsukamoto *et al.*, 1992, Lin *et al.*, 1993) caused by strains similar to the European "very virulent" strains.

By comparing 582 bases in the VP2 coding region, Brown *et al.* (1994) found that the "very virulent" UK isolates were closely related to each other, but differed by at least four amino acids from the standard strains. On the other hand, the UK isolates were very similar to a highly virulent Japanese isolate with no amino acid changes in the VP2 coding region. Lin *et al.* (1993) has suggested that the prevailing highly virulent strains of IBDV in Japan might have originated from the "very virulent" European strains of IBDV, possibly in the imported attenuated IBDV vaccines. In the highly virulent Japanese isolates, Lin *et al.* (1993) identified specific amino acid exchanges at two positions (position 2 and 74) within the variable region but there were no common amino acid substitutions associated with the virulence of the virus.

Among some American strains, a 14-amino acid segment containing 11 amino acid mismatches between the non-pathogenic serotype 2 OH-IBDV and pathogenic serotype 1 strains were observed in the first hydrophilic area of VP2

that may correspond to the serotype-specific antigenic site associated with virulence (Dybing, 1992). Serotype 2 OH-IBDV also lacks the conserved amino acid sequence motif, S-W-S-A-S-G-S (Bayliss *et al.*,1990) found in the VP2 region of all virulent strains. However, mutations present in other parts of the viral genome such as the noncoding regions may also contribute to the virulence of IBDV especially of the recently emerging pathotypic variants but this remains to be determined.

1.2.4.3 Control of IBDV

Traditionally a combination of live and inactivated vaccines have been used in the parent breeder flocks to induce the production of high levels of maternal antibody in the broiler progeny (McFerran *et al.*, 1981, Wyeth and Chettle, 1990). The maternal antibody prevents early infections thereby eliminating immunosuppression. However, since 1988, highly virulent strains of IBDV have emerged and are becoming a major problem in the poultry industry. Therefore, the need for developing newer vaccines has become crucial.

With increased knowledge in the molecular structure and immunology of IBDV, better attenuated and genetically engineered vaccines are being continuously developed. Structural protein genes of IBDV have been expressed in fowlpox and baculovirus-vector systems. VP2 from a virulent IBDV strain 52/70, has been expressed as a β-galactosidase fusion protein in a recombinant fowlpox

virus, fpIBD 1, and shown to protect against mortality, but not against damage to the bursa of Fabricius (Bay'iss *et al.*, 1991). Recombinant FPV-VP2 containing the VP2 encoding region under the control of the fowlpox early/late promoter inserted immediately downstream of the thymidine kinase gene was expressed in chicken embryo skin cells (CES) and a significant level of protection was provided by FPV-VP2 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-derived IBDV antigens were found to confer 79% protection against subclinical IBDV infection. The entire structural protein genes (the large ORF), of variant strain GLS, were expressed using a recombinant baculovirus vIBD7 (Vakharia *et al.*, 1993; 1994). A chimeric cDNA clone of the large segment A of GLS, encoding VP3, VP4 and the VP2 modified to encode the B69 epitope, has been expressed in a recombinant baculovirus. This subunit vaccine has been found to confer active protection to specific-pathogen free chickens against virulent challenge with the standard IM and STC strains as well as against the variant E/Del and GLS IBDV strains (Snyder *et al.*, 1994).

The ability of many of the recombinant vaccines described above to induce high titer, long lasting VN antibody responses in hens primed by an injection of subunit vaccine would result in the protection of their progeny for many weeks

after hatching. Recombinant proteins used in these studies can also be produced relatively cheaply. Yet these vaccines have to be constantly modified for production of the VP2 of variants of IBDV since highly virulent variant field strains are emerging. Unfortunately, some of these variant strains do not even exhibit significant antigenic shift and the molecular basis for their variation is not clearly understood.

Another major concern with the subunit vaccines is that the immune response induced by them is so specific that they may exert a selective pressure on the wild virus, resulting in even more antigenic variants (Kibenge *et al.*, 1988a). Also there are other limitations as to the duration and magnitude of the immune response and the cost and convenience of administering these vaccines.

With the better understanding of the molecular basis of virulence and recent advances in recombinant DNA technology (Chanock, 1982), it is possible to construct modified viruses with defined mutations (Kit *et al.*, 1985) that are safe and immunogenic. For developing such genetically engineered viruses for control of IBDV infection, it is essential to identify parts of IBDV genome that are likely to contain the virus virulence determinants and regulatory elements.

1.2.4.4 Diagnosis of IBDV

Despite improved vaccination programmes, outbreaks of IBD still occur. Prompt and early diagnosis of IBD in commercial poultry operations is important for effective control. Methods generally employed for IBDV diagnosis include detection of antibodies by agar gel precipitation, VN, ELISA and fluorescent antibody staining (Lukert and Saif, 1991; Rosenberger, 1989; Snyder *et al.*, 1988; Lee *et al.*, 1992). With increased knowledge of sequence data of the IBDV genome of various isolates, more sensitive and rapid methods are becoming available using either radiolabeled (Jackwood *et al.*, 1989, Kibenge, 1992) or nonisotpic labeled (Jackwood *et al.*, 1990, Lee *et al.*, 1992) cDNA probes. The polymerase chain reaction (PCR) also provides extremely sensitive methods for IBDV diagnosis (Wu *et al.*, 1992; Lee *et al.*, 1992,1994; Stram *et al.*, 1994). For developing rapid and effective control strategies, it is also important to determine the subtype specificity of IBDV. Jackwood and Jackwood (1994) have reported that it is possible to differentiate some antigenic variant strains of IBDV from the standard strains by PCR amplification of a relatively conserved sequence region of VP2 followed by restriction analysis. The variant strains (variant A, MD and 1048E) were found to have a unique pattern of restriction sites not present in standard strains like STC, 52/70, PBG98, Cu-1 and 002-73.

1.3 Nucleotide Sequencing Methods Used for IBDV

Analysis of the IBDV genome in relation to gene expression and antigenic diversity has been facilitated by the availability of improved sequencing techniques. The dideoxy-chain termination sequencing method (Sanger *et al.*, 1977b) using the modified bacteriophage T7 DNA polymerase (Tabor and Richardson, 1987) has been most commonly used. This method is simpler and faster than the Maxam and Gilbert chemical sequencing method (Maxam and Gilbert, 1977). Bayliss *et al.* (1990) have used Maxam and Gilbert chemical degradation method in addition to the dideoxy-chain termination method to determine the sequence of IBDV strain 52/70.

Regardless of the method used for sequence analysis of IBDV, the dsRNA genome was first converted into cDNA. For this, the first strand cDNA was initially synthesized from denatured dsRNA by extension of random primers with reverse transcriptase. Second strand cDNA synthesis is with DNA polymerase I (Gubler and Hoffman, 1983). Kibenge *et al.* (1991) ligated the ds cDNA into the *PstI* site of plasmid pUC9 via GC-tailing, followed by transformation of competent *E.coli* cells (Sambrook *et al*, 1989). IBDV specific cDNA clones identified by colony blot hybridization were subsequently analyzed for plasmid size. The sequencing was carried out on plasmid DNA isolated from selected clones until the complete ORFs of genome segments A (Kibenge *et al.* 1991) and B (Dybing, 1992) were obtained from overlapping cDNA inserts. Bayliss *et*

al. (1990) also used random primers to construct the cDNA clones. The sequences at the 5' end of the coding strand of the strain 52/70 were determined by primer extension and Maxam and Gilbert sequencing method. Yet Bayliss *et al.* (1990) were unable to identify unambiguously the extreme terminal end nucleotides. Vakharia *et al.* (1992) amplified the large genome segment A of the two IBDV variant strains, DS326 and E/DEL, by PCR using specific primers that are homologous to the conserved regions of variant strain GLS and cloned the cDNA into a pCR 1000 vector for sequence analysis. The sequence of the variant GLS strain was initially obtained from clones made by random priming of reverse transcription and standard cloning procedures. None of the protocols used to date resulted in isolation of clones containing the complete terminal sequences of the genome segments.

Generally, it has been difficult to determine the 5' and 3' terminal sequences. The terminal cDNA clones are missing in the cDNA libraries either due to premature termination of the reverse transcriptase or due to the presence of extended secondary structures in the terminal regions or simply because of insufficient amounts of high quality RNA molecules to start with. Incomplete removal of VP1, the genome-linked viral protein, from the terminal ends of IBDV might also have resulted in viral RNA molecules with inaccessible termini.

1.3.1 Improved Methods for Terminal Sequence Determination

Many improved methods for determining terminal sequences are becoming available. Various modifications of RNA-PCR based primarily on reverse transcription of RNA followed by PCR (RT-PCR or RNA-PCR) (Hultman *et al.*, 1989) have been employed to determine the terminal sequences of genes.

For rapid amplification of cDNA ends (RACE), anchor sequences can be added to single-stranded cDNA either by homopolymeric tailing with terminal transferase or by ligating oligonucleotides to the cDNA with T4 RNA ligase. Such anchor sequences then serve as suitable primer binding sites for subsequent PCR (Edwards *et al.*, 1991). The tailing of the 3' ends of ssRNA is carried out with *E. coli* poly(A) polymerase. It is then reverse transcribed to cDNA with oligo $(dT)_{20}$ primer and reverse transcriptase. The resulting cDNA is subsequently amplified by PCR with oligo $(dT)_{20}$ and a sense primer. The 3' end can also be tailed with poly(C) using terminal deoxynucleotide transferase and further PCR amplification can be carried on the tailed template using gene-specific primer from the known terminal viral sequences along with an oligo $(dG)_{12\text{-}18}$ primer (Christ, 1992). For determining the 5' end of Hepatitis C virus (HCV), it was initially tailed with dATP by calf thymus terminal deoxynucleotide transferase. It was then reverse transcribed and amplified by PCR with oligo $(dT)_{20}$ and an antisense primer deduced from the 5' noncoding regions of HCV isolates (Okomoto *et al.*, 1991).

It is also possible to anchor an RNA molecule to a linearized plasmid DNA and to advance initiation of cDNA synthesis by reverse transcriptase (Mueller *et al.*, 1993). Briefly, ssRNAs ending with 3' OH group are covalently linked to the 5' monophosphate overhang of an *Eco*RI linearized vector DNA by T4 RNA ligase. The cDNA synthesis is then directly initiated at the 3' OH group of the restriction site by reverse transcriptase. PCR amplification of the cDNA product is further carried out with a specific vector plus-strand primer and a 5' terminally labeled primer complementary to the 3' region of the cDNA product, using *Taq* DNA polymerase. The resulting PCR product is then directly sequenced with a nested, vector specific plus-strand primer using T7 DNA polymerase.

A protocol that is considered to be particularly useful for the analysis of terminal sequences of RNAs that are otherwise difficult to obtain involves circularization of RNA molecules by ligation of the 3' and 5' ends of the viral genomic RNA strands using T4 RNA ligase (Romaniuk and Uhlenbeck, 1983) followed by cDNA synthesis. PCR amplification (Saiki *et al.*, 1988) across the ligated 3'-5' end junction is then carried out using two specific primers, one from the available 5' sequence and the other from the available 3' sequence, flanking the ligated sequences containing the unknown terminal region (Ochman *et al.*, 1990). The PCR products are subsequently cloned and sequenced by dideoxy-chain termination method (Sanger *et al.*, 1977). Termini of many closely related RNA molecules can be determined by using the same set of primers.

In the past few years, variations of such a protocol have been successfully used to obtain the terminal sequences of single-stranded RNA of flavivirus (Mandl *et al.*, 1991), pestivirus (Brock *et al.*, 1992) and arterivirus (Chen *et al.*, 1994). The genomic RNA of many of these single-stranded positive RNA viruses carry a type 1 cap structure (m^7GpppAmp) at its 5' ends. The capped end of the genomic RNA was removed using tobacco acid pyrophosphatase (TAP), leaving a 5' monophosphate for ligation by T4 RNA ligase to the 3' OH end. The genomic RNA thus circularized or tandemly ligated was then heat-denatured, reverse transcribed and amplified across the ligated ends by PCR. For PCR, a set of primers selected from the available sequences bracketing the unknown terminal sequences were used. Widmer (1994) also determined successfully the terminal sequences of single segmented, double-stranded RNA genome of Leishmania RNA virus (LRV1) by using the same protocol as above with slight modifications. Since the genomic dsRNA is not capped, TAP was not used. To date none of the researchers have adapted this novel protocol of 3'-5' end ligation to obtain the terminal sequences of segmented RNA viruses containing more than one genomic segment and particularly of double-stranded segmented RNA viruses such as reoviruses, rotaviruses and birnaviruses. Of particular interest in the present study are the terminal sequences of bisegmented double stranded RNA genome of IBDV. Furthermore, the genome segment A and B of IBDV and a closely related member of the same group IPNV are rather unique when compared to other segmented dsRNA viruses, in having a viral protein

VP1 attached to their terminal ends (Müller and Nitschke, 1987; Calvert *et al.*, 1991; Dobos, 1993). Hence, in the present study the protocol of 3'-5' end ligation was adapted, appropriately modified and developed further to obtain the terminal sequences of genome segments A and B of IBDV. Moreover this method was considered to be the method of choice for determining terminal sequences since both 5' and 3' terminal sequences would be obtained simultaneously.

1.4 Research Objectives

1) To determine the terminal sequences of genome segments A and B of six strains of IBDV by 3'-5' end ligation; four strains of serotype 1 and two strains of serotype 2;

2) To compare these nucleotide sequences and to identify their 5' and 3' ends;

3) To analyze the terminal sequences for blocks of conserved sequences that may contain regulatory signals important in viral replication; specifically to find any structures that may be associated with virus specific functions.

4) To establish the complete nucleotide sequence of IBDV strain OH, for both segments A and B by finding the missing extreme terminal sequences.

2. MATERIALS AND METHODS

2.1 Viruses

Six IBDV strains were used in the present study (Table 1). Strains SK140a, QC-2, IN and GLS are of serotype 1 whereas OH and SK9 are of serotype 2. The eleventh cell culture passage of the OH strain was kindly supplied by Dr. Y. Saif of Ohio State University and the third cell culture passage of the IN strain was kindly supplied by Dr. A. Silim, University of Montreal, St.Hyacinthe. OH-IBDV is nonpathogenic in chickens (Ismail *et al.*,1988) and was originally isolated from a turkey flock in Ohio (Jackwood *et al.*, 1982). The IN strain is a serotype 1 antigenic variant that was isolated from a large commercial layer flock in Indiana (Ismail *et al.*, 1990) which had adequate levels of maternal antibodies at hatch to standard IBDV and was vaccinated with a live IBD commercial vaccine (strain UV) at 28 and 35 days of age. Strains SK140a and SK9 were field isolates obtained in the second egg passage from Sackville Animal Pathology Laboratory, Sackville, New Brunswick. Strain GLS is a variant of serotype 1 which was kindly provided by Dr. J. Giambrone, Auburn University. Strain GLS is characterized by the absence of MAb-defined neutralizing sites R63 and B69, present on all standard IBDV strains and considerable economic losses have been reported since its emergence (Snyder *et al.*, 1992). Strain QC-2 was kindly supplied by Dr. A. Silim, University of Montreal, St. Hyacinthe. It was isolated in Quebec from the bone marrow of commercial layer flocks showing respiratory

Table 1. List of Selected IBDV Strains

Strain	Serotype	Reference
OH	2	Jackwood *et al.* (1982)
SK9	2	Kibenge, (1992)
SK140a	1	Kibenge, (1992)
QC-2	1	Reddy *et al.* (1991b)
GLS	1 variant	Snyder *et al.* (1988c)
IN	1 variant	Ismail *et al.* (1990)

symptoms. Strain QC-2 was found to induce low B69 neutralization titers (Reddy *et al.*, 1991b).

2.2 Virus Propagation

Monolayers of Vero cells (American Type Culture Collection, Rockville, Madison, USA) were treated with 1 ml of 0.25% trypsin for 5 min at 37^{0}C and the desired number of cells were seeded into 25 ml tissue culture flasks. Growth medium contained Eagle's minimum essential medium (EMEM), 10% inactivated fetal bovine serum (FBS), 100 I.U. penicillin/ml, 100 μg streptomycin/ml and 25 units of mycostatin/ml. The flasks were incubated at 37^{0}C in a humidified 5% CO_2-in-air atmosphere incubator until the monolayer was confluent. After discarding the medium, the monolayer was inoculated with 10^4 median tissue culture infective dose ($TCID_{50}$) of IBDV in 0.1 ml of EMEM. The inoculated culture was incubated at 37^{0}C for 1 h to adsorb the virus onto the cells. Maintenance medium (same as growth medium with FBS reduced to 2%) was subsequently added and incubated for approximately 5-7 days. The cultures were examined microscopically every day until complete cytopathic effect (CPE) was manifest (Kibenge *et al.*, 1988).

2.3 Extraction of dsRNA

The dsRNA was extracted using the method of Jackwood *et al.* (1989) with minor modifications. Briefly, the cells from infected cultures were disrupted by

freezing and thawing three times and the cell suspension was centrifuged at 3,200 g in Beckman rotor GH 38 for 20 minutes at 4°C to remove the cell debris. The virus in the resulting supernatant was pelleted by ultracentrifugation at 141,000 g using Beckman rotor SW 28 for 6 h at 4°C to yield partially purified virus. The viral pellets were then digested with proteinase K (GIBCO BRL, Canada) at a final concentration of 2.0 mg/ml in presence of 0.5% sodium dodecyl sulfate (SDS) (Boehringer Mannheim, Laval, PQ) at 37°C for 2 h to release the virus dsRNA. The dsRNA was extracted with phenol-chloroform and ethanol precipitated in presence of 3 M sodium acetate (pH 5.2). Any contaminating DNA was removed by treatment with RNase free DNase I (Pharmacia, Baie d'Urfe, PQ) at a concentration of 100 units/mg of total nucleic acid for 1 h at 37°C. It was again phenol-chloroform extracted and ethanol precipitated. The RNA pellet was suspended in TSE buffer (50 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 6.9) containing 35% ethanol. The dsRNA was separated from any contaminating ssRNA by passing the solution in a CF-11 cellulose column (Mellor *et al.*, 1985). For this, a 5 ml syringe plugged with silicone-treated glass wool was filled with sterile CF-11 cellulose powder (Whatman Biosystems Ltd, England). The sample was loaded on top of a hydrated column and ssRNA was eluted first by washing twice with 5 ml portions of TSE buffer containing 15% ethanol. The dsRNA was then eluted with 4 ml of TSE buffer without ethanol and precipitated with 2.5 vol of cold absolute ethanol and 0.1 vol of 3 M sodium acetate (pH 5.2).

2.4 Purification of IBDV Segments A and B

For each viral strain used, the two viral RNA segments were separated by electrophoresis in 1.2% low melting agarose gel at 120 V for 2 h. The ethidium bromide stained bands (segments A and B) were cut out separately and purified as described by Favre (1992). Briefly, the tubes containing the weighed gel slices were placed in a 65°C water bath for 10 min after adding an equal volume for weight of TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA). The melted agarose solution was extracted twice with 1 vol of Tris-buffered phenol at room temperature and the aqueous phase was again extracted with 0.1 vol of 4 M lithium chloride at 4°C. The supernatant was subsequently transferred to a new tube and 1μl containing 20 mg/ml glycogen solution (Boehringer Mannheim) was added as a carrier molecule in order to improve the final yield of recovered dsRNA. The dsRNA was precipitated with 2.5 vol of cold ethanol in the presence of 0.1 vol of 3 M sodium acetate (pH 5.2) at -70°C overnight. The pellet was washed with 70% ethanol and dried in vacuum.

2.5 Preparation of 5' Ends for Ligation

The proteinase K digestion was carried out as described by Müller and Nitschke (1987a) with modifications, in attempts to remove any residual VP1 sticking to the ends of genome segments A and B. A 100 μl reaction volume consisted of 0.1 M Tris-HCl, pH 7.5, 150 mM NaCl, 12.5 mM EDTA, 1% SDS and 20 μg of proteinase K, and was incubated at 37°C for 30 min. The reaction mixture was

extracted with phenol-chloroform and dsRNA was ethanol precipitated from the aqueous phase and dried under vacuum as before.

Phosphorylation of any available 5'-OH group at the terminus of viral RNA (segments A and B) was carried out by polynucleotide kinase reaction according to the manufacturer's instructions (USB, Cleveland, Ohio, USA). The enzyme T4 polynucleotide kinase catalyzes the transfer of the phosphate group from ATP to a 5'-OH group at the terminus of an RNA or DNA molecule (Richardson, 1981). A 10 μl reaction mixture containing 1-3 μg of viral RNA, 0.5 mM ATP and 6 units of T4 polynucletide kinase was incubated at 37^{0}C for 30 min and then inactivated by heat at 65^{0}C for 5 min.

2.6 Ligation of Viral RNA

Immediately following the phosphorylation reaction, the individual viral RNA segments containing the unknown sequences at the terminal ends were ligated using T4 RNA ligase (USB). T4 RNA ligase catalyzes the ATP dependent formation of phoshodiester bonds between the 5' phosphoryl group of a donor molecule and the 3' hydroxyl group of an acceptor molecule, either intra- or intermolecularly (Romaniuk and Uhlenbeck, 1983). A 20 μl reaction mixture consisting of 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 1 mM ATP, 60 μg/ml BSA, and 31 U of enzyme was incubated at 17^{0}C overnight and the ligated viral RNA

was then extracted with phenol/chloroform, ethanol precipitated and resuspended in diethylpyrocarbonate (DEPC)-treated water prior to further use.

2.7 Primer Directed cDNA Synthesis

The segments A and B dsRNA from ligated reactions described above were used as templates for cDNA synthesis with IBDV-specific primers. The sequences of the oligonucleotide primers used are shown in Table 2. They were designed from sequence information of OH-IBDV segments A (Kibenge *et al.*, 1991) and B (Dybing, 1992) by using the Primer Detective computer program (Version 1.01, Clonetech Labs, USA) and were purchased from either the Regional DNA Synthesis Lab, University of Calgary, Alberta, or from Bio/Can Scientific Inc., Mississauga, Ontario.

The segments A and B dsRNA from the ligated reactions were separately denatured in 90% dimethyl sulphoxide (DMSO) (Qian and Kibenge, 1994) at 65^0 C for 90 min, chilled on ice immediately and the ssRNA was precipitated with 2.5 vol of cold ethanol in presence of 0.1 vol of sodium acetate. The cDNA synthesis was carried out following the manufacturer's protocols with the TimeSaver^{TM} cDNA Synthesis Kit (Pharmacia) with slight modifications. Two specific primers SA05 and SA06 (Table 2) for segment A, and SB02 and SB03 (Table 2) for segment B were used. Since each viral dsRNA yielded two templates following denaturation in 90% DMSO, 5' and 3' terminal ends of both

Table 2. The Oligonucleotide Primers Used in the Viral cDNA Synthesis

Name and size of primer	Sequence	Location
SA05 (20 mer) (sense)	5'GCCTAAAGTCCCCATCCATG	2179-2198[1]
SA06 (20 mer) (antisense)	5'TCTCGATTTGCATGGGCTAG	1520-1539[1]
SB02 (20 mer) (antisense)	5'CGAGTTGCTTGTCCCATGTA	594-613[2]
SB03 (20 mer) (sense)	5'CTTCGCCATGAACATTGCCC	1454-1473[2]

[1]The location refers to map positions on genomic segment A sequences of IBDV strain OH (Kibenge *et al.*,1991)
[2]The location refers to map positions on genomic segment B sequences of IBDV strain OH (Dybing, 1992)

(+) and (-) strands were amplified. Briefly, the DMSO denatured RNA was heated at 65°C for 10 min prior to cDNA synthesis and chilled immediately on ice. For segment A, 1 μl each of 20-mer oligonucleotide primers SA05 and SA06 were used as specific reverse transcription primers at an approximate concentration of 25 μM in a 50 μl first strand reaction mixture. The reaction mixture contained in addition deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP), bovine serum albumin (BSA), RNAsin, murine reverse transcriptase and dithiothreitol (DTT) in Tris-HCl buffer and was incubated at 37°C for 1 h. The first strand cDNA was initially synthesized from denatured IBDV dsRNA by extension of specific primers with reverse transcriptase. At the end of the incubation period, the contents were transferred to a second reaction mix containing *E.coli* DNA polymerase I, *E.coli* RNase H and deoxynucleotide triphosphates (dNTPs) and incubated at 12°C for 30 min and subsequently at 22°C for 1 h. The RNA templates were degraded by RNase H and cDNA was synthesized with DNA polymerase I in the second strand reaction mix. Samples of ds cDNA synthesized were extracted with phenol chloroform, precipitated with 2 vol of cold absolute ethanol in presence of 0.1 vol of 3 M sodium acetate (pH 5.2) and suspended in 100 μl of water prior to use in PCR. The same procedure for cDNA synthesis was repeated for segment B using specific 20-mer oligonucleotide primers SB02 and SB03 (Table 2) in the first strand reaction.

2.8 Polymerase Chain Reaction (PCR)

A modified method of inverse PCR (Section 1.3.1) was used to generate sufficient product of ligated terminal regions for molecular cloning. In inverse PCR, it is possible to amplify unknown DNA sequences lying outside a pair of primers, if one primer sequence is at the end of the known DNA sequence (Triglia *et al.*, 1988; Ochman *et al.*, 1988). Instead of primers directing extension towards each other, the primer sequences are selected in such a way that extension takes place away from each other. In the modified method used here, the terminal ends containing the unknown sequences were ligated. PCR amplification of the ligated junction was carried out using a set of primers complementary to the known sequences bracketing the unknown target region. A linear amplified product was obtained containing the targeted unknown sequences flanked by known primers. This was cloned into a suitable vector and subsequently sequenced.

2.8.1 Amplification Across 3' and 5' Ends of Viral RNA

The different sets of primers used for PCR amplification of the 3' and 5' ligated junctions of segments A and B are shown in Table 3. For all the strains except GLS segment A, two primer pairs, SA08/SA09 for segment A and SB07/SB08 for segment B were used at 0.5 μM concentration. Primers SA08 and SA09 were selected from the published sequences of segment A of OH strain (Kibenge *et al.*, 1991) and primer pairs SB07 and SB08 were designed from the

Table 3. The Oligonucleotide Primers Used for the PCR Amplification of IBDV Terminal Sequences

Name and size of primer	Sequence	Location
SA08 (20 mer) antisense	5'CCTGGAACAAGGCGGTGATT	[illegible]
SA09 (20 mer) sense	5'TGACGAGGACTTGGAGTGAG	3133-3152[1]
SB07 (24 mer) sense	5'CCAAAAAGAGAGCCGCCAATAGCC	2713-2736[2]
SBO8 (24 mer) antisense	5'CGCCCCTAGTAGCCACGTTAATTC	29-52[2]
SAI-03 (20 mer) sense	5'TGATGAGGACCTTGAGTGAG	3133-3152[3]
SAI-04 (20 mer) antisense	5'CCTGGAACAAGGCCTTGACG	28-47[3]

[1]Location refers to map postions on genomic segment A sequences of IBDV strain OH (Kibenge *et al.*, 1991).
[2]Location refers to map positions on genomic segment B sequences of IBDV strain OH (Dybing, 1992).
[3]Location refers to map positions on genomic segment A of IBDV strain GLS which appear in the GenBank database with the Accession Number M97346

published sequences of segment B of OH strain (Dybing, 1992) using the Primer Detective computer program (Version 1.01, Clonetech Labs, USA). For strain GLS, primer pairs SAI-03 and SAI-04 at equivalent positions as primers SA08 and SA09 were selected from the published GLS sequence of segment A (Vakharia *et al.*, 1994).

Reaction conditions for PCR were as previously described (Qian and Kibenge, 1994). Briefly, 1-5 μl of cDNA synthesized from ligated dsRNA was used as a template. PCR was performed in a final 100 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 μM of each primer; 4 units of *Taq* DNA polymerase (BIO/CAN Scientific Inc., Mississauga, Ontario) with 100 μl mineral oil overlay. Mixtures were subjected in each cycle to the following temperature parameters: template denaturation at 94°C for 1 min, primer annealing at 55°C for 30 sec and extension at 72°C for 1 min for a total of 35 cycles of amplification. A final extension was carried out at 72°C for 10 min to complete the amplification reaction. Ten μl of the PCR products were loaded onto a 1.5% agarose gel and electrophoresed for 2 h at 100 V.

2.8.2 Subcloning of PCR Products

The PCR products were purified by extraction from the low melting-temperature agarose gels as described by Qian and Kibenge (1994). They were subsequently ligated to the pCR™II vector using the TA cloning™ Kit, Version

2.1 (Invitrogen, San Diego, USA). Enzymatic modification of the PCR product to create blunt ends or the use of PCR primers which contain restriction sites is not required with this kit. The technique is based on the principle that the thermostable *Taq* polymerase used in PCR adds a single A residue to the 3'-ends of duplex molecules of DNA regardless of the template used. These 3' A overhangs present in the PCR product are then used to insert the product into pCR™II vector which contains 5' T overhangs. Complementary A's and T's in the 1-base overhangs facilitate the ligation of insert to the vector (Finney, 1994).

For the ligation with the pCR™II vector, the reaction was set up at 12°C overnight at a 1:1-1:3 molar ratio of vector:PCR product as recommended by the manufacturer. The ligated mixture was subsequently used to transform supplied *E.coli* strain INV F' competent cells (Invitrogen). Uptake of plasmid based recombinant DNA by chemically pre-treated competent cells was carried out by a short 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 $MgCl_2$ 10 mM $MgSO_4$ and 20 mM glucose) and shaking at 225 rpm for 1 h at 37°C. The transformants were then plated on YT agar plates (0.8% bactotryptone, 0.5% bactoyeast extract, 0.5% NaCl and 1.5% agar) containing 50 μg/ml ampicillin and 0.005% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Since the cloned DNA was inserted into *Eco*RI restriction site within the *lacZ* gene of the vector, disrupting the reading frame of the *lacZ* gene,

the recombinants were amp^r *lacZ*$^-$ and formed white colonies whereas the nonrecombinants were amp^r *lacZ*$^+$ and formed blue colonies by cleaving the substrate X-gal by the enzyme galactosidase encoded by the lacZ gene. White colonies containing the recombinant plasmids were selected for further analysis.

2.8.3 Screening for Recombinant Plasmids

Selection of white colonies carrying the recombinant plasmids was done by either one of the following two methods. In the first method, plasmid DNA isolation was carried out from selected white colonies, followed by restriction with *Eco*RI. The size of the inserts were then identified by 1.5% agarose gel electrophoresis. In the second method, the direct PCR screening of cDNA clones (Sandhu *et al.*, 1989) was done. For this, specific primer pairs SA08/SA09 for segment A and SB07/SB08 for segment B (Table 3) were used. Briefly, the putative recombinant colonies were touched with the tip of a sterile micropipette and resuspended in the PCR mix which was heated at 100^{0}C for 10 min to lyse the bacterial cells and denature the DNA. They were subsequently cooled to room temperature to allow the primers to anneal followed by 25 cycles of PCR with *Taq* polymerase and the reaction products were examined by 1.5% agarose gel electrophoresis.

2.9 Plasmid DNA Isolation

The selected recombinant plasmids containing the inserted DNA were isolated from the host *E.coli* cells in sufficient quantity and purity for sequencing. For this, the alkaline lysis method (Birnboim and Doly, 1979) as modified by Kibenge *et al.* (1990) was used. Briefly, cells picked from plates streaked with single colonies were inoculated into 50 ml of 2X YT broth (0.1% bactotryptone, 0.1% yeast extract, 0.05% NaCl) containing 100 μg/ml ampicillin and grown overnight with vigorous shaking at 37^{0}C. Harvested cells were lysed by treatment with lysozyme-EDTA followed by SDS-NaOH treatment. RNase A (Pharmacia, Baie d'Urfe, PQ) was subsequently added (5 mg/ml) to remove any contaminating cellular RNA. The cellular proteins were precipitated with 7.5 M ammonium acetate. Plasmid DNA was recovered from the supernatant by ethanol precipitation. The pellet resuspended in TE buffer (pH 8.0) was precipitated with 0.4 vol of 30% polyethylene glycol (PEG, MW 7,000-9,000, Fisher Scientific, Nepean, Ontario) in 1.8 M NaCl. The PEG was removed by phenol chloroform extraction and the plasmid DNA was precipitated with 2.5 vol cold absolute ethanol in presence of 3 M sodium acetate (pH 5.2). The pellet was suspended in 200 μl TE buffer, pH 8.0. The quantity and purity of plasmid DNA was assessed spectrophotometrically (Pharmacia, GeneQuant RNA/DNA Calculator) by using OD 260/280 readings. The plasmid DNA was stored at -70^{0}C until used in sequencing reactions.

2.9.1 Plasmid DNA Sequencing

Plasmid DNA was sequenced by a modification of the Sanger dideoxychain-termination method (Chen and Seeburg, 1985) according to the protocols of Sequenase™ Version 2.0 DNA Sequencing kit (Amersham Life Science, Oakville, Ontario). For each strain, two clones of PCR product containing the terminally ligated region of segment B were sequenced. For segment A, three clones were analysed by sequencing. The sequencing method basically consists of two separate reactions; an initial reaction in which the primer is annealed to the denatured template DNA and a termination reaction in which extension of the labeled primer by Sequenase modified T7 DNA polymerase takes place in presence of one of four dideoxynucleotide triphosohates (ddNTP). Once the modified analog, ddNTP is incorporated into the growing chain of DNA, the chain is unable to extend as it will not have an OH group at the 3' position for the next nucleotide to be joined. Hence incorporation of a dideoxynucleotide terminates the chain. Four such sets of chain terminated fragments (one for each dideoxy analog) are generated and electrophoresed, and the base sequence of the new DNA is read from the autoradiogram of the four lanes.

Briefly, the double-stranded plasmid DNA template was first denatured to yield single-stranded DNA. For this, alkaline-denaturation was carried out by denaturing 3-5 μg of plasmid DNA in 0.1 vol of 2 M NaOH and 2 mM EDTA and incubating at 37^{0}C for 30 min. DNA was precipitated with 2-4 volumes of

ethanol at -70°C in presence of 0.1 volume 3 M sodium acetate for at least 15 min. The pelleted DNA was washed with cold 70% ethanol and dried in vacuum. The pelleted denatured DNA was dissolved in 7 μl of distilled water and to this, 2 μl of reaction buffer and 1 μl of primer were added. Either the forward (Universal primer M13/pUC) or reverse primer (Reverse sequencing primer M13/pUC) (Boehringer Mannheim) was used. Additional specific primers used to obtain terminal sequences of IBDV genome segments A and B were SA08 and SA09 and SB07 and SB08 (Table 3), respectively. Annealing was carried out by heating at 65°C for 2 min and cooling slowly for about 30 min to 35°C. The annealed DNA mixture was incubated for 5 min at room temperature in presence of Sequenase, DTT and all four dNTPs of which dATP was radiolabeled (redivue™ [α^{35}S] dATP, 10mCi/ml, Amersham Canada Ltd, Oakville, Ontario). 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 incubated at 37°C for 5 min and stopped by adding 4 μl of stop solution (xylene cyanol 0.1%, bromophenol blue 0.1%, 2ml 0.5 M EDTA in formamide) and stored at - 70°C until ready to load onto the sequencing gel.

The sequencing gel solution containing 6% acrylamide and 8 M urea (Ansorge and Labeit, 1984) was freshly prepared in TBE sequencing buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) and filtered. To 200 ml of the filtered

solution, 1.6 ml of 2.5% (w/v) ammonium persulphate and 80 μl of TEMED (N,N,N',N'-tetramethyl-1,2-diaminoethane) were quickly added and the mixture was poured in between two sealed glass plates. The wells for loading the samples were formed by inserting the slot former immediately into the gel solution between the two glass plates and the gel was allowed to polymerize for 1 h. The slot former and the sealing tape from the bottom of the plates were removed and the wells of the gel were flushed with TBE buffer to wash away any unpolymerized acrylamide and urea. The gel plates were placed into the bottom chamber of a vertical electrophoresis unit (GIBCO BRL, Model S2) in which both chambers were filled with TBE buffer.

The sequence reaction products stored at -70^{0}C were thawed on ice and heated to 75-80^{0}C for 2 min just before loading into the wells and the electrophoresis was carried out at 55 W for at least 5 hours. The gel was transferred onto one of the two glass plates at the end of electrophoresis and soaked in a solution containing 10% methanol and 10% acetic acid for 1 h to remove urea. It was then dried on Whatman chromatograghy paper under vacuum at 80^{0}C for 2-3 h in a Hoeffer Scientific SE1160 slab gel dryer and subjected to direct autoradiograghy by exposing to Kodak X-Omat film at room temperature for approximately 36 h.

2.9.2 Sequence Analysis

The nucleotide sequences were read manually from the autoradiographs and the data were entered into a computer and analyzed either manually or by using LaserGene software (DNASTAR, Inc., Madison, Wisconsin, U.S.A.) and the FASTA program package for microcomputers (Pearson and Lipman, 1988). Analysis for sequence homology with published sequences was carried out using the FASTA program under default settings. For secondary structure prediction in the terminal sequences, analysis was done either manually based on complementary base pairing between adjacent inverted repeat sequences and inverted terminal repeat sequences (see page 70) or by using the RNA MFold computer program based on complementary base pairing and minimum free energy of foding at physioliogical temperature under default settings (Zuker *et al.*, 1989).

3. RESULTS

3.1 PCR Amplification Across 3'-5' End Junctions of Viral RNA Strands

Two pairs of primers, SA05 (sense) and SA06 (antisense) for segment A, and SB02 (antisense) and SB03 (sense) for segment B (Table 2, Fig. 1), were used in cDNA synthesis across the 3' end-5' end junctions for all the six IBDV strains. Each viral dsRNA segment yielded two templates following denaturation in 90% DMSO carried out just prior to cDNA synthesis. Therefore, 5' and 3' terminal ends of both (+) and (-) strands were amplified. A different set of primers was selected for PCR (Table 3, Fig. 1). The DNA analysis of the clones of the resultant PCR products for segments A and B are shown in Figures 2 and 3, respectively. The sizes of amplified PCR products obtained of 3' and 5' terminal regions for segment A and segment B of all the six strains are summarized in Table 4. The PCR amplification with primer pair SA08/SA09 yielded products of size 155 bp long for segment A of IBDV strains OH, IN, and SK140a, and ~ 265 bp long for strains SK9 and QC-2. The PCR amplification of segment A of strain GLS with specific primer pair SAI-03/SAI-04 yielded a product 226 bp long. For segment B, primer pair SB07/SB08 yielded an almost an identical size product ~ 260 bp long for all the six IBDV strains.

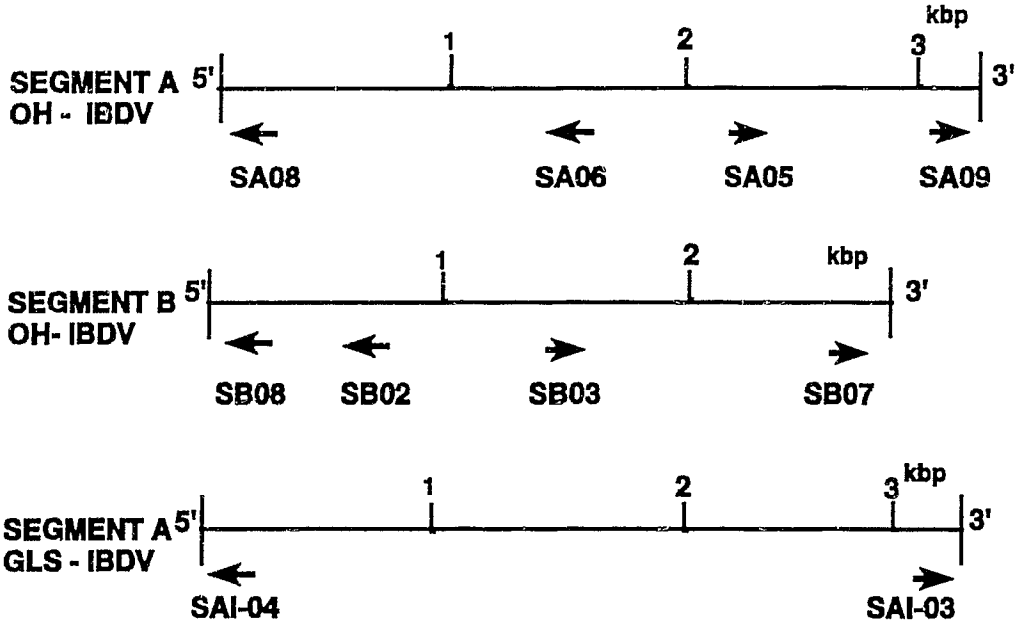


Fig. 1: Approximate location of upstream (→) and downstream (←) primer sequences on the genomic segment A of IBDV strains OH and GLS and segment B of strain OH. The segment specific primers are identified with the suffix SA for segment A and suffix SB for segment B. The sequences and exact map locations are given in Tables 2 and 3.

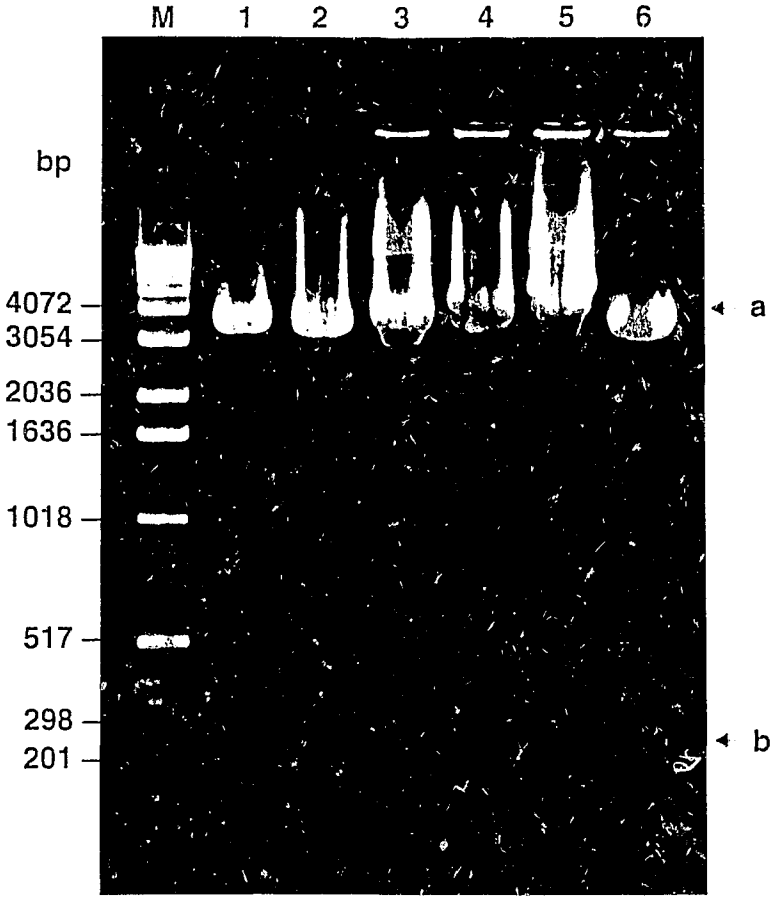


Fig. 2: Agarose gel electrophoresis of *Eco*RI digested DNA from recombinant clones of segment A of IBDV strains. Lane M is 1 kb ladder. Lanes 1-6 are recombinant clones containing PCR products of IBDV strains SK140a, IN, SK9, QC-2 and GLS, respectively. Arrow a, indicates the band of plasmid DNA. Arrow b, indicates positions of the cloned PCR products.

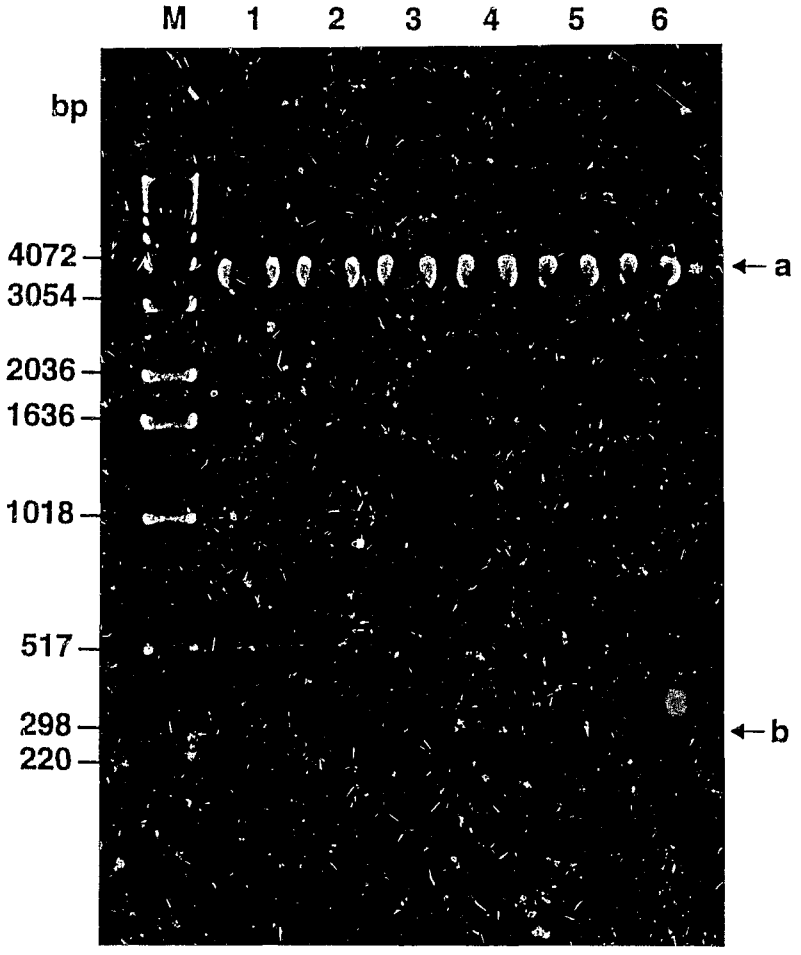


Fig. 3: Agarose gel electrophoresis of *Eco*RI digested DNA from recombinant clones of segment B of IBDV strains. Lane M is 1 kb ladder. Lanes 1-6 are recombinant clones containing PCR products of IBDV strains OH, SK140a, IN, SK9, QC-2 and GLS, respectively. Arrow a, indicates the large band of plasmid DNA. Arrow b, indicates positions of the cloned PCR products.

3.2 Sequence Analysis of IBDV Terminal Sequences

The sizes of the PCR products determined by gel electrophoresis were similar to those obtained by nucleotide sequencing. The terminal nucleotide sequence analysis of 3'- 5' ligated regions of genome segments A and B of all the six IBDV strains are schematically represented in Fig. 4.

3.2.1 Segment A and B of IBDV Strains OH, IN and SK140a

By sequencing it was found that the DNA inserts of segment A clones of strains OH, IN and SK140a were 155 nucleotides long (Fig. 5). The segment B clones of all the strains had DNA inserts ranging from 258 to 262 nucleotides long (Fig. 6).

Sequences showing 99-100% identity were obtained for segment A of strains OH, SK140a and IN (Fig. 5, Table 5, 6). From the published segment A sequences of IBDV strain OH (Kibenge *et al.*, 1991), about 46 nucleotides on the 5' end (OH 5', nts 1-46) and about 39 nucleotides from the 3' end (OH 3', nts 3133 - 3171) were 100% homologous with the sequence of the segment A clones (Fig. 7a and 7b respectively). In further sequence analysis, it was found that the 3' and 5' termini of segment A were homologous to those of segment B in a region 34 nucleotides long (Fig. 5 and Fig. 6). The location of this consensus sequence in the segment A PCR products indicated that 14

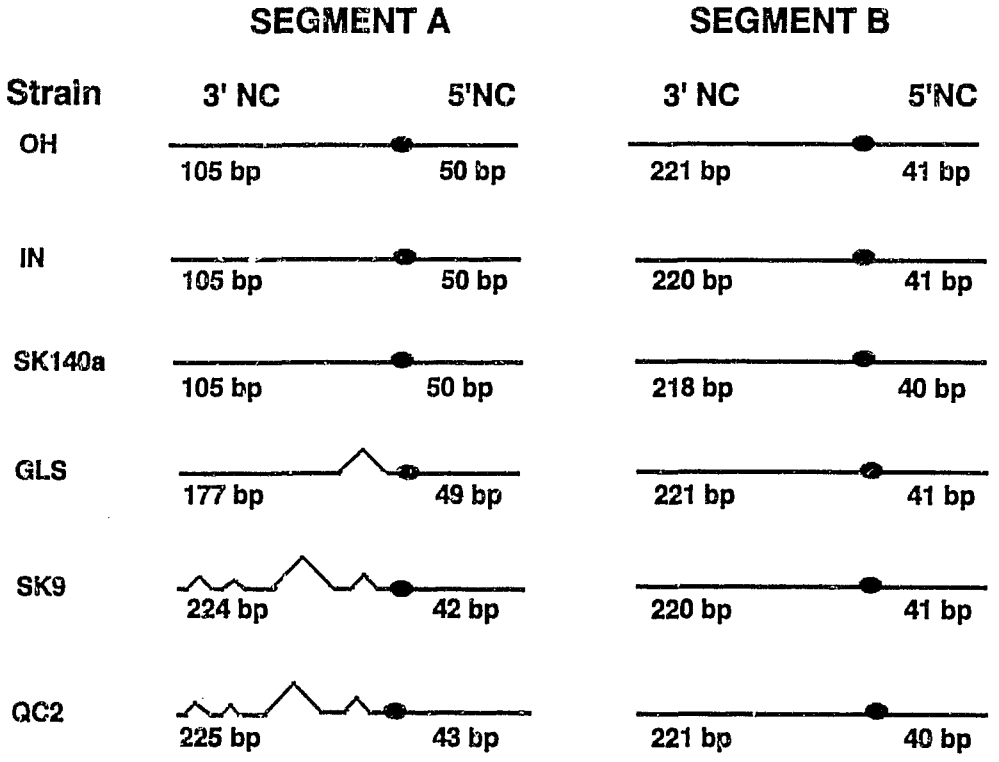


Fig. 4: 5' and 3' ligated terminal regions of six selected IBDV strains. •, indicates point of intramolecular junction of 3' and 5' ends. Λ, indicates regions of additional sequences of variable length present on genome segment A of IBDV strains GLS, SK9 and QC-2.

Table 4: PCR product sizes of 3' and 5' ligated regions of IBDV strains

Strain	Segment A	Segment B
OH	155 bp	262 bp
IN	155 bp	261 bp
SK140a	155 bp	258 bp
GLS	226 bp	262 bp
SK9	266 bp	261 bp
QC-2	268 bp	261 bp

bp, denotes basepairs

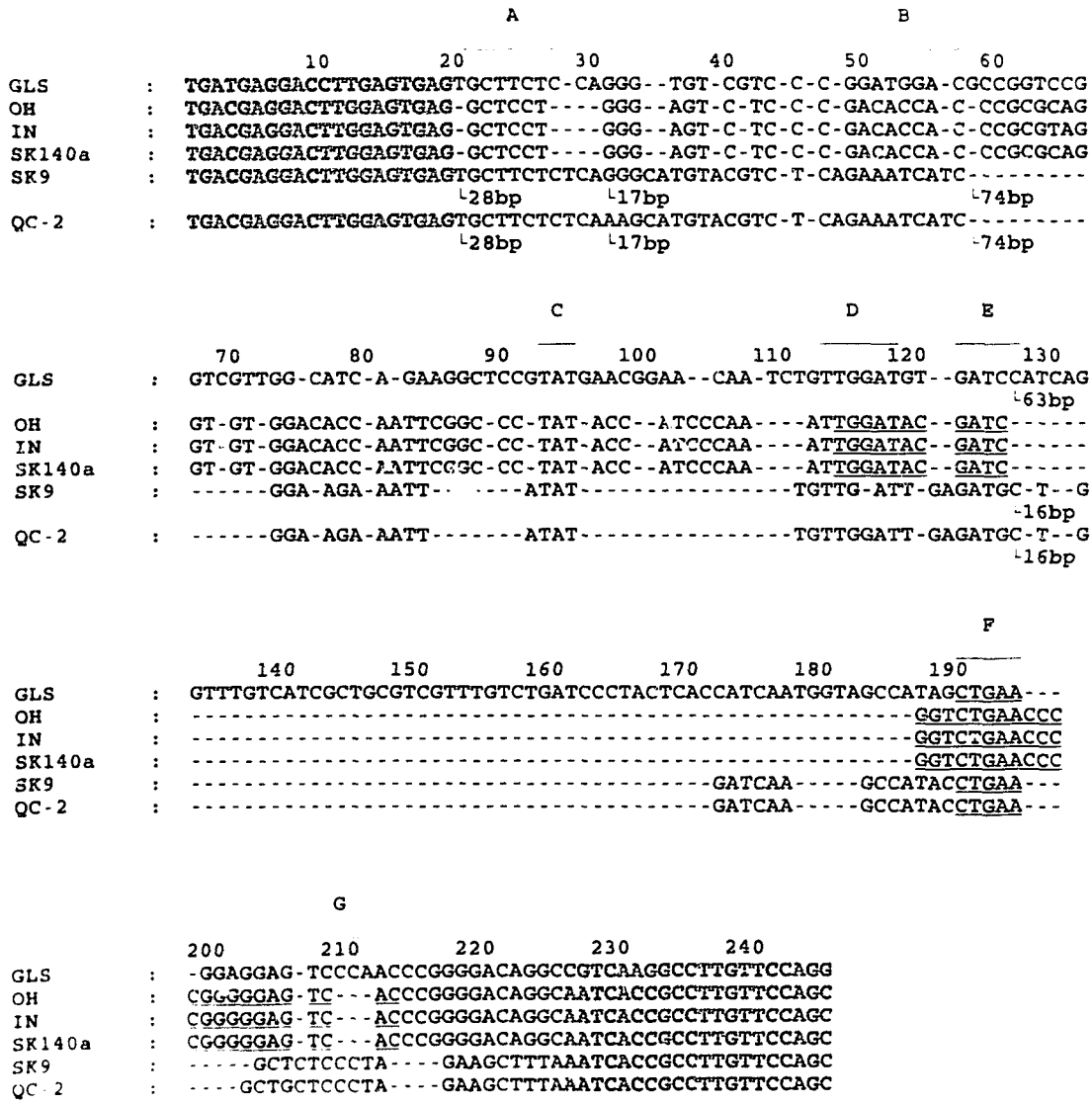


Fig. 5: IBDV segment A terminal cDNA sequences. The sequences of the segment A of IBDV strains GLS, OH, IN, SK140a, SK9 and QC-2 are shown. (The coordinates of the PCR product sequence are given above the sequence). Hyphens are introduced to align the sequences to maximum. The sequences for the primers SA09, SA08, SAI-03 and SAI-04 (Table 3) are shaded. A stretch of 34 nt sequence showing homology between segment A and B terminal sequences of strains OH, IN and SK140a is underlined. Regions A, B, C, D, E, F and G represent blocks of conserved sequences between the six IBDV strains. L, indicates positions where there are additional sequences of variable lengths in base pairs (bp).

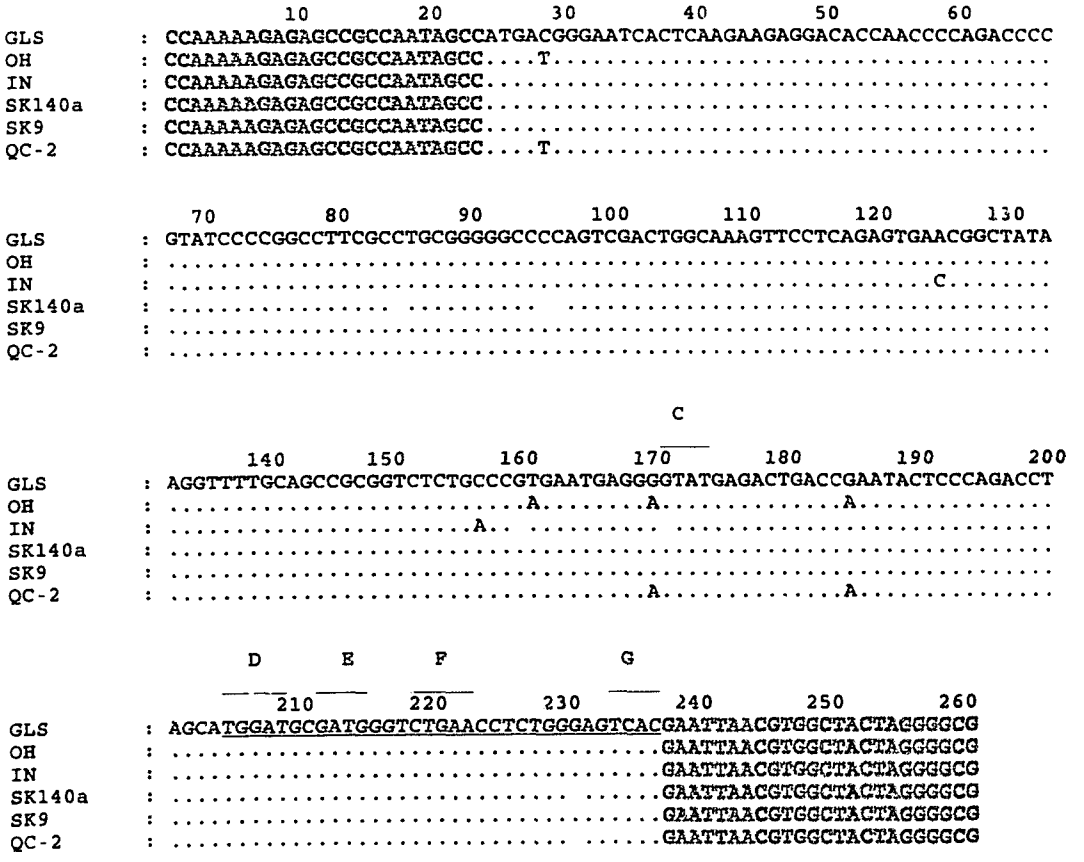


Fig. 6: IBDV segment B terminal cDNA sequences. The sequences of the segment B PCR products of IBDV strains GLS, OH, IN, SK140a, SK9 and QC-2 are shown. The sequence of IBDV strain GLS is written in full. Dots indicate homologous sequence while gaps indicate deletions/insertions. The sequence for primers SB07 and SB08 is shaded. A stretch of 34 nt sequence showing homology between segment A and B terminal sequences is underlined. Regions C, D, E, F and G represent common sequences on segment A and B of all the six IBDV strains.

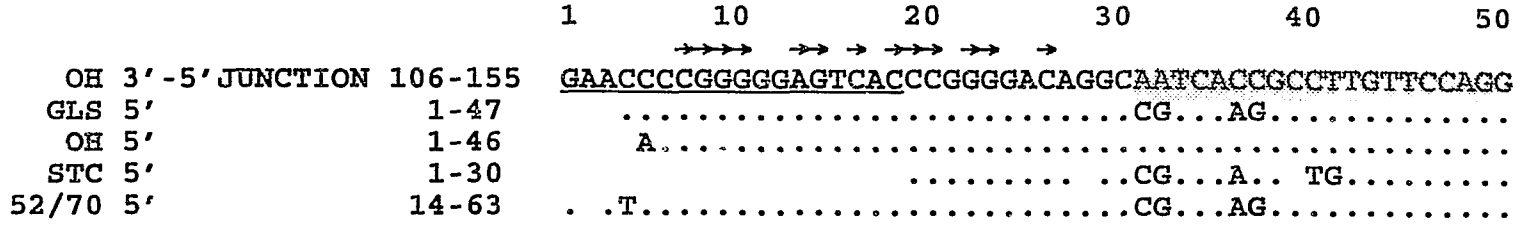


Fig 7a: Alignment of the segment A 5' terminal sequence of IBDV strain OH with the published IBDV segment A sequences of strains OH (Kibenge *et al.*, 1991), STC (Kibenge *et al.*, 1990), 52/70 (Bayliss *et al.*, 1990), and GLS (Vakharia *et al.*, 1994). The coordinates of the complete sequence of genome segment A of OH are given above the sequence. Dots indicate homologous sequence while gaps indicate undetermined sequence. The primer sequences are shaded. The area of sequence homology between segment A and B terminal sequences is underlined. Arrows indicate inverted repeats present in both Figs. 7a and 7b as represented in the predicted secondary structure for segment A in Fig. 13 on page 71.

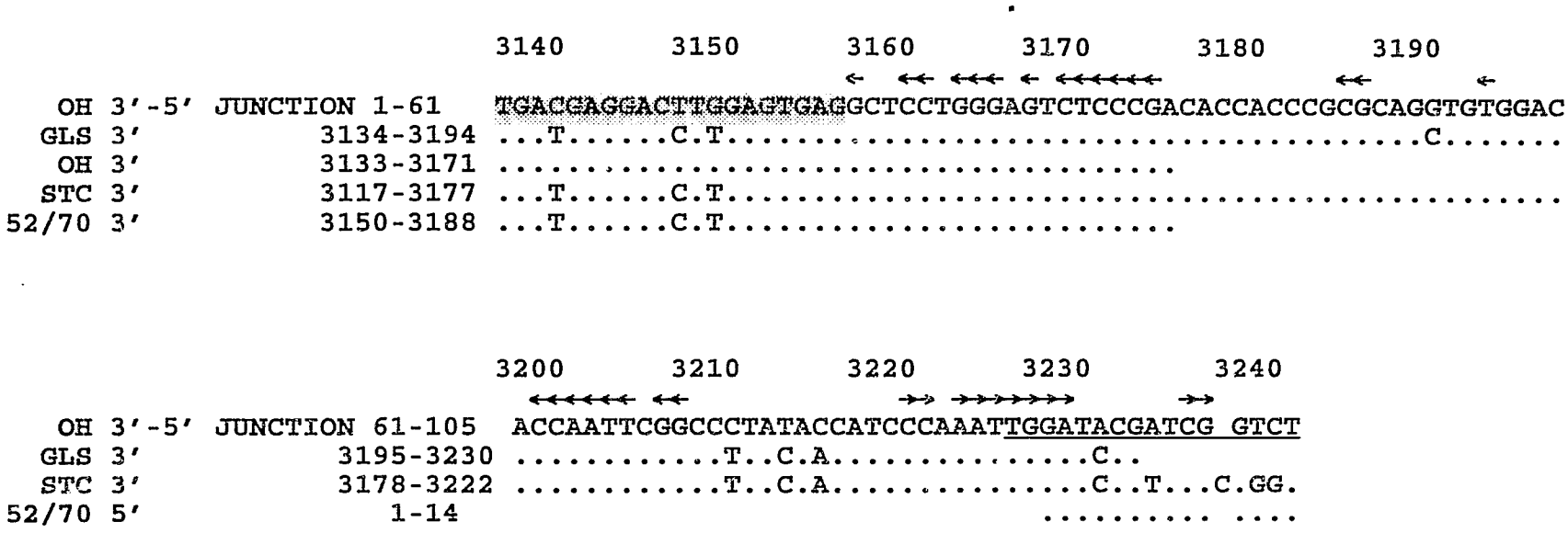


Fig 7b: Alignment of the segment A 3' terminal sequence of IBDV strain OH with the published IBDV segment A sequences of strains OH (Kibenge *et al.*, 1991), STC (Kibenge *et al.*, 1990), 52/70 (Bayliss *et al.*, 1990), and GLS (Vakharia *et al.*, 1994). The coordinates of the complete sequence of genome segment A of OH are given above the sequence. Dots indicate homologous sequence while gaps indicate undetermined sequence. The primer sequences are shaded. The area of sequence homology between segment A and B terminal sequences is underlined. Arrows indicate inverted repeats present in both Figs. 7a and 7b as represented in the predicted secondary structure for segment A in Fig. 13 on page 71.

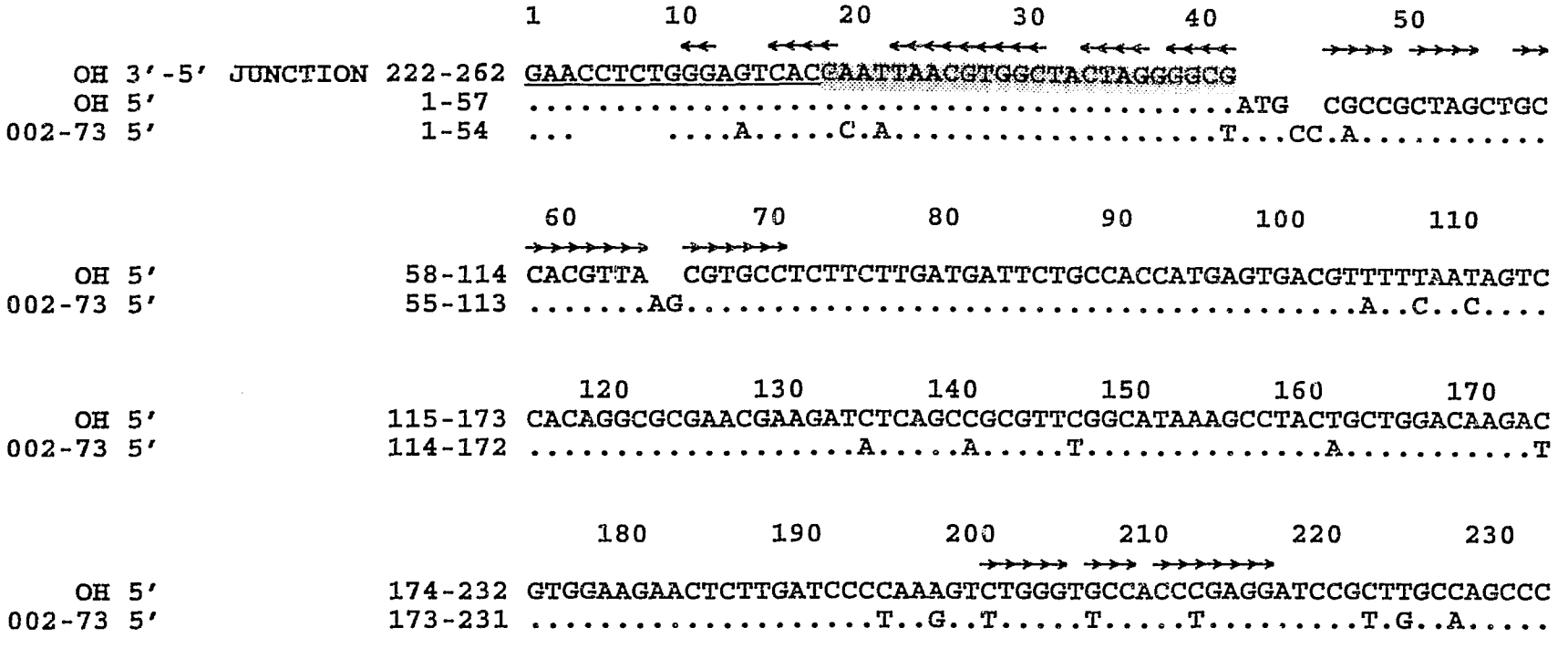


Fig. 8a: Alignment of the segment B 5' terminal sequence of IBDV strain OH with the published sequence of strain 002-73 (Morgan *et al.*, 1988). The coordinates of the complete sequence of genome segment B of OH are given above the sequence. Dots indicate homologous sequence while gaps indicate undetermined sequence. The primer sequences are shaded. The area of sequence homology between segment A and B terminal sequencs is underlined. Arrows indicate inverted repeats present in both Figs. 8a and 8b as represented in the predicted secondary structure for segment B in Fig. 13 on page 71.

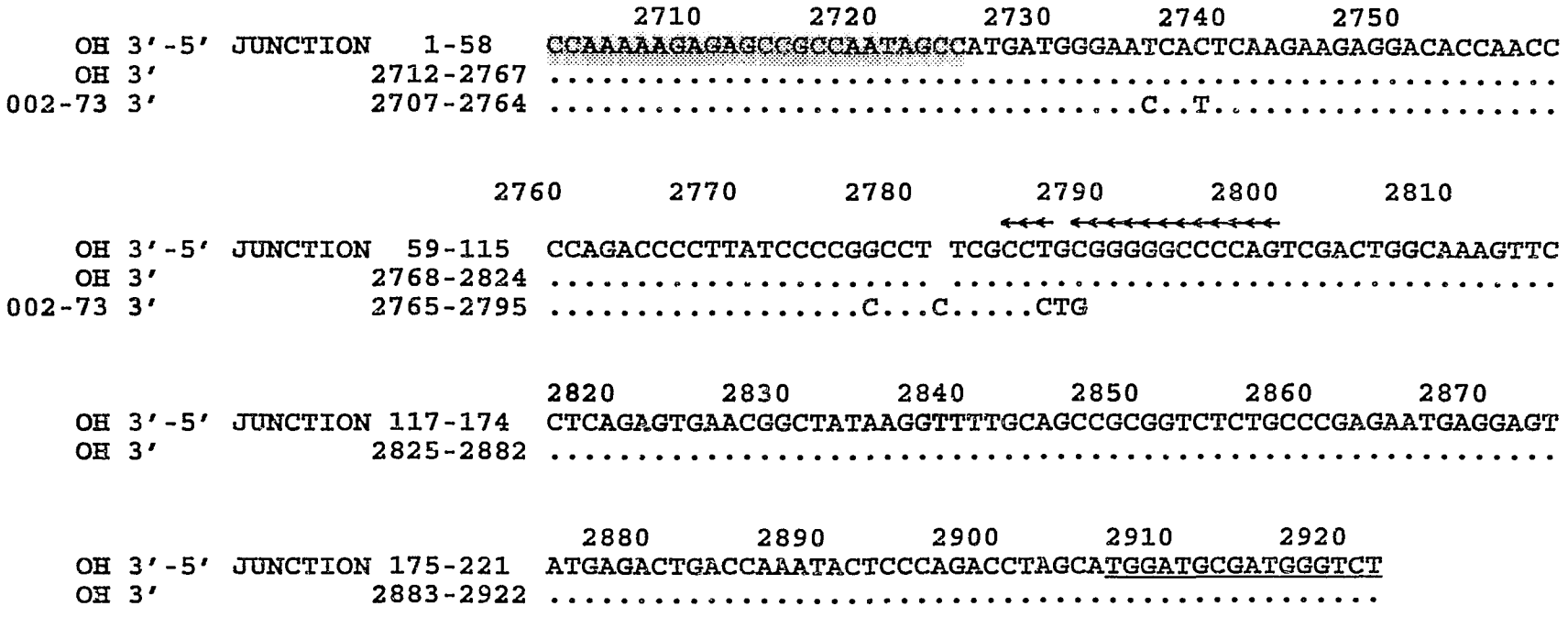


Fig. 8b: Alignment of the segment B 3' terminal sequence of IBDV strain OH with the published sequence of strain 002-73 (Morgan *et al.*, 1988). The coordinates of the complete sequence of the genome segment B of OH are given above the sequence. Dots indicate homologous sequence while gaps indicate undetermined sequence. The primer sequences are shaded. The area of sequence homology between segment A and B is underlined. Arrows indicate inverted repeats present in both Figs. 8a and 8b as represented in the predicted secondary structure for segment B in Fig. 13 on page 71.

nucleotides (nts 1-14) of the 5' end of the IBDV strain 52/70 (Bayliss *et al.*, 1990) extend into the proposed 3' consensus sequence (Fig. 7b).

By aligning the sequence of the segment A of strain OH with IBDV strains STC, 52/70,and GLS, it was established that there were additional 70 nucleotides on segment A of IBDV strain OH (OH 3'-5' junction, nts 106-109, Fig. 7a; OH 3'-5' junction, nts 40-105, Fig. 7b) although these nucleotides were present in the combined published sequences of IBDV strains STC, 52/70 and GLS (Fig. 7a, 7b). Therefore, the total length of the genome segment A of strain OH is 3,241 bp.

Similar to segment A of IBDV strains OH, SK140a and IN, the segment B of these strains showed 98-99% identical sequence data (Fig. 6). Alignment of additional sequences (about 172 nucleotides) in segment B of strain OH with that already determined following sequencing of the OH cDNA library (Dybing, 1992) established the total length of genome segment B of strain OH to be 2,922 bp (Figs. 8a and 8b). Sequence comparisons of segment A and B PCR products for the three strains revealed 85.3% homology between segment A and B. By further analysis, a homologous sequence between segment A and B was identified and it is 34 nucleotides long (TGG AT(A/G) CGA T(C/G)G GTC TGA ACC (C/T)C(G/T) GG(G/-) GAG TCA C) (Fig. 5 and Fig. 6). In segment B it is immediately upstream of primer SB08 (Fig. 6).

5' TERMINUS

```
                1
M - GLS   :   GAA........

V - GLS   :      ........
```

3' TERMINUS

```
                     3230       3240       3250       3260
M - GLS   :   ........ATCCATCAGGTTTGTCATCGCTGCGGTCGTTTGTCT

V - GLS   :   ......

                3270       3280       3290       3300
M - GLS   :   GATCCCTACTCACCTCAATGGTAGCCATAGCT
```

Fig. 9: Additional sequences found at 5' and 3' termini of segment A of IBDV strain GLS (M-GLS) not present in the published sequences of V-GLS (Vakharia *et al.*, 1994). The terms M-GLS and V-GLS were used just to denote sequences determined by Nagarajan **M** and Vakharia *et al.* respectively.

3.2.2 Segment A and B of IBDV strain GLS

Attempts at PCR amplification across 3'-5' ligated terminals of segment A of strain GLS using OH specific primers SA08 and SA09 did not yield any product. A PCR product of 226 bp was obtained for segment A by using GLS specific primers SAI-04 and SAI-03. Segment A clones of GLS had DNA inserts of 226 nucleotides long (Fig. 5) while those in segment B clones had inserts of 262 bp long (Fig. 6). Segment A sequence of GLS was found to have an additional sequence of 39 nucleotides at position 135 (Fig. 5) not present in segment A sequences of the strains OH, SK140a, IN, SK9 and QC-2 (Fig. 5).

By aligning the sequences of segment A of strain GLS with those of the published sequences of GLS (Vakharia *et al.*, 1994), 71 additional nucleotides were identified on segment A of GLS (Fig. 9), out of which 3 nts belonged to the 5' end and 68 nts to the 3' end. The total length of the genome segment A of strain GLS is therefore 3,300 bp. The segment B sequence of strain GLS was 100% identical to that of strain SK9. The strains OH, QC-2 and IN had two to four nucleotide substitutions, SK140a had four nucleotide deletions and QC-2 had one nucleotide deletion compared to segment B of GLS (Fig. 6).

3.2.3 Segment A and B of IBDV Strains SK9 and QC-2

Segment A clones of strain SK9 had a DNA insert of 266 nucleotides and those of strain QC-2 had a DNA insert of 268 nucleotides. The sequences of segment

```
                10        20        30        40
SK9   :  TGACGAGGACTTGGAGTGAGCTCTAAGTGTCCTTGCTACCAAGAATGAT

QC-2  :  ..........................................

         50        60        70        80        90
SK9   :  GCTTCTCTCAACTTAGACATACTAAGAGGGCATGTAGGTCT CAGAAATC

QC-2  :  ........................AA..................

          100       110       120       130       140
SK9   :  ATCATCTTACGAACAAGAAGGGAGAGTTTCAGTTACAAGAGAAATATTG

QC-2  :  ............G.....................................

           150       160       170       180       190
SK9   :  CTGAATAAATGGAGGGAAAAAAACGAAAGGGAGGAAGAAATTATATTGT

QC-2  :  ............................A...................

             200       210       220       230       240
SK9   :  TGATTGAGATGCTGGATCAAGCCATACCTGAAGCT CTCCCTAGAAGCT

QC-2  :  ..................................G.............

             250       260
SK9   :  TTAAATCACCGCCTTGTTCCAGC

QC-2  :  ......................
```

Fig. 10: Terminal cDNA sequence of 3'- 5' junction of segment A of strains SK9 and QC-2. Dots indicate sequences identical to SK9. Gaps indicate deletions or insertions. Shaded areas are primer sequences.

A of SK9 and QC-2 were 98% identical except for few point mutations and deletions (Fig. 10). But they differed considerably from the terminal sequences of segment A of the other four strains (Fig. 5). Strains SK9 and QC-2 were found to have stretches of additional sequences at four positions, 28 nts at position 20, 17 nts at position 31, 74 nts at position 58 and 16 nts at position 128 (Fig. 5). The additional sequences had no homology with segment A sequences of the other four strains even though they were 98% identical among themselves (SK9 and QC-2) (Fig. 10). Segment B clones of SK9 and QC-2 had DNA inserts of 261 nucleotides long and their sequences were 98% identical to sequences of segment B of the other four strains OH, SK140a, IN and SK9 (Fig. 6).

3.3 Identification of the 3'-5' junction in the viral RNA strands

The 3'-5' junction in the ligated viral strands was identified primarily on the basis that the 5' ends of both segments begin with a guanine base attached to VP1 (Dobos, 1993). Since genome segments A and B were homologous in a 34 nucleotide stretch TGG AT(A/G) CGA T(C/G)G GTC TGA ACC (C/T)C(G/T) GG(G/-) GAG TCA C (Figs. 5 and 6), this was considered to contain the 3' and 5' termini of the viral RNA strands. There are several Gs in this consensus sequence that could serve as the 5' terminus nucleotide, including the GG at the beginning of the consensus sequence, which are within two nucleotides to the 5' terminus of segment A in IBDV strain 52/70 (Bayliss *et al.*, 1990). However, the G right in the middle (nucleotide position 17) of the consensus sequence is

considered to be the more likely 5' terminus since at this location both 3' and 5' termini are 100% identical between genome segments A and B, a feature of segmented RNA viruses. As for example, all ten segments of double stranded RNA of reoviruses (Antczak *et al.*, 1985; Anzola *et al.*, 1987; Yan *et al.*, 1992) have conserved tetranucleotide and pentanucleotide sequences at their 5' and 3' ends respectively. All eight negative sense ssRNA segments of influenza A viruses (Stockle *et al.*, 1987; Desselberger *et al.*, 1980) have conserved sequences of 12 and 13 nucleotides at their 3' and 5' ends. The 5' terminal sequence of the plus strands in both segments of IBDV is proposed to begin with GAACC- (Fig. 11). The 3' terminal sequence in both segments is proposed to end with -GGUCU (Fig. 12). These 5' and 3' terminal sequences are conserved in both genome segments A and B of strains OH, SK140a and IN suggesting that they are characteristic of the genus *Avibirnavirus*.

However, the 5' termini of segment A of the strains GLS, SK9 and QC-2 begin with GAA- (Fig.11) and do not have the two nucleotides CC after GAA. The 3' termini of segment A of GLS, SK9 and QC-2 end with -UA(G/C)CU (Fig. 12) instead of GGUCU as noted in strains OH, SK140a and IN. Segment A of strain GLS has an additional sequence of 63 nucleotides between nucleotide residues C and U and this additional sequence lies within 5 nts from the 3' end (Fig. 12). Terminal segment A of strains SK9 and QC-2 have additional sequences of 16 nts between nucleotide residues G and U and this additional sequence lies

	SEGMENT A	SEGMENT B
Strain	5′ end	5′ end
OH	GAACCCGGGGGAGUC	GAACCUCUGGGAGUC
SK140a	GAACCCGGGGGAGUC	GAACCUCUG-GAGUC
IN	GAACCCGGGGGAGUC	GAACCUCUGGGAGUC
GLS	GAA---GGAGGAGUC	GAACCUCUGGGAGUC
SK9	GAA---GCU---CUC	GAACCUCUGGGAGUC
QC-2	GAA---GCUG--CUC	GAACCUCUG-GAGUC

Fig. 11: Common features of the 5' end sequence of IBDV genome. Regions common to both genome segments A and B in a stretch of 15 nucleotides at the 5' termini are shaded. ---, are introduced to align the sequences to maximum.

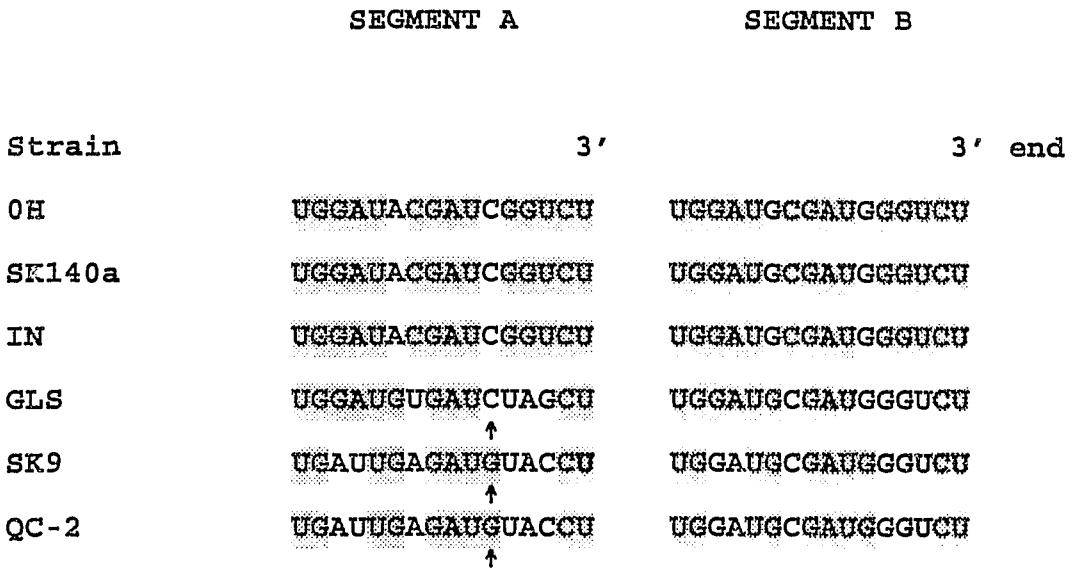


Fig. 12: Common features of the 3' end sequence of IBDV genome. Regions common to both genome segments A and B in a stretch of 16 nucleotides at the 3' termini are shaded. ↑ , indicates a region where there is an insert of variable length in strains GLS, SK9 and QC-2.

within 5 nucleotides from the 3' end (Fig. 12). However it is pertinent to note that all the six strains of IBDV have their nucleotide sequences of segment A begin with GAA- and end with -CU (Fig. 11 and 12).

Nucleotide sequences of segment B of IBDV strains GLS, SK9 and QC-2 all begin with 5' GAACC and end with GGUCU 3' similar to strains OH, SK140a and QC-2 (Fig 11 and 12).

3.3.1 Terminal Sequence Analysis of Segment A of Six IBDV Strains

The 5' noncoding region of segment A of IBDV strains OH, SK140a and IN are highly conserved with 100% sequence identity (Table 5) among them, 84% identity with strain GLS, and 60% identity with strains SK9 and QC-2. IBDV strains SK9 and QC-2 share 97.6% sequence identity in this region but only around 60% sequence identity with the other five strains.

In the 3' noncoding region, IBDV strains OH and SK140a are 100% identical (Table 6) and the sequence of strain IN is almost identical with strains OH and SK140a except for position 65, where there is a substitution (Fig. 5). IBDV strain GLS has about 44% sequence identity with strains OH, SK140a and IN and 34% sequence identity with strains SK9 and QC-2 (Table 6), and a 39-bp additional sequence at position 128 (Fig. 5) not present in any of the other five IBDV strains. IBDV strains SK9 and QC-2 have 98% sequence identity except for few

Table 5. Percent Nucleotide Identity in 5' Noncoding Region of Segment A of Six IBDV Strains

STRAIN	GLS	OH	IN	SK140a	SK9
GLS					
OH	84.0				
IN	84.0	100			
SK140a	84.0	100	100		
SK9	57.1	60	60	60	
QC-2	57.1	60	60	60	97.6

Table 6. Percent Nucleotide Identity in 3' Noncoding Region of Segment A of Six IBDV Strains

STRAIN	GLS	OH	IN	SK140a	SK9
GLS					
OH	43.4				
IN	43.4	99.5			
SK140a	43.4	100	99.0		
SK9	33.9	27.6	27.6	27.6	
QC-2	34.4	27.6	27.6	27.6	98.2

nucleotide substitutions (Fig. 9) but only 34% identity with IBDV strain GLS and 27% identity with strains OH, SK140a and IN (Table 6). The low percentage sequence identity in this region is mainly attributed to a number of additional nucleotides present in IBDV strains SK9 and QC-2 as detailed in Section 3.2.3 (Fig. 4).

Even though considerable sequence heterogeneity was noticed in the terminal regions of segment A of six IBDV strains, highly significant conserved sequence motifs were also identified among all the six IBDV strains at regions A, B, C, D, E, F and G (Fig. 5).

3.3.2 Terminal Sequence Analysis of Segment B of Six IBDV Strains

Highly conserved terminal sequence identity (96%-100%) was observed for segment B of all six IBDV strains in both the 5' and 3' noncoding regions (Fig. 1 and 6). In the 5' noncoding region, there is nearly 100% sequence identity except for IBDV strains QC-2 and SK140a, where there is a deletion of one nucleotide at position 232 (Fig. 6). In the 3' noncoding region, there are nucleotide substitutions, at positions 29, 126, 158, 162, 171 and 186 (Fig. 6) between segment B of six IBDV strains. At positions 29, 171 and 186, strains OH and QC-2 have a different nucleotide substitution; at position 162, strain GLS has a different nucleotide substitution; at positions 126 and 158, strain IN has different nucleotide substitutions that are not present in any other five strains and

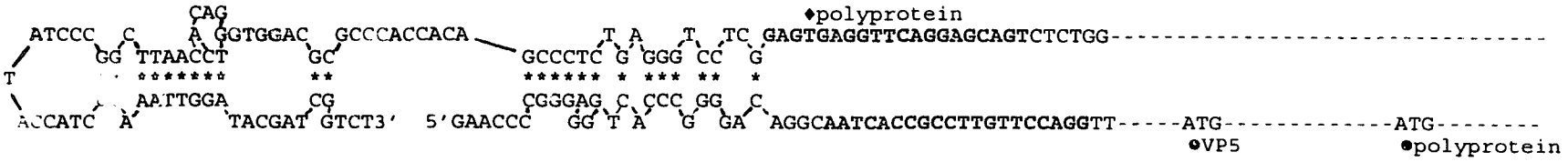
has deletion of nucleotides at positions 161 and 172 (Fig. 6). Almost identical conserved sequence motifs as were found in regions C, D, E, F and G of segment A existed in segment B of all the six strains of IBDV (Figs. 5 and 6).

3.4 Secondary Structures

The segment A terminal sequences of OH strain of IBDV have the potential to form a stem and loop structure (or hairpin loop) (Fig. 13), in which the single stranded RNA folds back onto itself in structures stabilized by hydrogen bonds between complementary bases forming the stem, and the unpaired bases the loop. Such structure is predicted manually for terminal sequences of strain OH (Fig. 13) by the folding of adjacent inverted repeat sequences present at the 3' terminus of the molecule, which are continuous with inverted terminal repeats at the 5' terminus (Fig 7a and 7b). The published segment A sequences of IBDV strain OH (Kibenge *et al.*, 1991) contain some of the inverted terminal repeats in nucleotide positions 3-22 and 3153-3170. The adjacent inverted repeat sequences are analogous to the inverted and complementary repeat sequence structure previously reported in segment A of IBDV strain STC (Kibenge *et al.*, 1990).

The segment B terminal sequences of OH strain is also proposed to have a stem and loop structure similar to segment A (Fig. 13), that can be folded from adjacent inverted repeat sequences spanning nucleotide positions 10-41 and 45-

Segment A (IBDV strain OH)



Segment B (IBDV strain OH)

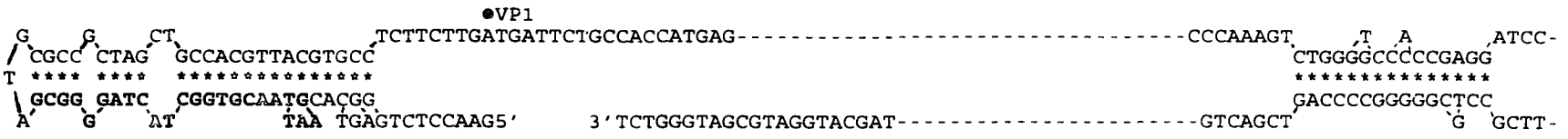
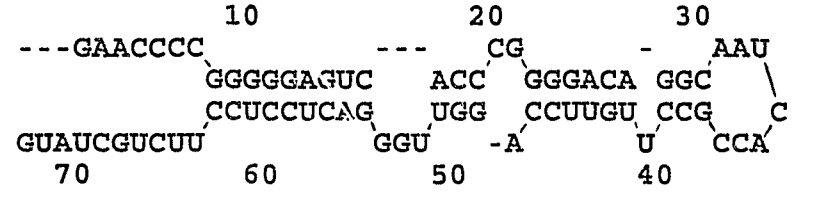


Fig 13: Predicted secondary structures for terminal sequences of segments A and B of IBDV strain OH. The bolded sequences denote PCR primers. (*) denotes H-bonding. Hyphens indicate long stretches of sequence. The proposed 3' and 5' termini are indicated. (♦) denotes the polyprotein stop codon and (•) denotes initiation codons of the VP5 and polyprotein ORFs on segment A (Kibenge *et al.*, 1991) and the VP1 ORF on segment B (Dybing, 1992).

70, terminal inverted repeat sequences in nucleotide positions 200-216 and 2793-2808 (Figs. 8a and 8b).

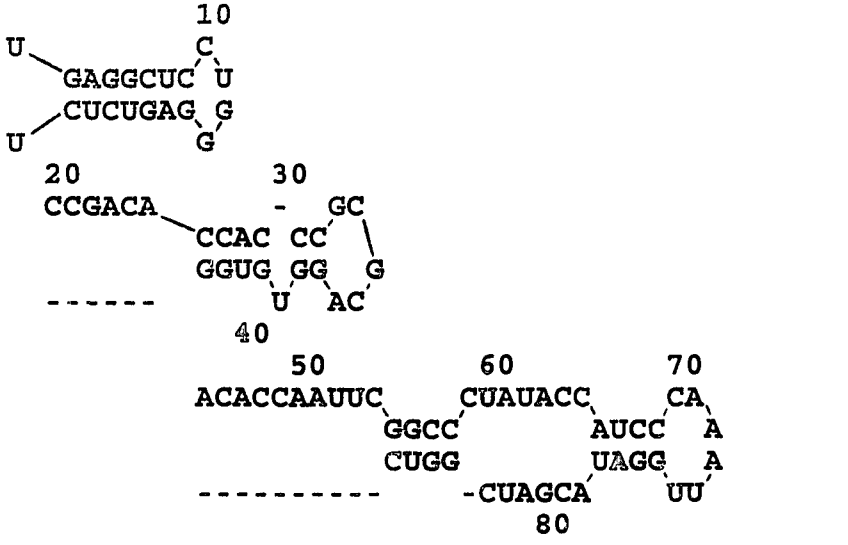
The 5'- and 3'- noncoding sequences of segment A of IBDV strains OH and GLS were also analyzed for secondary structures in the noncoding region by the RNA MFold computer program based on complementary base pairing and based on free energy of folding at physiological temperature 37^{0}C (Zuker *et al.*, 1989). For the 5' noncoding region of segment A of strains OH and GLS and segment B of strain OH, only one structure was generated by the RNA MFold program (Figs. 14, 15 and 16). For the 3' noncoding region of segment A of strain OH, a total of five structures were generated. Among them the one that was predicted with minimum free energy of folding was selected to represent the 3' noncoding region of segment A of strain OH (Fig. 14). Similarly, four structures were generated for the 3' noncoding region of segment B of strain OH. Of these, the one with minimum free energy prediction and therefore likely to be stable is presented in Fig. 16. For the 3' noncoding region of segment A of strain GLS, a total of seven structures were generated. As before, the one which had minimum free energy prediction for folding and therefore likely to have a relatively stable structure is presented in Fig. 15. Interestingly, this structure had similar hairpin loop motifs as were predicted for the 3' noncoding region of segment A of OH strain (Figs.14 and 15). Also, the structure predicted for the 5' noncoding region of segment B RNA MFold program was very similar to the

OH - A 5' NONCODING REGION



Temperature = 37.0 Energy = -19.5 kcal

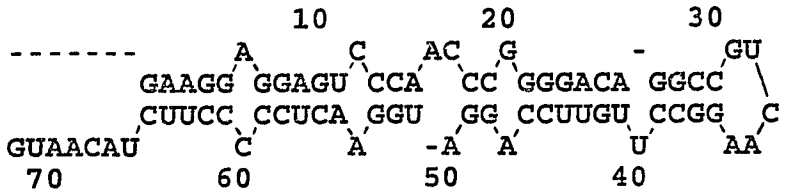
OH - A 3' NONCODING REGION



Temperature = 37.0 Energy = -19.5 kcal

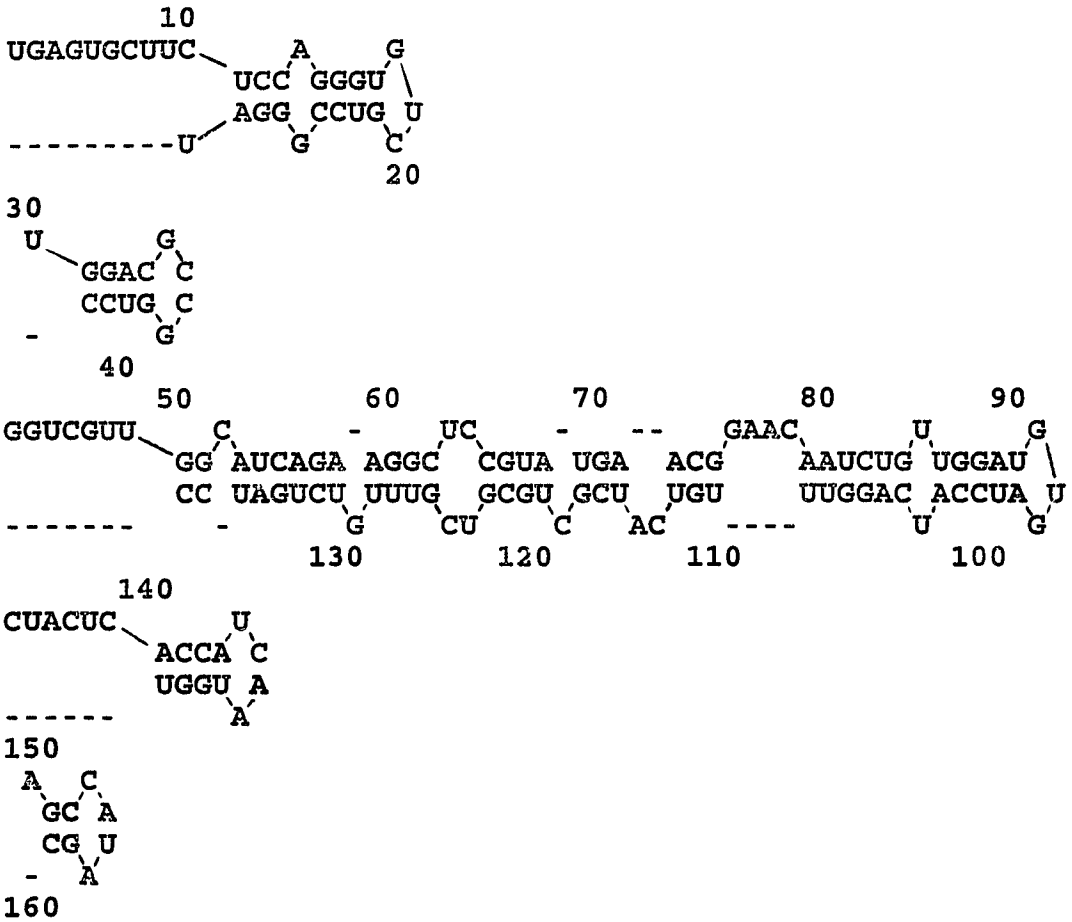
Fig. 14: Secondary structure prediction for 5' and 3' noncoding regions of segment A of strain OH by RNA MFold computer program (Zuker *et al.*, 1989).

GLS - A 5' NONCODING REGION



Temperature = 37.0 Energy = -31.2 kcals

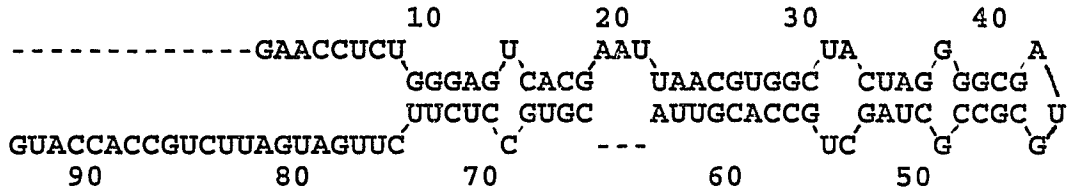
GLS - A 3' NONCODING REGION



Temperature = 37.0 Energy = -31.5 kcal

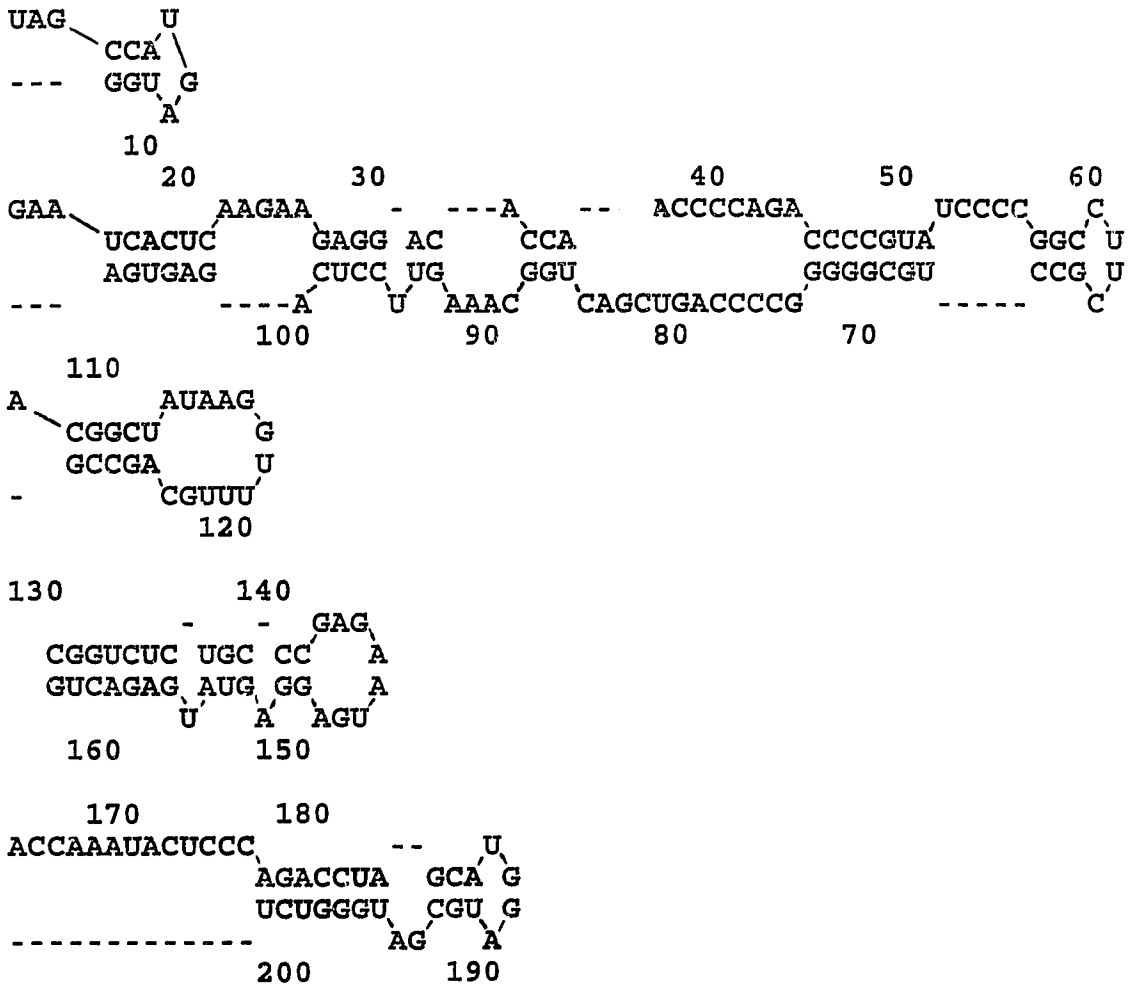
Fig. 15: Secondary structure prediction for 5' and 3' noncoding regions of strain GLS by RNA MFold computer program (Zuker *et al.*, 1989).

OH - B 5' NONCODING REGION



Temperature = 37.0 Energy = -27.5 kcal

OH - B 3' NONCODING REGION



Temperature = 37.0 Energy = - 48.5 kcal

Fig 16 : Secondary structure prediction for 5' and 3' noncoding regions of segement B of strain OH by RNA MFold computer program (Zuker *et al.*, 1989).

one predicted manually in sharing the same hairpin loop motif (Fig. 13 and Fig.16).

The predicted secondary structures for the 5'- and 3'-noncoding regions of segment A of OH strain of IBDV were very similar to the structures predicted for strain GLS (Figs. 14 and 15). Similar secondary structures as were predicted for the 5'- and 3'-noncoding regions of segment B of OH strain by RNA MFold computer program (Fig. 16) are likely to exist in the 5'- and 3'-noncoding regions of segment B of all the other five IBDV strains because terminal sequences of segment B are highly conserved among the strains showing more than 97% sequence identity (Fig. 6).

4. DISCUSSION

4.1 Terminal Sequence Analysis of Segment A

IBDV strains OH, SK140a, IN showed 100% sequence identity in the 5' terminal region and with the strain GLS shared a sequence identity of 84% (Table 5). The 5' terminal sequences of strains SK9 and QC-2 were identical but differed from the other four strains by 40%. The 5' terminals of strains GLS, SK9 and QC-2 were missing a few nucleotides, especially some Cs and Gs in this region when compared to strains OH, SK140a and IN (Fig. 5). It is possible that these missing nucleotides are not essential for maintaining regulatory functions in the 5' noncoding region and only the optimum length nucleotide sequences required for virus specific functions are conserved among the strains. Interestingly, segment B of all the strains have their 5' ends start with an identical sequence of GAA as was reported for segment B of IBDV strain 002-73 (Morgan *et al.*, 1988). This trinucleotide sequence at the 5' termini is highly conserved in segment A and B of all the six IBDV strains (Fig. 11). Similar conservation of a trinucleotide sequence 5' GAA- was reported for segment A and B of IPNV strains (Duncan *et al.*, 1991). It may function as a recognition signal for packaging the viral genome segments as has been observed with influenza A viruses where all the eight viral genome segments start and end with the conserved sequence of 12 to 13 nucleotides at their 5' and 3' ends (Desselberger *et al.*, 1980). The conserved 5' terminal sequence GAA- in IBDV

and IPNV genomes may act as a primer binding site for VP1, attaching to the terminal ends to initiate replication (Dobos, 1993; 1995).

The 3' terminal sequences of IBDV strains OH, SK140a and IN were essentially the same except for a single base substitution of T for G at position 65 in strain IN (Fig. 5). Other than the primer sequences, a number of conserved sequence motifs (regions A, B, C, D, E, F and G) were identified in the noncoding sequences among the six IBDV strains (Fig. 5). These sequences may be of some functional importance such as recognition signal for initiating transcription by viral RNA polymerase, as has been observed with viruses such as infectious bronchitis virus (Sethna *et al.*, 1989). Of particular interest are the conserved sequence motifs identified at the 3' terminal regions D (TTGGAT), E (GATC), and F (CT) (Fig. 5 and 6). Any one of these or some other specific conserved sequence motifs at the 5' terminus may contain the promoter sequence for binding of viral RNA polymerase to initiate transcription since two full length transcripts are made from IBDV genome segments A and B (Spies *et al.*, 1987). In influenza A virus, a promoter located at the 3' ends of viral RNA segments is suggested to be involved in viral RNA polymerase binding for initiating the synthesis of full-length cRNA (Fodor *et al.*, 1993, 1994).

Nonetheless considerable sequence heterogeneity was identified in the 3' terminal regions of segment A of some of the strains studied. An unusual

observation was the presence of a stretch of 39 nucleotides at position 128 in the strain GLS only (Fig. 5). The origin of this additional sequence in GLS and its significance are presently not known. IBDV strains SK9 and QC-2 also had a number of additional sequences at the 3' noncoding region (Fig. 5) which are not present in the other four IBDV strains. It is also interesting to note that serotype 1 strain QC-2 and serotype 2 strain SK9 in spite of their different serotype specificity shared almost identical terminal sequences. Computer analysis of the additional sequences in strains SK9, QC-2 and GLS for identity with the reported sequences in the GenBank database using the FASTA program did not reveal any significant sequence identity.

It may be speculated that these additional sequences have originated from a recombination event between viral RNAs of closely related IBDV strains, for example between GLS or SK9 or QC-2 and strains that have not been sequenced yet, by a mechanism similar to that suggested for coronavirus RNA (Banner and Lai, 1991) and Sindbis virus RNA (Weiss *et al.*, 1991). Recombination between the viral RNA and the host cell RNA could also account for such sequence variability as has been observed for infectious bronchitis virus (Williams *et al.*, 1993), potato leafroll virus (Mayo and Jolly, 1991), turnip mosaic virus (Sano *et al.*, 1992), and plumpoxvirus (Cervera *et al.*, 1993).

It is not uncommon for strains of the same virus to have variable 3' terminal sequences or 5' terminal sequences. For example in flaviviruses, some strains of tick-borne encephalitis viruses were reported to have variable 3' terminal sequences (Mandl *et al.*, 1991; Meyer *et al.*, 1993) and some strains of hepatitis C virus were found to have variable 5' terminal sequences (Simmonds *et al.*, 1993). Extensive sequence variability were also noted in the 3' noncoding regions of segment A of IBDV strains SK9 and SK140a (Fig. 5) which were isolates from the same geographical region. Similar observations were reported for the alphaviruses such as Ross River virus isolates from the same region, having rearrangements, deletions, insertions and substitutions in the 3' untranslated region (Faragher *et al.*, 1986).

In infectious bronchitis virus, Williams *et al.* (1993) have identified in the 3' noncoding regions of US strain Beaudette and a Japanese strain, a region of about 184 bases that was absent in the antigenically closely related strain Massachusetts 41. In these strains, the likely site for recombination was flanked by highly conserved sequences. Interestingly, comparable observations were made in the terminal regions of segment A of IBDV strains GLS, SK9 and QC-2 where regions flanking the positions of additional sequences were highly conserved and were essentially of the same sequence as in segment A of other three strains OH, IN and SK140a (Fig. 5). Such observations seem to provide compelling evidence that recombination events have occurred in these strains.

Vakharia *et al.* (1994) have found that IBDV strain GLS is not only a distinct variant but is also genetically distant from other serotype 1 and serotype 2 strains. The functional importance, if any, of additional sequence present in the 3' noncoding region of segment A of this virulent variant strain has to be further determined. It remains to be shown if this sequence has any effect on the virulence or pathogenesis of this strain. This would need to be further characterized, taking into account other sequence variations present in the VP2 region of this strain.

Generally, genetic recombination in RNA viruses is supposed to play an important role in RNA repair and in generation of diversity among virus strains. Environmental pressures placed on the virus contribute to genetic variability and these include point mutations and recombination events (Wang *et al.*, 1994). The lack of proof-reading in viral RNA polymerases is also considered to be responsible for the high frequencies of point mutations among the RNA viruses. This also provides a mechanism for antigenic and pathogenic evolution (Holland *et al.*, 1982; Truyen *et al.*, 1995). More rapid modifications in the viral genome as a result of RNA recombination have been associated with severe diseases in chickens immunized with the related vaccine strain of infectious bronchitis virus (Lai, 1992; Banner and Lai, 1991). In fact, some of the recently emerging European and Japanese IBDV isolates from vaccinated broiler flocks are highly virulent without showing changes in their antigenic characteristics (Lin *et al.*,

1993; Brown *et al.*, 1994). It might be speculated that in these strains the changes in virulence may have been a consequence of mutations in the noncoding region and this possibility needs further investigation.

It is possible that IBDV strains SK9 (serotype 2) and QC-2 (serotype 1), in spite of their serotype differences are genetically and evolutionarily closely related. They exhibit more than 95% sequence identity in their 5' and 3' noncoding regions. Similarly, serotype 2 strain OH and the serotype 1 strains SK140a and IN may be closely related as they exhibit close to 100% sequence identity in the 5' and 3' noncoding regions.

The significance of additional sequence present at 3' termini of segment A of IBDV especially in the variant strain GLS may be viewed from an entirely different perspective, such as absence or deletion of this particular sequence in the three strains OH, IN and SK140a. Segment A of GLS strain shares with these strains 85% sequence identity at the 5' terminus and only 44% sequence identity at the 3' terminus (Tables 5 and 6). The relatively low percentage of sequence identity at the 3' terminus is mainly attributed to the presence of 60 additional nucleotides in the strain GLS.

Little is known about the effect of cell-culture adaptation and passage on the pathogenicity and immunogenicity of IBDV. Tsai and Saif (1992) have found that

the attenuation of IBDV variant strain IN grown in BGM-70 cell line resulted in loss of its pathogenicity to specific pathogen free (SPF) chicks, without changes in its immunogenicity. Also the live IN strain vaccines prepared from attenuated virus did not protect chickens from challenge with the wild-type IN strain. It is likely that adaptation of IBDV strains to Vero cells may involve maintaining only the sequences necessary for replication. Any additional sequences that may be necessary for establishing infection by recognizing cell-type specific proteins in the bursa might have been lost in subsequent passages in Vero cells. It is tempting to speculate here that the variant strain GLS being more virulent than the variant strain IN (Snyder 1990) maintained its additional 3' terminal sequence even after serial passages in Vero cells. Willcocks *et al.* (1994) have reported such observations with astroviruses that are adapted to grow in primary human embryo cells by serial blind passage. These viruses had a 45-nucleotide deletion compared to fecal virus isolates grown in a cell line derived from a human colonic carcinoma. It has been suggested that the deletion resulted in the loss of a few amino acids in a nonstructural protein of unknown function and in the virus ability to grow in primary human embryo cells. Passage of human hepatitis A virus (HAV) in fetal rhesus monkey kidney cells has also resulted in attenuation of the virus and it has been mapped to mutations in the 5' untranslated region at bases 152 and/or 203-207 facilitating enhanced replication of HAV in a highly host cell-specific fashion (Day *et al.*, 1992).

4.2 Terminal Sequence Analysis of Segment B

Sequences in the noncoding regions of segment B of all six IBDV strains are highly conserved (Fig. 6) in spite of sequence divergence identified in segment A of some of these strains. Conservation of an optimum length sequence in this region may be important for vital processes such as replication. It also highlights the essential role of this region as this segment encodes the dsRNA-dependent RNA polymerase (Spies *et al.*, 1987). Any significant changes in this sequence may result in a virus that is unable to replicate. Zou and Brown (1992) have shown that the smallest fragments of M1 genome segment of deletion mutants of reovirus produced by serial passage at high multiplicity of infection contained minimum essential sequences. These sequences were from both termini of the segment implicated in genome replication and assembly.

However, there were few nucleotide substitutions in the noncoding regions of segment B of some IBDV strains and a single nucleotide deletion in strain IN (Fig. 6). Similar observations were made in the terminal regions of segment A of all six IBDV strains (Fig. 5). Some of these point mutations and deletions might have occurred as a result of adaptation to growth in cell lines and might be implicated in loss of virulence but this needs to be further elucidated.

Even though many of the attenuating mutations cause amino acid substitutions in the structural proteins of the virus thereby modulating virus entry into a

particular population of cells, some attenuating mutations that map to control elements in the noncoding region have also been reported. The attenuated phenotype of Sabin 3 poliovirus largely results from mutations in the genome at nucleotide position 472 in the noncoding region and 2034 in the coding region (Westrop *et al.*, 1989). It has been shown further that these mutations affect the translation efficiency of poliovirus RNA *in vitro* (Svitkin *et al.*, 1990). The attenuation of neurovirulence of Theiler's virus (TMEV) has been mapped to a single nucleotide (C residue) deletion out of four Cs between positions 876 and 879 in the 5' noncoding region (Pritchard *et al.*, 1992).

4.3 5' and 3' End Sequence Identity Determining Generic Specificity

The genome segment B of all six IBDV strains begins and ends with conserved pentanucleotide sequences GAACC and GGUCU, respectively, identical to that of genome segment A of strains OH, SK140A and IN (Fig. 11 and 12) suggesting they are characteristics of the genus *Avibirnavirus*. The genome segment A of IBDV strains GLS, SK9 and QC-2 also begins with GAA- and ends with the same consensus sequence -CU. In reoviruses, which are similar to birnaviruses in having a dsRNA genome, all the ten segments of reovirus serotype 3 (strain Dearing) share a common tetranucleotide sequence at the 5' terminus and a common pentanucleotide sequence at the 3' terminus of their plus strands (Antczak *et al.*, 1982). The common terminal end sequences of segmented genomes of RNA viruses belonging to the same genus such as

reoviruses (Anzola *et al.*, 1987), La Crosse viruses (Obijeski *et al.*,1980) and influenza viruses (Stockle *et al.*, 1987) suggest that these conserved sequences might serve as recognition signals for viral polymerase and assembly of genome segments into the progeny virus (Yan *et al.*, 1992; Zou and Brown, 1992). All eight negative-sense single-stranded RNA segments of influenza A viruses have conserved sequences of 12 and 13 nucleotides at their 3' and 5' ends, respectively. These sequences are partially complementary and are capable of forming a panhandle structure that can serve as a regulatory signal for transcription and replication, as well as for packaging of RNA into virus particles (Hsu *et al.*, 1987; Fodor *et al.*, 1994).

Mundt and Müller (1995) have reported the terminal sequences of segment A and B of four European strains (P2, Cu-1, Cu-1M, 23/82) of IBDV to begin with GGA- instead of GAA- as was observed with strains in the present study. Interestingly, 3' termini of segment A of both the European strains and the strains studied here end with the same dinucleotide sequence -CU 3'. This conserved dinucleotide sequence is present not only at the 3' termini of segment A but also at the 3' termini of segment B of all the six IBDV strains studied here. However, the 3' termini of segment B of strains studied by Mundt and Müller (1995) seem to be considerably shorter than that of segment B of strains studied here and the significance of this observation contributing to the genetic diversity of IBDV strains has to be further elucidated.

It is possible that segment B of the European strains are missing the additional sequences found on the strains studied here mainly because of the method that was adapted by Mundt and Müller (1995b) to obtain the 3' terminal sequences. Homopolymeric dA-tailing method with poly (A) polymerase has been used and it is considered to be rather a relatively inefficient and less reliable method, if secondary structures are present at the 3' end (Mandl *et al.*, 1991). In addition, PCR amplification following dA-tailing requires a relatively low annealing temperature, such as 37^{0}C, favouring nonspecific binding of the oligo-dT primer to other A- rich sequences resu g in nonspecific products (Hirzman *et al.*, 1993; Zhiner and Gehring, 1994) 5' RACE method used by Mundt and Müller (1995) to determine the 5' term sequences has to be modified to obtain the terminal ends of segment A of IBDV which have VP1 attached to their 5' ends (Dobos, 1993). The re terminal sequences of European strains have additional 15 nucleotides a' ends that are placed at the 3' termini in the present study. Here the pr consideration had been given to the finding of a stretch of conserved hc ogous sequence between segment A and B of IBDV strains OH, IN and SK140a and aligning the sequences of strains GLS, SK9 and QC-2 which shar e same common end sequence identity with the other three strains. The proposed 5' and 3' ends of the ligated terminal sequences for all the strains were assigned based on the observation of reported terminal sequences of segmented RNA viruses sharing common termini between all the segments (Antczak *et al.*, 1982; Obijeski *et al.*, 1980; Stockle *et al.*, 1987;

Yan *et al.*, 1992). However the 5' RACE method could be used to confirm the proposed 5' ends of genomic segments A and B of IBDV strains as suggested by Hirzman *et al.* (1993).

Segment A of published sequence of GLS strain (Vakharia *et al.*, 1994) is missing the terminal three nucleotides 5' GAA- and the strain OH (Kibenge *et al.* 1991) the terminal two nucleotides 5 'GA-. It is interesting to note here that segment B of IBDV strain 002-73 (Morgan *et al.*, 1988) also begins with 5' GAA. The IPNV consensus sequence (Duncan *et al.*, 1991) is similar to IBDV for the first three nucleotides (5' GAA-) at the 5' termini of segment A ad B. This trinucleotide sequence 5' GAA- is likely to serve as a recognition signal for primer (VP1) binding to initiate replication. However, there is no sequence identity in the noncoding region beyond that and none at all at the 3' termini between IBDV and IPNV. This further justifies the re-classification of the family *Birnaviridae* into three different genera (Dobos, March 1995, Personal communication).

4.4 Secondary Structures

The panhandle structures predicted manually for the 3'-5' terminal junction (Fig 13) of the prototype strain OH originate as a result of adjacent inverted repeats in the 3' terminus in segment A and in the 5' terminus in segment B. Segment A hairpin loop structure becomes a mirror image of the one in segment B, which

might be significant in facilitating the packaging of both segments A and B in the viral capsid. These panhandles may also be responsible for the sequence in this region being placed either at the 5' end or the 3' end as in IBDV strain 52/70 (Fig 7a, Fig 7b). In Sindbis virus, the first 44 nucleotides in the 5' nontranslated region which are capable of forming a hairpin structure were found to be important for virus replication. All deletions tested in this region were found to be lethal or resulted in poor growth of the virus when compared to the parental virus (Niesters and Strauss, 1990). In influenza virus, a terminal panhandle structure is implicated in the initiation of viral RNA transcription (Fodor *et al.*, 1994).

Secondary structures were also predicted by the RNA MFold program. In the 5' noncoding regions of segment A of IBDV strains OH and GLS, there exist similar hairpin loop motifs from bases 30 and 40 (Fig. 14 and Fig. 15). The conservation of such a hairpin loop motif rather than the actual nucleotide sequence in this region might be of some functional importance, possibly serving as a recognition signal for virus specific functions. In calicivirus genomes, the 3' terminal untranslated regions are quite variable in their length, yet they all were suggested to form stem and loop or hairpin structures (Seal *et al.*, 1994). These areas of the genome are believed to be important in providing the replication signals for amplification of RNA molecules (Weiner *et al.*, 1987). Such conserved structures seem to exist as well in the 3' noncoding regions of

segment A of IBDV strains OH and GLS. There are two hairpin loop structural domains between base 10 and 40 and another hairpin loop structure in strain OH between base 70 and 80 and in strain GLS between base 140 and 150 (Fig. 14 and Fig. 15). Similar hairpin loop structures were predicted for the 5' and 3' noncoding regions of segment B of strain OH (Fig. 16) by the RNA MFold computer program. Such structures may presumably exist in segment B of all the IBDV strains sequenced in this study as well as in segment B of IBDV strain 002-73 sequenced by Morgan *et al.* (1988), since the nucleotide sequences for segment B are highly conserved (Figs. 6 and 7) (Qian and Kibenge, 1994). The validity of the proposed stem and loop structural models for the noncoding regions of viral genome could be assessed further by other biochemical approaches. Single and double stranded regions of these structures could be characterized by determining the sites at which transcripts expressed by synthetically constructed viral RNA would be cleaved with single- and double-stranded specific nucleases. This would allow determining the existence of any stem and loop structure involved in viral specific functions as has been shown for other viruses such as hepatitis C virus (Brown *et al.*, 1992).

In the enteroviruses and rhinoviruses, an RNA hairpin loop structure located at the 5' end of the viral RNA is highly conserved and is shown to affect the translation efficiency of poliovirus RNA in infected cells (Simoes and Sarnow, 1991). Such structures are proposed to be important for replication of RNA of

flaviviruses (Brinton *et al.*, 1986; Hahn *et al.*, 1987) such as hepatitis C virus (Hans *et al.*, 1992). Similar structures were presumed to interact with the viral polymerase during viral RNA amplification (Jacobson *et al.*, 1993) or with cell type-specific proteins (Chang *et al.*, 1993). RNA secondary structures were implicated in viral virulence also, such as the 5' noncoding region of polioviruses playing a significant role in neurovirulence (Skinner *et al.*, 1989). The neurovirulence of Theiler's virus has also been shown to be attenuated by a three nucleotide insertion at the stem and loop structure of the 5' untranslated region (Bandyopadhyay *et al.*, 1993).

4.5 General Discussion and Conclusion

The genomes of several IBDV strains had been previously sequenced. But they may not represent the complete genomic RNA sequence since 5' and 3' ends were not determined. Common end sequences usually characteristics of segmented RNA viruses and consensus sequence motifs harbouring regulatory signals were not identified. Since 1988, alterations in IBDV characteristics such as antigenic and virulence variations have been associated with problems in vaccination practices. Some of the recent European and Japanese isolates were shown to alter their virulence without showing changes in their antigenicity and overcome immunity induced by vaccines of standard strain vaccines. The location of sites in the viral genome involved in such virulence variations and the underlying molecular mechanism are not presently known.

At the present time considerable attention has been focused on the coding regions of the IBDV genome which has facilitated better understanding of the molecular basis for antigenic variations. Not much information is available about the noncoding regions of the IBDV genome. In order to gain a better understanding of the recent changes of IBDV in molecular terms, this study focused on the noncoding regions of the viral genome as they contain important regulatory signals for viral replication, transcription, assembly and encapsidation and possibly virus virulence determinants.

A novel method of PCR amplification across the ligated 3' and 5' end junctions of the viral RNA strand was developed to obtain the terminal sequences of dsRNA genome segments of six selected IBDV strains. In this method an attempt was made to remove the terminal protein before ligation of 5' phosphoryl end to the 3' OH end. A specific sense and a specific antisense primers were used for the cDNA synthesis following ligation since viral dsRNA yielded two templates following denaturation. 5' and 3' ends of both (+) and (-) strands were amplified yielding more templates for subsequent PCR amplification of the ligated region. By this modified method, the terminal sequences of both the genome segments of six strains of IBDV were successfully determined and the common consensus terminal nucleotide sequences which may be characteristics of bisegmented genome of IBDV were identified. Even though the majority of the 5' termini might have existed as blocked termini due to incomplete removal

of VPg, it is possible that there existed a small proportion of 5' termini accessible to phosphorylation by polynucleotide kinase, facilitating subsequent ligation of 5' end to 3' OH end by T4 RNA ligase. 5' end labeling studies with [γ^{32}P] ATP of both viral RNA segments of IBDV OH strain (Kibenge and Qian, personal communication) showed only a small fraction of the viral RNA after the end labeling, indicating that the majority of the viral RNA 5' ends were blocked and inaccessible to phosphorylation. A similar observation had been made with 5' termini of adenovirus DNA molecules which have viral protein (VPg) attached to the 5' termini (Carusi, 1977).

Conserved terminal sequences at the 5' and 3' ends of genome segments A and B that may be characteristics of the genus *Avibirnavirus* were identified. This sequence lies in a 34-base homologous sequence between the segment A and B of IBDV strains OH, SK140a and IN. These sequences are in regions of inverted terminal repeats and adjacent inverted repeats which readily form panhandle structures. The total lengths of genome segment A and B of IBDV strain OH were determined to be 3,241 bp and 2,922 bp, respectively, after finding the additional extreme terminal sequences. The complete nucleotide sequence thus determined will facilitate reverse genetics studies on IBDV which may allow construction of functionally active IBDV from molecularly cloned DNA copies of genomic sequences.

A high degree of conservation of terminal sequences of genome segment B of all six IBDV strains reflects the essential role of this segment which encodes the putative viral RNA polymerase. The conservation of an optimum length terminal sequence may be essential for maintaining important functions such as viral replication and infectivity. Substantial variations identified in the 3' noncoding sequences of segment A of IBDV strains may contribute to the genetic diversity of IBDV and possibly to virus virulence determination. Likely, recombination events and cell culture adaptations were suggested to account for such variations. Obviously, additional strains of IBDV need to be sequenced so as to fully characterize the range of variations. No definite sequence pattern differentiating serotype 1 strains from serotype 2 strains was observed in the noncoding regions of genome segment A and B of the six IBDV strains. Regardless of terminal sequence heterogeneity identified in genome segment A of IBDV strains GLS, SK9 and QC-2, consensus sequence motifs were identified between segment A and B of all six IBDV strains. These sequences are likely to play a vital role in regulatory functions such as replication, genome expression and assembly of the progeny virus and possibly harbour determinants for virulence.

This study has provided further insights into the molecular organization of the IBDV genome. Regions of virus-specific sequences and secondary structures likely to be important in regulatory events and possible virulence factors have

been identified. These findings would likely facilitate better understanding of the replication strategy of IBDV, its evolutionary pattern, mode of generation of diversity and its pathogenicity and the development of better and more effective approaches for disease control.

4.6 Future Studies

This study has added new information to the existing knowledge on the molecular organization and diversity of the IBDV genome. As an outcome of the present study, two approaches for future studies could be envisaged. Probable regulatory sequences to be targeted for site-directed mutagenesis and deletion studies have been identified. There is considerable challenge ahead to map the exact nucleotide residues involved in regulatory processes such as viral replication, viral genome expression and phenotypic changes such as virulence. Such endeavour would be facilitated by the construction of functionally active IBDV from molecularly cloned DNA copies of genomic sequences since direct mutational studies on IBDV RNA are difficult. Better understanding of genome replication and assembly of IBDV is especially important in the context of recent emergence of highly virulent strains of IBDV. Improved recombinant viruses could be constructed. Other alternative therapeutic approaches such as antisense oligonucleotides to intervene viral regulatory processes could be further explored. For example, if packaging signal sequences involved in

recognition by capsid protein are identified, they may serve as potential targets for antiviral compounds.

Another approach worth pursuing would be the study of the effect of successive cell culture passage on the pathogenicity of IBDV in relation to covariations in the coding and noncoding sequences of IBDV genome. It is evident from the present study that variations in the noncoding regions contribute to the genetic diversity of IBDV isolates. In some of the variant strains studied such as GLS and IN, additional sequences, deletions and substitutions have been observed in the terminal region. Such studies may provide important clues as to the mechanism involved in IBDV virulence and facilitate construction of better recombinant and attenuated vaccine strains of IBDV for control of infectious bursal disease.

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APPENDIX

COMMONLY USED BUFFERS AND SOLUTIONS

40% Acrylamide

38 g Acrylamide
2 g Bis-acrylamide

DNase I buffer

50 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
50 μg/ml BSA

Lysozyme Buffer

50 mM Glucose
10 mM EDTA
25 mM Tris base
adjusted to pH 8.0

30 % PEG 8000 in 1.8 M NaCl
make it up and autoclave

RNase solution

5 mg/ml Type 1 panceatic RNase in
0.1 x SSC in sterile DEPC water
Incubate at 90 C for 5 min just prior to use.

5X Sequenase Buffer

200 mM Tris-HCl, pH 7.5
100 mM $MgCl_2$
250 mM NaCl

Sequencing Gel

84 g Urea
29 ml 40% acrylamide
20 ml TBE
81 ml dd H_2O
Filter and add

1.6 ml 10% ammonium persulphate
80 μl TEMED

S O C Medium

2.0% Bactotryptone
0.5% Bacto yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM Glucose

Stop solution

95% Formamide
20 mM EDTA
0.05% Xylene cyanol

TBE electrophoresis buffer

89 mM Tris base adjusted to pH 8.0
89 mM Boric Acid
2 mM EDTA

TBE Sequencing buffer

100 mM Tris adjusted to pH 8.3
83 mM Boric Acid
1 mM EDTA

TE buffer

10 mM Tris base adjusted to pH 8.0
1 mM EDTA

TSE buffer

50 mM Tris base
100 mM NaCl
1 mM EDTA
adjusted to pH 6.9

X-gal

100 mg X-gal
5 ml dimethylformamide

2X YT broth

16% Bacto. yptone
10% Bactoyeast Extract
5% NaCl

YT agar plates

0.8% Bactotryptone
0.5% Bactoyeast Extract
0.5% NaCl
1.5% Agar